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Analysis of Fenhexamid in Caneberry, Blueberry, and Pomegranate by Liquid Chromatography–Tandem Mass Spectrometry

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An analytical method for the determination of fenhexamid [*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide] in caneberry, blueberry, and pomegranate was developed utilizing acetone extraction, column cleanup, liquid−liquid partitioning, and liquid chromatography−tandem mass spectroscopy (LC-MS/MS) for detection. Method validation recoveries ranged from 91 to 96% for caneberry, from 80 to 91% for blueberry, and from 74 to 95% for pomegranate. Control samples collected from IR-4 trials for all matrixes had residue levels of <0.020 ppm. Fenhexamid-treated field samples had residue levels that ranged from 0.46 to 16.11 ppm (caneberry), from 0.87 to 2.91 ppm (blueberry), and from 1.59 to 1.85 ppm (pomegranate). The method was validated to a limit of quantitation of 0.020 ppm, and the limit of detection was 0.009 ppm.

KEYWORDS: **Fenhexamid; Elevate; fungicide; LC-MS/MS**

INTRODUCTION

Botrytis cinerea, or gray mold, is one of the most commonly found fungal pathogens and can greatly reduce both the quantity and quality of field crops (*1*). In California, high-value crops such as strawberries, pears, and grapes are particularly susceptible to gray mold, both in the field and during transport and storage (*2*, *3*). In addition to being nearly ubiquitous in crops, *B. cinerea* is classified as a high-risk pathogen due to its ability to develop resistance to various control agents (*2*). As such, the identification of new compounds to control gray mold is of great importance.

Fenhexamid [*N*-(2,3-dichloro-4-hydroxyphenyl)-1-methylcyclohexanecarboxamide], a foliar fungicide of the hydroxyanilide class, was developed by Bayer AG in 1989 (*3*, *4*). This compound has been demonstrated to be highly efficacious against *B. cinerea* and related pathogens such as *Sclerotinia sclerotiorum* and *Monilinia* spp. on grapes, stone fruit, citrus, vegetables, strawberry, raspberry, black currant, and sweet cherries (*1*, *2*, *4*−*9*). The unique mode of action of fenhexamid shows no evidence of heightened risk of resistance (*10*). As such, fenhexamid would be useful in antiresistance strategies for fungal control (*10*−*12*). In addition, fenhexamid has been shown to break down rapidly in the environment and to be ecologically benign and nonvolatile (*13*, *14*). Because of the above characteristics, fenhexamid has been judged as safe for the environment and is classified as a "reduced-risk pesticide" by the U.S. EPA (*3*, *4*).

Methods for the analysis of fenhexamid include gas chromatography−nitrogen−phosphorus detection (GC-NPD) (*15*) and high-performance liquid chromatography−electrochemical detection (HPLC-ELCD) and HPLC-UV detection (*16*).

In the present study a selective and sensitive analytical method for fenhexamid residues in caneberry, blueberry, and pomegranate has been developed for use in residue enforcement as well as risk assessment. This method involves extraction with acetone, column cleanup, acetonitrile/hexane partition, and liquid chromatography−tandem mass spectrometry detection (LC-MS/MS).

Residues of fenhexamid found in crops collected from U.S. Department of Agriculture Interregional Research Project 4 (USDA IR-4) testing fields have been summarized. IR-4 is a federal agriculture program that carries out the research needed for the registration of pest control materials on minor crops.

MATERIALS AND METHODS

Materials. Fenhexamid (CAS Registry No. 126833-17-8, 99.8% purity, lot M00759) was acquired from Tomen Agro, Inc. (San Francisco, CA). All solvents and reagents were of residue grade or better. Water was prepared using a Milli-Q reagent water system. Specifications for columns used for analysis are cited below.

