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Determination of Fipronil and Four Metabolites in Foodstuffs of Animal Origin Using a Modified QuEChERS Method and GC–NCI–MS/MS

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Abstract

A sensitive, effective, and reliable method for the accurate determination of fipronil and four metabolites (fipronil carboxamide, fipronil sulfone, fipronil sulfide, and fipronil desulfinyl) in foodstuffs of animal origin (egg, milk, beef kidney, beef liver, chicken, and chicken liver) was developed by isotope dilution–gas chromatography–negative chemical ionization–tandem mass spectrometry (GC–NCI–MS/MS). Samples were purified by a modified QuEChERS method. Four isotopically labeled internal standards were added in the sample extraction process to compensate for the matrix effect. The average recoveries were 78.2–107.1% with RSD ≤ 8.5%. The limits of quantification (LOQs) were 0.2 μg kg^{-1} for fipronil carboxamide and fipronil sulfone and 0.1 μg kg^{-1} for fipronil, fipronil sulfide, and fipronil desulfinyl. At the same time, the fragmentation mechanism of the five target compounds was analyzed via mass spectral data to help identify the compounds.

Keywords Fipronil; metabolites · GC − NCI − MS/MS · Isotope dilution · Fragmentation mechanism

Introduction

Food safety has drawn increasing attention worldwide. Veterinary drug residues, pesticide residues, and food authenticity are important factors in food safety. Fipronil is a neurotoxic insecticide that can damage insecticide nerves by blocking γ-aminobutyric acid (GABA)-regulated chloride pathways. Fipronil insecticide can inhibit chloride ion inflow into nerve cells because GABA receptors are ligand-gated chloride ion channels, resulting in overexcitation of the pest nervous system [Li et al. 2015; Liu et al. 2015]. At the same time, fipronil can be degraded into metabolites of different structures, such as fipronil sulfone, fipronil sulfide, and fipronil desulfinyl, in the environment. These metabolites are more toxic and more stable than the parent compound [McMahen et al. 2015]. For example, fipronil desulfinyl, a photolysis product of fipronil in the environment, is very stable and much more toxic for most animals than fipronil [Hainzl et al. 1998]. Another metabolite, fipronil sulfone, is 3.3 times more toxic than fipronil [Gunasekara et al. 2007].

Fipronil has been banned in the USA and France due to its very high toxicity and metabolites. At the same time, some countries and regions have established maximum residue limits (MRLs) for fipronil and its metabolites in certain foods. The EU has set an MRL of 5 μg kg^{-1} for the fipronil residue in tea, orange, and chicken egg samples to address this issue [EU Pesticides Database]. China has set temporary MRLs of 10 μg kg^{-1} and 20 μg kg^{-1} for fipronil and its metabolite residues, respectively, in poultry meat, poultry offal, egg, and milk [GB 2763–2019]. Codex Alimentarius has set MRLs of 20 μg kg^{-1}, 100 μg kg^{-1}, 20 μg kg^{-1}, and 20 μg kg^{-1} for fipronil in cattle kidney, cattle liver, cattle milk, and eggs, respectively (Codex Alimentarius Pesticides Database n.d.). Since food of animal origin is the main source of food consumed by people worldwide, it is necessary to establish a fast, accurate, sensitive, and specific analysis method for the determination of fipronil and its metabolites in food of animal origin to ensure and evaluate food safety.

To date, there have been many reports on the analysis and determination of fipronil and its metabolites in different

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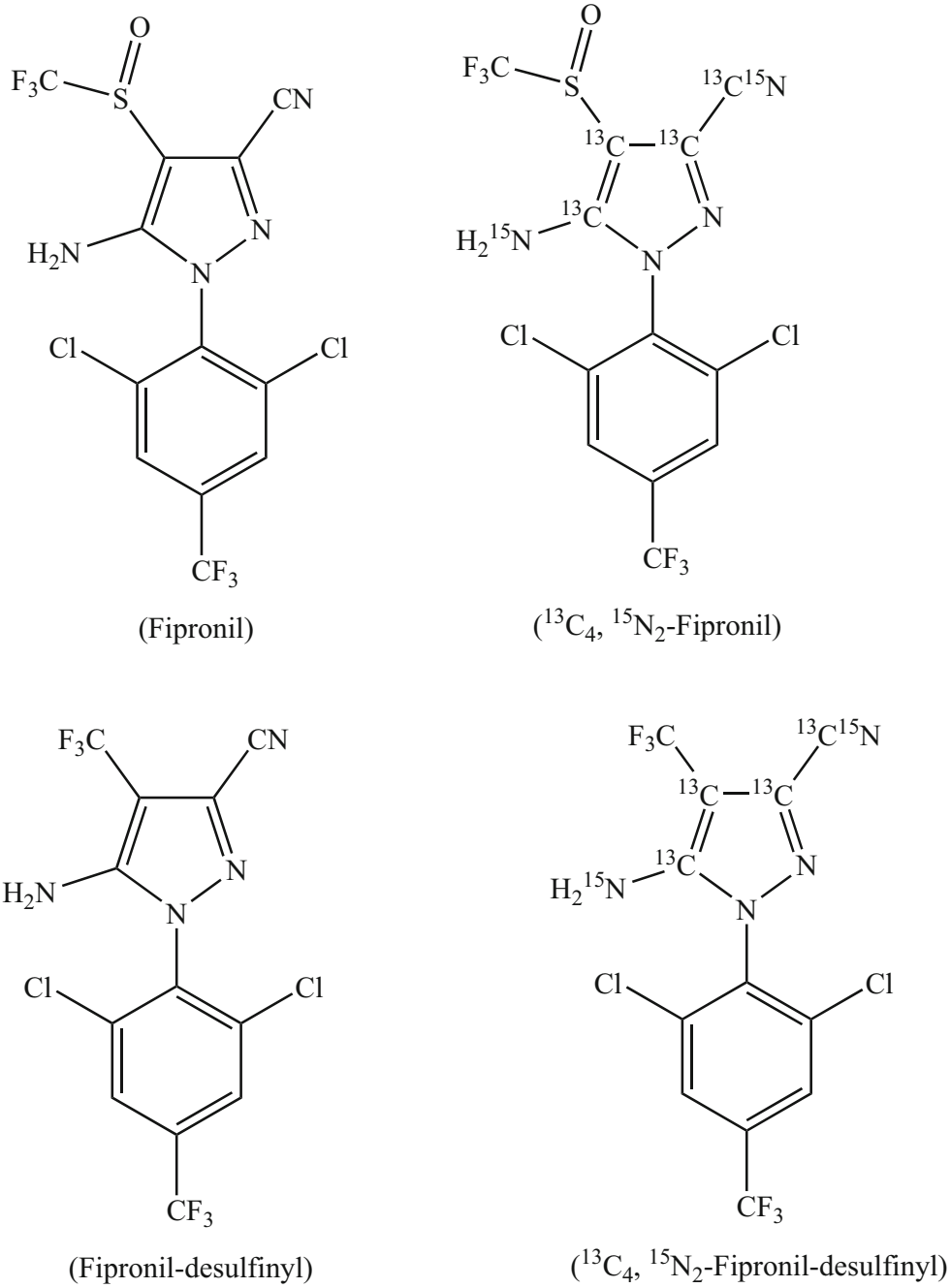

