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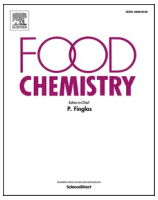

Analytical Methods

Dissipation of penconazole formulation in horticultural crops by ultrahigh performance liquid chromatography-high resolution mass spectrometry: From the active substance to metabolites

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ABSTRACT

The present study describes the dissipation and metabolism of penconazole in horticultural products by a method based on ultra-high performance liquid chromatography-quadrupole-orbitrap (UHPLC-Q-Orbitrap). Targeted and suspected analysis were carried out. Two independent trials were performed under laboratory conditions (on courgette samples), and under greenhouse conditions (on tomatoes) during 43 and 55 days, respectively. In both studies, a pesticide formulation (TOPAS® EW) containing penconazole was used. The results showed that penconazole was relatively short-lived (<30 days) in horticultural products. The proposed method allowed for the tentative identification and semi-quantification of nine metabolites. In addition, the potential toxicity of these metabolites was evaluated, observing that some of them are even more toxic than penconazole, as triazole lactic acid. This research may provide a starting point for understanding the dissipation process of penconazole, the formation pathways of its main metabolites, their concentrations and toxicity to ensure food safety and the environmental protection.

1. Introduction

Pesticide dissipation in plants may involve physicochemical (volatilization, hydrolysis, photolysis, oxidation, reduction, etc.) and/or biochemical processes (Chau, Son, & Van Hop, 2020). Additionally, several factors can influence pesticide dissipation, including climatic conditions (temperature, sunlight, humidity, etc.), pesticide properties, application method, time of harvest, dosage, plant species, time between applications, and food storage and preparation (Chau et al., 2020; Hassan, Ahmed, & Arief, 2013; Heshmati et al., 2019, 2020; A. Rahimi, Heshmati, & Nili-Ahmadabadi, 2022; Romeh, Mekky, Ramadan, & Hendawi, 2009; Saadaoui, Boujelbane, Serairi, Ncir, & Mzoughi, 2021).

Penconazole [(RS)-1-[2-(2,4-dichlorophenyl)pentyl]-1H-1,2,4-triazole] is a member of the triazole family, and contains the 1,2,4-triazole moiety (Abd-Alrahman & Ahmed, 2012). It is considered a systemic compound that causes a potential inhibition of fungal ergosterol biosynthesis (Abd-Alrahman & Ahmed, 2013; Zhang, Wang, Luo, Sheng, Zhou, Zhong, Lou, Sun, Yang, Cui, & Chen, 2019), due its rapid absorption and distribution into plant leaves when it is directly applied (Abd-Alrahman & Ahmed, 2012; Hassan et al., 2013; Romeh et al., 2009).

Penconazole is authorised to be used on crops that may be grown in rotation (Brancato et al., 2017b). It is commonly used to prevent and control powdery mildew in horticultural products, particularly in tomato and courgette (Li et al., 2022a; Li et al., 2022b). The European Food Safety Authority (EFSA) has established a maximum residue limit (MRL) for penconazole as the sum of this fungicide and three metabolites that are isomers (CGA 132465, CGA 190503 and CGA 127841), and MRLs are expressed as penconazole. The MRLs are set at 0.06 and 0.1 mg/kg in tomato and courgette, respectively (European Commission, 2019). These crops are widely cultivated in south-eastern Spain (Martínez-Granados et al., 2022b), and are among the most commonly consumed horticultural products (Martínez-Dalmau et al., 2022a).

The persistence of penconazole, expressed as half-life ($t_{1/2}$), in soil under field and laboratory (at 20 °C) conditions is 89.1 and 117.2 days, respectively, while the Residual Level (RL_{50}) in crops ranges from 1.5 to

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14.0 days (University of Hertfordshire. (2022), 2022).

In recent decades, the dissipation of penconazole has been a topic of interest, and this fungicide has been analyzed by gas chromatography-tandem mass spectrometry (GC–MS/MS) (Heshmati, Nili-Ahmadabadi, Rahimi, Vahidinia, & Taheri, 2020) or by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC has turned into the principal technique for the analysis of penconazole residue dissipation in different crops, as tomato, using diode array detector (Abd-Alrahman & Ahmed, 2012), or triple quadrupole mass spectrometer (Wang et al., 2014) (Romeh et al., 2009) as detection systems. However, none of these methods have used high-resolution mass spectrometry (HRMS), which is becoming a major and essential technique for the identification of a wide range of compounds at very low concentration in complex samples (López-Ruiz, Romero-González, Martínez Vidal, Fernández-Pérez, & Garrido Frenich, 2017), performing different working modes as targeted and un-targeted.

Despite the increasing use of penconazole in horticultural products (Li, Han, et al., 2022) and its reported dissipation in tomato samples (Abd-Alrahman & Ahmed, 2012; Romeh et al., 2009; Wang et al., 2014), few studies have been focused on the metabolism of this fungicide, and the dissipation of penconazole in courgette has not yet been evaluated.

The most relevant metabolites of the triazole family are known as triazole derivate metabolites (TDMs): 1,2,4-triazole (1,2,4-T), triazole acetic acid (TAA), triazole alanine (TA) and triazole lactic acid (TLA) (Ströher Kolberg et al., 2016). Based on this information, the metabolism of penconazole in field, particularly in primary crops, has been investigated in horticultural products, and EFSA has evaluated these metabolites in different matrices. For instance, the dissipation of penconazole was studied in cucurbits and grapes, identifying seven metabolites: CGA 179944, three isomers (CGA 127841, CGA 132465 and CGA 190503), 1,2,4-T, TAA and TLA. However, the results showed low residues of TDMs and no other significant metabolites were found (State & Gmbh, 2008). Metabolites of penconazole were also studied in blackberries and raspberries (European Food Safety Authority, 2014) or grapes (Brancato et al., 2017a), yielding a total of seven metabolites in each study. Another study determined penconazole in apple and tomato samples, finding six metabolites: the three isomers, TAA, TA and TLA (Brancato et al., 2017b). The main conclusion of these studies is that penconazole metabolites were identified and quantified at very low concentrations.

