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Improved Analytical Methods for Determination of Residues of Nitrapyrin and 6-Chloropicolinic Acid in Different Crop Matrices by Liquid Chromatography-Tandem Mass Spectrometry

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Abstract

Novel analytical methods were developed for the determination of nitrapyrin and 6-chloropicolinic acid (6-CPA) residues in agricultural products. Nitrapyrin was extracted with hexane: toluene (1:1, v/v) and 6-CPA with 0.1 N sodium hydroxide (NaOH). For nitrapyrin, samples were cleaned with silica solid phase extraction (SPE) and for 6-CPA with C18 or carbon SPE. Analysis was performed by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) with external calibration (0.010 to 0.0010 $\mu g \cdot mL^{-1}$ for nitrapyrin and 0.0050 to 0.00025 $\mu g \cdot mL^{-1}$ for 6-CPA). Recoveries for nitrapyrin and 6-CPA were 68% - 102% and 66% - 90%, respectively. Nitrapyrin was detected in celery and onion (<0.020 to 0.15 $\mu g \cdot g^{-1}$) and 6-CPA in onion, mustard greens and lettuce (<0.050 - 0.19 $\mu g \cdot g^{-1}$). Lower level method validation (LLMV) for nitrapyrin and 6-CPA was 0.020 $\mu g \cdot g^{-1}$ and 0.050 $\mu g \cdot g^{-1}$, respectively. The methods presented in this study are robust and were utilized for the registration of nitrapyrin on agricultural commodities in the United States.

Keywords

Herbicide, Agricultural Products, External Calibration, LC-MS/MS, Registration

1. Introduction

The worldwide production of vegetables continues to increase as consumer demand increases. The estimated production of vegetables in the United States

(U.S.) for 2022 was 36,848,000 tons, with a harvested area of 809,389 hectares (ha). Lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), onion (*Allium cepa L.*), celery (*Apium graveolens*), cabbage (*Brassica oleracea*) and broccoli (*Brassica oleracea var. italica*) are some of the 26 vegetables listed [1]. These represent around 28% of the total area planted (249,210 ha) and area harvested (247,106 ha). Crop productivity is strongly influenced by the nutrients available in the soil, and nitrogen plays an important role applied as fertilizer. The use of nitrogen fertilizers has increased dramatically in the last century, and this trend is expected to continue [2]. However, increased fertilizer application can create the potential for nitrogen loss before plant uptake [2]. Nitrapyrin [2-chloro-6-(trichloromethyl)-pyridine] (CAS No. 1929-82-4) was identified as a nitrification inhibitor in 1962 and has been registered in the U.S. since 1974 as a bacteriostatic agent [3]-[7]. Nitrapyrin is the active ingredient in commercially available nitrogen stabilizers, efficiently delaying the conversion of ammonium (NH_4^+) to nitrate (NO_3^-) by inhibiting nitrifying bacteria in soil. This improves nitrogen retention in the soil, reduces nitrate leaching and nitrous oxide emissions, improves nitrogen use efficiency, and improves crop yields [2] [8]-[21]. Environmental factors such as temperature, soil organic matter, pH and fertilization parameters can affect the efficiency of nitrapyrin [2] [22]. Nitrapyrin has multiple routes of degradation in the environment (hydrolysis, aerobic and anaerobic metabolism). The main identifiable metabolite of nitrapyrin in plants, soil and animals is 6-chloropicolinic acid (6-CPA). In plants, degradation to 6-CPA can occur directly or via 2-chloro-6(dichloromethyl)pyridine intermediate [23]. The 6-CPA metabolite is a more mobile compound compared to nitrapyrin. Some studies have reported the presence of 6-CPA in plant foodstuffs such as corn, lettuce, tomatoes (*Solanum lycopersicum*), oats (*Avena sativa*) and carrots (*Daucus carota* subsp. *sativus*) [24]. Nitrapyrin has the potential to affect the physiology, function, and health of living organisms both directly and via its metabolite 6-CPA [2]. Nitraprin can exhibit low mammalian toxicity and affect the function of liver and kidneys in mice and is classified as "suggestive evidence of carcinogenic potential" [2] [6] [25] [26]. Due to its positive effect on crop yields, the agricultural use of nitrapyrin has increased over time, making it one of the most widely used inhibitors in the U.S. [27]-[31]. Nitrapyrin use has been registered on corn (*Zea mays* subsp. *mays*), wheat (*Triticum aestivum* subsp. *aestivum*), and sorghum (*Sorghum bicolor* (L.) Moench) [7] [11]. U.S. growers have identified additional crops that would benefit from the expanded use of nitrapyrin, including lettuce, spinach, celery, onion, cabbage, broccoli and mustard greens (*Brassica juncea*). However, extending the registration of nitrapyrin additional crops will require increased monitoring of nitrapyrin and 6-CPA residues in the selected agricultural products.

In this work, we present improved analytical methods and generated field residue data for nitrapyrin application to a range of agricultural commodities in support of U.S. registration. Whereas previous analytical methods were time consuming and costly due to greater sample processing and derivatization re-

quirements [5] [32]-[34], we present two fast and efficient methods for analysis for nitrapyrin via LC-MS/MS. The residue data generated was used to establish tolerances or maximum residue limits (MRLs) that allow for the harmonization of U.S. tolerances with international standards for food safety standards and agricultural practices.

2. Materials and Methods

The present study was conducted under *Good Laboratory Practices 40 CFR Part 160 and EPA Residue Chemistry Test Guidelines OPPTS 860* [35] [36].

Chemicals and Reagents

Nitrapyrin (CAS Registry No. 001929-82-4; 99.6%; Figure 1) and 6-CPA (CAS Registry No. 4684-94-0; 99%; Figure 1) were obtained from Dow AgroSciences LLC (Indianapolis, IN). Solvents were of LC-MS and pesticide grade. Reagents were of ACS grade and solutions were prepared using water 18.2 MΩcm.