Fig. 1 Chemical structure of fipronil, four metabolites, and four isotope internal standards

samples [Biswas et al. 2019; Chen et al. 2018; Li et al. 2019a, b, 2020; Montiel-León et al. 2018; Kadar and Faucon 2006; Peng et al. 2016; Vasylieva et al. 2015; Wang et al. 2019]; however, previous studies have focused on fipronil or its metabolites, such as fipronil sulfone, fipronil sulfide, and fipronil desulfinyl, but few of them have reported about the simultaneous determination of fipronil carboxamide residue.

Since fipronil and its metabolites all contain 6 F atoms, two Cl atoms, and a cyanogroup, which are highly electronegative, in their molecular structures, it is undoubtedly the best choice to use negative chemical ionization (NCI) technology to determine the specific responses of electronegative groups or elements. Compared with electron impact (EI) ionization, NCI has a lower noise background because it is a soft ionization technique. In general, the more electronegative, the higher the sensitivity.

An increasing number of pesticide residues in food are analyzed using gas chromatography coupled with tandem mass spectrometry (GC–MS/MS) because of its high selectivity and sensitivity [Jadhav et al. 2019; Song et al. 2019; Zhu

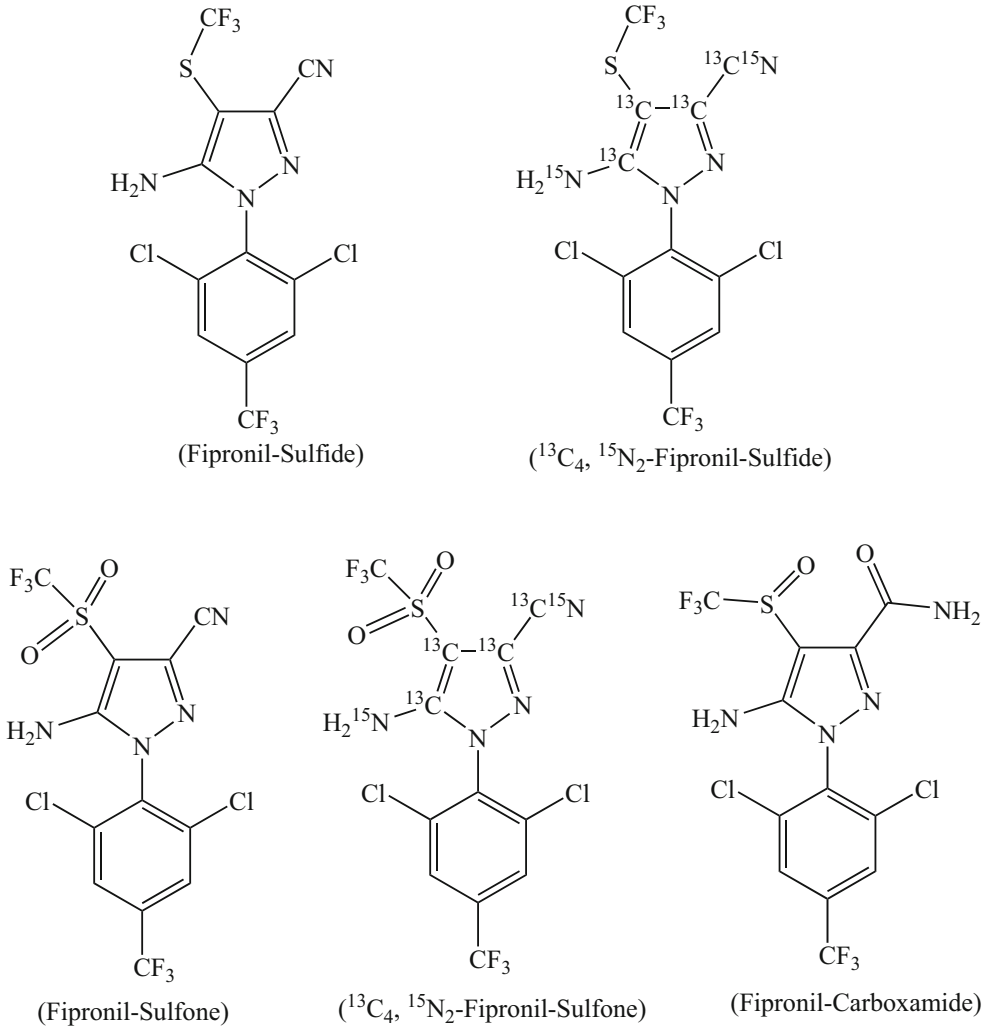

Fig. 1 (continued)

Table 1 Analytical conditions for GC-NCI-MS/MS

Compound	Retention time (min)	Parent ion (m/z)	Product ion (m/z)	Collision energy (eV)
Fipronil desulfinyl	8.51	352.0	145.0*	18
			317.0	10
$^{13}C_4$, $^{15}N_2$-Fipronil-desulfinyl	8.51	358.1	151.1*	18
			323.0	10
Fipronil sulfide	9.35	384	314.9*	6
			244	24
$^{13}C_4$, $^{15}N_2$-Fipronil-Sulfide	9.35	390	320.9*	8
			247	24
Fipronil	9.45	366.0	318.0*	6
			250.0	10
$^{13}C_4$, $^{15}N_2$-Fipronil	9.45	372.0	324.0*	10
			252.0	14
Fipronil sulfone	10.15	416.0	283.0*	10
			244.0	10
$^{13}C_4$, $^{15}N_2$-Fipronil-Sulfone	10.15	422.0	289.0*	10
			247.1	8
Fipronil carboxamide	11.20	409.9	253.0*	18
			339.9	6