In addition to these known metabolites of penconazole, one investigation reported new metabolites detected in aqueous solutions of penconazole that had been treated with gamma irradiation (Saadaoui et al., 2021). In that laboratory trial, the irradiated solutions of penconazole were determined using high performance liquid chromatography-tandem mass spectrometry with Quadrupole Time-of-Flight (HPLC-QTOF-MS/MS), detecting ten metabolites, and three of which were the three isomers detected by EFSA.

To date, 15 known metabolites of penconazole have been reported. On the one hand, EFSA has identified eight: CGA 127841 ($C_{13}H_{15}Cl_2N_3O$), CGA 132465 ($C_{13}H_{15}Cl_2N_3O$), CGA 190503 ($C_{13}H_{15}Cl_2N_3O$), 1,2,4-T ($C_2H_4N_3$), TAA ($C_4H_5N_3O_2$), TA ($C_5H_8N_4O_2$), TLA ($C_5H_7N_3O_3$) and CGA 179944 ($C_{11}H_9Cl_2N_3O_2$) (Brancato et al., 2017a, 2017b; European Food Safety Authority, 2014; State & Gmbh, 2008). On the other hand, Saadaoui et al. (Saadaoui et al., 2021) identified ten metabolites: TPP1 ($C_8H_{13}N_3O_2$), TPP2 ($C_{13}H_{17}N_3$), TPP3 ($C_{13}H_{14}ClN_3$), TPP4 ($C_{13}H_{16}ClN_3O$), TPP5 ($C_{13}H_{16}ClN_3O$), TPP6 ($C_{14}H_{17}Cl_2N_3$), TPP7 ($C_{13}H_{15}Cl_2N_3O$), TPP8 ($C_{13}H_{15}Cl_2N_3O$), TPP9 ($C_{13}H_{15}Cl_2N_3O$) and TPP10 ($C_{13}H_{13}Cl_2N_3O_2$).

Due to the limited research and lack of knowledge regarding the dissipation process of penconazole in horticultural products using HRMS and the potential for detecting relevant metabolites during its dissipation, a study was needed to understand the behaviour of this fungicide to ensure the safety of horticultural products acquired by consumers. Therefore, the aim of this study was to analyse the behaviour of penconazole in courgette and tomato, and its dissipation into TDMs and other metabolites over a period of >40 days. To do this, two trials were designed to evaluate the dissipation and metabolism of penconazole in horticultural products under two diverse conditions: laboratory and field (greenhouse). The laboratory trial was carried out to simulate the dissipation of penconazole (under controlled and specific conditions) after harvesting a vegetable, in this case courgette, while the greenhouse trial was performed to evaluate this process in a real-life situation, growing tomatoes. This is the first time that an analytical method based on LC-HRMS was developed for the dissipation study of penconazole (under two different situations) in horticultural products, and to explore its pathway through the resulting metabolites.

2. Materials and methods

2.1. Chemical and reagents

Penconazole (CAS registry No. 66246-88-6, purity of 98.85%) was supplied by Sigma Aldrich (St. Louis, MO, USA), and triazole acetic acid (purity ≥98%) by Supelco (Buchs, Switzerland) (CAS registry No. 28711-29-7).

Acetonitrile and methanol (99.9% of purity) were provided by Honeywell Riedel-de-Haen (Seelze, Germany), and water by J.T. Baker (Deventer, The Netherlands), all of which were of LC-MS grade. Formic acid (>98% of purity) was purchased from PanReac AppliChem (Barcelona, Spain). Syrange filters Econofilter Nylon 0.2 μm, 13 mm were obtained from Agilent Technologies (Santa Clara, CA, USA).

Two mixtures, one for ESI positive and other for ESI negative, supplied by Thermo Fisher Scientific (Waltham, MA, USA), were used to calibrate the UHPLC-Q-Orbitrap: a mixture of acetic acid, caffeine, Met-Arg-Phe-Ala-acetate salt and Ultramark 1621 (ProteoMass LTQ/FT-hybrid ESI positive), and a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrate, and Ultramark 1621 (fluorinated phosphazenes) (ProteoMass LTQ/FT-hybrid ESI negative).

The representative formulated product (pesticide formulation) was TOPAS® EW 19.4% penconazole (*w/w*), provided by Syngenta (Madrid, Spain).

2.2. Preparation of standard solutions

A stock standard solution was prepared at 1000 mg/L, by dissolving 10 mg of pure penconazole in 10 mL of methanol. From this solution, a working standard solution was prepared at 10 mg/L and a solution at 1 mg/L was also prepared, both in methanol. The stock and working standard solutions were stored at −18 °C.

2.3. Analysis by UHPLC-Q-Orbitrap-HRMS

The analysis was performed using a Thermo Fisher Scientific Vanquish Flex Quaternary LC (Thermo Scientific Transcend™, Thermo Fisher Scientific, San Jose, CA, USA), coupled to a Q-Exactive Orbitrap hybrid mass spectrometer, both from Thermo Fisher Scientific (Exactive™, Thermo Fisher Scientific, Bremen, Germany). A heated electrospray interface (HESI) (HESI-II, Thermo Fisher Scientific, San Jose, CA, USA), in positive and negative modes, was also used.

