Combination of High Specific Activity Carbon-14 Labeling and High Resolution Mass Spectrometry to Study Pesticide Metabolism in Crops: Metabolism of Cycloxaprid in Rice

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Combination of high specific activity carbon-14 labeling and high resolution mass spectrometry to study pesticide metabolism in crops: Metabolism of cycloxaprid in rice

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Abstract

The study of pesticide metabolism in crops is critical for assessing the mode of action and environmental risks of pesticides. However, the study of pesticide metabolism in crops is usually complicated and it is often a daunting challenge to accurately screen the metabolites of novel pesticides in complex matrices. This study demonstrated a combined use of high-specific activity carbon-14 labeling and high-resolution mass spectrometry (HSA-14C-HRMS) for metabolism profiling of a novel neonicotinoid cycloxaprid in rice. By generating the characteristic radioactive peaks on the liquid chromatogram, the use of 14C can eliminate the severe interference of complex matrices and quickly probe target compounds; by producing ion pairs with unique abundance ratios on HRMS, high-specific activity labeling can effectively exclude false matrix positives and promote the elucidation of metabolite structure. The structures of 15 metabolites were identified, three of which were further confirmed by authentic standards. Based on these metabolites, a metabolic profile of cycloxaprid was established, which includes denitrification, demethylation, imidazolidine hydroxylation and ring cleavage olefin formation, oxidation and carboxylation reactions. The strategy of combining high-specific activity 14C labeling and HRMS offers unique advantages and provides a powerful solution for profiling unknown metabolites of novel pesticides in complex matrices, especially when traditional non-labeling methods are not feasible.

1. Introduction

Understanding pesticide metabolism in crops is essential for pesticide development, safe use, and developing environmental remediation strategies (Zhang and Yang, 2021). High-resolution mass spectrometry (HRMS) is a state-of-the-art tool for the qualitative identification of the structure of unknown metabolites, based on analysis of accurate mass precursors and fragment ions (Kern et al., 2009; Fenner et al., 2013; Liberatore et al., 2020; Flasch et al., 2020). However, the biggest challenge in elucidating metabolites with HRMS, is the rapid and efficient selection of target peaks from numerous MS features in the total ion chromatogram (TIC). This is particularly restrictive in biological matrices such as plant tissues, where the metabolites of chemicals such as pesticides generally occur at trace levels, along with many much more abundant endogenous biomolecules (Fu et al., 2018). Furthermore, standards for metabolites are not usually commercially available, which makes their characterization even more challenging. The presence of characteristic structures (e.g., Cl, Br) can provide further assistance; for example, when a compound contains a Cl atom, its metabolites can be easily detected by the isotopic ratio of 35Cl/37Cl in mass spectra (Cariou et al., 2016). However, this still has significant disadvantages: first, the natural presence of Cl-containing compounds still leads to partial false-positive peaks (Qiu et al., 2021); second, Cl atoms may detach during metabolism, which leads to dechlorinated metabolites, which are not easy to be detected (Xue et al., 2017; Feung et al., 1975).

Recently, the combination of stable isotope labeling and HRMS has been considered as a promising option for chemical metabolite analysis (Fu et al., 2018). The use of stable isotope labeling can create artificial isotope patterns that can easily be detected with HRMS by introducing heavy isotopes of common elements (e.g., 12C, 15N, 2H, 34S) (Bueschl

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et al., 2014; Zachleder et al., 2018; Doppler et al., 2019). Subsequently, some of the automated analytical software, such as HTIME and MetExtract, enables the extraction of “twin-ion” from HRMS data to help screen metabolites (Kuhl et al., 2011; Kluger et al., 2013; Bueschl et al., 2012; Ceraní et al., 2020). By applying $^{13}$H or $^{13}$C labeling, the combination of stable isotope labeling and HRMS has been used to elucidate the metabolism of pesticides and pharmaceuticals in various biological matrices (Kluger et al., 2013; Takahashi et al., 2018). However, the combination of stable isotope labeling and these software still has great limitations and uncertainties in practical applications (Chokkathukalam et al., 2014; Fu et al., 2018). For example, a large number of false-positive peaks may be screened out from the MS data, and further manual investigation of these peaks is very time-consuming in practical use (Qiu et al., 2021; Tian et al., 2018).