* The MS/MS transition was used for quantitation

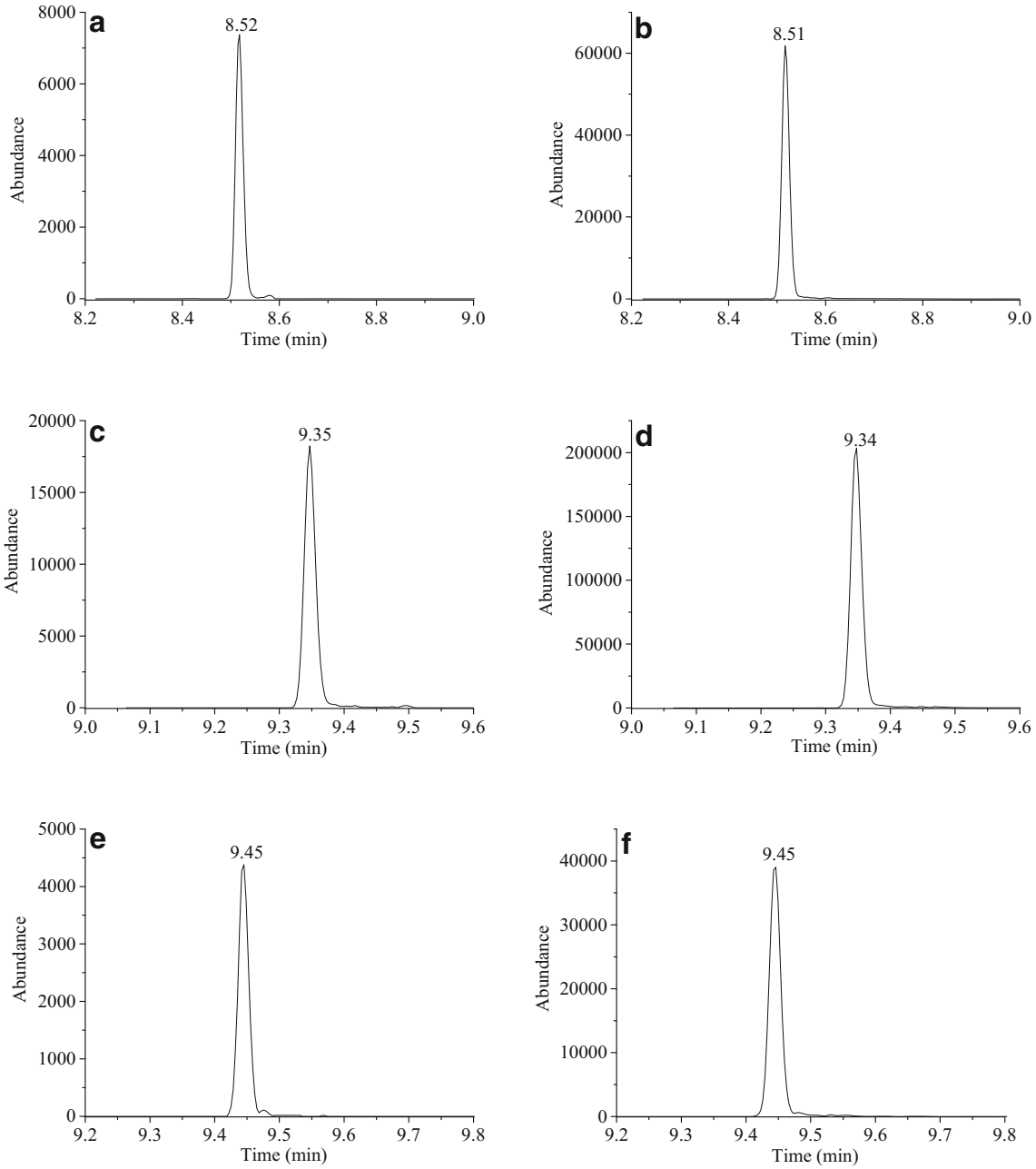

Fig. 2 GC–NCI–MS/MS MRM chromatograms of a beef liver sample spiked with 2.0 μg kg^{-1}. (**a** fipronil desulfinyl; **b** $^{13}C_4$, $^{15}N_2$-Fipronil-desulfinyl; **c** fipronil sulfide; **d** $^{13}C_4$, $^{15}N_2$-Fipronil-Sulfide; **e** fipronil; **f** $^{13}C_4$, $^{15}N_2$-Fipronil; **g** fipronil sulfone; **h** $^{13}C_4$, $^{15}N_2$-Fipronil-Sulfone; **i** fipronil carboxamide)

et al. 2019]. To our knowledge, GC–NCI–MS/MS has not been used previously to simultaneously monitor the residue levels of fipronil and four metabolites (fipronil carboxamide, fipronil sulfone, fipronil sulfide, and fipronil desulfinyl) in foodstuffs of animal origin.

The quick, easy, cheap, effective, rugged, and safe (QuEChERS) method is an extraction and purification method, and as an environmentally friendly pretreatment, QuEChERS is widely used in the determination of pesticide residues in food [Daniel and Lucio do Lago 2019; Li et al. 2019a, b].

Thus, we developed a modified QuEChERS method combined with GC–NCI–MS/MS for the simultaneous identification of fipronil and four metabolites (fipronil carboxamide, fipronil sulfone, fipronil sulfide, and fipronil desulfinyl) in foodstuffs of animal origin (egg, milk, beef kidney, beef liver, poultry meat, and poultry offal). To obtain accurate quantitative results, four isotopically labeled internal standards were

