Analysis of Flonicamid and Its Metabolites in Dried Hops by Liquid Chromatography–Tandem Mass Spectrometry

MATT J. HENGEL* AND MARION MILLER

Department of Environmental Toxicology, University of California at Davis, One Shields Avenue, Davis, California 95616

An analytical method was developed for the determination of the neo-nicotinoid insecticide flonicamid (*N*-cyanomethyl-4-trifluoromethylnicotinamide) and its metabolites *N*-(4-trifluoronicotinoyl) glycine (TFNG), 4-trifluoronicotinic acid (TFNA), and 4-trifluoromethylnicotinamide (TFNA-AM) in dried hops. The method utilized C18 and polymeric solid phase extraction (SPE) column cleanups, liquid–liquid partitioning, and liquid chromatography (LC) with mass spectrometry (MS/MS). Method validation and concurrent recoveries from untreated dried hops ranged from 66 to 119% for all compounds over five levels of fortification (0.005, 0.02, 0.2, 2.0, and 4.0 ppm). Flonicamid-treated hop samples collected from three field sites had the following residues: flonicamid levels of 0.561–2.85 ppm, TFNA levels of 0.302–0.470 ppm, TFNA-AM levels of 0.038–0.177 ppm, and TFNG levels of 0.098–0.204 ppm. Untreated hop samples from all fields had residues <0.005 ppm for flonicamid, TFNA, TFNA-AM, and TFNG. The limit of quantitation and limit of detection for all compounds were 0.005 and 0.0025 ppm, respectively.

INTRODUCTION

As United States growers strive to be more competitive in the global market, crop yield, quality, and safety are a priority. In the case of hops, the control of hop aphids (*Phorodon humuli*) has become increasingly difficult as more traditional pesticides (organophosphates and carbamates) are no longer registered for use (*1*). As a result, more pressure is placed upon newer, reduced risk compounds to control the hop aphid. One class of reduced risk compounds that has shown good efficacy on aphids are the neo-nicotinoids; these include imidacloprid, thiacloprid, thiamethoxam, and flonicamid.

Flonicamid (*N*-cyanomethyl-4-trifluoromethylnicotinamide) was developed by Ishihara Sango Kaisha, Ltd. (ISK) (*2*). In 2000, flonicamid was presented as a novel selective systemic pesticide that is highly effective against aphids and other sucking insects (*3*). The mode of action has been identified as a blocking of the A-type potassium channel (*4*). Biological effects include loss of directed movement and suppression of feeding by aphids (*2–4*). In addition, the mode of action is not via acetylcholine esterase or nicotinic acetylcholine receptors, thus reducing cross-resistance with similar insecticides (*3, 5*). With minimal cross-resistance characteristics and lack of toxicity to beneficial arthropods, flonicamid lends itself for use in integrated pest management programs (*2, 3, 5*). Currently, flonicamid is produced and distributed by FMC and ISK Biosciences (*6*). The

United States Department of Agriculture Interregional Project No. 4 (USDA IR-4) program is in the process of submitting data to the Environmental Protection Agency (EPA) for the purpose of registering the use of flonicamid on hops.

Separation and analysis of flonicamid and metabolites (**Figure 1**) can be accomplished using a high-pressure liquid chromatograph (HPLC) coupled to a mass spectrometer (MS) (*7, 8*). Few analytical methods are available in the literature that describe flonicamid with different agricultural matrixes (*7, 8*). Generally, the methods available are geared towards fruits and vegetables with high moisture content and are not well-suited to the complex matrix of hop cones. Improved cleanup steps are required to reduce the resins and oils associated with the hop extract, which can complicate analyses by causing enhancement or suppression of the ionization process in the ionization source (*9*).

In the present study, a rugged and sensitive method for the detection of flonicamid and its metabolites in dried hop samples was developed to be used as an enforcement method to support pesticide registration and risk assessment. The new method utilizes acetonitrile (MeCN)/water extraction, solid phase extraction (SPE), liquid–liquid partition, and hydrophilic interaction chromatography-tandem mass spectrometry detection (HILIC-MS/MS).