Separations were carried out using a Hypersil GOLD™ aQ column (100 mm × 2.1 mm; 1.9 μm particle size), from Thermo Fisher Scientific, with a flow rate of 0.2 mL/min. The injection volume was 10 μL and the column temperature was set at 30 °C.

The mobile phase consisted of an aqueous solution containing 0.1% formic acid (phase A) and methanol (phase B). The elution gradient started with 95% of A and was kept constant for 1 min. Then, it was decreased to 0% of A and was held constant for 6 min, before returning to the initial conditions in 0.5 min and remaining constant for 3.5 min, for a total running time of 14 min (Prata et al., 2022).

The ESI parameters used were spray voltage at 4 kV; sheath gas (N_2, 95%), 35 (arbitrary units); auxiliary gas (N_2, 95%), 10 (arbitrary units);

S-lens RF level, 50 (arbitrary units); capillary temperature, 300 °C; and heater temperature, 305 °C. The mass spectra were acquired using four different acquisition functions: (1) full MS, ESI^+, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 70,000 Full Width at Half Maximum (FWHM); AGC target = 1e6, (2) full MS, ESI^-, without fragmentation (the higher collisional dissociation (HCD) collision cell was switched off), mass resolving power = 70,000 FWHM; AGC target = 1e6, (3) data independent mass spectrometry fragmentation (DIA-MS/MS), ESI^+ (HCD on, collision energy = 30 eV), mass resolving power = 35,000 FWHM; AGC target = 2e5, (4) DIA-MS/MS, ESI^- (HCD on, collision energy = 30 eV), mass resolving power = 35,000 FWHM; AGC target = 2e5. The full scan-MS mode was ranged between *m/z* 60 and 900.

The chromatograms were obtained using external calibration mode and subsequently processed with Xcalibur™ version 4.3.73, with Quan and Qual Browser. Additionally, the Mass Frontier™ 8.0 in-silico software (Thermo Fisher Scientific, Les Ulis, France) was used to confirm the fragment ions.

Finally, the Toxicity Estimation Software Tool (T.E.S.T.) software (United States Environmental Protection Agency. (2022), 2022) was used to estimate the toxicity of metabolites.

2.4. Laboratory trials

A laboratory trial was carried out during the autumn of 2021 (from November to December) to evaluate the dissipation of penconazole on courgette samples treated with the pesticide formulation. The samples were kept for 43 days under normal laboratory conditions, which means ambient temperature (15±5 °C) and at least 8 h of sun exposure. Courgette samples were treated with the pesticide formulation of penconazole, TOPAS® EW 19.4% penconazole (*w/w*) at the corresponding recommended dose (SD), 0.02%, and at double dose (DD), 0.04%. DD was monitored to determine any differences in the penconazole dissipation process, including the number of metabolites identified at lower levels, compared to SD.

Samples were stored at room temperature, and three replicates with an approximate size of 500 g were analysed, at 2 and 6 h, and at 1, 2, 5, 10, 15, 22, 29, 36 and 43 days.

Some courgette samples were considered as blank and were not treated with the pesticide formulation. During the experimental period, the weight of two blank courgettes was checked to control for water loss, and the data was considered in the calculation of the concentration during the dissipation process.

2.5. Greenhouse trial

In addition, an agricultural field trial using a hydroponic system was carried out. In this context, the dissipation study was performed in a greenhouse located in Almería (Spain) in 2022 (from January to March), over 55 days on tomato samples.

The experimental field (234 plants distributed in 161.6 m^2 in area) was divided into six blocks or plant lines (39 plants for each block). Three of them were sprayed with TOPAS® EW (19.4% penconazole), at the recommended dose (0.02%) and no other phytosanitary products were sprayed during the experiment, whereas the remaining three blocks were selected as a control and were not irrigated with the pesticide formulation. Every block was divided into three plots, which were considered as three replicates. The characteristic parameters of greenhouse experiment are shown in Table S1 (see supplementary material).

According to the instructions provided by the TOPAS supplier, two applications were performed to the crops, with a 7-day interval between them. Finally, tomato samples, taken in triplicate with an approximate size of 500 g (4–5 tomatoes), were collected at 2 h and 1, 2, 3, 4, 7, 14, 24, 38 and 55 days after the second application.

2.6. Sample preparation and extraction

Fresh courgette samples were purchased from a local store in Almería, Spain, and they were immediately treated with the commercial product as they arrived to the laboratory, while tomatoes were directly harvested from the greenhouse. After collecting the samples (either courgettes or tomatoes), they were crushed and homogenized (including the peel). The samples were then treated using the previously published procedure (Hergueta-Castillo, López-Rodríguez, López-Ruiz, Romero-González, & Garrido Frenich, 2022), which involved solid–liquid extraction (SLE). This extraction consisted of adding 10 mL of acetonitrile to 10 g of sample into a 50 mL centrifuge tube and shaking the mixture for 1 min. The mixture was then centrifuged at 3700 rpm (3061 *g*) for 10 min and 1 mL of the supernatant, which had been filtered through a 0.2 μm filter, was collected and injected by UHPLC-Q-Orbitrap-HRMS.

2.7. Data calculation

The dissipation of penconazole was determined by plotting the residue concentration of this fungicide versus time. In laboratory trials, penconazole was fitted to a biphasic kinetic model, while in greenhouse it was fitted to a Single First-Order (SFO). The residual concentration, or final concentration (Ct) and the half-life time (DT_{50} or $t_{1/2}$) were calculated using Eqs. (1) and (2) for the biphasic model, and Eqs. (2) and (3) for the SFO model:

$$C(t) = C_0 e^{-k_1 t} + C_1 e^{-k_2 t} \quad (1)$$

$$DT_{50} = \frac{\ln 2}{k} \quad (2)$$

$$C(t) = C_0 e^{-kt} \quad (3)$$

where C_0 and C_1 represent the concentration (μg/kg) of the fungicide in the sample (either courgette or tomato) at time t (day), while k, k_1 and k_2 are the dissipation kinetic rate constants ($days^{-1}$).