Radioisotope labeling, combined with chromatographic separation and HRMS, can quickly locate the retention time of metabolites in chromatograms to eliminate the interference of nontarget organic compounds (Meermann et al., 2012; Zhao et al., 2020). However, in the conventional radioisotope labeling, the specific radioactivity of compounds is usually low and cannot form ion pairs in the mass spectrum as stable isotope labeling does (Fu et al., 2013; Li et al., 2013). Therefore, it is still difficult to achieve fast and simple screening of unknown metabolites from a large number of candidates by conventional radioisotope labeling. Adding further diagnostic characteristics to compounds is a very efficient strategy for achieving higher detection. High specific activity $^{14}$C labeling is supposed to create unique isotopic ion pairs in mass spectra and overcome the disturbing ion suppression effects caused by matrices such as Cl. Therefore, the high-specific activity $^{14}$C labeling is likely to combine the advantages of conventional radioisotope labeling and stable isotope labeling and greatly improve the efficiency and accuracy of metabolite identification. Even if there is no halogen in the parent, possible metabolites can be quickly targeted by monitoring ion pairs with unique abundance ratios in the mass spectrum. However, up to now, few studies have utilized the advantages of the unique isotopic characteristics created by high-specific activity $^{14}$C labeling for metabolic studies.

In this study, we evaluated the feasibility of combining high-specific activity carbon-14 labeling and HRMS (HSA-$^{14}$C-HRMS) to study pesticide metabolism in crops. Cycloxaprid, a promising novel neonicotinoid insecticide developed for agricultural pest control due to its low toxicity to mammals and bees, was selected as the model compound and labeled with $^{14}$C at the high-specific activity. Rice, a common staple crop and the main crop to which cycloxaprid is applied, was selected as the model plant matrix. The workflow includes the rough probing of target compounds by the radioactive peaks of $^{14}$C on the liquid chromatogram, the screening of metabolite molecular ions by the ion pairs with unique abundance ratios on HRMS generated by high-specific activity labeling, and the metabolite structure elucidation by MS/MS ion fragments with unique abundance ratios. A total of 15 metabolites were identified and the possible metabolic mechanism and pathways of cycloxaprid in rice were proposed. The established method is expected to be broadly applied to numerous new compounds in various environmental matrices.

2. Materials and methods

2.1. Chemicals

The $^{14}$C-cycloxaprid (5-(6-chloro-pyridin-3-ylmethyl)-7-nitro-11-oxa-2,5-diaza-tricyclo[6.2.1.0$^\text{1,6}$]undec-6-ene) was synthesized in the lab and the synthesis route is shown in Figure S1. The specific activity of $^{14}$C-cycloxaprid was 29.50 mCi mmol$^{-1}$, and the radiochemical and chemical purity were both $>$98%. Here, a specific activity of $\geq$20 mCi mmol$^{-1}$ was defined as high-specific activity labeling for a single $^{14}$C atom labeling by a series of previous experiments, which was shown to be sufficient to creat unique isotopic ion pairs in mass spectra and overcome the disturbing ion suppression effects caused by impurities such as chlorine-containing compounds. The non-labeled cycloxaprid was provided by Shanghai Shengnong Pesticide Co., Ltd (Shanghai, China). The standard of (nitromethylene)imidazole (NMI) was provided by the Key Lab of Chemical Biology, School of Pharmacy, East China University of Science and Technology (Shanghai, China). N-(6-chloropyridin-3-yl)methyl) formamide and 1-(6-chloropyridin-3-yl)methyl imidazolidin-2-one were synthesized by Kainou Chemical Technology Inc. (Shanghai, China). The preparation methods of cocktail A (used for quantifying the radioactivity of the liquid extracts) and B (used for trapping $^{14}$CO$_2$) are described in the previous literature (Wang et al., 2013). All solvents used were HPLC grade purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China).

2.2. Experimental setup

The metabolism of cycloxaprid in rice experiment was designed and conducted according to OECD (Organization for Economic Co-operation and Development) guideline for the testing of chemicals (OECD501: Metabolism in Crops) (OECD, 2007). At the beginning of the rice heading stage, the $^{14}$C-cycloxaprid (concentration of 17.77 mCi L$^{-1}$) was uniformly sprayed on rice plants with two times the maximum field recommended dosage (active ingredient 90 g ha$^{-1}$ for rice) using a calibrated automatic sprayer. Detailed information on rice cultivation is provided in the Support information (SI). The average dose per plant after treatment was 0.0173 mCi, and no phytotoxic effects of cycloxaprid on rice were observed during cultivation. Plant tissues were collected at 3 h, 5 d, 10 d, 15 d, 21 d and 35 d after the insecticide applications, with three replications per time point and eight plants per replication. The entire rice plants (rootless) were collected randomly using scissors, and divided into leaves and straw (LS) and panicle. The LS tissues were cut into small pieces of $<$1 cm, freeze-dried at $-$80 °C for 72 h and ground into a homogenized powder. The panicle samples were oven-dried at 50 °C for 48 h, and the 0, 5, 10 and 15 d samples were directly ground into powder, while 21 and 35 d samples were hulled into husks and rice by an automatic rice husker (TR-250; Kett Electric Laboratory, Tokyo, Japan) before being ground. The rice tissues were combusted in an OX501 biological oxidizer (R.J. Harvey Instruments Co., NJ, USA) and radioactivity trapped as $^{14}$CO$_2$ was quantified by a liquid scintillation counter (LSC; Tri-Carb 2910; PerkinElmer, Turku, Finland). The remaining powder was stored at $-$80 °C until further sample preparation.