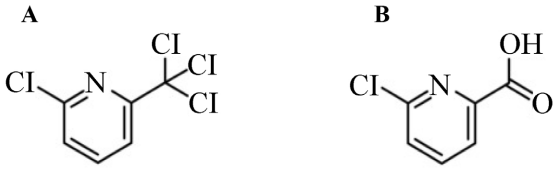

Figure 1. Chemical structure of nitrapyrin (A) and 6-CPA (B).

Standard Solutions

Stock solutions (1.0 mg·mL^{-1}) of nitrapyrin and 6-CPA were prepared by adding 50 mg (corrected for purity) of the analytical reference compound to a 50 mL volumetric flask. Nitrapyrin was brought to volume with 4:1 hexane/toluene (v/v) and 6-CPA with methanol.

Fortification solutions were prepared at 100, 10 and 1.0 μg·mL^{-1} by serial dilutions in 4:1 hexane/toluene (v/v) for nitrapyrin and methanol for 6-CPA. A solution of 0.10 μg·mL^{-1} of nitrapyrin was prepared in acetone for preparation of calibration solutions.

For nitrapyrin, calibration solutions for LC-MS/MS analysis were prepared by taking different volumes of the 0.10 μg·mL^{-1} solution and diluting them to volume in 1:1 methanol/water (v/v). A four-point standard curve was prepared with concentrations at 0.010, 0.0050, 0.0020 and 0.0010 μg·mL^{-1}. For 6-CPA, calibration solutions (0.0050, 0.0020, 0.0010, 0.00050 and 0.00025 μg·mL^{-1}) were prepared in 25:75 methanol/water (v/v). All solutions were stored in amber bottles. Solutions of nitrapyrin were stable for 148 days at ~5˚C. Solutions of 6-CPA were stable for 365 days at ~5˚C for calibration solutions and at ~0˚C for stock and fortification solutions [33].

Field Sample Collection

Samples of lettuce (head and wrapper leaves), spinach (leaves), broccoli (flower head), celery (untrimmed leaf stalk), cabbage (head with wrapper leaves), mustard greens (leaves) and onion (plants and bulbs) from the 2013, 2014, 2015 and

2016 growing seasons were provided by the U.S. Department of Agriculture-National Institute of Food and Agriculture Interregional Research Project No. 4 (USDA-NIFA IR-4 program). A total of 384 samples were collected from crop production regions in California (Region X), Texas (Region VI), South Carolina (Region II), Ohio (Region V), Wisconsin (Region V), Arkansas (Region IV), New York (Region I), Georgia (Region II), New Mexico (Region VIII), Florida (Region III), Maryland (Region IX and II), Colorado (Region IX), Washington (Region XI) and Idaho (Region XI) [37].

Nitrapyrin was applied twice to the experimental fields following local commercial fertilizer application practices (Table 1). Each field site included one untreated and one treated plot. Adequate buffer zones were employed between plots to prevent contamination. Duplicate samples were harvested from each plot. The untreated samples were harvested before the treated samples. In total, there were 7 field trials (42 samples) for mustard greens, 14 field trials (90 samples) for lettuce, 6 field trials (36 samples) for spinach, 6 field trials (36 samples) for broccoli, 9 field trials (54 samples) for celery, 6 field trails (44 samples) for cabbage and 11 field trials (82 samples) for onion. Samples were collected, placed into properly labeled plastic-lined cloth bags, frozen and then transferred to the laboratory for analysis.

Table 1. Treatments, application of nitrapyrin in the experimental commodities and collection of samples.

Commodity	Target rate of active ingredient (ai)	Treatment/ Application type	Collection of samples**
Lettuce*, cabbage, broccoli, mustard greens and celery	0.56 kg ai·ha^{-1}	#1 soil side dress banded	45 ± 3 days after application
		#2 soil side dress banded	30 ± 3 days after application
Spinach	0.56 kg ai·ha^{-1}	#1 preplant as a banded application 14 days before planting	45 ± 3 days after application
		#2 preplant as a banded application the day of planting	30 ± 3 days after application
Onion*	0.56 kg ai·ha^{-1}	#1 banded over the top (2 applications)	75 ± 3 days after application 45 ± 3 days after application
	1.12 kg ai·ha^{-1}	#2 banded over the top	45 ± 3 days after application

*A declined field trial for lettuce and onion (plants and bulbs) was included. For lettuce, the trial consisted of 5 collection dates at 23 ± 1, 27 ± 1, 30 ± 3, 33 ± 1 and 37 ± 1 days after the last application of nitrapyrin. For onion there were 6 collection dates at 7 ± 1, 14 ± 1, 30 ± 1, 45 ± 1, 60 ± 1 and 75 ± 1 days after the last application of nitrapyrin; **Collection of samples is expressed as the number of days after the last application of nitrapyrin with a margin of ±1 to 3 days. Samples were collected on different days according to the sampling day.

Sample Preparation

Samples were homogenized in the presence of dry ice using either a Blixer food processor (Robot-Coupe USA, Inc.) or Hobart food chopper (Hobart Corp., Troy, OH). Homogenized samples processed using the Hobart food processor were sifted through a #6 wire mesh screen, and stored in labeled pint jars

(~16 oz) at −20˚C.

Storage Stability Study

Since samples couldn't be analyzed immediately after collection, it was necessary to determine any possible degradation of nitrapyrin and 6-CPA in field treated samples during the storage period in the freezer. Two sets of storage stability samples were prepared. Each set consisted of six untreated samples of each commodity studied fortified at 0.20 μg·g^{-1} with either nitrapyrin or 6-CPA. For nitrapyrin, 5.0 g of homogenized frozen sample contained in a 50-mL polypropylene centrifuge tube was fortified by adding 100 μL of 10 μg·mL^{-1} fortification solution to the sample. For 6-CPA, 2.5 g of homogenized frozen sample was fortified by adding 50 μL of 10 μg·mL^{-1} fortification solution to the sample. Tubes containing these samples were capped and stored at −20˚C in the same freezer as field treated samples. Three samples of each group were analyzed after a storage period equivalent to the longest interval between sampling and extraction of field-treated samples. The remaining samples were retained for long-term storage.