MATERIALS AND METHODS

Materials. Flonicamid (CAS Registry No. 158062-67-0, 99.7% purity, Lot No. 272785), 4-trifluoromethylnicotinic acid (TFNA, CAS
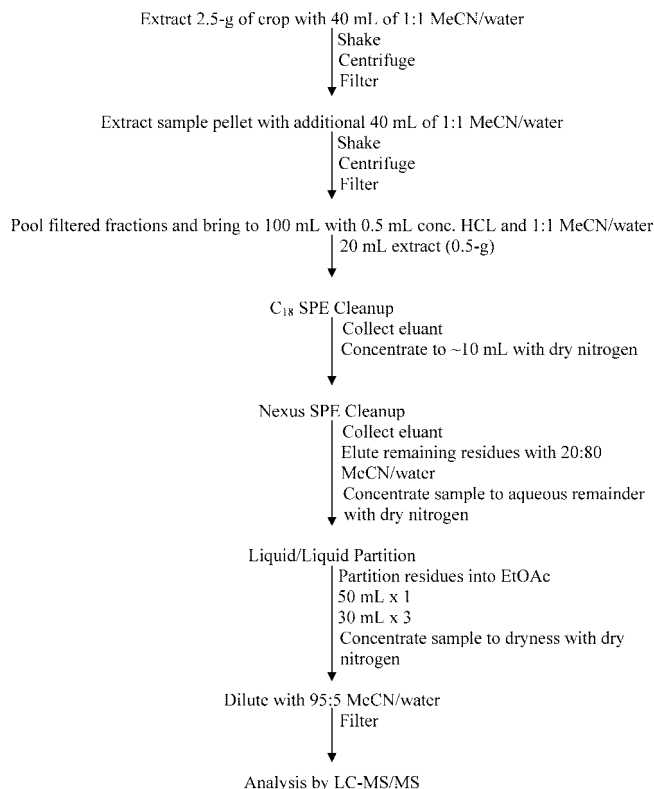
* Author to whom correspondence should be addressed (telephone (530) 752-2402, fax (530) 754-8556, e-mail mjhengel@ucdavis.edu.
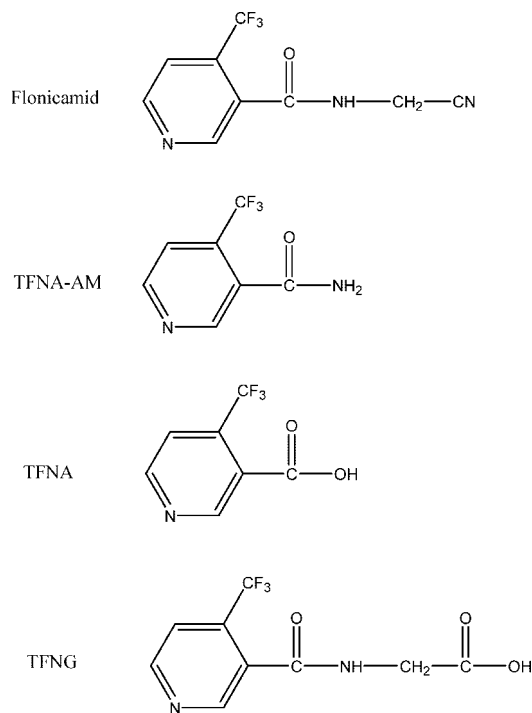
Figure 1. Molecular structures for flonicamid and metabolites.

Figure 2. Basic sample flowchart for analysis.

Registry No. 158063-66-2, 99.4% purity, Lot No. 404593), 4-trifluoromethylnicotinamide (TFNA-AM, CAS Registry No. 158062-71-6, 100% purity, Lot No. 275591), and *N*-(4-trifluoromethylnicotinoyl)glycine (TFNG, CAS Registry No. 207502-65-6, 99.4% purity, Lot No 404594) were acquired from FMC Agricultural Products Group (Princeton, NJ). All solvents and reagents were of residue grade or better. Water was prepared using a Milli-Q reagent water system. Specifications for SPE and filtration are cited below.

Preparation of Standard Solutions. Stock solutions (1.00 mg/mL) of each compound of interest were prepared by adding ∼100 mg (adjusted for purity) of pure compound to separate 100 mL volumetric flasks and bringing up to volume with MeCN. The stock solutions were stored generally at −20 °C and were stable for 1 year. A high-level fortification solution was prepared by taking 5 mL aliquots of each stock solution and diluting up to volume in a 50 mL volumetric flask with MeCN, resulting in a 100 μg/mL mixed solution. A mid-level fortification solution was prepared by taking a 5 mL aliquot of the 100 μg/mL mixed solution and diluting up to the volume in a 50-mL volumetric flask with MeCN, resulting in a 10 μg/mL mixed solution. A low-level fortification solution was prepared by taking a 5 mL aliquot of the 10 μg/mL mixed solution and diluting up to the volume in a

50-mL volumetric flask with MeCN, resulting in a 1.0 μg/mL mixed solution. Calibration solutions for LC-MS/MS analysis were prepared by taking various volumes of the 10 and 1 μg/mL mixed solutions and diluting up to the volume in 95:5 MeCN/water, resulting in calibration standards over a range of 0.1–40 pg/μL. Fortification and calibration solutions were stored at ∼5 °C and were stable for 6 months. Stability of standard solutions was previously determined by FMC Corporation (7).

Collection of Field Samples. A total of 12 hop samples (6 treated and 6 untreated) were collected from IR-4 field trial sites in Washington, Idaho, and Oregon. Treated samples received three flonicamid applications at a rate of 0.1 kg (active ingredient)/ha (0.089 lb active ingredient/acre). The final application was 10 ± 1 days prior to harvest. Following sample collection, the hop cones were dried in a manner consistent with commercial drying methods (heated air kilns) and transferred, frozen, to our facility.

Sample Preparation. Hop samples (∼300 g each) were chopped with equal portions of dry ice using a Hobart food chopper (Hobart

Table 1. Compound-Specific Information for Chromatography and Mass Spectrometry Conditions

compound	Q1[a] mass (AMU)	Q3[a] mass (AMU)	DP[b] (V)	FP[c] (V)	EP[d] (V)	CEP[e] (V)	CE[f] (V)	CXP[g] (V)	RT[h] (min)
flonicamid	229.8	202.8	26	370	9.5	18	27	16	3.90
TFNA-AM	190.8	147.8	31	370	10.5	17.8	33	8	4.47
TFNA	191.8	147.8	31	350	9.5	16	35	12	3.76
TFNG	248.8	202.8	26	370	8.5	16	31	16	4.29

[a] Q1 and Q3 represent compound-specific transitions monitored. [b] Declustering potential. [c] Focusing potential. [d] Entrance potential into first quadrupole. [e] Collision cell entrance potential. [f] Collision energy. [g] Collision cell exit potential. [h] Retention time.