2.3. Sample extraction and cleanup

The obtained powder (LS, panicle, husk [1 g], and rice [5 g]) was extracted with 15 mL of the following solutions, sequentially: water (six times), acetonitrile (twice) and methanol. The samples were vortexed for 2 min, mechanically shaken for 1 h, extracted by ultrasonication for 30 min, and then centrifuged at 10,000 rpm at 4 °C for 10 min. The organic and water extracts were collected to 50-mL and 100-mL volumetric flasks and made up to volume with acetonitrile and water, respectively. An aliquot (1 mL) of each extract was mixed with cocktail A to measure radioactivity with LSC. The extraction recovery was shown to be $>$95% after the sequential solvent extraction by the radioactivity analysis. The sum radioactivity of all extracts was defined as the extractable residue (ER), and the remaining $^{14}$C residue was defined as bound residue (BR).

The aqueous extracts (100 mL) were freeze-dried and re-dissolved in 1 mL methanol. The organic extract was concentrated to near dryness in a rotary evaporator at 35 °C and then re-dissolved in 1 mL methanol. The above extracts were combined and transferred to a ProElut CARB column (Dikma, Beijing, China), and eluted with 10 mL of methanol: water (8:2, v/v), methanol and methanol: toluene (3:1, v/v). The eluted fractions were collected and 0.5 mL was taken to measure the radioactivity. The remaining fractions were evaporated to dryness in a nitrogen
evaporator. All the radioactive fractions were re-dissolved with 1 mL of acetonitrile:water (1:1, v:v) and filtered through a 0.22-μm nylon syringe filter (Jinteng, Tianjin, China). The final samples were stored in 2-mL amber borosilicate vials at 4 °C before analysis.

2.4. HPLC-LSC analysis

A 20-μL aliquot of the concentrated extract was analyzed using a Waters 2695 HPLC system, equipped with Waters 2998 photodiode array detector (Waters, MA, USA) and a reversed-phase C18 column (Diamonsil, 5 μm, 250 × 4.6 mm; Dikma, Beijing, China). The mobile phase started with 95% water/0.1% acetic acid (A) and 5% acetonitrile/0.1% acetic acid (B). The gradient was held for 10 min and changed to 10% B at a constant rate after 60 min; after 2 min the mobile phase changed to 100% B and held for 10 min before returning to starting conditions. The column temperature was kept at 30 ± 2 °C, and the flow rate was maintained at 1 mL min⁻¹. The post-column eluent was collected using a Waters Fraction Collector III (Waters, MA, USA) at 1 min intervals and mixed with 10 mL of cocktail A. After dark acclimation for 24 h, the 13C radioactivity was measured by LSC. The radiochromatograms were plotted based on the radioactivity content and retention time of each fraction on the chromatogram.

2.5. HPLC-MS/MS analysis

To identify the structures of metabolites, the final samples were analyzed by coupling the Agilent 1100 series HPLC system (Agilent Technologies, CA, USA) with an ultra-high-definition quadrupole time-of-flight mass spectrometer (Agilent 6530; Agilent Technologies, CA, USA) equipped with an electrospray ionization source. The HPLC mobile program was the same as shown in Section 2.4. The mass spectrometer was operated in positive ion mode, using the following operation parameters: ion source temperature of 250 °C, capillary voltage of 3.5 kV, drying gas-flow of 10 L min⁻¹ and nebulizer pressure of 30 psi. The mass spectra were collected using full scan mode, in the range of 20–1000 m/z. After screening, the eligible precursor ions were subjected to target MS/MS analysis. The isolation width was set to be 4 m/z, and the collision energy range was 5–50 eV.