2.8. Method validation

The validated method was evaluated in the courgette samples of the laboratory trial (at SD) according to Guidance Sante 11312/2021 (Commission, 2021). Table S2 shows the main parameters of penconazole and triazole acetic acid (TAA) (compounds with available standards), including matrix effect, limit of quantification (LOQ), recovery and inter- and intra-day precision values.

3. Results and discussion

3.1. Dissipation study of penconazole

The natural behaviour and dissipation profile of penconazole (when treated with the pesticide formulation TOPAS®) was evaluated. The dissipation kinetics of penconazole under two conditions (laboratory and greenhouse) were determined by plotting the residue concentration of this fungicide over time.

Different kinetic models were tested, including zero order, half order, and second order, but the data did not fit properly to these models. Nevertheless, the dissipation process was well-fit by the biphasic dynamic kinetic model in the laboratory trials ($R^2 \geq 0.972$) (Fig. 1a and Fig. 1b) and by the SFO model ($R^2 = 0.978$), in the greenhouse trial (Fig. 1c).

Under laboratory conditions, the penconazole concentration gradually increased until it reached the highest value at 535.6 μg/kg (in the case of SD on the fifth day of the study), and 1062.1 μg/kg (in DD on the first day after application). Then, the values started to decrease, as it was

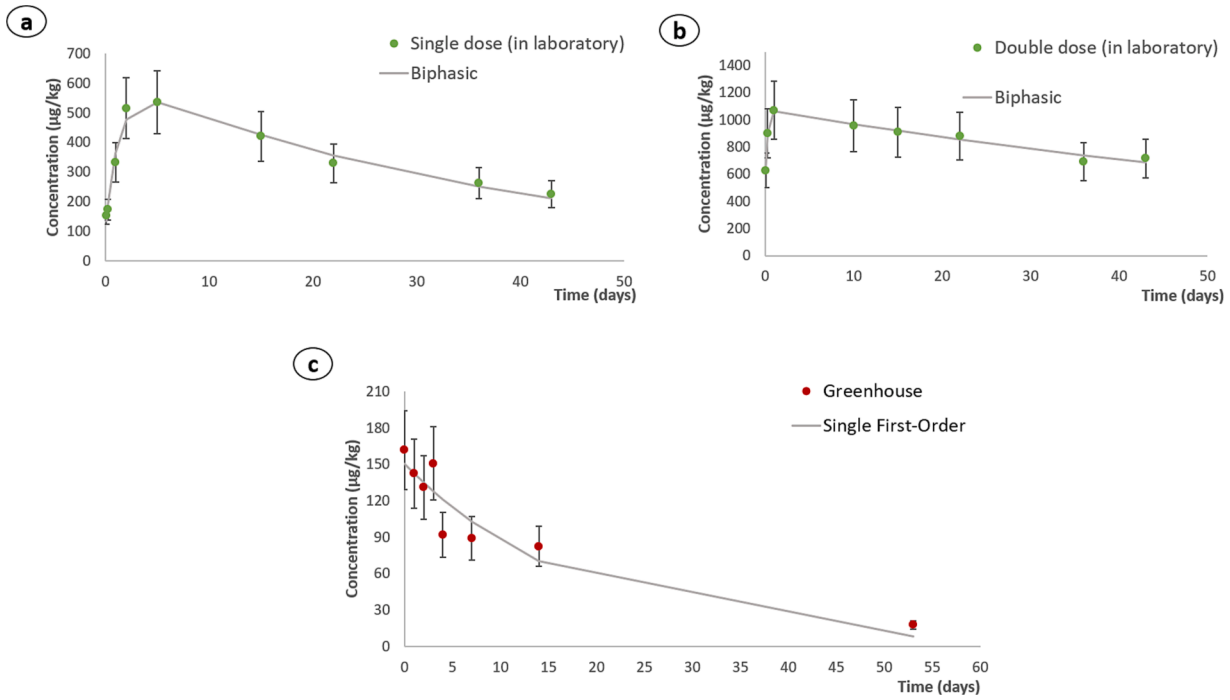

Fig. 1. Kinetics of penconazole in laboratory trials at two concentration levels: a)single dose (recommended dose by the manufacturer at 0.02%) and b) double dose (at 0.04%); and c) in greenhouse trial (0.02%) (n = 3).

illustrated in Fig. 1a and Fig. 1b. The initial concentration of penconazole (C_0) was higher in DD (352.1 µg/kg) compared to SD (102.9 µg/kg), as expected, although the maximum concentration achieved at DD was double than at single dose for this systemic compound, observing a different absorption capacity depending on the tested concentration. The k_1 and k_2 values showed that the dissipation process was slightly lower at the beginning (k_1 = 0.03 and 0.01 $days^{-1}$, in SD and DD, respectively) than at the end (k_2 = 0.8 and 5.8 $days^{-1}$, in SD and DD, respectively) as it is shown in Table 1. It should be noted that the dynamic dissipation significantly increased at the end of the experiment in the case of DD. In terms of the persistence of half-life ($t_{1/2}$), differences were observed between k_1 and k_2, with quicker values at the beginning of the study compared to the end of the trials. In this context, $t_{1/2}$ (k_1) for SD was 27.7 days, and $t_{1/2}$ (k_2) was 0.9 days, whereas for DD the values were different (66.1 and 0.1 days in k_1 and k_2, respectively), as shown in Table 1. The results revealed that there were significant differences in the dissipation of penconazole under laboratory conditions, with $t_{1/2}$ (k_1) in SD being more than twice that in DD, and $t_{1/2}$ (k_2) for SD being 9 times higher than in DD. Therefore, the dissipation trend depends on the dose of the pesticide formulation.