Extraction of Nitrapyrin

Two extraction methods were used due to the different physicochemical properties (mainly solubility in water and log P_{ow}) of nitrapyrin (solubility in water 72 mg·kg^{-1} at 25˚C, and measured log octanol/water partition coefficient (log P_{ow}) 3.32) and 6-CPA (solubility in water 3.40 g·L^{-1}) [38] [39]. The extraction methods included solvents with different polarities to extract each compound. The extraction of nitrapyrin was based on the method proposed by Claussen [32]. Five grams of frozen sample were weighed into a 50 mL polypropylene centrifuge tube. Recovery samples were fortified with nitrapyrin and then 20 mL of 1:1 hexane/toluene (v/v) and 10 mL of type 1 (milli-Q) water were added. For fortified samples, the extraction solvent was added 20 min after the samples were spiked in order to allow the solvent from the fortifying solution to evaporate. Samples were shaken for 90 minutes using a mechanical shaker at 180 rpm (Max^{Q} 3000, Barnstead/Lab-line, USA). Following extraction, the samples tubes were centrifuged at 2000 g for 8 min.

Solid Phase Extraction of Nitrapyrin

An aliquot of 10 mL (equivalent to 2.5 g of crop material) of the sample extract was transferred to a 200 mL TurboVap tube and concentrated under nitrogen to approximately 1 mL at 35˚C using a TurboVap II evaporator (Zymark®, USA). The sample extract was then cleaned using a silica SPE cartridge (1 g/6mL, Biotage, San Jose, CA). Silica SPE cartridges were conditioned with one column volume (CV) of hexane before the concentrated sample extract was loaded onto the SPE cartridge. The TurboVap tube was rinsed with 2 mL of toluene and the rinse was loaded onto the SPE cartridge. Residues of nitrapyrin were eluted from the SPE cartridge with 1 CV of toluene. The eluate was transferred into a 200 mL TurboVap tube containing 0.50 mL of keeper solution (1% 1-decanol in acetone, w/v) and evaporated with nitrogen to near dryness at 35

˚C using a TurboVap II evaporator. Final extracts were dissolved in 1:1 methanol/water (v/v) and sonicated for approximately 1 min. Sample extracts were diluted to final volumes of 25,100 and 1000 mL for nitrapyrin fortification levels of 0.020, 0.20 and 2.0 $\mu g \cdot g^{-1}$, respectively. Final extracts were submitted to LC-MS/MS analysis.

Extraction of 6-CPA

The extraction of 6-CPA was based on the method proposed by Claussen [33]. For analysis of 6-CPA, 2.5 g of frozen sample was weighed into a 50 mL polypropylene centrifuge tube. Recovery samples were fortified and 25 mL of 0.1N NaOH were added. For fortified samples, the extraction solvent was added 20 min after the samples were spiked in order to allow the solvent from the fortifying solution to evaporate. Samples were shaken for 2 h using a mechanical shaker at 200 rpm (Max^{Q} 3000, Barnstead/Lab-line, USA). Following extraction, the sample tubes were centrifuged at 2600 g for 12 min.

Solid Phase Extraction of 6-CPA

A 2 mL aliquot (equivalent to 0.20 g of crop material) of the sample extract was removed and transferred into a 15 mL polypropylene centrifuge tube. 5 mL of 1.0 N hydrochloric acid (HCl) was added, the sample was vortex mixed and then centrifuged for 5 min at 1800 g. For cabbage, spinach and mustard greens, a C18 SPE (1 g/6mL, BondElut, Agilent, Folsom, CA) was used and for celery, broccoli, lettuce and onion, a carbon SPE (Enviro-Clean, UCT (200 mg/6mL, Bristol, PA) was used. The SPE cartridges were conditioned with one CV of acetonitrile and then one CV of 0.1 N HCl. The supernatant was loaded onto the SPE cartridge. The pellet contained in the 15 mL polypropylene centrifuge tube was rinsed with 2 mL of 0.1 N HCl, vortex mixed and then centrifuged for 5 min at 1800 g. The rinse was loaded onto the SPE cartridge and 6-CPA was eluted from the cartridge using either 1 CV (for C18 SPE) or 3 CVs (for carbon SPE) of 99:1 acetonitrile/water (v/v). The eluates were collected and quantitatively transferred to 200 mL TurboVap tubes and evaporated to near dryness at 35˚C using a TurboVap II evaporator. The final extract was dissolved in 1:1 methanol/water (v/v) and sonicated for approximately 1 min. Sample extracts were diluted to final volumes of 20, 40 and 400 mL for 6-CPA fortification levels of 0.050, 0.20 and 2.0 $\mu g \cdot g^{-1}$, respectively. The final extracts were then submitted to LC-MS/MS analysis.

Sample Analysis

The analysis of nitrapyrin was performed using an Agilent 1200 series LC coupled to an Agilent 6430 triple quadrupole tandem mass spectrometer (Santa Clara, CA). Electrospray Ionization (ESI) and Atmospheric Pressure Chemical Ionization (APCI) ionization sources were tested, and APCI was found to achieve better ionization. The analysis of 6-CPA was performed using an Agilent 1260 series LC coupled to an Agilent 6460 triple quadrupole tandem mass spectrometer equipped with an Agilent Jet Stream ESI ion source. See **Table 2** for instrument-specific conditions.

Table 2. Mass spectrometer instrument parameters.