2.6. Isotope abundance calculation

The radioactivity is the number of decays per unit time, which can be calculated from the equation (Kratz and Lieser, 2013):

\[
A = \frac{dN}{dt} = \lambda N
\]  

(1)

where \(A\), \(\lambda\), and \(N\) were the radioactivity (Bq, 1 Bq = 3.7 × 10⁻¹⁰ Ci), the decay constant, and the number of atoms, respectively. The decay constant \(\lambda\) is a probability per unit time that an individual atom decays, and which can be expressed in terms of the half-life \(T_{1/2}\) of the substance, \(\lambda = \ln 2/T_{1/2}\). The total atoms \(N\) can be obtained by multiplying the amount of substance \(n\) with the Avogadro’s number \(N_A\), \(N = nN_A\). According to eq (1), the formula for calculating isotopic abundance of carbon-14 (δ¹⁴C) in a molecule can be deduced (eq (2)):

\[
\delta = \frac{SA \times T\%}{\ln 2 \times N_A}
\]  

(2)

The SA is the specific activity of the radionuclide (Bq mol⁻¹). The δ¹⁴C was calculated to be 47.28%, and the δ¹²C value was correspondingly determined to be 52.72%. Therefore, the abundance ratio of 12C:13C was about 10.9. The isotopic distribution of 14C-cycloxaprid could be described by the following product of polynomials (Yerget, 2020):

\[
(a_1 + a_2)^m (b_1 + b_2)^n
\]  

(3)

where \(a_1\), \(a_2\) and \(b_1\), \(b_2\) respectively represent the abundance of each isotope of each element in the molecule (i.e., the abundance of 35Cl, 37Cl, 13C, and 14C). The exponents \(m\) and \(n\) were the numbers of atoms of each isotope, respectively. There is only one chlorine atom and one carbon-14 atom in one 14C-cycloxaprid molecule, so the \(m\) and \(n\) are both 1. The natural abundances of 13C, 2H, 17O, 18O, and 15N were not included in this calculation due to their negligible contributions to the calculation. The natural isotopic ratio of 35Cl:37Cl is 75.77%:24.23%. According to this binomial expansion shown above, the abundance ratio of M:(M + 2):(M + 4) was determined to be 39.95%:48.50%:11.46%. The M + 4 ion peak was not considered for target spectrum screening due to its low relative abundance. Therefore, for 14C-cycloxaprid, the abundance ratio of M: M + 2 was 39.95%:48.50%, about 4:5, which could be used for metabolite screening.

2.7. Data processing

For the identification of metabolites, the retention time of metabolites in mass spectra was first determined by radioactivity, and then the data files from MS were manually screened using Agilent Mass Hunter software. Potential candidate metabolites can be easily screened according to the following criteria: (1) peaks detected at a time period consistent with radioactivity; (2) the presence of M and M + 2 ion pairs; (3) the M/(M + 2) abundance ratio of 4:5; (4) good peak shape. In addition, if the Cl atom is lost, the compound would lose the isotopic contributions of Cl in its mass spectrum. In this case, the ratio of M/(M + 2) only depends on the abundance ratio of 12C:14C, that is, dechlorinated compounds need to be screened at the 10:9 ratio. From this, the exact m/z of each candidate was obtained. A reasonable formula was calculated by a molecular formula generator, and the exact mass differs from the theoretical postulated mass by less than 5 ppm. The eligible precursor ions underwent target MS/MS analysis to obtain the fragmentation spectra. The compound structures were derived from analysis of the fragmentation spectra, based on the nitrogen-containing rule and reasonable loss of debris. The preliminary structure of each candidate was proposed by using MassBank and MassHunter databases to simulate the fragmentation patterns, and comparison with experimentally-derived fragmentations. The metabolites were selected for custom synthesis to confirm the authenticity of the proposed structure.

All the samples were taken in triplicate. Statistical analysis, by one-way analysis of variance (ANOVA) and Duncan test, was performed with SPSS 24.0 (IBM SPSS Statistics, NY, USA). Graphs were prepared using Origin 9.0 (MicroCal Software, MA, USA).

3. Results and discussion

A sketch map was utilized to demonstrate the expected advantages and superiorities of using HSA-13C-HRMS. As shown in Fig. 1, the use of high-specific activity 14C labeling was expected to provide unique diagnostic characteristics for profiling the metabolites of cycloxaprid. Comparing to the traditional non-labeling method or stable-isotope labeling, the HPLC retention time of 14C-metabolites can be quickly determined by radioactivity. Comparing to the conventional 14C labeling method, the use of high-specific activity of 13C labeling can achieve further screening in the MS features within the retention time by creating a specific proportion of ion pairs (M and M + 2), thus significantly improving the efficiency and accuracy of screening. For instance, the ion pairs of M and M + 2 should be consistent with δ¹³C:δ¹⁴C for the metabolites labeled 13C but without Cl. Similarly, the abundance ratio of M:(M + 2) should be about 4:5 for the metabolites with both labeled 14C and Cl atom, as depicted in Section 2.6.
3.1. Workflow for the combined use of high-specific activity carbon-14 labeling and HRMS for metabolite probing

A stepwise framework is presented in Fig. 2 to demonstrate the workflow for metabolite probing using a combination of high-specific activity carbon-14 labeling and HRMS. First, the extracts were subjected to HPLC-LSC analysis, and retention time and the radioactivity of metabolites were determined, which could be used for roughly probing the

Fig. 1. Schematics of studying the metabolism of pesticide using strategies of non-labeling (A), conventional 14C labeling (B), and high-specific activity 14C labeling (C).