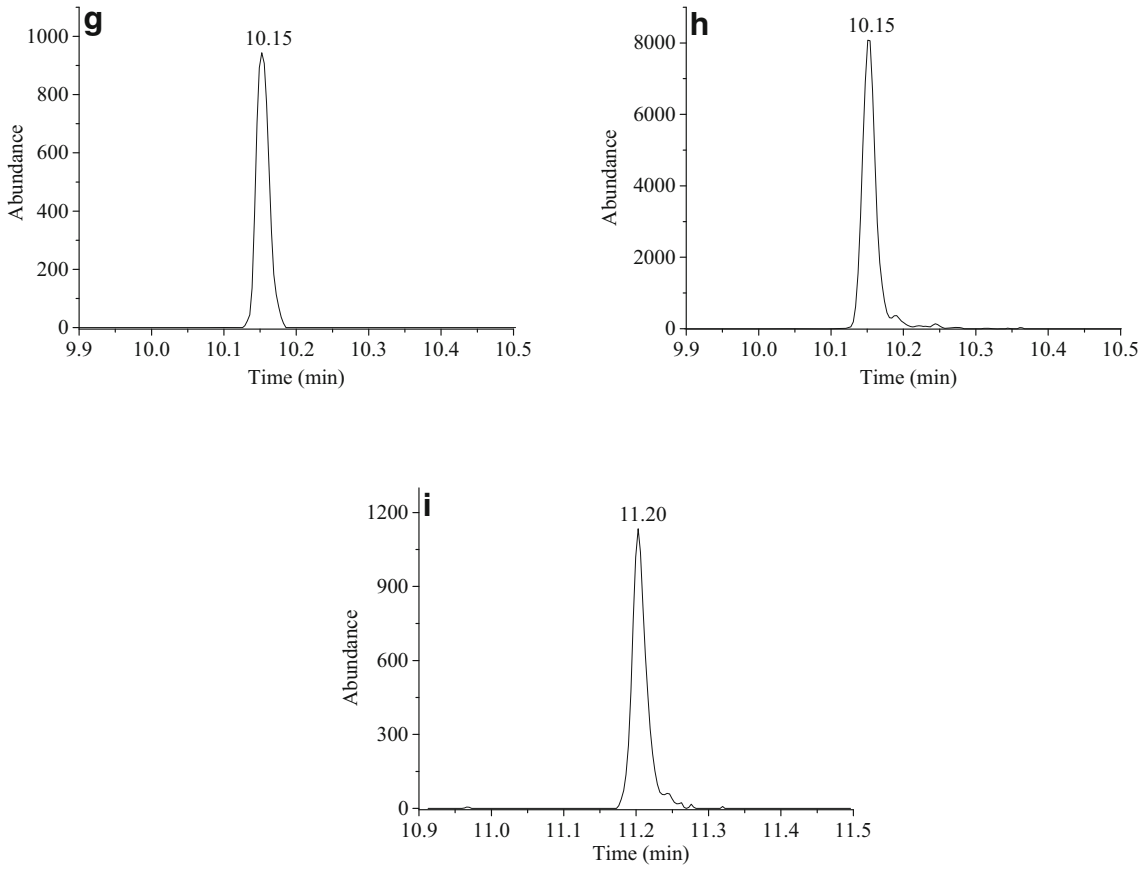

Fig. 2 (continued)

used for quantitative analysis. Additionally, the fragmentation mechanism of target compounds on NCI–MS/MS was analyzed to help identify objects.

Materials and Methods

Reagents and Materials

A certified standard of fipronil (CAS No. 120068-37-3, 96.5% purity) was purchased from LGC Labor GmbH (Augsburg, Germany), and fipronil carboxamide (CAS No. 205650-69-7, 96.5% purity) was acquired from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Fipronil sulfone (CAS No. 120068-36-2, 98.5% purity), fipronil sulfide (CAS No. 120067-83-6, 98.0% purity), and fipronil desulfinyl (CAS No. 205650-65-3, 98.0% purity) were acquired from the Ministry of Agricultural Environmental Protection Research and Monitoring Institute, China.

$^{13}C_4$,$^{15}N_2$-Fipronil (100 μg mL^{-1}), $^{13}C_4$,$^{15}N_2$-Fipronil-Sulfone (100 μg mL^{-1}), $^{13}C_4$,$^{15}N_2$-Fipronil-desulfinyl (100 μg mL^{-1}), and $^{13}C_4$,$^{15}N_2$-Fipronil-Sulfide (100 μg mL^{-1}) were purchased from Cambridge Isotope Laboratories, Inc. (USA). Figure 1 shows the structural formula of the standard product. Acetonitrile, n-hexane, and acetic acid were acquired from Merck (Darmstadt, Germany). Salt-out packages (containing 4 g of magnesium sulfate, 1 g of sodium citrate, 0.5 g of sodium citrate semihydrate, and 1 g of sodium chloride) and purification tubes (containing 150 mg of anhydrous magnesium sulfate, 50 mg of PSA, and 50 mg of C_{18}) were acquired from Yuexue Technology Co., Ltd. (Shanghai, China). Ultrapure water was obtained from a Milli-Q water purification system from Millipore (Bedford, MA, USA).

QuEChERS Method Procedure

All samples (approximately 200 g, except milk) were first homogenized with a GM 200 (Retsch, Germany) Grindomix and stored at − 18 °C before analysis. A 10-g sample was placed in a 50-mL centrifuge tube, and the first step was to add 20 μL of a mixed solution containing four internal standards (10 μg mL^{-1}) and 10 mL of acetonitrile (saturated with n-hexane). The sample was homogenized at a high speed of 15,000 r min^{-1} for 2 min. A salt-out package (containing 4 g of magnesium sulfate, 1 g of sodium citrate, 0.5 g of sodium citrate semihydrate, and 1 g of sodium chloride) was added, shaken vigorously, and centrifuged for 5 min at

