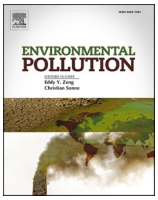
Environmental Pollution 349 (2024) 123924

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol



Tracing the dissipation of difenoconazole, its metabolites and co-formulants in tomato: A comprehensive analysis by chromatography coupled to high resolution mass spectrometry in laboratory and greenhouse trials☆

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ARTICLE INFO

Keywords:
Difenoconazole
Metabolite identification
Co-formulants
Dissipation kinetics
Greenhouse and laboratory trials

ABSTRACT

The study evaluated Ceremonia 25 EC®, a plant protection product (PPP) containing difenoconazole, in tomato crops, to identify potential risks associated with PPPs, and in addition to this compound, known metabolites from difenoconazole degradation and co-formulants present in the PPP were monitored. An ultra high performance liquid chromatography coupled to quadrupole-Orbitrap mass analyser (UHPLC-Q-Orbitrap-MS) method was validated with a working range of 2 μg/kg (limit of quantification, LOQ) to 200 μg/kg. Difenoconazole degradation followed a biphasic double first-order in parallel (DFOP) kinetic model in laboratory and greenhouse trials, with high accuracy ($R^2 > 0.9965$). CGA-205374, difenoconazole-alcohol, and hydroxy-difenoconazole metabolites were tentatively identified and semi-quantified in laboratory trials by UHPLC-Q-Orbitrap-MS from day 2 to day 30. No metabolites were found in greenhouse trials. Additionally, 13 volatile co-formulants were tentatively identified by gas chromatography (GC) coupled to Q-Orbitrap-MS, detectable up to the 7th day after PPP application. This study provides a comprehensive understanding of difenoconazole dissipation in tomatoes, identification of metabolites, and detection of co-formulants associated with the applied PPP.

1. Introduction

Pesticides are used to combat phytopathogens, avoiding severe crop losses, with devastating nutritional and economic consequences (Savary et al., 2019). The Food and Agriculture Organisation of the United Nations (FAO) estimates that up to 20–40% of world crops are lost due to pests, representing an economic impact of more than EUR 200 billion per year. Pesticides have excelled in allowing for the production of larger amounts of food with fewer losses, but if label practices are not followed properly, pesticide exposure may present some side effects on organisms (López-Ruiz et al., 2020; Kenko et al., 2023).

Fungicides, along with bactericides, accounted for 43% of total pesticide sales in the European Union (EU) in 2020 (Eurostat, 2022) Triazole derivatives are the most common type of fungicide, whose market is projected to reach $ 4.90 billion by 2028 (Data Bridge Market Research, 2021). Difenoconazole, a broad-spectrum systemic triazole fungicide, is one of the most frequently used fungicides due to its effectiveness in dealing with certain types of fungal pests and as a result, it remains of great analytical interest (Liu et al., 2021a).

Difenoconazole, as any other active substance, is regulated extensively, covering its registration process, maximum residue levels (MRLs), and its use in PPPs. It has been authorised in the European Economic Area (EEA) since January 2009, under EC Regulation 1107/2009 (European Commission, 2009) and it is currently approved by all EU member states, except Italy (European Commission, n.d.) According to EC Regulation 2019/552 (European Commission, 2019) the MRL for difenoconazole in the EU has been set at 2 mg/kg in tomatoes, one of the most characteristic agricultural products grown in Southern Spain. The requirements for the approval of PPPs are set by EC Regulation 284/2013 (European Commission, 2013a), linked to EC Regulation 1107/2009, whereas the requirements for the approval of active substances are set by EC Regulation 283/2013 (European Commission,

☆ This paper has been recommended for acceptance by Charles Wong.

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https://doi.org/10.1016/j.envpol.2024.123924
Received 5 February 2024; Received in revised form 29 March 2024; Accepted 2 April 2024
Available online 3 April 2024

2013b) For registration (approval) purposes, the residue of concern (RoC) for food of plant origin is defined as difenoconazole and its triazole derivative metabolites (TDMs), which are tested for their amount and toxicity to calculate a hazard. However, for monitoring purposes, the definition of RoC is clearly different, as it is limited to difenoconazole and does not take metabolites into account (no MRL for metabolites), as agreed upon by all agents involved, most likely because they are not present at an appreciable concentration, or they have shown no greater toxicity than difenoconazole during registration studies. The peer review of the European Food Safety Authority (EFSA) concluded that difenoconazole residues are stable in tomatoes for at least 2 years if stored at −20 °C (European Food Safety Authority, 2011).