Table 2. Average Recoveries of Flonicamid, TFNA, TFNA-AM, and TFNG from Hops

fortification level (ppm)	flonicamid[a]	TNFA[a]	TFNA-AM[a]	TFNG[a]
0.005	77 ± 5 (n = 4)	68 ± 2 (n = 4)	86 ± 3 (n = 4)	102 ± 10
0.02	84 ± 8 (n = 6)	97 ± 12 (n = 6)	91 ± 11 (n = 6)	104 ± 11 (n = 6)
0.20	77 ± 6 (n = 7)	81 ± 7 (n = 7)	86 ± 5 (n = 7)	88 ± 8 (n = 7)
2.0	92 ± 4 (n = 3)	93 ± 2 (n = 3)	97 ± 2 (n = 3)	109 ± 5 (n = 3)
4.0	69 ± 3 (n = 3)	82 ± 3 (n = 3)	81 ± 3 (n = 3)	91 ± 8 (n = 3)

[a] Values are mean percent recovered ± standard deviation; n is the number of replicates.

Analysis of Flonicamid and Its Metabolites in Dried Hops

J. Agric. Food Chem., Vol. 55, No. 20, 2007 **8035**

Table 3. Residue Results of Flonicamid-Treated Hops

sample ID	flonicamid[e]	TFNA[e]	TFNA-AM[e]	TNFG[e]
A[a]-ID01[b]	<0.005	<0.005	<0.005	<0.005
B[a]-ID01	<0.005	<0.005	<0.005	<0.005
C-ID01	2.85	0.312	0.177	0.110
D-ID01	2.78	0.302	0.165	0.098
A-OR01[c]	<0.005	<0.005	<0.005	<0.005
B-OR01	<0.005	<0.005	<0.005	<0.005
C-OR01	1.10	0.470	0.139	0.204
D-OR01	1.20	0.442	0.153	0.204
A-WA03[d]	<0.005	<0.005	<0.005	<0.005
B-WA03	<0.005	<0.005	<0.005	<0.005
C-WA03	0.561	0.335	0.038	0.156
D-WA03	0.565	0.334	0.038	0.168

[a] Prefix A and B denote untreated samples. [b] Samples from Idaho field site. [c] Samples from Oregon field site. [d] Samples from Washington field site. [e] Values in ppm.

Corp., Troy, OH). Each chopped sample was stored in a labeled ~1-L jar, and a lined lip was loosely closed on top to allow the dry ice to dissipate during storage at −20 °C.

Stability Study. Six control samples were fortified with flonicamid, TFNA, TFNA-AM, and TFNG at the 0.2 ppm level and were collocated with the field samples generally at −20 °C. Three samples were analyzed after a storage period equivalent to the longest interval of sampling and analysis of field-treated samples. The remaining samples were retained for long-term storage.

Extraction. A 2.5-g aliquot of crop was weighed into a 50 mL disposable tube (flonicamid and metabolite recoveries were fortified at this point), and 40 mL of extraction solution (MeCN/water, 1:1, v/v) was added. The sample was capped and placed horizontally on a Max-Q 3000 platform shaker for 30 min at 100 rpm (Barnstead-LabLine, Melrose Park, IL) and then centrifuged for 10 min at ~1500 rpm using a FXD centrifuge (International Centrifuge, Boston, MA). The supernatant fluid was filtered using a vacuum flask and a Büchner funnel fitted with a Whatman #1 filter (Whatman International Ltd., England). An additional 40 mL of extraction solution was added to the sample pellet, and the sample was shaken vigorously to break up the pellet. The sample was returned to the platform shaker for 30 min at 100 rpm and subsequently centrifuged for 10 min at ~1500 rpm. The supernatant fluid was filtered and pooled with the first supernatant extraction and transferred to a 100 mL graduated cylinder. The sample volume was made to 100 mL with the addition of 0.5 mL of concentrated hydrochloric acid and 1:1 MeCN/water. The sample was mixed thoroughly and 20 mL, equivalent to a 0.5-g aliquot, was measured out into a 25 mL graduated cylinder.