Preparation of Standard Solutions. Stock solution (1.00 mg/mL) was prepared by dissolving 0.0501 g of fenhexamid into 50 mL of methanol. The stock solution was stored generally at −20 °C and was stable for 1 year. Standard dilutions for fortification were prepared by diluting 5 mL of the stock solution to 50 mL with methanol, resulting in a 100 μg/mL solution. A low-level fortification solution was prepared by taking 5 mL of the 100 μg/mL solution and diluting up to 50 mL with methanol, resulting in a 10 μg/mL solution. Calibration solutions for LC-MS/MS analysis were prepared by taking 0.25, 1.0, 0.50, 0.25, and 0.125 mL of the 10 μg/mL solution and diluting each aliquot to 50 mL in mobile phase (40:60, 0.1% acetic acid/methanol, v/v). The dilutions resulted in 500, 200, 100, 50, and 25 pg/μL solutions, respectively. Fortification and calibration standard solutions were stored in the refrigerator (<5 °C) and were stable for 6 months.

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Table 1. Average Recoveries of Fenhexamid in Caneberry, Blueberry, and Pomegranate

crop/matrix	level 1 (ppm)	% ± SD[a]	level 2 (ppm)	% ± SD	level 3 (ppm)	% ± SD	level 4 (ppm)	% ± SD	level 5 (ppm)	% ± SD
caneberry	0.020	94.50 ± 2.29 ($n = 3$)	0.50	91.36 ± 1.08 ($n = 5$)	2.00	91.83 ± 0.58 ($n = 3$)	5.0	96.27 ± 3.95 ($n = 5$)	20.0	95.78 ± 3.78 ($n = 6$)
blueberry	0.020	85.10 ± 8.84 ($n = 5$)	0.50	91.44 ± 1.64 ($n = 5$)	2.00	79.95 ± 7.16 ($n = 3$)	5.0	88.46 ± 4.63 ($n = 5$)		
pomegranate	0.020	74.38 ± 11.19 ($n = 4$)	0.50	83.17 ± 10.38 ($n = 4$)	2.00	95.23 ± 3.96 ($n = 3$)	5.0	88.30 ± 4.17 ($n = 4$)		

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

Collection of Field Samples. Fenhexamid formulation of TM-402 50% WDG (EPA Reg. No. 66330-35, CAS Registry No. 126833-17-8) was used for application in these field studies. For caneberry and blueberry, fields were treated with four applications at 7 ± 1 day intervals (0.75 lb of active ingredient/acre) with the final application made on the day of harvest. For pomegranate, fenhexamid was used as a postharvest dip (corresponding to 0.75 lb of active ingredient/100 gal of water). For dipped fruit, fenhexamid was allowed to dry prior to collection of the treated samples. Samples were collected from IR-4 field testing sites throughout the United States and Canada: six fields of caneberries, eight fields of blueberries, and one field of pomegranate. (For specific information, contact IR-4 Project, Center for Minor Crop Pest Management, Technology Centre of New Jersey, 681 U.S. Highway 1 South, North Brunswick, NJ 08902−3390.)

Each test site consisted of one untreated (control) and one treated plot. Individual plots were of adequate size to ensure that no more than 50% of the plot would be needed to provide the necessary plant material for residue samples. Adequate buffer zones were employed between plots to prevent contamination.

For caneberry and blueberry, duplicate samples were harvested from each plot. For pomegranate, 4 samples of 24 fruits each were harvested for dipping. Each sample was collected in a manner to ensure a representative, impartial sample. The sample was placed in a plastic-lined cloth bag that was labeled with complete identification. After collection, samples were frozen within 24 h of harvest. Samples were kept frozen during shipping and held generally at −20 °C at the laboratory until analysis.

Sample Preparation. The crop was chopped with equal amounts of dry ice using a Hobart food chopper (Hobart Corp., Troy, OH). Each chopped sample was stored in a labeled ∼1 L jar, and a lined lid was loosely closed on top to allow the dry ice to dissipate during storage generally at −20 °C. After 24 h, the lids were tightened to seal the jar.

Stability Study. Six control samples were fortified with fenhexamid at the 2.00 ppm level for each crop matrix and were collocated with the field samples generally at −20 °C. Three samples were analyzed after a storage period equivalent to the interval between harvest and analysis of field-treated samples. The remaining samples were retained for long-term storage.