In relation to the dissipation of penconazole in the greenhouse (Fig. 1c), the initial concentration of this fungicide was 150.8 µg/kg, which was the maximum observed during the experiment, indicating that the concentration gradually decreased during the study. Besides, the k value was 0.05 $days^{-1}$ and the persistence was 12.7 days, as shown in Table 1. The residue level of penconazole at the end of the study (day 55) was 17.6 µg/kg, which is lower than the corresponding MRL of 60 µg/kg set for tomato. Therefore, the samples could be safely consumed without any risk for the human health.

In relation to the preharvest interval (PHI) for safe consumption of penconazole-treated tomato, EFSA has set 3 days after application of the pesticide formulation (Brancato et al., 2017b), while another study suggested a period of 15 days (Abd-Alrahman & Ahmed, 2012). Nevertheless, in the present study, the PHI for tomato was calculated, and it was 18 days. These differences may be due to the type of field, as tomato was cultivated under greenhouse conditions in this study, whereas in the other researches penconazole was applied to tomato cultivated in open fields.

According to previous studies, the SFO kinetic has been commonly reported for the dissipation process of penconazole in tomato samples. For instance, Wang et al. obtained a $t_{1/2}$ of 1.90–1.98 days under open field conditions (Wang et al., 2014), which means that penconazole dissipated more quickly than in the present study (12.7 days). In another study, the dissipation of penconazole was estimated over a period of 21 days in open field, finding that $t_{1/2}$ was 5.61 days (Abd-Alrahman & Ahmed, 2012), while in this study the persistence of penconazole was longer. The study carried out by Romeh et al. over a period of 14 days showed a $t_{1/2}$ of 5.21 days, while in this study, the persistence was higher (12.7 days) (Romeh et al., 2009). These differences may be due to various factors related to the agronomic conditions, such as the season in which the study was conducted or the type of field, as this study is the only one in which tomatoes were cultivated under greenhouse conditions. It must be emphasized that this investigation is the longest one compared to other studies focused on the dissipation of penconazole.

Other researchers have also studied the dissipation kinetics of various pesticides in horticultural products, which were fitted to a biphasic kinetic order, similar to the laboratory trials in the present study. For example, when malathion was evaluated in aqueous extracts

Table 1
Kinetic model parameters of penconazole dissipation in both experiments: laboratory in courgette samples (biphasic kinetic model) and greenhouse in tomato samples (single-first order).[a]

Experiment Kinetic parameters	Laboratory trials		Greenhouse study
	Single dose	Double dose	
C_0 (µg/kg)	102.9	352.1	150.8
k_1 ($days^{-1}$)	0.03	0.01	0.05
k_2 ($days^{-1}$)	0.8	5.8	NA
$t_{1/2}$ (k_1) (days)	27.7	66.1	12.7
$t_{1/2}$ (k_2) (days)	0.9	0.1	NA
DT_{90} (days)	3.0	0.4	42.0
R^2	0.972	0.975	0.978

[a] Abbreviations: C_0: initial concentration; $t_{1/2}$: half-life; NA: Not applicable; k_1 and k_2: dissipation kinetic rate constants; R^2: determination coefficient.

of different fruits and vegetables in field (S. Rahimi, Talebi, Torabi, & Naveh, 2015) or endosulfan in tomato fields (Ntow, Ameyibor, Kelderman, Drechsel, & Gijzen, 2007).

Finally, we would like to indicate that the differences observed between the kinetic models in laboratory and greenhouse trials could be explained because the different matrix selected, environmental conditions in both situations although in the laboratory study, the "experimental" conditions were mimic, and different stress responses.

3.2. Tentative identification of penconazole metabolites

Penconazole metabolites have been described in bibliography by EFSA (Brancato et al., 2017a, 2017b; European Food Safety Authority, 2014; State & Gmbh, 2008) and by Saadaoui et al. (Saadaoui et al., 2021). A total of 15 metabolites may be considered as suspected metabolites of penconazole. In order to identify them, a suspected approach was applied using UHPLC-Q-Orbitrap-HRMS. Table 2 shows the HRMS parameters of tentatively identified penconazole metabolites.

On the one hand, in the laboratory trial, eight metabolites were tentatively identified: three isomers (CGA 127841, CGA 132465 and CGA 190503), TLA, TAA, TA, TPP6 and TPP10. On the other hand, in the greenhouse trial, seven metabolites were detected: three isomers (CGA 127841, CGA 132465 and CGA 190503), CGA 179944, TLA, TAA and TPP10. Thus, six metabolites were determined in both experiments, and the chemical structures are shown in Fig. 2.

Regarding the three isomers, two peak signals were observed approximately at 7.45 and 7.79 min (Fig. 3a), which means that three options are possible: (1) each peak could correspond to one of these metabolites; (2) there could be an overlap of two metabolites in one peak and the other peak may be another metabolite; or (3) the two peaks may correspond to the same compound (double peak). Since, it was not possible to determine which specific isomers were detected, the three isomers have been refereed to collectively in the study.

As it is observed in Fig. 3b–d, the TDMs were the metabolites that eluted first, with retention time <2 min, while the three isomers and TPP6 eluted at 7 min approximately (Table 2). For each metabolite, different fragment ions were obtained using Data Independent Acquisition (DIA), and they were compared with those provided by Mass Frontier™ software.