Solid Phase Extraction (SPE). Mega Bond Elut-C_{18} SPE columns (1 g/6 mL, Varian Inc., Harbor City, CA) were preconditioned with 5 mL of methanol followed by 5 mL of 0.25 N hydrochloric acid. When
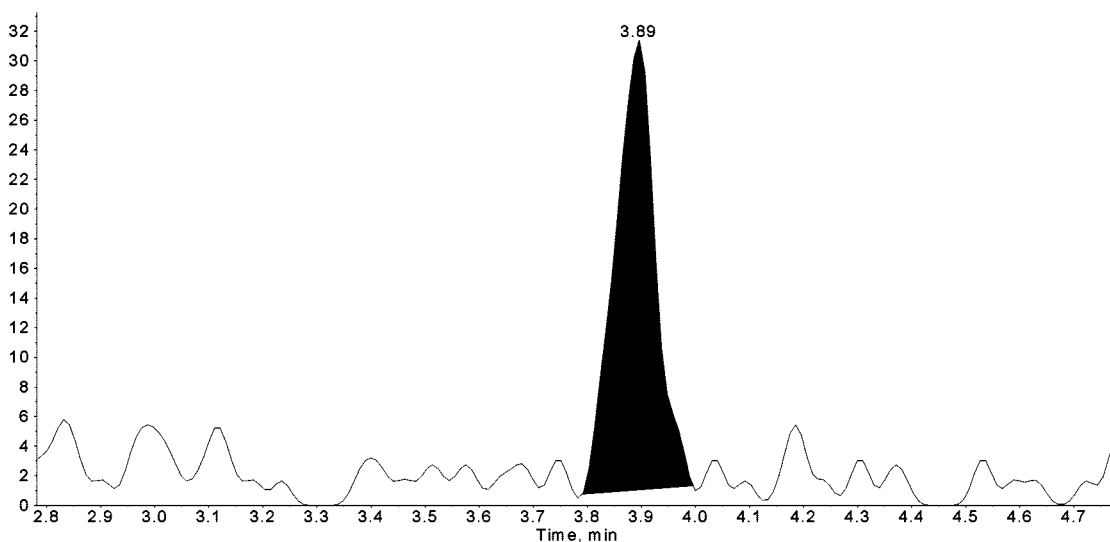
Figure 3. Flonicamid chromatogram of 0.1 pg/μL (equivalent to 0.0025 ppm) calibration standard (MRM = 229.8/202.8).
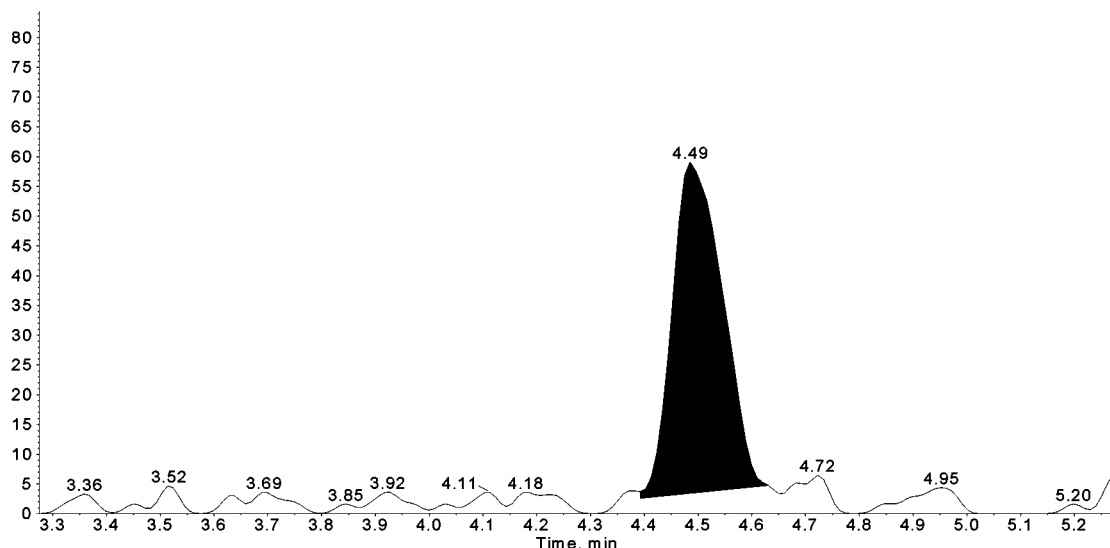
Figure 4. TFNA-AM chromatogram of 0.1 pg/μL (equivalent to 0.0025 ppm) calibration standard (MRM = 190.8/147.8).
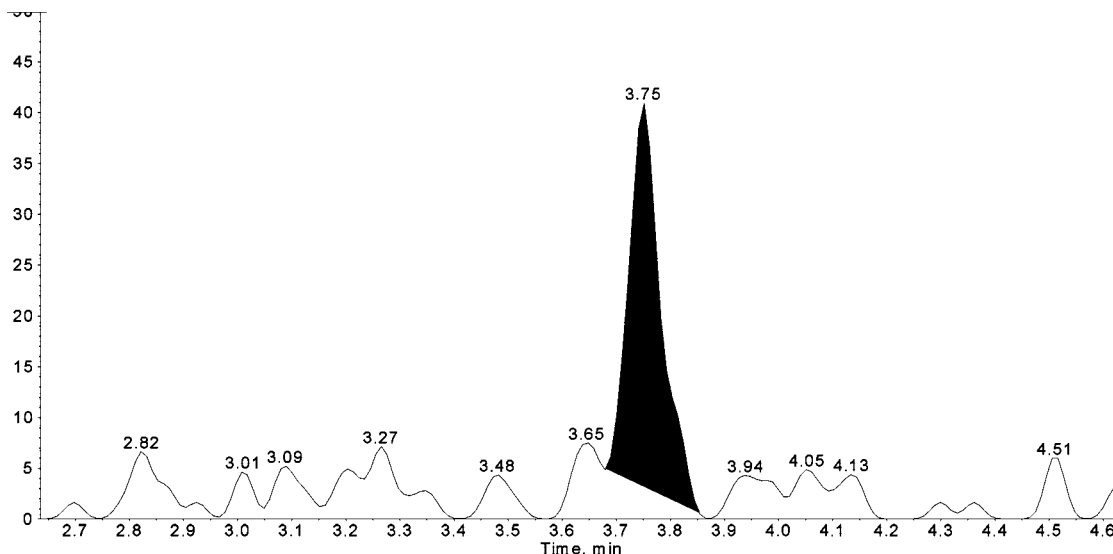
Figure 5. TFNA chromatogram of 0.1 pg/μL (equivalent to 0.0025 ppm) calibration standard (MRM = 191.8/147.8).
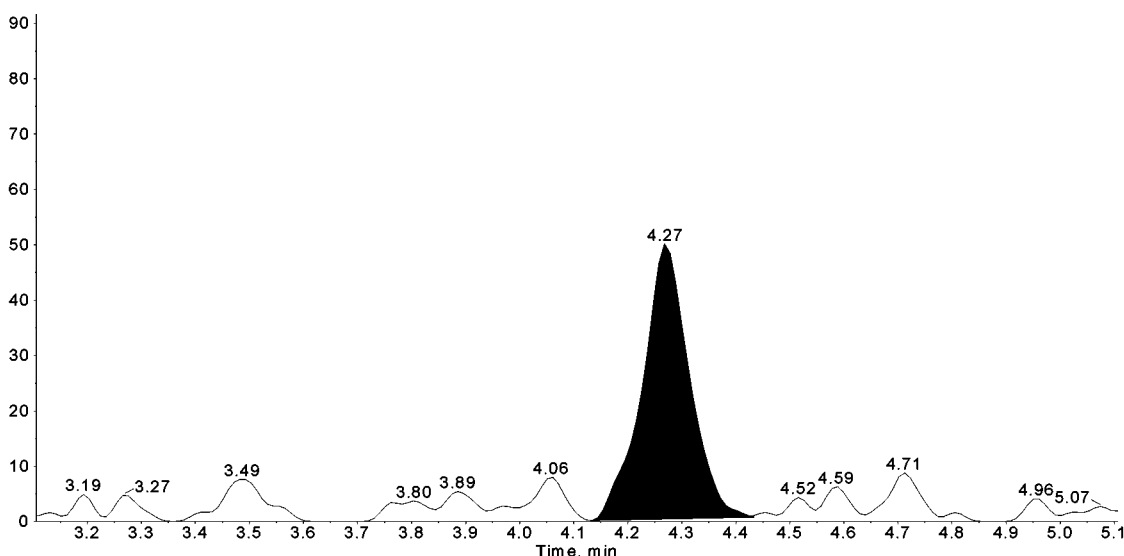
Figure 6. TFNG chromatogram of 0.1 pg/μL (equivalent to 0.0025 ppm) calibration standard (MRM = 248.8/202.8).