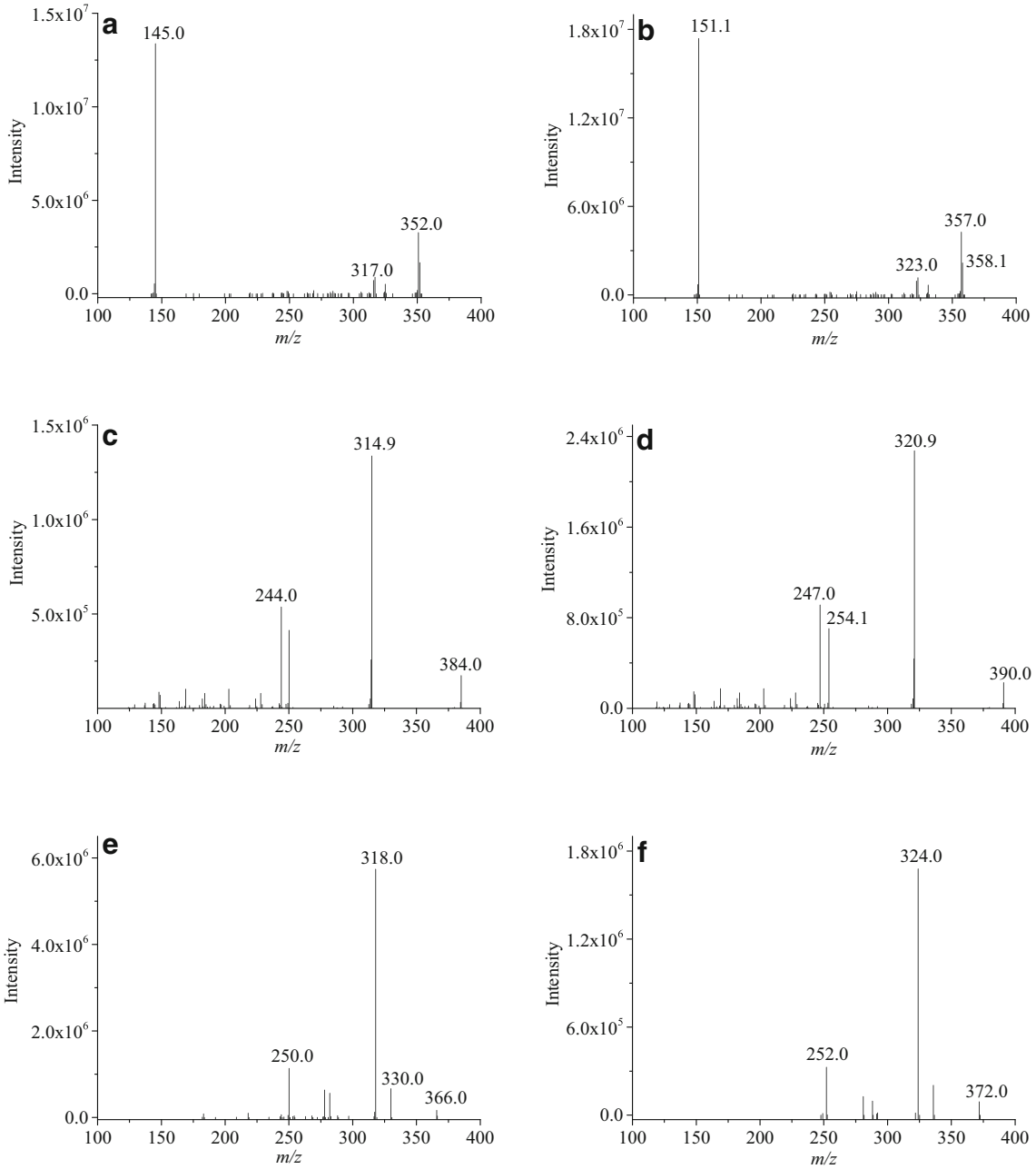

Fig. 3 GC–NCI–MS/MS product scan spectrum of fipronil, four metabolites and four isotope internal standards. (**a** fipronil desulfinyl; **b** $^{13}C_4$, $^{15}N_2$-Fipronil-desulfinyl; **c** fipronil sulfide; **d** $^{13}C_4$, $^{15}N_2$-Fipronil-Sulfide; **e** fipronil; **f** $^{13}C_4$, $^{15}N_2$-Fipronil; **g** fipronil sulfone; **h** $^{13}C_4$, $^{15}N_2$-Fipronil-Sulfone; **i** fipronil carboxamide)

10000 r min^{-1}. A total of 1 mL of the acetonitrile layer was added to a 10-mL glass centrifuge tube, and then 1 mL of n-hexane (saturated acetonitrile) was added; the mixture was swirled and then stratified statically, the n-hexane layer was discarded, and then 1 mL of n-hexane (saturated acetonitrile) was added to repeat the above step once more. The acetonitrile layer was added to a 2-mL purification tube (containing 150 mg of anhydrous magnesium sulfate, 50 mg of PSA, and 50 mg of C_{18}). The mixture was vortexed for 2 min and centrifuged for 5 min at 10000 r min^{-1}. Then, the supernatant (acetonitrile layer) was transferred to a 2-mL sample vial for GC–MS/MS determination.

Preparation of Standard Solutions

The individual standard stock solutions of five standards prepared in acetonitrile were at a concentration of

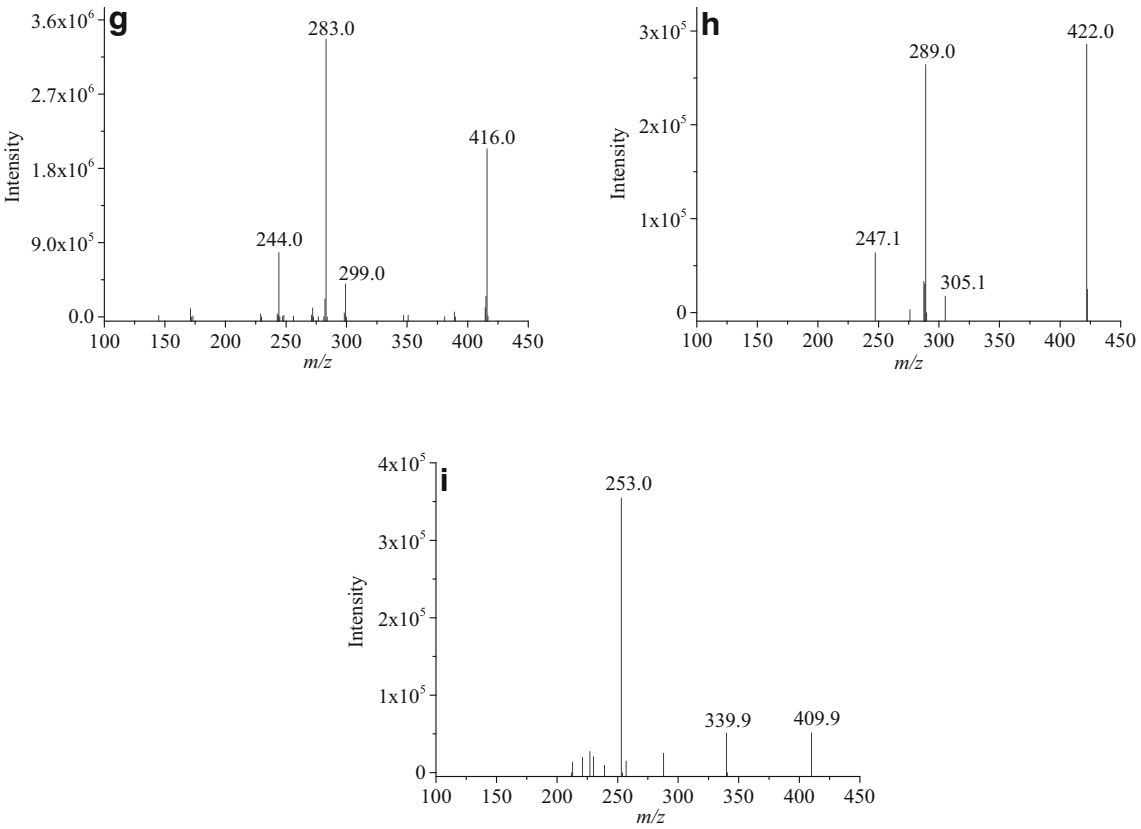

Fig. 3 (continued)

100 μg mL^{-1} and stored in a refrigerator at − 18 °C in the dark.

For the standard working solution, a proper amount of the standard stock solution was mixed and then diluted to 1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 ng mL^{-1} with acetonitrile.

The standard isotopically working solution (0.1 μg mL^{-1}) mixed 0.1 mL of four isotopically internal standard solutions (100 μg mL^{-1}) in a 100-mL volumetric flask, and then diluted to scale with acetonitrile.

Matrix-matched calibration solution were prepared by taking blank samples without fipronil or the four metabolites and processing them according to the "QuEChERS method procedure" step to obtain six "supernatant acetonitrile layers," and then 0.2 mL of the standard isotopically working solution (0.1 μg mL^{-1}) was added into each of six "the supernatant acetonitrile layer." "The supernatant acetonitrile layer" were evaporated to dryness with nitrogen at 30 °C. Then, 1.0 mL of the standard working solutions was added to the dried extract, and the final concentration levels of the matrix-matched calibration standards were 1.0, 2.0, 5.0, 10.0, 20.0, and 50.0 ng mL^{-1}. All four isotopically labeled internal standards had concentrations of 20 ng mL^{-1}.