Application of PPP with difenoconazole can lead to residues in crops, including coformulants, essential components of PPPs, and difenoconazole metabolites produced during pesticide dissipation (López-Ruiz et al., 2017).

The use of difenoconazole-based PPPs may have a significant impact on the environment. After its application, difenoconazole can infiltrate soil and water systems through runoff and leaching processes (Wang et al., 2020). It can then undergo dissipation, generating several metabolites that can persist and lead to accumulation in the environment. Furthermore, the presence of difenoconazole residues and its metabolites in water can have negative effects on aquatic ecosystems, as they can show a potential impact on organisms and their habitats (Nataraj et al., 2023). In addition to the impact on aquatic ecosystems, difenoconazole and its metabolites can also affect terrestrial organisms, such as soil microorganisms (H. Zhang et al., 2021), which is likely to decrease soil health and fertility over time. Furthermore, plants grown in soils contaminated with difenoconazole residues may absorb these compounds, resulting in inhibition of their growth, as well as potential food safety concerns (Liu et al., 2021b).

Moreover, the co-formulants present in difenoconazole PPPs can also contribute to environmental contamination. For example, surfactants, which are usually added to PPPs, can persist in the environment and pose risks to both terrestrial and aquatic ecosystems (Badmus et al., 2021).

Most of the available studies focus on the determination of the active substance (Nasr et al., 2009; Lehel et al., 2022) and among those that address the analysis of its metabolites, most analyse environmental samples rather than vegetable ones, where difenoconazole PPPs are applied. Regarding vegetable samples, EFSA listed several metabolites of difenoconazole as a part of the Draft Assessment Report (DAR) on its approval along with their metabolic pathways. In this way, triazole alanine, triazole acetic acid, CGA 205374, CGA 205375 and CGA 189138 were generated after the application of ^{14}C-difenoconazole labelled to various vegetables (European Food Safety Authority, 2011). Zhang et al. (Y. Zhang et al., 2021) determined one difenoconazole metabolite in an urban river by liquid chromatography coupled to time of flight mass spectrometry (LC-Q-TOF-MS), while Man et al. (2021) identified 14 difenoconazole metabolites in water and soils by LC-Q-TOF-MS, with a limit of quantification (LOQ) for difenoconazole of 10 μg/kg in both water and soils. Regarding the determination of difenoconazole metabolites in fruits and vegetables, Hergueta-Castillo et al. (2022) developed an analytical method for the determination of difenoconazole-alcohol, a difenoconazole metabolite, in orange, courgette, strawberry and grape samples by UHPLC-Q-Orbitrap-MS/MS, with LOQs ranging from 5 to 50 μg/kg. Li et al. (2012) determined difenoconazole and difenoconazole-alcohol, in cucumbers and tomatoes using LC with ultraviolet detector (UV), and reported a LOQ of 100 μg/kg for difenoconazole, and 40 μg/kg for difenoconazole-alcohol. Previous studies have evaluated the kinetics of difenoconazole, yet none has determined its metabolites in varied vegetable scenarios, including laboratory and greenhouse settings with different dissipation conditions, which could lead to distinct metabolite profile (European Food Safety Authority, 2011; Rapporteur Member State: Sweden, 2010)

In terms of co-formulants, they may also show toxicological effects (Feiertag et al., 2021), but likewise metabolites, their analysis in vegetables is not covered typically in scientific literature. Co-formulants can be more toxic than the active substance, making them analytes of great concern (Feiertag et al., 2023). Despite this, co-formulants are also systematically overlooked in routine analyses. Moreover, there is no current EU legislation establishing MRLs for co-formulants in tomato fruits, as they were not taken into account in the original registration assessment. So far only two studies have worked on the analytical determination of co-formulants in vegetable treated with PPPs. However, they are actually considered during the approval or renewal process of active substances in the EU (Data collection, 2022). Balmer et al. (2021) determined the presence of co-formulants on various crops treated with PPPs using different formulations and the occurrence of anionic surfactants and solvents was determined by LC coupled to tandem mass spectrometry (LC-QqQ-MS/MS). Additionally, Marín-Sáez et al. (2022) confirmed the presence of volatile and non-polar co-formulants in laboratory trials in tomato using gas chromatography coupled with Q-Orbitrap high resolution mass spectrometry (GC-Q-Orbitrap-MS).