the solvent reached the top of the packing, the sample was loaded to the SPE with the aid of a 30 mL reservoir. Mild vacuum was applied, and the eluant was collected in a 40 mL conical tube. The graduated cylinder containing the sample was rinsed with 5 mL of 1:1 MeCN/water and added to the SPE. The eluted sample was transferred to a TurboVap tube and concentrated to ∼10 mL with dry nitrogen and water bath at 50 °C using a TurboVap II workstation (Caliper Life Science, Hopkinton, MA). The concentrated sample was subjected to a second cleanup by SPE. ABS Elut-Nexus SPE columns (0.5 g/12 mL, Varian Inc., Harbor City, CA) were conditioned with 5 mL of methanol followed by 5 mL of 0.25 N hydrochloric acid. When the solvent reached the top of the packing, the sample was loaded to the SPE. Mild vacuum was applied, and eluant was collected in a 40 mL conical tube. The TurboVap tube from the previous concentration step was rinsed with 5 mL of MeCN/water (20:80, v,v) and added to the SPE. Sample elution was completed with an additional 10 mL of MeCN/water (20:80, v,v). The resulting sample was transferred to a clean TurboVap tube and concentrated to the aqueous remainder using the TurboVap II workstation mentioned above.

Liquid/Liquid Partition. The aqueous sample remainder from the SPE was transferred to a 125 mL separatory funnel. The TurboVap tube was rinsed with 50 mL of ethyl acetate (EtOAc), and this volume was added to the separatory funnel containing the aqueous sample. The funnel was shaken vigorously for 1 min, with venting. The lower layer was drained off, and the EtOAc was collected in a clean TurboVap

tube. The aqueous layer was partitioned three more times with 30 mL aliquots of EtOAc. All EtOAc fractions were pooled and concentrated to dryness using the TurboVap II workstation. The final sample was dissolved into an appropriate volume with MeCN/water (95:5, v,v) and filtered through a 0.2 μm Acrodisc syringe filter (Pall Corporation, Ann Arbor, MI) prior to analysis by LC-MS/MS.

Sample Analysis. Sample analysis (see **Figure 2**) was conducted with a Perkin-Elmer Series 200 autosampler and binary micropumps (Perkin-Elmer, Shelton, CT) coupled to an Applied Biosystem API-2000 tandem mass spectrometer via a Turbo Ionspray source (Applied Biosystem, Palo Alto, CA). The Turbo Ionspray source was operated in positive ionization mode with drying nitrogen gas at 450 °C. Curtain gas, ion source gas #1, and ion source gas #2 were operated at 50, 30, and 80 psi, respectively. The mass spectrometer was operated in multiple reactant monitoring mode (MRM). See **Table 1** for compound conditions. Hydrophilic interaction chromatographic separation was accomplished with a Agilent Zorbax RX-Sil column (150 × 4.6 mm ID, 5 μm particle size, Agilent Technologies, Palo Alto, CA). Initial mobile phase composition was 95:5 0.2% formic acid in MeCN/0.2% formic acid with a flow rate of 500 μL/min. The mobile phase program consisted of 0–1 min 95:5, 1–2 min ramp gradient to 60:40, 2.0–7.0 min hold 60:40, 7.0–7.1 min ramp gradient back to 95:5 while increasing the flow rate to 1.5 mL/min, 7.1–14.0 min hold 95:5 at 1.5 mL/min, 14.0–14.1 min. ramp flow rate to 500 μL/min, 14.1–16.0 hold