Parameter	Nitrapyrin (Agilent 6430)	6-CPA (Agilent 6460)
Source temperature	300˚C	300˚C
Source gas flow (N_2)	10 L·min^{-1}	10 L·min^{-1}
Nebulizer gas flow (N_2)	60 psi	45 psi
Capillary voltage	1750 V (+)	3500 V (−)
Collision gas	N_2 (24 m Torr)	N_2 (8 m Torr)
Primary transition (m·z^{-1})	114.0→78.0	156.0→111.9
Fragmentor (V)	100	84
Collision energy (V)	24	8
Dwell time	20	100
Sheath gas temperature	Not applicable	325˚C
Sheath gas flow (N_2)	Not applicable	12 L·min^{-1}
Nozzle voltage	Not applicable	1000 V

For nitrapyrin, a Poroshell 120 EC-C8 LC column (30 × 3.0 mm i.d., 2.7 µm particle size, Agilent, Folsom, CA) was used and operated at ambient temperature (~25˚C). The mobile phase was 0.05% formic acid in water (A) and methanol (B) at flow rate of 0.50 mL·min^{-1}. The gradient program is shown in Table 3. The injection volume was 20 µL.

Table 3. Gradient program for analysis of nitrapyrin (Agilent 6430).

Step	Total time (min)	Mobile phase A (%)	Mobile phase B (%)
0	0.0	80	20
1	0.5	80	20
2	0.6	5	95
3	3.0	5	95
4	3.1	80	20
5	5.5	80	20

Divert flow to waste from 0.00 to 2.40 min. Direct flow to the MS detector from 2.40 to 3.40 min. Divert flow to waste after 3.40 min.

For 6-CPA, a Poroshell 120 EC-C18 LC column (50 × 3.0 mm i.d., 2.7 µm particle size, Agilent, Folsom, CA) was used with a column heater at 40˚C. The mobile phase was 0.05% acetic acid in water (A) and 0.05% acetic acid in methanol (B). The gradient program is shown in Table 4. The injection volume was 10 µL. The mass spectrometers were operated in multiple reaction monitoring mode (MRM). Two transitions were tested for each compound, 114/78 m/z and 114/51 m/z for nitrapyrin and 156/111.9 m/z and 158/114 m/z for 6-CPA. The transitions used for quantitation of nitrapyrin and 6-CPA were 114/78 m/z and 156/111.9 m/z, respectively. These transitions showed more abundance and less

background with all the commodities studied. For data anlysis, MassHunter Quantitative Analysis version b.06 was used. Nitrapyrin and 6-CPA residues were quantified using a linear standard curve method. Retention times for nitrapyrin and 6-CPA were 3.2 min and 5.3 min, respectively.

Table 4. Gradient program for analysis of 6-CPA (Agilent 6460).

Step	Total time (min)	Flow rate ($mL \cdot min^{-1}$)	Mobile phase A (%)	Mobile phase B (%)
0	0.50	0.30	97	3
1	2.00	0.30	5	95
2	5.30	0.30	5	95
3	5.40	0.60	97	3
4	9.00	0.60	97	3
5	9.10	0.30	97	3
6	11.00	0.30	97	3

Divert flow to waste from 0.00 to 4.80 min. Direct flow to the MS detector from 4.80 to 5.80 min. Divert flow to waste after 5.80 min.

3. Results and Discussion

The method detection limit (MDL) was calculated as described in EPA Method 314.0 [40]. Six replicate injections of samples fortified at 0.020 $\mu g \cdot g^{-1}$ with nitrapyrin and 0.050 $\mu g \cdot g^{-1}$ with 6-CPA were analyzed by LC/MS-MS, and an MDL was calculated on the basis of the Student's *t* value at the 99% confidence interval. The MDLs for all the commodities studied are shown in **Table 5**. For nitrapyrin the method was validated at 0.020, 0.20 and 2.0 $\mu g \cdot g^{-1}$. The recoveries obtained for all the matrices studied were in the range of 76% - 102% (**Table 5**) except for lettuce (at 2.0 mg/kg) with a recovery of 68%. The standard deviation (SD) was less than or equal to 15% in all cases. **Figure 2** shows example chromatograms of all control and fortified matrices at 0.020 $\mu g \cdot g^{-1}$. The coefficient of determination (R^2) was ≥ 0.99 in all cases. Iwata *et al.* [5] reported similar results for nitrapyrin on strawberry (validation levels of 0.10 $\mu g \cdot g^{-1}$, 0.50 $\mu g \cdot g^{-1}$ and 1.0 $\mu g \cdot g^{-1}$), recoveries of 83% - 94% with SD ≤ 4%. Linghui *et al.* [34] reported recoveries of nitrapyrin (validation levels of 0.050 $\mu g \cdot g^{-1}$, 0.10 $\mu g \cdot g^{-1}$ and 0.20 $\mu g \cdot g^{-1}$) for wheat, sorghum, maize and popcorn of 83.1% - 96.4% (SD ≤ 9%). The protocol for sample preparation used in our study was based on a previously reported method [32]. However, in this study we utilize a smaller sample size (5 g *vs.* 100 g) and less extraction solvent (30 mL *vs.* 300 mL) compared to the method used by Iwata *et al.* [5]. Also, unlike the method of Linghui *et al.* [34] our method does not involve derivatization or the use of internal standards. In our method, the cleanup step was modified by using commercial silica SPE without pretreatment, which makes the method safer, more efficient and faster than the original method using silica gel baked for 2 h at 100 ˚C and treated with a solution of sulfuric acid [32]. In this study, residues of nitrapyrin were eluted from silica SPE with approximately 6 mL

of toluene. The elution pattern of the silica SPE was consistent for all the matrices studied, even when using SPE columns from different lot numbers. In the method proposed by Claussen [32], the use of in-house packed columns required a redetermination of the elution pattern for each batch of deactivated silica gel, with significant time and material costs. Alternatively, commercial cartridges in this research represent significant time and solvent savings. Likewise, the quantitation of residues by LC-MS/MS is almost 3-times faster than the reference method (5.5 min vs. 16 min), enabling a much higher sample throughput. In a regulatory environment, these savings translate to a greater number of samples tested with significantly improved reproducibility and efficiency.