Assessing the fate and behaviour of difenoconazole, its metabolites and co-formulants is vital for evaluating the overall environmental impact of difenoconazole-based PPPs. By means of comprehensive monitoring, it is possible to evaluate the potential risks linked to the application of those PPPs and implement appropriate mitigation measures to minimise their adverse effects on the environment. Hence, the present study aims to provide relevant experimental information on the dissipation kinetics of difenoconazole, identifying those metabolites generated during the process, as well as the co-formulant residues presented in the PPP, to broaden the current knowledge on food safety in vegetables treated with difenoconazole PPPs. To this end, laboratory and greenhouse studies were carried out on tomatoes to cover different scenarios, after foliar application of Ceremonia 25 emulsifiable concentrate (EC)®, a PPP containing difenoconazole. Sample analyses were carried out by ultra-high performance liquid chromatography coupled to Q-Orbitrap high resolution mass accuracy spectrometry (UHPLC-Q-Orbitrap-MS), for difenoconazole and its metabolites, and by GC-Q-Orbitrap-MS, for co-formulants, providing more reliable results than conventional low resolution mass spectrometry (LRMS) techniques. Data was acquired in Full Scan MS and data independent acquisition (DIA) modes and processed by suspect screening. The UHPLC-Q-Orbitrap-MS method for difenoconazole was validated, and difenoconazole, its identified metabolites (UHPLC- Q-Orbitrap-MS) and Ceremonia 25 EC® co-formulants (GC-Q-Orbitrap-MS) were monitored.

2. Materials and methods

2.1. Materials and equipment

Ceremonia 25 EC® is an emulsifiable concentrate PPP that contains 25% (*w/w*) of difenoconazole. The product was purchased from FMC (Philadelphia, PA, USA), while the analytical grade difenoconazole standard (≥99.5%) was provided by LGC Standards (Teddington, United Kingdom). The preparation of the mobile phase involved the use of LC-MS methanol (Chromasolv™, ≥99.9%) from Honeywell (Charlotte, NC, USA), LC-MS water (LiChromasolv®) acquired from Merck (Darmstadt, Germany), and LC-MS grade formic acid (99.0%) from Fisher Scientific (Waltham, MD, USA). Sample extraction was carried out using LC-MS acetonitrile (Chromasolv™, ≥99.9%) obtained from Honeywell.

A 100 μm polydimethylsiloxane (PDMS) fibre was used for solid-phase microextraction (SPME) for GC-Q-Orbitrap-MS analysis, purchased from Supelco (Bellefonte, PA, USA). The sample extracts were shaken using a 444–1372 vortex supplied by VWR International (Darmstadt, Germany), while the extraction process was carried out using a Polytron homogenizer provided by Kinematica (Luzern, Switzerland).

External and mass-lock calibration for UHPLC-Q-Exactive-MS, ESI +

involved the use of an infused ProteoMass LTQ/FT-hybrid ESI mixture containing Ultramark 1621, acetic acid, caffeine, and Met-Arg-Phe-Ala-acetate salt, while ESI- external mass calibration was performed using an LTQ/FT-Hybrid ESI negative mixture containing Ultramark 1621, sodium dodecyl sulphate, sodium salt hydrate of taurocholic acid, and acetic acid. Additionally, mass-lock calibration was carried out in both negative (*m/z* 265.14790; 212.07489) and positive modes (*m/z* 414.98098; 391.24429; 279.15909; 214.08963; 112.98559). For GC-Q-Exactive-MS perfluorotributylamine was used as the mass calibrant.

2.2. Laboratory studies

According to the Spanish Ministry of Agriculture's PPP fact sheet for Ceremonia 25 EC®, the preharvest interval (PHI) in tomato is 7–14 days, and laboratory studies were conducted using a single and double dose. For a single dose, the application rate of Ceremonia (250 g of difenoconazole/L) was 10 L/ha, so the application rate of difenoconazole was 2500 g of difenoconazole/ha. For the double dose, the application rate was 20 L Ceremonia/ha, so the application rate of difenoconazole was 5000 g of difenoconazole/ha.