Analysis of Flonicamid and Its Metabolites in Dried Hops

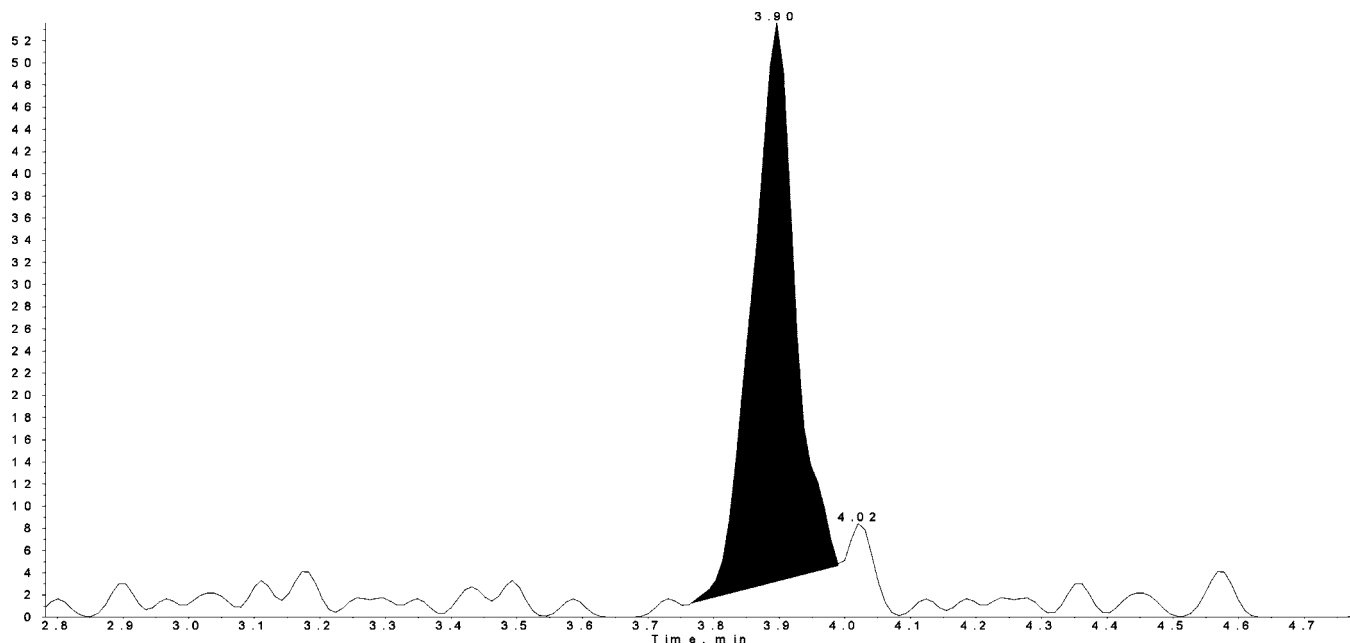*J. Agric. Food Chem.*, Vol. 55, No. 20, 2007 **8037**

Figure 7. Flonicamid chromatogram of 0.005 ppm recovery (77%) from a hop sample (MRM = 229.8/202.8).

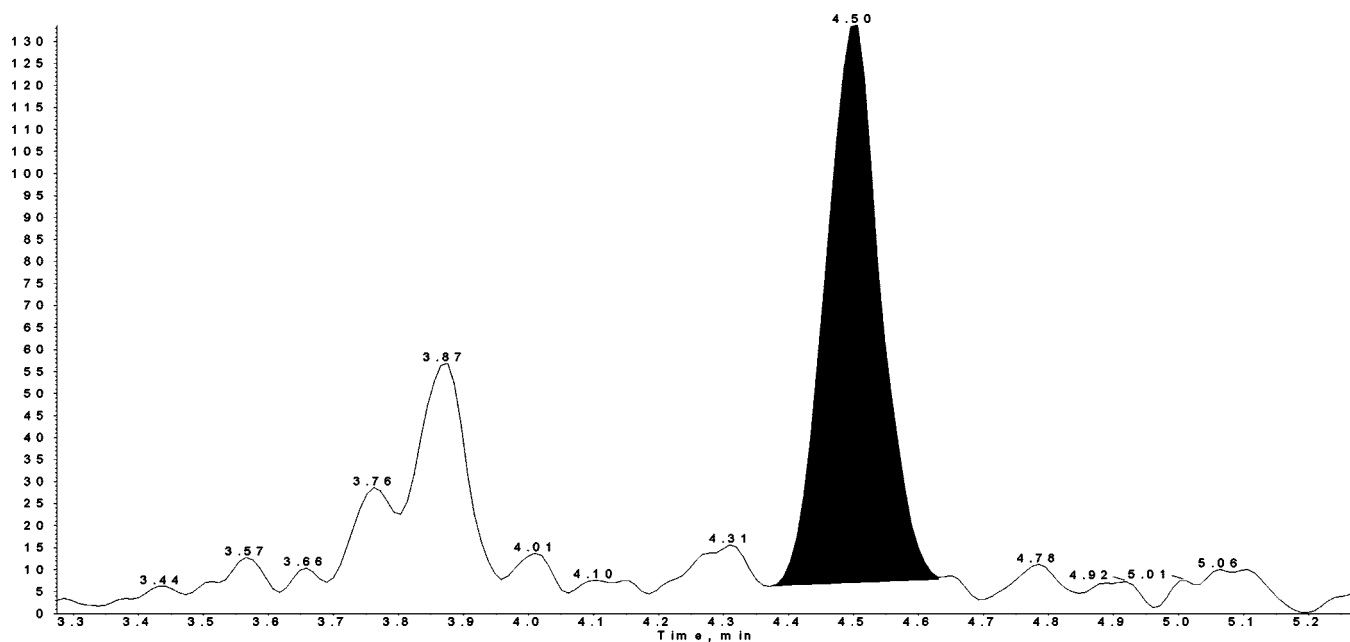**Figure 8.** TFNA-AM chromatogram of 0.005 ppm recovery (83%) from a hop sample (MRM = 190.8/147.8).

95:5 at 500 μL/min. Sample residues were quantified using a linear standard curve method ($R^2 = 0.98$ or better for all compounds).