GC – NCI – MS/MS Analysis Conditions

A Thermo Fisher Trace 1300 GC coupled with a Thermo Fisher TSQ 8000 triple quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was used for GC–NCI–MS/MS analysis. Analytes were separated on an HP-5 MS 30 m × 0.25 mm × 0.25 μm capillary column. The carrier gas was helium (99.9999% purity) at a flow rate of 1.0 mL min^{-1}. Methane acted as the reaction gas. The oven temperature was initially 60 °C (held for 1 min), and the temperature was increased to 200 °C at 30 °C min^{-1}, and then increased to 300 °C at 15 °C min^{-1} and held for 5 min. The injector temperature was set to 240 °C. The injection mode was splitless, and the injection volume was 1.0 μL.

The mass spectrometer was operated in negative chemical ionization source (NCI) mode, and the reaction gas was methane. The temperatures of the ion source and transfer line were 200 °C and 250 °C, respectively. Multiple reaction monitoring (MRM) mode was used for quantitative analysis; each compound had one precursor ion and two product ions. Table 1 shows the analytical conditions for GC–NCI–MS/MS, including the retention times, precursor ions, product ions, and their optimal collision energies.

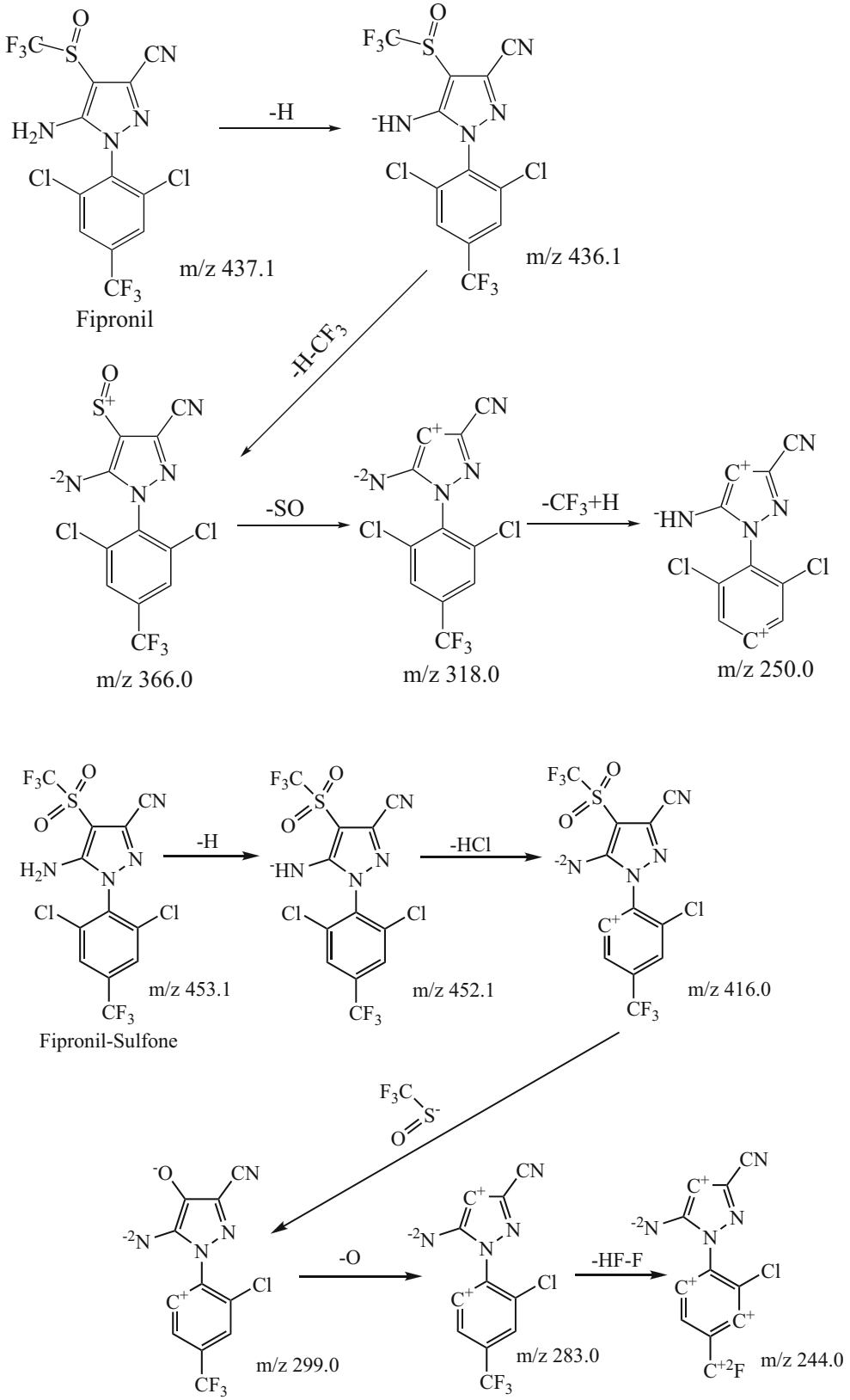

Fig. 4 Proposed fragmentation pattern with structures for the productions of fipronil and four metabolites