Extraction. A 50-g aliquot of crop was weighed into a 500 mL flask (recoveries were fortified at this point) and 250 mL of acetone was added. The sample was homogenized for 3 min (∼16000 rpm) using an Ultra-Turrax T25 with an 18G rotor/stator (Janke & Kunkel). The homogenized sample was filtered using a vacuum flask, through Whatman no. 934-AH filter paper. If necessary, Celite 545 (not acid washed) was utilized to improve filtration speed. The blending flask was rinsed three times with 50 mL portions of acetone and each wash was added to the filter cake, the extracts were pooled and made up to 500 mL with acetone. A 250 mL aliquot (25 g) was measured into a 500 mL round-bottom flask and rotary evaporated to the aqueous remainders, roughly 5 mL (water bath at 40 °C). The extracts may be stored overnight in a refrigerator (<5 °C).

Sample Cleanup. After rotary evaporation, 20 mL of water was added to the concentrated sample extract. The extract was added to a 20/50 Chem Elut column (Varian Sample Preparation Products, Harbor City, CA, part 1219-8009) and allowed to equilibrate for 10 min. Fenhexamid residues were eluted, by gravity, with 4 × 50 mL aliquots of 15:85, v/v, ethyl acetate/cyclohexane. The eluate was collected in a clean 250 mL round-bottom flask. Elution can take up to 2 h to complete. The sample was rotary evaporated to dryness, and the residue was dissolved into 50 mL of acetonitrile. The dissolved residue was transferred to a 125 mL separatory funnel containing 50 mL of hexane. The separatory funnel was shaken for 30 s, and the phases were allowed to separate (hexane on top). The acetonitrile was drained into the original round-bottom flask, and the hexane was discarded. The partition

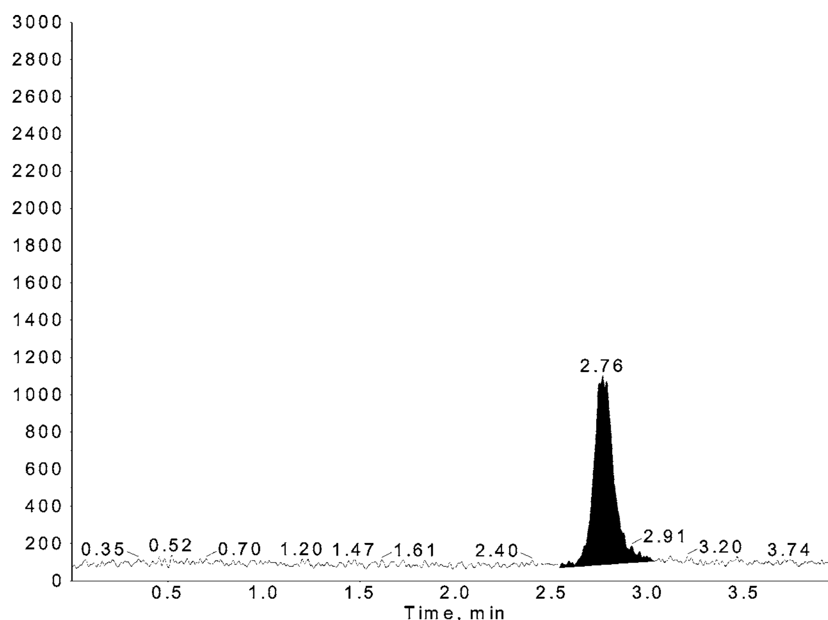

Figure 1. Selected ion chromatogram (m/z 97) of 25 pg/μL calibration standard.