All metabolites were ionized in positive mode, except CGA 179944, which was ionized in negative mode (Fig. 3e). This compound was only found in the greenhouse trial.

In addition, all compounds were identified using a mass error lower than 5 ppm for the characteristic ion, detecting at least one fragment ion with a mass error lower than 5 ppm as well (Table 2). A common fragment, *m/z* 70.03997 ($C_2H_4N_3$), was observed in five metabolites, TAA, TA and the isomers (CGA 127841, CGA 132465 and CGA 190503). This fragment corresponded to 1,2,4-triazole, another TDM, and it was common for the compounds belonging to the triazole family (Abd-Alrahman & Ahmed, 2012), so it was expected that it appeared in penconazole metabolites. In the study of Saadaoui et al. (Saadaoui et al., 2021), *m/z* 70.03997 was also found as a fragment of TPP6 and TPP10 metabolites, while it was not detected in this study. This difference may be due to the type of mass analyser used, which was a QTOF in Saadaoui's study and a Q-Orbitrap in this one, as well as the low concentration of this compound in the current study.

Once these metabolites were tentatively identified, a semi-quantitative approach was carried out to determine their concentration. For that purpose, the metabolite TAA was used as standard for the TDMs, and the parent compound (penconazole) was used for the other metabolites, due to the similarity of their structures. In the case of TAA, as standard was available, this metabolite could be properly confirmed and quantified.

In laboratory trials, the most prevalent metabolites were the isomers (CGA 127841, CGA 132465 and CGA 190503), as they were detected from the first day (after pesticide formulation application) to the last day of the study. The concentration showed a clear downward trend in both application doses (Table 3). On the first day, values of 27.3 μg/kg (at SD)

Table 2
Characteristic parameters for tentatively identified metabolites of penconazole.[a]

Compound	Toxicity value	RTW (min)	Molecular formula	Theoretical mass (*m/z*)	Mass error (ppm)	Ionization mode	Fragment ions			References
							Molecular formula	Theoretical mass (*m/z*)	Mass error (ppm)	
Triazole lactic acid (TLA)	ARfD: 0.3 mg/kg bw	1.69–1.74	$C_5H_7N_3O_3$	158.05601	2.74	ESI (+)	$C_4H_7N_2O_3$ $C_5H_5N_3O_2$	131.04512 139.03763	1.25 −2.81	Brancato et al., 2017a; Brancato et al., 2017b; European Food Safety Authority, 2014; State & Gmbh, 2008
Triazole acetic acid (TAA)	ARfD: 1.0 mg/kg bw	1.27–1.30	$C_4H_5N_3O_2$	128.04545	1.97	ESI (+)	$C_2H_4N_3$	70.03997	0.86	
Triazole alanine (TA)	ARfD: 0.3 mg/kg bw	1.52–1.69	$C_5H_8N_4O_2$	157.07202	3.21	ESI (+)	$C_2H_4N_3$ $C_5H_7N_4O$	70.03997 139.06144	2.96 3.84	
CGA 127841	LD_{50}: 1452.15 mg/kg	7.48–7.79	$C_{13}H_{15}Cl_2N_3O$	300.06649	−2.51	ESI (+)	$C_2H_4N_3$	70.03997	−1.32	
CGA 132465	LD_{50}: 2129.18 mg/kg						$C_7H_{10}N_3$	136.08692	2.68	
CGA 190503	LD_{50}: 1936.36 mg/kg						$C_{11}H_{11}Cl_2$	213.02323	−2.31	
CGA 179944	LD_{50}: 985.20 mg/kg	3.56–4.02	$C_{11}H_9Cl_2N_3O_2$	283.9999	1.74	ESI (-)	$C_2H_3O_9$ $C_{11}H_8Cl_2N_3O$	171.98498 269.01172	1.05 2.23	
TPP6	–	7.52–7.62	$C_{14}H_{17}Cl_2N_3$	298.08723	0.33	ESI (+)	$C_{12}H_{15}$ $C_{15}H_{28}ClN$	159.11692 257.18993	−1.22 −1.64	Saadaoui et al., 2021
TPP10	LD_{50}: 941.12 mg/kg	2.15–2.34	$C_{13}H_{13}Cl_2N_3O_2$	314.04575	−4.42	ESI (+)	$C_4H_5O_5$ $C_5H_3Cl_2$	133.01333 252.96084	−4.21 2.25	

[a] Abbreviations: ARfD: acute reference dose; ESI (+): electrospray interface in positive mode; ESI (-): electrospray interface in negative mode; predicted lethal dose 50% in oral rats (LD_{50}) (United States Environmental Protection Agency. (2022), 2022); RTW: retention time window; –: compound whose LD_{50} could not be predicted.