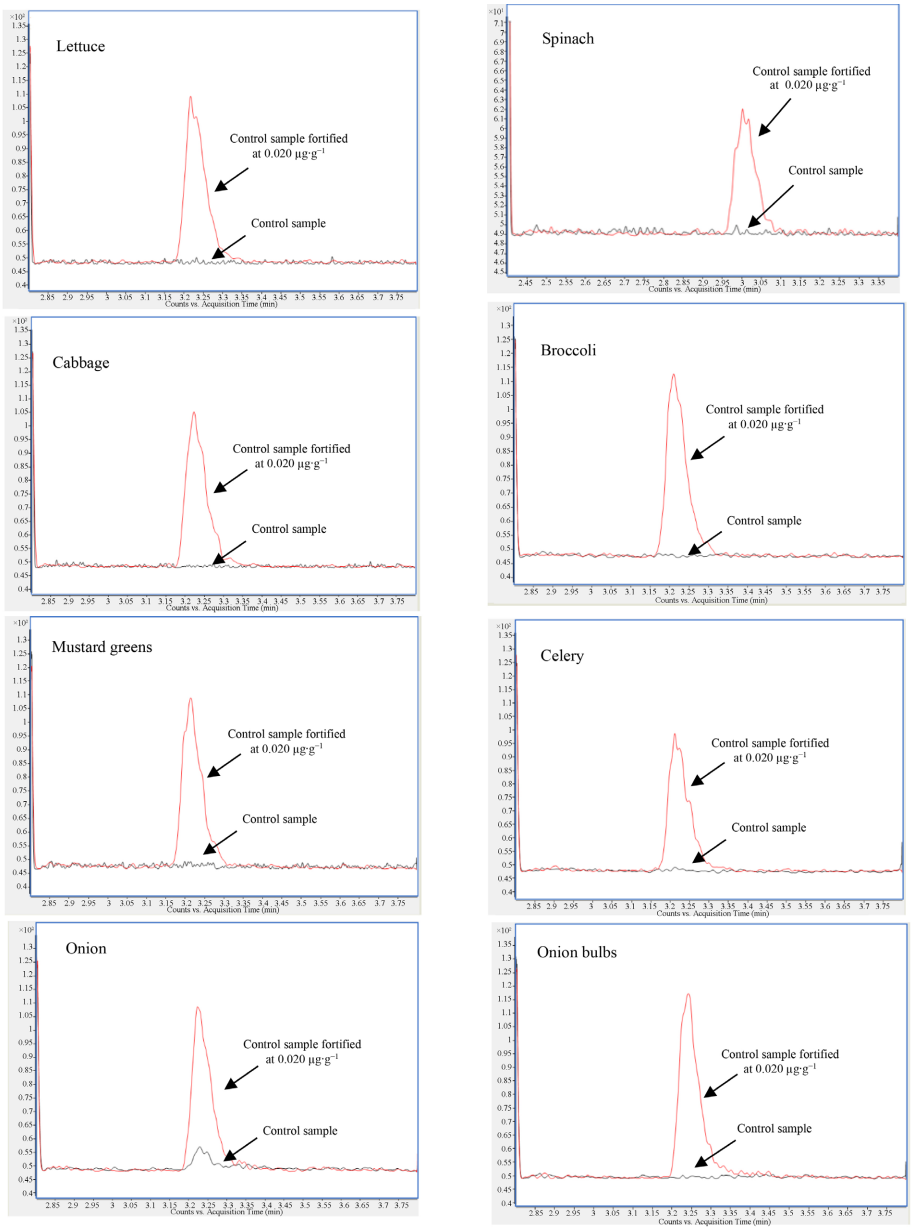

Figure 2. Extracted ion chromatograms of control samples and fortified samples at 0.020 µg·g^{-1} with nitrapyrin. Calibration standard from Agilent 6430: MRM transition 114→78.

Table 5. MDLs and recovery of nitrapyrin and 6-CPA from different sample matrices.

	Nitrapyrin Fortification[a]				6-CPA Fortification[a]			
Sample Matrix	0.020 μg·g^{-1}	0.20 μg·g^{-1}	2.0 μg·g^{-1}	MDL[b] (μg·g^{-1})	0.050 μg·g^{-1}	0.20 μg·g^{-1}	2.0 μg·g^{-1}	MDL[b] (μg·g^{-1})
Lettuce	76 ± 9 (n = 7)	86 ± 7 (n = 6)	68 ± 4 (n = 2)	0.0062	74 ± 3 (n = 10)	79 ± 7 (n = 9)	81 ± 4 (n = 6)	0.0053
Spinach	91 ± 7 (n = 6)	96 ± 2 (n = 6)	102 ± 10 (n = 5)	0.0049	66 ± 7 (n = 6)	72 ± 1 (n = 6)	79 ± 2 (n = 5)	0.013
Cabbage	91 ± 4 (n = 6)	87 ± 8 (n = 8)	95 ± 3 (n = 3)	0.011	73 ± 3 (n = 7)	80 ± 5 (n = 7)	85 ± 4 (n = 3)	0.0098
Broccoli	86 ± 15 (n = 7)	89 ± 8 (n = 9)	90 ± 4 (n = 3)	0.011	74 ± 5 (n = 7)	79 ± 6 (n = 9)	79 ± 2 (n = 3)	0.0081
Mustard greens	84 ± 14 (n = 7)	92 ± 7 (n = 9)	83 ± 2 (n = 3)	0.0039	66 ± 5 (n = 7)	75 ± 7 (n = 9)	88 ± 2 (n = 3)	0.0072
Celery	79 ± 6 (n = 7)	83 ± 8 (n = 10)	87 ± 7 (n = 3)	0.0023	70 ± 4 (n = 7)	70 ± 4 (n = 10)	90 ± 4 (n = 3)	0.0060
Onion plants	83 ± 9 (n = 6)	86 ± 8 (n = 5)	80 ± 8 (n = 3)	0.0058	74 ± 6 (n = 6)	79 ± 6 (n = 5)	86 ± 5 (n = 3)	0.011
Onion bulbs	90 ± 8 (n = 6)	84 ± 6 (n = 9)	90 ± 8 (n = 3)	0.0052	74 ± 8 (n = 7)	80 ± 6 (n = 10)	89 ± 9 (n = 3)	0.012