Fig. 2. Workflow of the high-specific activity carbon-14 labeling combined with high-resolution mass spectrometry for metabolite probing. The asterisk represents the position of 14C labeling.
target compound (Fig. 2A). After then, the ion pairs of M and M + 2 with unique abundance ratios generated by high-specific activity 14C labeling were screened within a fixed time range in mass spectra and the molecular ion peaks of metabolites were determined (Fig. 2B). Subsequently, target MS/MS analysis was performed on the screened ion peaks, and the structures of candidates were elucidated and the false matrix positives were excluded by the ion fragments with unique abundance ratios (Fig. 2C). Finally, some of the proposed metabolites were selected for custom synthesis and used for metabolite structure verification by spectra comparison (Fig. 2D).

3.2. Locating of metabolites by radioactivity

The concentrated extracts from different rice sections were fractionated and analyzed with HPLC-LSC. The unknown metabolites of 14C-cycloxaprid were screened and targeted by their radioactive characteristics: 15 radioactive bands were detected. The retention time of the parent cycloxaprid was 64 min, and the metabolites were named in order of retention time. Figure 52 shows the radioactive metabolites extracted from the rice, 3 h, and 35 d after cycloxaprid spiking. The M1, M2, M4, M6, M9, M14, and M15 were present in the panicle extracts at 3 h, with retention times of 6, 8, 22, 34, 41, 54, and 69 min, respectively (Figure S2A). At 35 d, in husk extracts, there were eight additional metabolites: M3, M5, M7, M8, M10, M11, M12 and M13, with retention times of 17, 33, 35, 39, 42, 44, 48, and 52 min, respectively (Figure S2B).

The parent cycloxaprid was not detected, even 3 h after treatment, indicating the rapid metabolism of cycloxaprid after application. This could be attributed to the unstable oxabridged seven-member ring, in which the oxabridge bond is easy to break and form the open-chain product. The rapid degradation of cycloxaprid has also been observed in oxic and anoxic soils (Chen et al., 2017; Liu et al., 2015). Previous studies have shown that cycloxaprid is easily converted to the active metabolites. Chloropyridinylcarboxylic acid, a common metabolite of pesticides through amidation has rarely been reported before. In addition, thiamethoxam and clothianidin can be conjugated with glycine or reactions with glucoside and gentiobioside in plants (Casida, 2011), and usual way for molecules to increase in mass is by binding to another group, such as a glucoside or amino acid, which are two common secondary metabolites. Chloropyridinylcarboxylic acid, a common metabolite of chloropyridinyl neonicotinoids, undergoes various conjugation reactions with glucoside and gentiobioside in plants (Casida, 2011), and thiamethoxam and clothianidin can be conjugated with glycine or acetylated in spinach (Ford and Casida, 2008); however, the metabolism of pesticides through amidation has rarely been reported before.

Using the same strategy, 15 metabolites with the above ion pairs and diagnostic features were detected. Table 1 shows all the metabolites identified during the incubation period. Additional data on the structural identification is provided in SI (Figure S3-S18). The confidence levels for metabolites identification were defined according to the criteria established by Strickman et al. (Schymanski et al., 2014). Three metabolites were qualified for Level 1 (confirmed structure), nine metabolites were identified as Level 2b (probable structure), and the remaining three metabolites were assigned as Level 3 (tentative candidate). Except for M2 and M14, the other 13 metabolites were newly discovered in this study (Chen et al., 2017; Hou et al., 2017). The chloropyridinylmethyl moiety was detected in all metabolites, indicating that cycloxaprid might not be prone to dechlorination in rice. For organochlorine compounds, reductive dechlorination is often observed.
under anoxic conditions, which creates suitable microenvironments for microbial degradation (Chen et al., 2017). Dechlorinated products of cycloxaprid have been detected in anoxic soil but not in oxic soil (Chen et al., 2017; Liu et al., 2015). It should be mentioned that the metabolites cannot be detected if the carbon-14 atom is detached. The potential solution is to label multiple positions, which could lead to the discovery of additional metabolites in complex matrices (Wang et al., 2014).