RESULTS AND DISCUSSION

The method developed showed acceptable recoveries over several levels of fortification for each of the four compounds of interest (**Table 2**). The method limit of quantitation (LOQ) and detection (LOD) were determined to be 0.005 and 0.0025 ppm, respectively. LOD was defined as roughly five times the signal to noise and LOQ was defined as two times the LOD. The results of the storage stability experiment on untreated hops fortified at 0.2 ppm were determined to be 69 $\pm$ 3, 82 $\pm$ 3, 81 $\pm$ 3, 92 $\pm$ 1% ($n = 3$, 299 days of storage) for flonicamid, TFNA, TFNA-AM, and TFNG, respectively. Generally, the storage stability results correlate well with the spiked samples at the same fortification level (0.2 ppm), which suggests that

there was minimal degradation of the residues of concern during long-term storage at −20 °C. Untreated hop samples from all fields had residues <0.005 ppm for flonicamid, TFNA, TFNA-AM and TFNG. Field-treated hop samples collected from three field sites had residues that ranged from 0.561 to 2.85 ppm, 0.302 to 0.470 ppm, 0.038 to 0.177 ppm, and 0.098 to 0.204 ppm, for flonicamid, TFNA, TFNA-AM, and TFNG, respectively (**Table 3**). The field replicates for treated samples correlated very closely, while treated samples between field sites showed significant variability. This variability is to be expected given the different growing conditions each field site may have been subjected to during the growing season, such as rain events, overcast conditions, and pest pressures. The residues found in treated samples led EPA to establish a tolerance of 7.0 ppm for combined residues from flonicamid and its metabolites on hop
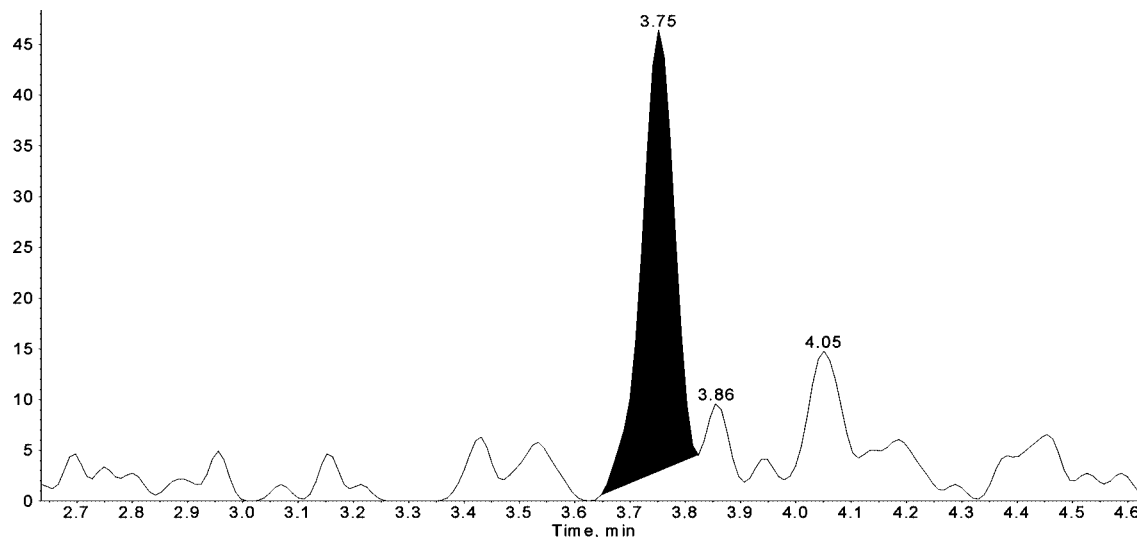
Figure 9. TFNA chromatogram of 0.005 ppm recovery (69%) from a hop sample (MRM = 191.8/147.8).
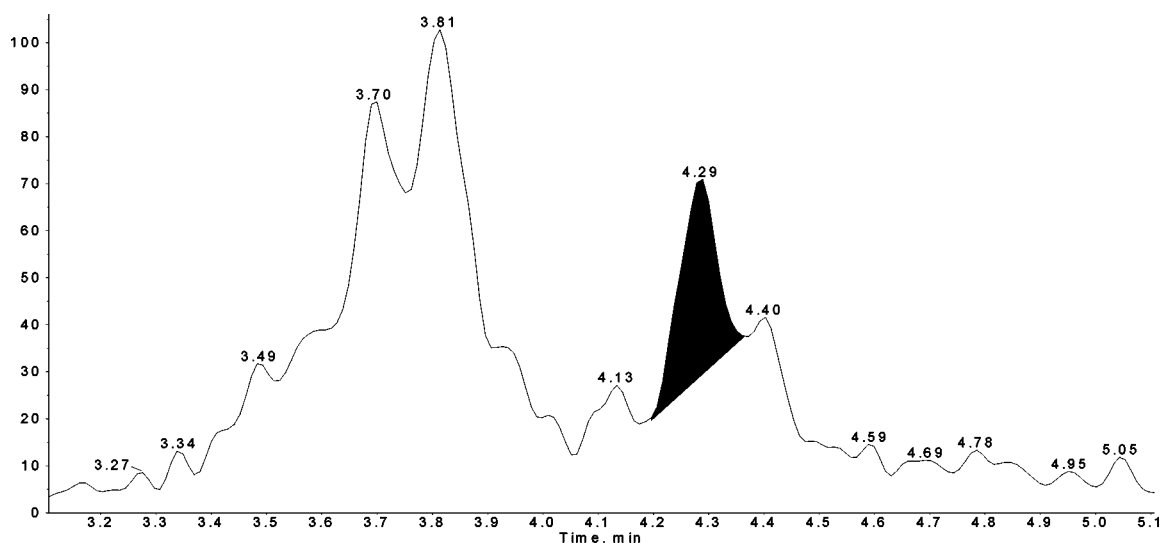

Figure 10. TFNG chromatogram of 0.005 ppm recovery (106%) from a hop sample (MRM = 248.8/202.8).

cones (*10*). Typical chromatograms of the analytical standard and hop extracts can be seen in **Figures 3–10**.