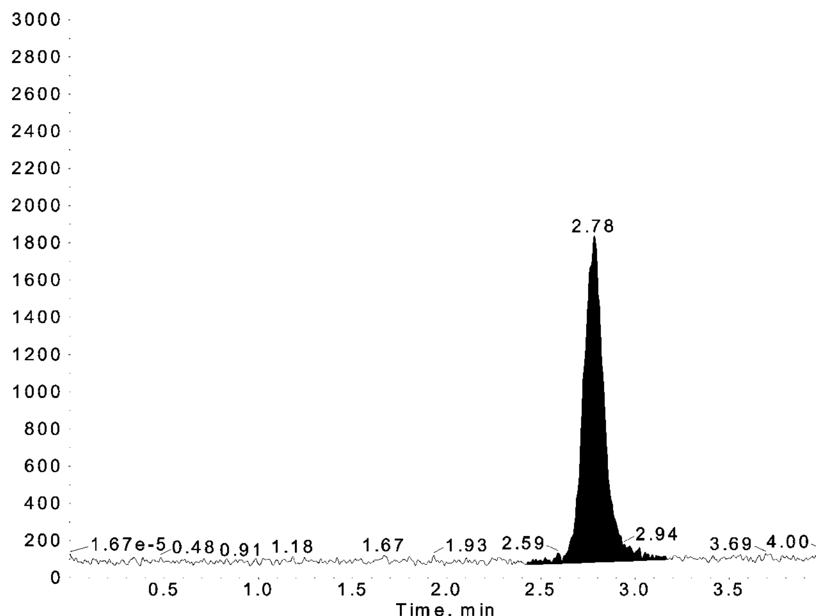

Figure 2. Selected ion chromatogram (*m/z* 97) of 0.020 ppm recovery (87%) from a pomegranate sample.

was repeated with another 50 mL of hexane. The acetonitrile fraction was collected in a clean 100 mL round-bottom flask and was rotary evaporated to near dryness (water bath at 40 °C). The residue was dissolved in an appropriate amount of 40:60, v/v, 0.1% acetic acid/methanol, and sonicated briefly. Just prior to analysis, a small portion (~1 mL) of the sample was filtered through a 0.2 μm HT Tuffryn filter disk (Acrodisc, Gelman Sciences, Ann Arbor, MI, part 4192) into an autosampler vial.

Sample Analysis. Sample analysis was conducted with a Perkin-Elmer Series 200 autosampler and a micropump (Perkin-Elmer, Shelton, CT) coupled to a PE Sciex API 2000 tandem mass spectrometer via an atmospheric pressure chemical ionization (APCI) source (PE Biosystems, Walnut Creek, CA). The APCI source was operated in positive ionization mode at 450 °C with nitrogen curtain gas pressure at 55 psi and ion source (nitrogen) gas 1 at 70 psi and gas 2 at 15 psi. The mass spectrometer was operated in multiple reactant monitoring mode (MRM) to observe the transition of *m/z* 302 to *m/z* 97 (via collision-induced dissociation with nitrogen gas). Ion *m/z* 302 represents the molecular ion, whereas *m/z* 97 suggests the methylcyclohexane fragment. Reverse-phase chromatographic separation was accomplished with a Restek Allure C_{18} column (50 × 3.2 mm, 5 μm particle size, Restek Corp., Bellefonte, PA). The autosampler was programmed to inject 5 μL. Initial mobile phase condition was 40:60% acetic acid (0.1%)/methanol with a flow rate of 800 μL/min. The mobile phase gradient consisted of 0−0.5 min 40:60, 0.5−1.0 min ramp to 25:75, 1.0−3.5 min hold at 25:75, and 3.5−4.0 min ramp back to initial conditions. Sample residues were quantified using a standard curve method ($R^2 \sim 0.990$), based on the response for *m/z* 97 (quantitation ion).