[a]Values are mean percent recovered ± standard deviation; n is the number of replicates; [b]MDL = (t) × (S_{n-1}), t = student's t value for 99% confidence level and standard deviation estimated with n−1 degrees of freedom (t = 3.365 for six replicates), S_{n-1} = sample standard deviation (n − 1) of the six replicate analyses.

For 6-CPA, the method was validated at 0.050, 0.20 and 2.0 μg·g^{-1}. A more sensitive instrument was used for analysis of 6-CPA (Agilent 6460) due to the suppression observed. The more sensitive Agilent 6460 system enabled greater sample dilution and reduced the observed matrix suppression. The recoveries for all the matrices were in the range of 70% - 90% except for spinach and mustard greens with recoveries around 66% (Table 5 and Figure 3). In all cases, the SD was ≤ 10% and the coefficient of determination (R^2) was ≥ 0.99. Similar results were reported on strawberry by Iwata *et al.* [5] with validation levels of 0.10 μg·g^{-1}, 0.50 μg·g^{-1} and 1.0 μg·g^{-1} and recoveries of 77% - 84% (SD ≤ 4 %). Linghui, *et al.* [34] reported recoveries at 0.050 μg·g^{-1}, 0.10 μg.g^{-1} and 0.20 μg·g^{-1} for wheat, sorghum, maize and popcorn of 80.4 - 98.4% (SD ≤ 10.1%). The preparation of samples for 6-CPA analysis was based on a previously reported method [33]. For samples with a high content of chlorophyll such as mustard greens, spinach and cabbage, the C18 SPE cartridge was efficient in removing the impurities and pigments present in these samples. For lettuce, broccoli, celery and onion, carbon SPE cartridges removed some of the impurities causing suppression. The elution pattern of the C18 and carbon SPE cartridges was consistent for all the matrices studied and no differences were observed between the lots of SPE cartridges used. In contrast to the method reported by Claussen [33], our method requires only one SPE cartridge for sample cleanup instead of two, and

it was not necessary to use internal standards. All samples were analyzed with external calibration for nitrapyrin and 6-CPA.