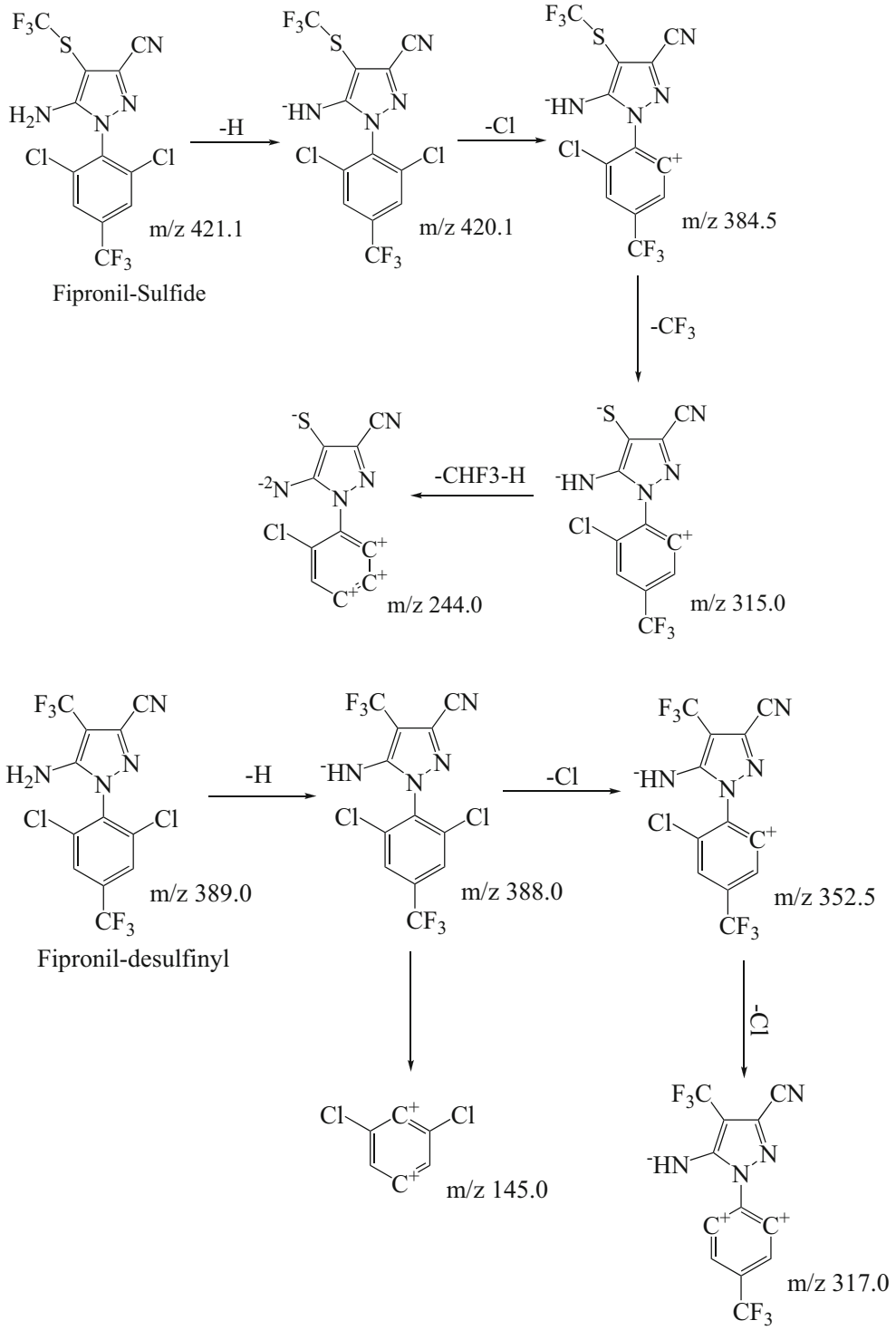

Fig. 4 (continued)

Results and Discussion

Optimization of GC – NCI – MS/MS Conditions

To accurately identify fipronil and four metabolite residues in foodstuffs of animal origin, mass spectrometric conditions were optimized first. Because fipronil and its metabolites have many halogen atoms, including fluorine and chlorine, they are very electronegative. Compared with EI, NCI is more suitable for analysis of electronegative groups, and NCI has a lower noise background and higher sensitivity. Therefore, we chose NCI as the ion source. Full GC–MS scans were performed in the m/z 100–500 range to determine the precursor ions for each standard. We chose the most abundant and most stable

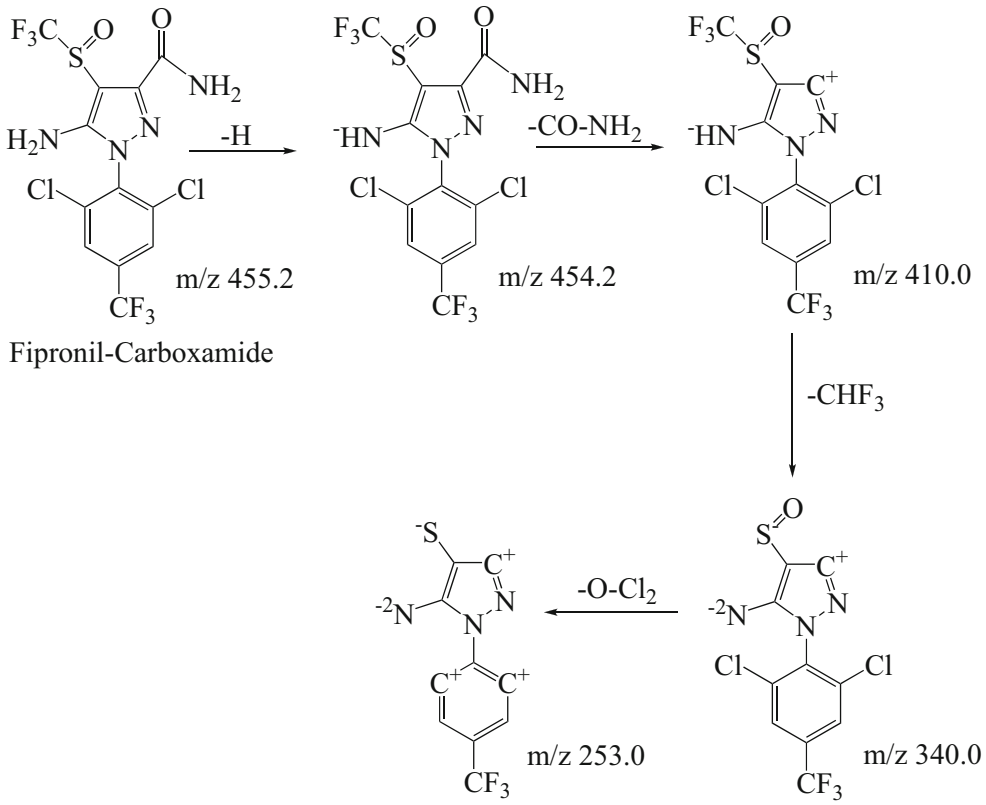

Fig. 4 (continued)

ions as precursor ions. In the process of selecting precursor ions, high-mass ions are preferred because they can effectively avoid the interference of isobaric masses caused by common molecular fragments [Rodríguez-Carrasco et al. 2012]. After selecting the precursor ion, the collision energy was optimized, the strongest fragment peak was searched, and the most abundant fragments were selected as qualitative and quantitative ions. This optimization procedure was strictly in accordance with the European Union directive [SANTE/11945/2015]. In accordance with this directive for quantitative mass spectrometric detection, a minimum of three identification points was required to meet this directive. In the GC–MS/

Table 2 Mean recoveries and precision of the developed method at three concentration levels with LOQs ($n = 6$)

Sample	Spiking Level (μg kg^{-1})	Recovery (%)	RSD (%)	LOQ (μg kg^{-1})
Fipronil	1.0	81.1	7.9	0.1
	5.0	89.5	5.3	
	10.0	93.2	3.4	
Fipronil sulfide	1.0	82.2	7.5	0.1
	5.0	90.3	5.4	
	10.0	105.4	3.8	
Fipronil desulfinyl	1.0	79.6	6.6	0.1
	5.0	85.4	4.8	
	10.0	95.3	2.9	
Fipronil sulfone	1.0	78.9	7.1	0.2
	5.0	88.5	5.5	
	10.0	107.1	3.6	
Fipronil carboxamide	1.0	78.2	8.5	0.2
	5.0	90.6	6.4	
	10.0	105.2	3.5	

MS analysis, a total of four points can be obtained (1 for the precursor ion and 1.5 for each product ion). Table 1 lists the optimized analytical conditions.