RESULTS AND DISCUSSION

The developed method showed acceptable recoveries using these various crop matrixes (**Table 1**). For caneberry, recoveries ranged from 90 to 105% over five levels of fortification (0.02, 0.5, 2.0, 5.0, and 20 ppm). For blueberry, recoveries ranged from 72 to 96% over four levels of fortification (0.02, 0.5, 2.0, and 5.0 ppm). For pomegranate, recoveries ranged from 72 to 96% over four levels of fortification (0.02, 0.5, 2.0, and 5.0 ppm). The results of storage stability tests on fenhexamid were 86 ± 1% for 253 days storage on caneberry, 77 ± 5% for 266 days storage on blueberry, and 91 ± 8% for 93 days of storage on pomegranate. Results from the storage stability study suggest that fenhexamid has minimal decomposition under extended storage times at approximately −20 °C. **Figures 1** and **2** show a typical chromatogram of an analytical standard and recovery from pomegranate, respectively.

Table 2. Residue Results of Fenhexamid Analysis in Caneberry, Blueberry, and Pomegranate

sample matrix	field	control samples (ppm)	treated samples (ppm)	
caneberry	BC05	<0.020	2.83	3.13
	NC20	<0.020	16.11	6.41
	NY24	<0.020	4.60	3.42
	ON05	<0.020	12.54	8.79
	OR05	<0.020	0.46	0.64
	OR06	<0.020	5.65	4.78
blueberry	GA*21	<0.020	0.87	1.61
	ME06	<0.020	1.81	4.00
	MI16	<0.020	2.76	2.91
	MI17	<0.020	1.69	1.54
	MI18	<0.020	1.87	1.52
	NC21	<0.020	1.57	1.23
	NJ29	<0.020	2.46	2.82
	OR07	<0.020	1.21	0.87
pomegranate	CA02	<0.020	1.71, 1.85	1.59, 1.72

Table 3. Stability Study Results of Fenhexamid Analysis of Caneberry, Blueberry, and Pomegranate

sample matrix	storage interval (days)	mean fenhexamid (ppm)	mean recovery[a] (% ± SD)
caneberry	253	1.72	86.17 ± 0.76
blueberry	266	1.54	77.17 ± 5.03
pomegranate	93	1.83	91.27 ± 7.63

[a] Values are mean percent ± standard deviation ($n = 3$).

Control samples collected from IR-4 trials for all matrixes had residue levels of <0.020 ppm. Field-treated samples ranged from 0.46 to 16.11 ppm, from 0.87 to 2.91 ppm, and from 1.59 to 1.85 ppm for caneberry, blueberry, and pomegranate, respectively (**Table 2**). **Figures 3** and **4** show typical chromatograms of fenhexamid isolated from blueberry samples (untreated and treated). The resulting field residue levels from the current study are in line with current U.S. EPA tolerances of 4.0, 3.0, 6.0, and 15 ppm on grape, strawberry, raisin, and pear, respectively (*3*, *17*, *18*). In addition, Cabras, using a similar application rate, found residue levels on grapes that correlate well with the results of our treated samples (*15*).

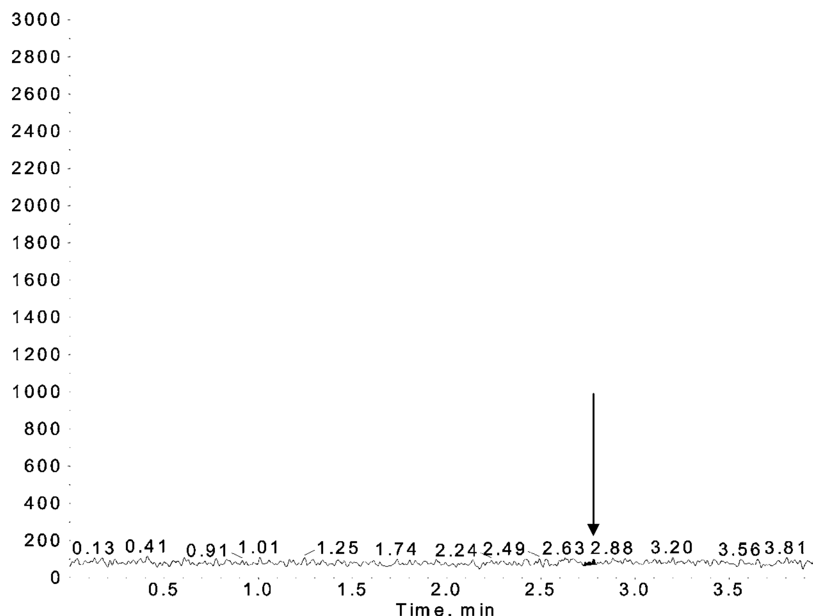

Figure 3. Selected ion chromatogram (*m*/*z* 97) of an untreated blueberry sample (<0.020 ppm).