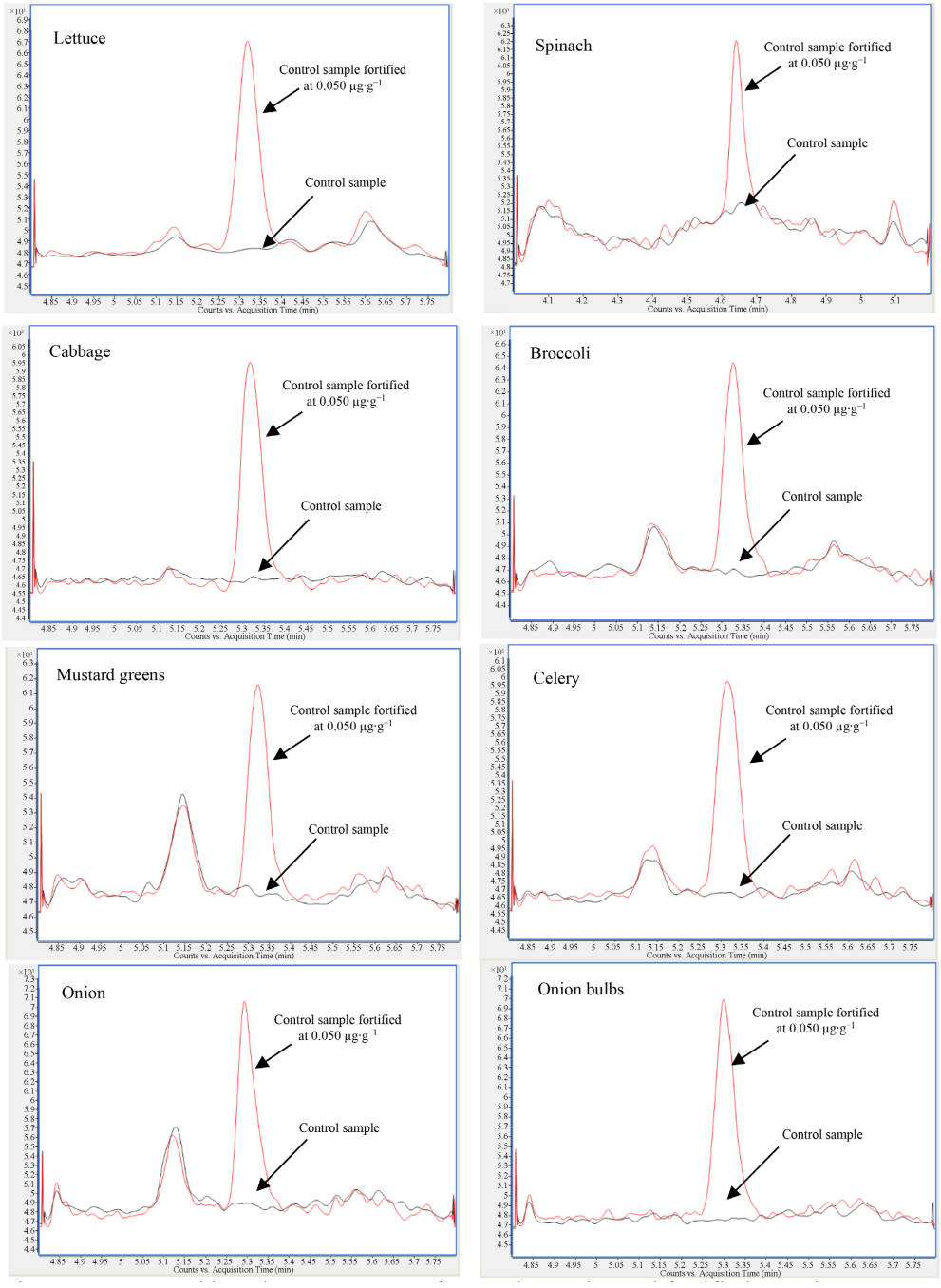

Figure 3. Extracted ion chromatograms of control samples and fortified samples at 0.050 µg·g^{-1} with 6-CPA. Calibration standard from Agilent 6460: MRM transition 156→111.9.

The results of storage stability experiments for nitrapyrin and 6-CPA on untreated samples fortified at 0.20 µg·g^{-1} are shown in **Table 6**. In all cases, these results align well with the spiked samples at the same fortification level. According to these results, no degradation on spinach, cabbage, broccoli, mustard

greens, lettuce or celery was observed from sample collection to analysis (one stability point). However, a 20% reduction of nitrapyrin was observed for onion (bulbs) over 636 days at −20˚C. Iwata *et al.* [5] conducted a stability study on strawberry (at −16˚C) and reported a 0.14 μg·g^{-1} decrease in nitrapyrin residues and a corresponding 0.18 μg·g^{-1} increase in 6-CPA residues after 223 days. In our study, storage stability samples fortified with nitrapyrin were not analyzed for residues of 6-CPA. Since onion bulbs were the only matrix that showed apparent degradation, a multiple point stability study with fortified control samples may be of future interest. It may be possible that onions contain compounds or enzymes that may contribute to the degradation of nitrapyrin even when samples are stored at −20˚C.

Table 6. Storage stability results of nitrapyrin and 6-CPA from different sample matrices.

Sample matrix	Nitrapyrin			6-CPA		
	Spiked sample[a]	Storage stability sample[a]	Storage period (days)	Spiked sample[a]	Storage stability sample[a]	Storage period (days)
Lettuce	80 ± 6	86 ± 8	819	75 ± 3	85 ± 5	806
Spinach	94 ± 2	95 ± 3	737	71 ± 1	72 ± 3	763
Cabbage	88 ± 5	84 ± 6	841	85 ± 3	81 ± 5	840
Broccoli	88 ± 3	93 ± 2	857	78 ± 5	70 ± 4	862
Mustard greens	94 ± 5	99 ± 4	834	68 ± 3	68 ± 1	841
Celery	79 ± 2	81 ± 4	616	78 ± 6	78 ± 4	616
Onion bulbs	89 ± 3	68 ± 7	636	81 ± 6	80 ± 9	670

[a]Values are mean percent recovered ± standard deviation with three replicates. Samples were fortified at 0.20 μg·g^{-1} of nitrapyrin and 6-CPA; spiked samples are untreated samples fortified the day of analysis. Storage stability samples are untreated samples fortified with nitrapyrin or 6-CPA and stored at −20˚C with treated field samples until analysis, these samples indicate any degradation of nitapyrin and 6-CPA that occurred in field treated samples during the storage period.

Residues of nitrapyrin on untreated and treated samples were <0.020 μg·g^{-1} for all the crops and experimental field sites, except for celery and onion. For celery, residues of nitrapyrin were found in treated samples from all field sites at < 0.020 to 0.15 μg·g^{-1}, except for field trial CA522 (**Table 7**). For onion, nitrapyrin residues were only found in plants from California. The residues were in the range of < 0.020 to 0.067 μg·g^{-1} (**Table 7**). Nitrapyrin residues have been reported by Kallio *et al.* [16] in red beets roots (*Beta vulgaris* L. var. *conditiva*) in the range of 0 - 1.19 μg·g^{-1}. Iwata *et al.* [5] did not detect nitrapyrin in strawberry fruits (detection limit was 0.040 μg·g^{-1}). The presence of nitrapyrin in celery and onion could be related to how it was applied in the field. According to literature, the main identifiable residue of nitrapyrin in plants is 6-CPA [24]. In some in-

stances, the formation of amide or ester conjugates can occur and then metabolize back to 6-CPA [5] [11]. Residues of 6-CPA were detected only in onion (bulbs and plants), mustard greens and lettuce. Residues of 6-CPA for onion bulbs and onion plants were in the range of < 0.050 to 0.18 μg·g^{-1} and < 0.050 to 0.098 μg·g^{-1}, respectively (Table 7). For mustard greens, 6-CPA residues were < 0.050 to 0.18 μg·g^{-1} and for lettuce < 0.050 to 0.19 μg·g^{-1} (Table 7). For the rest of the untreated and treated samples, residues of 6-CPA were < 0.050 μg·g^{-1}. There are few published studies regarding residues of nitrapyrin and 6-CPA in food crops [2]. However, the low levels of 6-CPA found here are consistent with the results reported by Iwata *et al.* [5] where 6-CPA was detected in strawberry fruits in the range of 0.04 to 0.09 μg·g^{-1}. Residues of 6-CPA have also been reported in sorghum (0.05 μg·g^{-1}) and potato (*Solanum tuberosum* subsp. *tuberosum*) (0.33 μg·g^{-1}). No detectable residues were found in corn, wheat or sugar beet (*Beta vulgaris* subsp. *vulgaris*) [11].

Table 7. Residues of nitrapiryn and 6-CPA in different sample matrices.