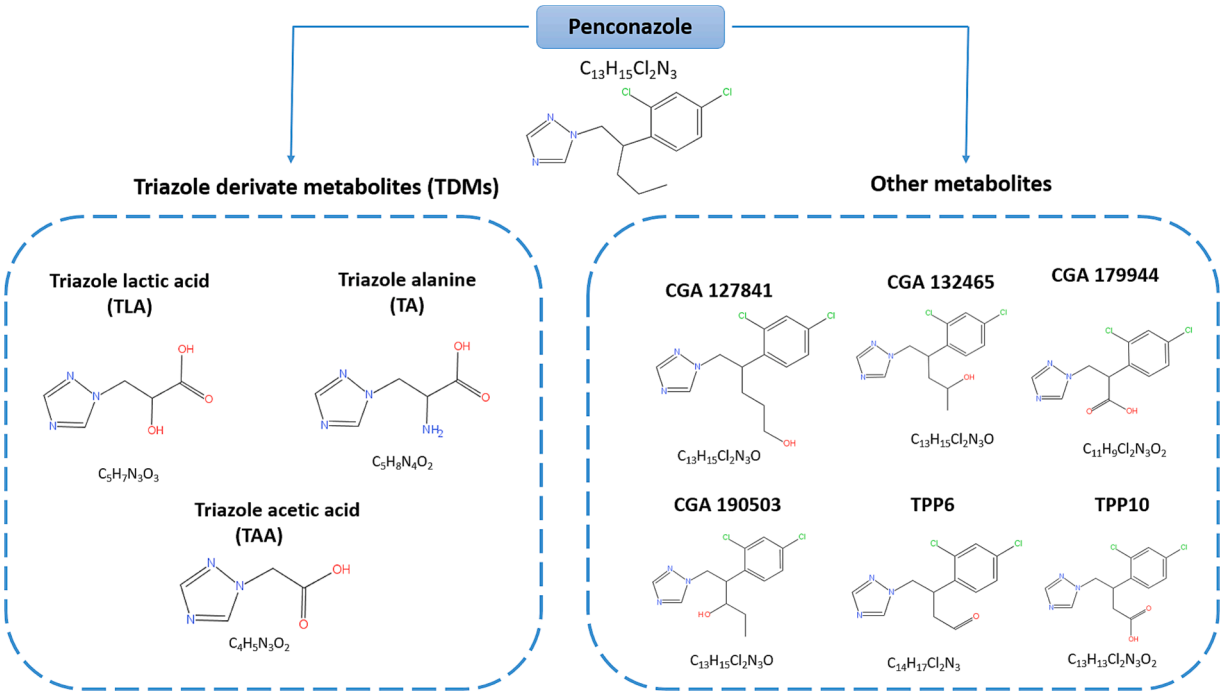

Fig. 2. Penconazole metabolites identified in this study.

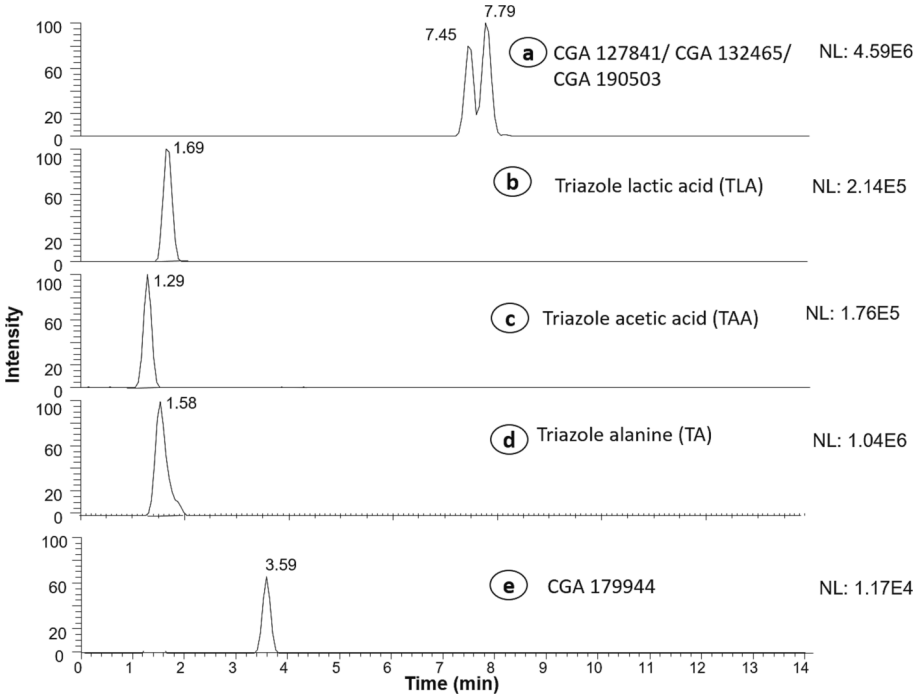

Fig. 3. Extracted Ion Chromatograms of penconazole metabolites: a) CGA 127841/CGA 132465/CGA 190503, b) triazole lactic acid (TLA) and c) triazole acetic acid (TAA) detected the 10th day after application in laboratory trial (at double dose); d) triazole alanine (TA) detected at laboratory trial (double dose) 2 days after spraying; and e) CGA 179944 detected at greenhouse trial 1 day after application.

and 73.7 μg/kg (at DD) were estimated, and they gradually decreased to 3.0 and 5.4 μg/kg, respectively. Additionally, the TA compound was only detected at 48 h after application at 14.9 μg/kg in SD, and at 20.1 μg/kg in DD (Table 3). The remaining metabolites were only identified at one day of the study, but at levels below the LOQ of TAA (in the case of TDMs, which was established at 2 μg/kg) or below the LOQ of penconazole (in the case of the other metabolites, whose LOQ was also set at 2 μg/kg).

In the greenhouse trial, the seven metabolites identified (three isomers (CGA 127841, CGA 132465 and CGA 190503), CGA 179944, TLA, TAA and TPP10) were mainly detected at the beginning of the study, but at concentrations below the LOQs of TAA and penconazole, in each case.