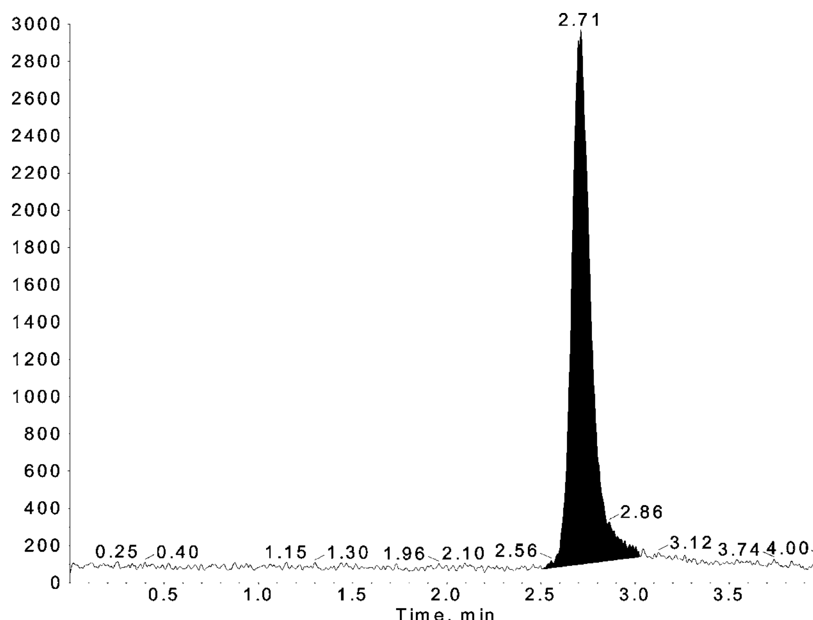

Figure 4. Selected ion chromatogram (*m*/*z* 97) of a treated blueberry sample (1.78 ppm).

Previously reported methods for the extraction of fenhexamid from field crops were few. The Cabras method employed a simple hexane extraction followed by determination using GC-NPD (*15*). The limit of quantitation (LOQ) for grapes was 0.1 ppm, and although rapid, the method did not offer the overall sensitivity required in our study. Another method was from Nüsslein, which utilized acetone extraction, Chem Elut cleanup, and determination by HPLC-ELCD (*19*). The use of ELCD allowed for a lower LOQ of 0.05 ppm in fruits and vegetables, as well as increased chemical specificity. The analytical method presented in this study was adapted from Nüsslein's method. Two major differences were incorporated into the current method, a liquid−liquid partition following the Chem Elut cleanup and the utilization of LC-MS/MS for residue determination. After cleanup by Chem Elut, much of the pigmentation from the fruits was carried through, along with the fenhexamid residues. Although these pigments do not interfere with the chromatography, they can adversely affect the ionization process in the APCI source. As a result, an enhancement of residues was seen in preliminary recovery samples. The quick liquid−liquid partition removed the majority of the pigments and greatly reduced the enhancement phenomena.

In addition to the liquid−liquid partition, the use of LC-MS/MS gave a fourfold advantage over detection by ELCD. First, the sensitivity was greatly increased, which allowed for an LOQ of 0.020 ppm to be obtained for all crop fractions. Second, the specificity was also increased, which minimized any potential chromatographic interferences. Third, because chromatographic interferences are minimized by using MS/MS detection, a shorter analytical column was used, which improved sample throughput with faster analytical runs. And finally, residues were quantitated and confirmed simultaneously.

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