3.4. Verification by comparison with the authentic standards

The structures of metabolites were further verified by comparison with the authentic standards, as far as possible. There were several major critical fragments in the MS/MS spectrum of M14. The m/z 221.0556 ion suggested the reduction of nitro to amino, and m/z 208.0635 suggested the loss of a nitro group. The m/z 126.0083 and 98.0726 ions were generated after the C-N bond connecting pyridine and imidazolidine in the m/z 221.0556 ion was broken. Based on the MS/MS analysis, M14 was presumed to be 1-(6-chloronicotinyl)-2-nitromethylene-imidazolidine (NMI). The MS/MS spectrum of M14 shows that when Cl and $^{14}$C were co-present in the fragment ions, there was an ion pair of M and M + 2, and the abundance ratio of M to M + 2 was about 4:5 (Fig. 3A1). The fragmentation pattern of M14 was consistent with that of the authentic standard, but the abundance ratio of the authentic standard is 3:1, which conforms to the isotopic ion abundance ratio of a Cl atom (Fig. 3A2). In addition, the retention time of M14 was the same as that of the standard. In conclusion, M14 was confirmed to be NMI. NMI is easily formed by cycloxaprid hydrolysis or photolysis, and is also the main metabolite of cycloxaprid in oxic soil (Chen et al., 2017; Hou et al., 2017). Furthermore, NMI is considered to be the insecticidal activity of cycloxaprid, and bioassays show that it has higher toxicity to nontarget organisms than cycloxaprid (Shao et al., 2013): NMI should, therefore, be taken into account when the limit standards for cycloxaprid residues are formulated.

M5 and M15 were also selected for custom synthesis to validate the proposed structure directly. The chromatographic behavior and mass spectral characteristics of the standards were the same as those of the metabolites. The abundance ratio of the ion pairs of metabolites was 4:5, and that of non-labeled standards was 3:1 (Fig. 3B and C): M5 and M15 were positively identified as N-((6-chloropyridin-3-yl)methyl)formamide and 1-((6-chloropyridin-3-yl)methyl)imidazolidin-2-one, respectively.
respectively. M5 and M15 are trace level metabolites in cycloxaclid metabolism, and the confirmation of their structures demonstrates the effectiveness of this approach in detecting low-abundance metabolites. The validation of these three metabolites provides compelling evidence that the high-specific activity $^{14}$C-labeling trace is an effective technique for identifying unknown metabolites, with high confidence in complex matrices.

3.5. The metabolism dynamics of cycloxaprid and metabolites

As an additional benefit, radiolabeling allows quantitative analysis of metabolites in the absence of reference standards, which simultaneously helps to elucidate the structure of metabolites and metabolism pathways (Nassar et al., 2003). The amounts of $^{14}$C-ER in different parts of rice were presented in Table S1. The metabolism dynamics of cycloxaprid and its metabolites in rice were estimated by the relative ratio of radioactivity in ER. As shown in Tables 2 and 3, cycloxaprid was rapidly transformed into various metabolites in rice tissue extracts, even after 3 h of treatment. In the panicle, M1 concentration increased until 15 d, and then gradually decreased from a maximum of 14.33 ± 1.31% of ER. However, in the LS, the M1 level remained consistent, suggesting that the metabolic processes of cycloxaprid may vary with tissue type. M2 increased to a maximum at 5 d of 39.59 ± 1.72% and 46.28 ± 4.30% of ER in LS and panicle, respectively, and then gradually decreased. M2 was the most abundant metabolite of cycloxaprid in rice metabolism and is one of the main metabolites of cycloxaprid in oxic soil. M3 was detected in panicle at 5 d, but only at 10 d in LS, and the maximum content was only 2.93 ± 0.28% of ER. M4 was formed in the initial stage of the experiment (3 h after treatment), and its content gradually decreased with time: it could not be detected at 10 d, indicating that it only existed as a transient intermediate and underwent a rapid further transformation. M5 was detected at 5 d, and then increased slowly to a maximum at 35 d. M6 was detected only in the panicle of rice at 3 h, and then rapidly disappeared. It could not be detected in the extract at 5 d, indicating that it only existed as a transient intermediate and underwent a rapid further transformation. It was not detected in LS, probably due to its quick transformation into other metabolites. M7 was detected at 5 d, and then fluctuated slightly over time. Both M8 and M10 were detected at 5 d, and their content increased to a maximum at 35 d. M9 only occurred in the panicle of rice at 3 h; like M6, M9 was rapidly converted to other metabolites, indicating that it is not a persistent metabolite of cycloxaprid. M11 and M12 exhibited similar dynamics to M7. In LS, the content of M13 fluctuated slightly, with the maximum content only being 2.94 ± 0.12% of ER, while in panicle, the content fluctuated with time, with the minimum content being 1.85 ± 0.36% and the maximum content being 7.58 ± 1.32%. The M14 content was highest at 3 h, which was 10.11 ± 3.05% of ER, and then was gradually converted into other metabolites. A previous bioassay experiment has shown that M14 is much more toxic to nontarget organisms than cycloxaclid (Shao et al., 2013); that M14 could not be detected at 10 d suggests the transient presence of M14 in rice may reduce its cumulative risk to beneficial organisms. M15 was detected in extracts throughout the experiment, and its content generally remained unchanged. Other metabolites have not been confirmed because there were many trace metabolites below the detection limit and the retention time was too long to identify.