		Nitrapyrin (μg·g^{-1})		6-CPA (μg·g^{-1})	
Sample matrix	**Field ID**	**Untreated samples**	**Treated samples**	**Untreated samples**	**Treated samples**
Lettuce	NY05, GA*05, SC*02, CA18, NM02, NM03, CA*02, CA*21, CA01, CA496, CA484, FL487, FL488	<0.020	<0.020	<0.050	<0.050
	CA17	<0.020	<0.020	<0.050	**<0.050 - 0.19**
Spinach	MD01, TX01, CA14, CA*15, NY19, CO491	<0.020	<0.020	<0.050	<0.050
Cabbage	NY03, SC*01, CA*06, CA*120, OH*527, TX489	<0.020	<0.020	<0.050	<0.050
Broccoli	CA*10, CA*11, CA*120, CA*13, OR05, TX490	<0.020	<0.020	<0.050	<0.050
Mustard greens	CA*22, CA*23, TX02, OH*341, WI461	<0.020	<0.020	<0.050	<0.050
	SC*16	<0.020	<0.020	<0.050	**0.10 - 0.18**
	AR11	<0.020	<0.020	<0.050	**<0.050 - 0.096**
Celery	CA*130	<0.020	**0.038 - 0.058**	<0.050	<0.050
	CA*27	<0.020	**<0.020 - 0.045**	<0.050	<0.050
	WI480	<0.020	**0.032 - 0.12**	<0.050	<0.050

Continued

Onion plants	FL159	<0.020	**<0.020 - 0.047**	<0.050	<0.050
	CA81	<0.020	**0.034 - 0.041**	<0.050	<0.050
	CA82	<0.020	**<0.020 - 0.051**	<0.050	<0.050
	CA522	<0.020	<0.020	<0.050	<0.050
	CA495	<0.020	**0.035 - 0.15**	<0.050	<0.050
	CA496	<0.020	**0.076 - 0.088**	<0.050	<0.050
	CA*127	<0.020	**0.034 - 0.067**	<0.050	<0.050
	CA91	<0.020	**<0.020 - 0.038**	<0.050	<0.050
	MD525	<0.020	<0.020	<0.050	**<0.050 - 0.098**
Onion bulbs	NY329, OH*342, CA*127, WA*454, WA*457, CA90, NM315	<0.020	<0.020	<0.050	<0.050
	ID210	<0.020	<0.020	<0.050	**<0.050 - 0.060**
	CA89	<0.020	<0.020	<0.050	**<0.050 - 0.18**

Samples from California field site: CA17, CA18, CA*02, CA*21, CA01, CA496, CA484, CA14, CA*15, CA*06, CA*120, CA*10, CA*11, CA*120, CA*13, CA*22, CA*23, CA*130, CA*27, CA81, CA82, CA522, CA495, CA496, CA*127, CA91, CA89, CA*127 and CA90. Samples from New York field site: NY05, NY19, NY03 and NY329. Samples from Georgia field site: GA*05. Samples from South Carolina field site: SC*02, SC*01 and SC*16. Samples from Florida field site: FL487, FL488 and FL159. Samples from Maryland field site: MD01 and MD525. Samples from Texas field site: TX01, TX489, TX490 and TX02. Samples from Colorado field site: CO491. Samples from Ohio field site: OH*527, OH*341 and OH*342. Samples from Oregon field site: OR05. Samples from Wisconsin field site: WI461 and WI480. Samples from Arizona field site: AR11. Samples from Washington field site: WA*454 and WA*457. Samples from Idaho field site: ID210. Some field IDs from certain regions are designated with an asterisk in the name.

Three major improvements were incorporated into our method for analysis of nitrapyrin: the utilization of commercial silica SPE cartridges without pretreatment, the transition to LC-MS/MS for residue determination and the use of external calibration standards for analysis. The use of LC-MS/MS with APCI detection greatly improved the sample throughput relative to GC-MSD analysis, which requires derivatization and significantly longer runs. For the analysis of 6-CPA, one SPE cartridge was used instead of multiple SPE cartridges which reduces the cost and time of analysis per sample. Also, the analysis by LC-MS/MS was conducted with external calibration instead of using costly isotopically labeled internal standards. One analyst can prepare a set of 22 samples in 8 h and the samples can be analyzed overnight. Sample throughput is dramatically increased and a total of 110 samples can be analyzed during an average 5-day workweek.

Data generated related to residues of nitrapyrin and 6-CPA reported in this study was used to register the use of nitrapyrin on the studied commodities and establish residue tolerances in the U.S. (**Table 8**) [21]. The present methods can

aid both domestic regulatory and international agencies involved in the monitoring of pesticide residues in foods. The application of these methods could also be extended to more agricultural crops of commercial importance and value around the world such a corn, spring wheat (including durum), winter wheat, barley, oats and canola, where the use of nitrapyrin has been approved in Canada, sorghum, maize and popcorn in China and grass in Costa Rica [41] [34] [42]. In addition, the method for analysis of 6-CPA could be applied in agricultural products where other picolinic acid herbicides such as clopyralid, picloram and aminopyralid have been applied and it is known that 6-CPA is one of the main metabolites [43].

Table 8. MRLs stablished by USEPA on the commodities studied.

	Group	MRL (ppm)*
Vegetable, *Brassica*, head and stem	5 - 16	0.1
Vegetable, bulb	3 - 07	0.3
Vegetable, leafy	4 - 16	0.4

*MRL is expressed as ppm ($\mu g \cdot g^{-1}$).

4. Conclusion

The analytical methods presented in this research were successfully tested on 7 crops that are commercially important. Data generated using the methods reported in this study was successfully used to register the use of nitrapyrin on the studied commodities and establish residue tolerances in the U.S. Although data related to the residues of nitrapyrin and 6-CPA in minor crops is limited, the generated data in the present study can serve as a valuable reference for future studies involving nitrapyrin use in real-world field systems.

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Conflicts of Interest

The authors declare no conflict of interest regarding the publication of this paper.

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