Figure 2 is an MRM chromatogram of a real blank beef liver sample spiked with all five standards and four isotopically labeled internal standards at 2.0 μg kg^{-1}. The background of the spiked sample is very clean, indicating that NCI can effectively avoid interference from impurities in the sample. However, the spectra obtained by GC–EI–MS/MS showed impurity peaks near the target peaks. Therefore, the selectivity of GC–NCI–MS/MS is better than that of GC–EI–MS/MS.

Figure 3 shows the GC–NCI–MS/MS product scan spectra of fipronil, four metabolites, and isotopically labeled internal standards. On the basis of the mass spectrometric fragmentation mechanism, we tried to derive the possible NCI–MS/MS fragmentation pathways of fipronil and its four metabolites (the isotopically labeled internal standards were not analyzed due to having the same structure) (Fig. 4). In the past, there was no relevant literature to report such a fragmentation mechanism. Thus, we are the first to propose the fragmentation mechanism of fipronil and its four metabolites in NCI–MS/MS.

Optimization of the QuEChERS Method

In the process of QuEChERS, the selection of extraction solvent is very important, as it can affect the efficiency of extraction and purification. Acetonitrile and a weakly acidic buffer are typically used as QuEChERS extraction solvents, so we compared different extraction solvents, including pure acetonitrile, 0.1% acetic acid in acetonitrile, and 0.5% acetic acid in acetonitrile. The extraction efficiency was highest when pure acetonitrile was used (82.5–107.4%). Therefore, pure acetonitrile was used as the QuEChERS extraction solvent.

The choice of adsorbent is another important factor of the QuEChERS method, as the adsorbent should effectively remove impurities and not affect the recovery rate of the target compounds. PSA and C_{18} are commonly used adsorbents for QuEChERS. PSA is mainly used to remove polar impurities, and C_{18} is mainly used to remove nonpolar impurities such as fats. $MgSO_4$ acts as a desiccant to remove residual moisture from organic extracts.

The object of this study was animal foodstuffs, which have a relatively high fat content, so fat removal was required. The first step in the extraction used acetonitrile (saturated with n-hexane) to reduce the amount of fat dissolved in acetonitrile; the second step used n-hexane (saturated acetonitrile) twice to remove fat with liquid-liquid separation, and the C_{18} adsorbent was added in the third step and used to further remove fats. The modified QuEChERS method in this study, including three steps to remove fat from animal foodstuffs, achieved a good purification effect. At the same time, a highly sensitive NCI source was used, and there was no sample matrix interference at the peak of the target compound, so the above purification steps were sufficient.

Matrix Effects

The matrix effect is a key parameter in mass spectrometry analysis and could cause the mass spectrometer signals to strengthen or weaken, thus affecting the accuracy of the analytical method [Lehotay et al. 2010; Yu and Xu 2012]. Isotopically labeled internal standards are one of the most effective methods to overcome the matrix effect [Hou et al. 2019]. All target analytes except fipronil carboxamide have commercially available isotopically labeled internal standards, because the structures of fipronil carboxamide and fipronil compounds are the most similar, and the $^{13}C_4$,$^{15}N_2$-fipronil isotopically labeled internal standard is also used in the quantitative analysis of fipronil carboxamide. In this study, four isotopically labeled internal standards were dissolved in matrix-matched calibration solutions, and good experimental results were obtained.

Linearity and Quantification Limits

The matrix-matched calibration standards curve had seven concentration points as follows: 1.0, 2.0, 5.0, 10.0, 20.0, 50.0, and 100.0 ng mL^{-1}. All four isotopically labeled internal standards had concentrations of 20 ng mL^{-1}. Under this condition, the standard curves obtained good linearity, and the linear coefficients ranged between 0.9988 and 0.9995. The LOQ was calculated as 10 times the signal-to-noise ratio, and it was 0.2 μg kg^{-1} for fipronil carboxamide and fipronil sulfone and 0.1 μg kg^{-1} for fipronil, fipronil sulfide, and fipronil desulfinyl (GC–NCI–MS/MS). The LOQ was 1.0 μg kg^{-1} for fipronil carboxamide and fipronil sulfone and 0.5 μg kg^{-1} for fipronil, fipronil sulfide, and fipronil desulfinyl (GC–EI–MS/MS).

Precision and Accuracy

Intraday and interday repeatability was tested with three spiked samples at different concentrations (low, medium, and high) to assess the accuracy of the method. The relative standard deviation (RSD) values were less than 3.2%, and the results showed good stability and repeatability.

The accuracy of the method was evaluated by adding a recovery experiment. The average recoveries at the three spiking levels were 78.2–107.1% (Table 2), and the relative standard deviation (RSD) was less than 8.5%, indicating that this method is accurate and has good precision.

Application to Real Samples

Using the method established in this study, 60 real foodstuffs of animal origin (egg, milk, beef kidney, beef liver, poultry meat, and poultry offal, with ten samples of each matrix) were purchased randomly from farmers' markets and supermarkets from different regions and analyzed. The results showed that residue of fipronil sulfone was detected in one egg sample, and the detected value was 10.2 μg kg^{-1}.

Conclusions

In this study, a modified QuEChERS method was used for extraction and purification, and a gas chromatography–negative chemical ionization–tandem mass spectrometry (GC–NCI–MS/MS) method was developed for the determination of fipronil and its four metabolites (fipronil carboxamide, fipronil sulfone, fipronil sulfide, and fipronil desulfinyl) in foodstuffs of animal origin. By selecting the NCI source, MRM mode, and quantification by isotopically labeled internal standards, matrix interference was reduced, and the method had high sensitivity and accuracy (recovery 78.2–107.1%, precision RSDs < 8.5%), which can satisfy the detection and quantitative analysis of fipronil and its four metabolite residues in foodstuffs of animal origin. Based on the NCI − MS/MS spectrogram of the compound, the structural analysis of fipronil and its four metabolites was carried out to help identify the compounds.

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Compliance with Ethical Standards

Conflict of Interest Chao Han declares that he has no conflict of interest. Beizhen Hu declares that she has no conflict of interest. Zhou Li declares that he has no conflict of interest. Caiqin Liu declares that she has no conflict of interest. Nan Wang declares that she has no conflict of interest. Changchun Fu declares that she has no conflict of interest. Yan Shen declares that she has no conflict of interest.

Ethical Approval This article does not contain any studies with human or animals.

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