In comparison to other investigations, some of the metabolites in this study were found at higher concentrations than in previous study, whereas in EFSA reports they were found at very low concentrations, being referred as trace levels (Brancato et al., 2017a, 2017b; European

Table 3
Concentration (μg/kg) of penconazole metabolites identified under laboratory conditions at single dose and at double dose (in brackets).[a]

Metabolite	Sampling period (hours or days)										
	2 h	6 h	1 day	2 days	5 days	10 days	15 days	22 days	29 days	36 days	43 days
Triazole lactic acid (TLA)	–	–	–	–	$<LOQ_t$	–	–	–	–	–	–
Triazole acetic acid (TAA)					–	–	$<LOQ_t$	–	–	–	–
Triazole alanine (TA)	–	–	–	14.9 (20.1)	–	–	–	–	–	–	–
CGA 127841/CGA 132465/CGA 190503	–	–	27.3 (73.7)	25.1 (16.8)	2.3 (7.5)	5.8 (3.9)	3.9 (6.5)	1.7 (3.8)	3.1 (6.7)	2.0 (5.7)	3.0 (5.4)
TPP6	–	–	–	–	–	–	–	–	–	$<LOQ_p$	–
TPP10	–	–	–	–	$<LOQ_p$	–	–	–	–	–	–

[a] Abbreviations: $<LOQ_t$: compound detected below LOQ of TAA (triazole acetic acid), but not quantified; $<LOQ_p$: compound detected below LOQ of penconazole, but not quantified; –: compound not detected.

Food Safety Authority, 2014; State & Gmbh, 2008). In the experimental study carried out by Saadaoui et al. (Saadaoui et al., 2021), where an aqueous solution of penconazole was evaluated, the detected metabolites were only identified, whereas in this study the metabolites were also quantified.

According to the levels of the identification of new compounds by HRMS proposed by Schymanski et al. (Schymanski et al., 2014), a level 2 of identification was achieved for all the metabolites, due to the structures were obtained. The TAA compound was suitably confirmed by the injection of the analytical standard and an identification level 1 was set.

3.3. Toxicity of penconazole and its metabolites

The toxic impact of pesticides on different horticultural crops is relevant to evaluate (Malhat, Badawy, Barakat, & Saber, 2014) in order to ensure food safety. In some cases, the metabolites detected during their dissipation may be even more toxic that the parent compound (Saber, Malhat, Badawy, & Barakat, 2016). Therefore, the effects of penconazole metabolites on human health were evaluated, in terms of toxicological values, and compared with penconazole to determine whether imply a higher risk than this fungicide for the consumer health. The Acute Reference Dose (ARfD) of penconazole was set at 0.5 mg/kg bw (Brancato et al., 2017a). Each TDM has its corresponding ARfD (Brancato et al., 2017a), and they are provided in Table 2. It should be noted that the values for TA and TLA are lower (ARfD = 0.3 mg/kg bw) than that provided by the parent compound, indicating that they could be more toxic. On the contrary, TAA is less toxic than the parent compound, but depending on the final concentration on the samples it could become a potential toxic metabolite.

Due to there is no available information for the remaining metabolites, the Lethal Dose 50% (LD_{50}) in oral rats has been predicted for each metabolite using a tool developed by the United States Environmental Protection Agency (United States Environmental Protection Agency. (2022), 2022). Table 2 shows the predicted values, except for TPP6, whose value could not be predicted. It can be observed that two of the penconazole isomers (CGA 179944 and TPP10) showed toxicity levels lower than 1000 mg/kg.

According to the results from toxicity, relevant values have been showed for the penconazole metabolites, as some of them may be more toxic, both for human health and for the environment, than the parent compound. For example, TA and TLA have a value of 0.3 mg/kg bw, compared to 0.5 mg/kg bw for penconazole, or LD_{50} <1000 mg/kg was observed for some compounds. Therefore, it can be concluded that this study provides relevant toxicological data that should be considered for accurate risk assessments in order to ensure food safety even to study the inclusion of some metabolites in the definition of MRL.

4. Conclusions

In this study, a sensitive and efficient method based on UHPLC-Q-Orbitrap-MS analysis was applied to determine penconazole and its metabolites in two horticultural products (courgette and tomato). The dissipation of this compound was carried out under both laboratory and greenhouse conditions and fit to a biphasic order kinetic model and to an SFO, respectively. The persistence or half-time ($t_{1/2}$) of penconazole was relatively low in both trials (<30 days in the laboratory and <15 days in the greenhouse).

The concentration of penconazole was gradually decreased in both horticultural products under both laboratory and greenhouse conditions, not exceeding the MRL of penconazole at the end of the study. It should be noted the dissipation trend was affected by the dose of pesticide formulation applied in the laboratory trials.

The proposed LC-HRMS methodology allowed for the suitable identification of up to nine penconazole metabolites in courgette and tomato samples. Eight compounds were detected in laboratory trials and seven in the greenhouse trial, six of them detected under both conditions: CGA 127841, CGA 132465, CGA 190503, TLA, TAA, and TPP10. After their identification, the concentration and toxicity of the detected metabolites were estimated and evaluated, observing that some of them were even more toxic than penconazole (TLA and TA). In consequence, they could be monitored and/or included in the MRL definition of this compound.

This research is of great significance in understanding the natural behaviour and dissipation process of penconazole, its derived metabolites, and its dissipation pathways to accurately perform risk assessments and ensure food safety.

CRediT authorship contribution statement

María Elena Hergueta-Castillo: Formal analysis, Investigation, Validation, Writing – original draft, Visualization. **Rosalía López-Ruiz:** Investigation, Software, Data curation, Supervision, Writing – review & editing. **Patricia Marín Membrive:** Methodology, Data curation. **Roberto Romero-González:** Methodology, Data curation, Supervision, Writing – review & editing. **Antonia Garrido Frenich:** Conceptualization, Resources, Writing – review & editing, Funding acquisition, Project administration.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2023.136266.

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