3.6. Proposed metabolism pathway

Based on the dynamic change of metabolites, and the structural logic, the probable metabolic pathways of cycloxaprid in the tested rice are proposed in Fig. 4. The degradation of cycloxaprid mainly occurred via two possible pathways.
Dynamics of metabolites of cycloxaprid in rice panicle (% of the total extractable radioactivity).

| Metabolites | Days after treatment |
|-------------|---------------------|
|             | 0                   | 5        | 10       | 15       | 21 (Husk) | 35 (Husk) | 21 (Rice) | 35 (Rice) |
| M1          | 4.00 ± 1.04c        | 6.48 ± 1.05b | 6.33 ± 1.72b | 14.33 ± 1.31a | 4.70 ± 0.36c | 3.99 ± 1.10c | 5.01 ± 0.24bc | 5.72 ± 0.26bc |
| M2          | 23.92 ± 2.55de      | 46.28 ± 4.30a | 34.85 ± 3.14b | 24.87 ± 4.59bcd | 32.41 ± 1.95bc | 22.77 ± 1.62c | 33.30 ± 0.88b | 29.40 ± 1.25bed |
| M3          | Nd                  | 2.28 ± 0.17ab | 1.87 ± 1.11b | 2.93 ± 0.28a | 2.38 ± 0.72ab | 2.60 ± 0.14ab | 1.81 ± 0.33b | 1.90 ± 0.36b |
| M4          | 8.53 ± 1.36a        | 2.08 ± 0.19b | Nd         | Nd         | Nd         | Nd         | Nd         | Nd         |
| M5          | Nd                  | 0.70 ± 0.28b | 2.00 ± 0.22ab | 2.22 ± 0.51ab | 3.05 ± 0.76a | 4.47 ± 0.23a | 2.45 ± 0.82a | 2.06 ± 0.49ab |
| M6          | 5.24 ± 1.00a        | Nd         | Nd         | Nd         | Nd         | Nd         | Nd         | Nd         |
| M7          | Nd                  | 2.08 ± 0.29c | 2.24 ± 0.17c | 2.84 ± 0.58bc | 3.65 ± 0.70a | 4.31 ± 0.05a | 3.48 ± 0.37ab | 3.94 ± 0.58a |
| M8          | Nd                  | 0.71 ± 0.77d | 1.94 ± 0.61d | 2.19 ± 0.52d | 4.59 ± 1.90c | 4.73 ± 0.62c | 10.40 ± 0.68b | 12.50 ± 1.01a |
| M9          | 1.39 ± 0.38a        | Nd         | Nd         | Nd         | Nd         | Nd         | Nd         | Nd         |
| M10         | Nd                  | 1.54 ± 0.44e | 2.54 ± 0.11de | 3.93 ± 0.87bc | 4.43 ± 0.25ab | 5.53 ± 0.94a | 3.57 ± 0.51bcd | 3.04 ± 0.59ed |
| M11         | Nd                  | 1.34 ± 0.05cd | 1.13 ± 0.19d | 2.12 ± 0.53bc | 2.46 ± 0.55ab | 3.41 ± 0.32a | 2.63 ± 0.45ab | 2.36 ± 0.94b |
| M12         | Nd                  | 1.24 ± 0.03c | 2.37 ± 0.35ab | 3.10 ± 0.70a | 2.42 ± 0.45a | 3.07 ± 0.52a | 1.49 ± 0.29bc | 1.62 ± 0.53bc |
| M13         | Nd                  | 1.85 ± 0.36b | 3.18 ± 0.61b | 2.63 ± 0.38b | 2.71 ± 0.44b | 3.45 ± 0.09b | 6.85 ± 1.55a | 7.58 ± 1.32a |
| M14         | 10.11 ± 3.05a       | 3.90 ± 1.11b | Nd         | Nd         | Nd         | Nd         | Nd         | Nd         |
| M15         | 3.54 ± 0.14a        | 2.21 ± 0.58bc | 2.58 ± 0.23abc | 2.55 ± 0.12abc | 2.10 ± 0.75bc | 2.90 ± 0.70ab | 1.55 ± 0.18bc | 1.81 ± 1.03bc |
| other       | 44.48 ± 1.50a       | 29.58 ± 3.35c | 40.83 ± 1.13ab | 39.22 ± 1.58b | 35.09 ± 0.19b | 38.87 ± 3.5ab | 27.47 ± 1.15c | 28.05 ± 0.69c |

Nd: not detected. Values are presented as percent of extractable residue of cycloxaprid. The comparison was made among days after treatment of each metabolite. Data followed by the same letter are not significantly different (P < 0.05).