In this study, the recovery values from the hop matrix are in agreement with recovery values determined by FMC Corp. for peach, potato, and wheat straw (*7*). The matrix that is closest to hops, in terms of moisture content, is the wheat straw, which had an LOQ and LOD of 0.02 and 0.01 ppm, respectively. However, although the straw is a dry material, it does not contain waxes, resins, and oils to the same degree as that found hop cones. The presence of these additional matrix constituents often complicates sample analysis because of chromatographic interferences and/or by ion suppression/enhancement in the ionization source of the mass spectrometer. To minimize these problems, an additional SPE cleanup was developed using the Nexus stationary phase. The Nexus polymeric material was chosen because of its unique properties to bind both polar and nonpolar compounds. Because of the difference in affinity for matrix components, as compared to the C_{18}, the Nexus material was able to retain much of the remaining matrix while allowing for the elution of flonicamid and metabolites with a lower concentration of MeCN in water. The result was a more selective cleanup.

In addition to SPE cleanups, atmospheric pressure chemical ionization (APCI) was attempted for residue determination. Typi-

cally, APCI does not suffer from suppression/enhancement issues that can be prevalent with electrospray ionization. Unfortunately, not all of the compounds were ionized with sufficient abundance to be useful in our study. Therefore, positive electrospray ionization was chosen. During initial method development, a typical reverse phase system was attempted to separate the four compounds. It was quickly determined that sensitivities varied greatly for each compound and were dependent on the mobile phase conditions such that as one compound was optimized another was hindered. The compound variability was exacerbated by the presence of crop matrix. To mitigate the mobile phase condition, our laboratory conducted compound separation by hydrophilic interaction chromatography. The hydrophilic interaction allowed for the separation of flonicamid and metabolites by more of an electrostatic mechanism rather than a hydrophobic mechanism (*11*). This technique allowed for all four compounds to elute relatively under the same mobile phase condition, which was high organic and low aqueous and yielded much higher sensitivity versus the reverse phase system. The increased sensitivity observed correlated well with the increased sensitivity reported by Naidong (*12*). The result of using hydrophilic interaction chromatography was a very rugged and sensitive system for analyses of flonicamid and flonicamid metabolites on hops.

ACKNOWLEDGMENT

LITERATURE CITED

(1) U.S. Environmental Protection Agency. Acephate, amitraz, car-baryl, chlorpyrifos, cryolite, et al. Tolerance Revocations. *Fed. Regist.* **2002**, *67* (147), 49606–49617.

(2) Tomlin, C. D. S., Ed. *The Pesticide Manual*; British Crop Protection Council: Surrey, U.K., 2003.

(3) Morita, M.; Ueda, T.; Yoneda, T.; Koyanagi, T.; Murai, S.; Matsuo, N.; Stratmann, B.; Ruelens, R. Ishihara Sangyo Kaisha, Ltd. IKI-220 - A Novel Systemic Aphicide. BCPC Conference - Pests & Disease, 2000; p 5965.

(4) Joost, H.; Staetz, C.; Black, B.; Hayashi, J.; Kinne, L.; Kelly, G.; Treacy, K. FMC Corporation. Flonicamid - A Novel Mode of Action for Piercing Sucking Insects. Proceedings - Beltwide Cotton Conferences, 2006.

(5) Dixon, A. OP Alternative Status Granted to New Insecticide, Flonicamid (F 1785 GH) for Use on Ornamentals Grown in Indoor Greenhouses. Alternative to OP's and other Chemistries. U.S. Environmental Protection Agency: Washington, DC, 2002.

(6) Meister, Richard, T., Ed. *Crop Protection Handbook*; Meister Media Worldwide: Willoughby, OH, 2007.

(7) Chen, A. W. Analytical Methodology for IKI-220 (F1785) and its Major Metabolites in/on Peach, Potato Tuber, and Wheat Straw. Analytical report (P-3561M) for FMC Corporation, Princeton, NJ, 2002.

(8) Zywitz, D.; Anastassiades, M.; Scherbaum, E. Simultaneous determination of neonicotinoid insecticides in fruits and vegetables by LC-MS and LC-MS-MS. *Dtsch. Lebensmittlel-Rundschau* **2003**, *99* (5), 188–196.

(9) Stueber, M.; Reemtsma, T. Evaluation of three calibration method to compensate matrix effects in environmental analysis is LC-ESI-MS. *Anal. Bioanal. Chem.* **2004**, *378* (4), 910–916.

(10) U.S. Environmental Protection Agency. Notice of Filing of Pesticide Petitions for Residues of Pesticide Chemicals in or on Various Commodities. *Fed. Regist.* **2007**, *72*, (123), 35237–35242.

(11) Bidlingmeyer, B. A.; Henderson, J. Investigation of retention on bare silica using reversed-phase mobile phases at elevated temperatures. *J. Chromatogr. A.* **2004**, *1060*, 187–193.

(12) Naidong, W. Bioanalytical liquid chromatography tandem mass spectrometry method on underivatized silica columns with aque-ous/organic mobile phases. *J. Chromatogr., B* **2003**, *796*, 209–224.