Fig. 4. Proposed metabolic pathway of cycloxaprid in rice plant. Solid arrow represents Pathway I; dotted arrow represents Pathway II. * Metabolites that have not been reported before; ** The sites of ketone are arbitrarily shown as carbon 10 or 11.

In Pathway I, the cleavage of the oxygen-bridge and dehydration initially produced M9, which further metabolized to form M14 through the loss of carbon chains on the epoxide ring. After M14 underwent cleavage of the C=C double bond between the nitro moiety and the imidazolidine ring, the hydroxyl radical attacked the original replacement site to form M2. M2 accounted for the largest proportion of all metabolites in cycloxaprid metabolism, formed at the initial stage of application, and was then gradually converted into other compounds. The hydroxyl group of M2 was further oxidized to the urea analogue M15. Alternatively, after the imidazolidine ring of M15 was cleaved and the nitrogen atom was lost, molecules were rearranged to form M1, or the terminal ketone group carboxylation formed M11. The vinyl of M1 was lost to form M5, and the ketone group attacked the C atoms of M11 to create M8.

In Pathway II, the oxabridged seven-member ring of cycloxaprid was rapidly cleaved, and the terminal methyl was aminated to form M4. The amide leaving M4 resulted in M6. The nitro and allyl alcohol were eliminated from M6, and then the hydroxyl radical simultaneously attacked the two methylene groups on the imidazolidine ring to create M12. This similar hydroxylation reaction, in which the 10- and 11-position carbon are hydroxylated, has also been found in the metabolism of cycloxaprid in mice. Alternatively, M12 underwent demethylation to M10 or further oxidation of one of the hydroxyl groups of M12 into a carbonyl to M13. M7 could be created by further oxidation of the hydroxyl of M10 or further oxidation of one of the hydroxyl groups of M12 into a ketone. The ketone sites from M7, M8, and M13 were arbitrarily shown as carbon 10 or 11.

4. Environmental implications

This study demonstrated the feasibility and superiority of combining high-specific activity carbon-14 labeling and HRMS to study pesticide metabolism in crops via studying the metabolism of cycloxaprid in rice. By generating the characteristic radioactive peaks on the liquid
chromatogram, the use of $^{14}$C can eliminate the severe interference of complex matrices and quickly probe target compounds; by producing ion pairs with unique abundance ratios on HRMS, high-specific activity labeling can effectively exclude false matrix positives and promote the elucidation of metabolite structure. With the support of high-specific activity carbon-14 labeling, the structures of 15 metabolites were elucidated, and the possible metabolic pathway of cycloxaprid in rice was proposed for the first time. The established method could be similarly applied to numerous new compounds in various environmental matrices.

It is undeniable that the strategy of using high-specific radioactive labeling still has some limitations, for example, $^{14}$C labeled compounds are expensive, sometimes it is difficult to achieve high-specific activity labeling, and many countries/areas have special safety requirements for the use of radioactive isotopes. However, the combination of high-specific activity carbon-14 labeling and HRMS could merit the advantages of stable isotope labeling and radioisotope labeling, and can serve as a powerful solution for profiling unknown metabolites in complex matrices at trace levels, especially when the conventional non-labeling methods are not feasible.

CRediT authorship contribution statement

Dahang Shen: Investigation, Methodology, Data curation, Writing – original draft. Zhijiang Lu: Software, Writing – review & editing. Jiayin Zhong: Software, Visualization, Investigation. Sufen Zhang: Visualization, Investigation. Qingfu Ye: Conceptualization, Funding acquisition. Wei Wang: Conceptualization, Writing – review & editing, Funding acquisition. Jay Gan: Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Detailed information about rice cultivation is provided in the text of SI. Table S1 shows the concentrations of $^{14}$C-ER in different parts of rice. Figure S1 shows the synthesis route of cycloxaprid. Figure S2 shows the representative radiochromatograms of the $^{14}$C-metabolites. Figures S3–S18 show the MS/MS spectra of cycloxaprid and its metabolites with proposed ion fragments. Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2021.106879.

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