Ceremonia was homogenised following Vinke's guidelines for liquid PPPs (Vinke, 2021). Afterwards, 1 kg and a half of already picked-up small-size local ecological tomatoes purchased at a local store were placed in separated trays (50 × 30 cm), sprayed homogeneously with the indicated solutions making sure that all sides of each tomato were sprayed, and kept at room temperature for up to 30 days. The tomatoes were randomly sampled (150 g per sample approx.) and 3 replicates were extracted and analysed at various time points (2 h, 8 h and 1, 2, 5, 12, 15, 21, and 30 days). Tomatoes underwent a series of morphological and compositional changes throughout the study as a consequence of the decomposition process. As part of such process, tomatoes slowly lost water, which concentrated the pesticide, and could interfere with kinetic studies. Therefore, the water loss in tomatoes was constantly assessed, which accounted for 26.5% of the initial weight after 30 days, and the results were adjusted accordingly.

2.3. Greenhouse studies

Concerning greenhouse studies (Table S1), a single dose (500 mL/ha) was applied to simulate real greenhouse conditions, resulting in an application rate of 125 g of difenoconazole/ha. Three separate crop lines were utilized to plant and grow tomatoes. The tomatoes were homogeneously sprayed twice with a solution containing Ceremonia 25 EC, with a 7-day interval between the first and last application. Furthermore, several blank crop lines were planted separated from the treated crop lines, to prevent cross contamination, and collected samples were used to ensure quality control of the analyses. For proper representativity, at least 1 kg of tomatoes showing resemblance to ripeness and shape were sampled at three randomly chosen spots, for every available crop line, and analysed 2 h after every application and, then at 1, 2, 3, 4, 7, 14, 24, 38 and 53 days.

2.4. Sample processing

The tomato samples were harvested and homogenised in a blender at 23 ± 2 °C, and subjected to two different extraction procedures. Three replicates were prepared for each processed sample. In the case of the UHPLC-Q-Orbitrap-MS analysis for difenoconazole and metabolites, samples were prepared in 50 mL centrifuge tubes weighing 10 g of tomato sample and pouring 10 mL of acetonitrile. The mixture was vortexed for 1 min. The mixture obtained was then centrifuged at 3700 rpm for 10 min, the supernatant was passed through 0.45 μm pore size nylon syringe filters, and 1 mL of the clean extract was taken to a LC glass vial for analysis. However, extraction efficiency of metabolites was not demonstrated, which should be done in further work before using in a regulatory environment. Regarding GC-Q-Orbitrap-MS analysis for co-formulants analysis, 10 g of tomato were weighed in a 22 mL SPME glass vial and directly analysed.

2.5. UHPLC-Q-Orbitrap-MS analysis

The UHPLC separation of difenoconazole and its metabolites was performed using a Hypersil GOLD aQ column (100 mm × 2.1 mm, 1.9 μm). The mobile phase was composed of an aqueous solution of 0.1% (*v/v*) formic acid in water (A) and methanol (B), which was pumped at a constant flow rate of 0.2 mL/min, while the injection volume was 10 μL. Analytes were eluted in gradient mode, with an initial composition of the mobile phase of 5% methanol from 0 to 1 min, then increased up to 100% methanol from 1 min to 4 min, and followed by a steady composition of 100% methanol from 4 min to 10 min. Finally, the composition was then reduced to 5% methanol from 10 to 10.50 min, and to reach column equilibrium, it was kept steady for an additional 3.5 min. Therefore, the total run time was 14 min.

Regarding analyte detection, a Q-Exactive-Orbitrap analyser operating in Full Scan MS and DIA (positive and negative ionization modes) was used. Electrospray ionization (ESI) conditions included capillary temperature of 300 °C, heater temperature of 305 °C, spray voltage of 4 kV, S-lens radio frequency (RF) level of 50, and use of 95% purity N_2 as auxiliary and sheath gas. Full Scan MS data was acquired in the *m/z* range of 60–900, at a resolution of 70,000 at *m/z* 200, and an AGC target of 10^6 for both positive and negative modes. Furthermore, the DIA acquisition was performed at a resolution of 35,000 at *m/z* 200, an isolation window of *m/z* 50.0, an AGC target value of 10^5, and loop count 5. Xcalibur 4.3 software (Thermo Scientific™) was used for data acquisition and processing.

2.6. GC-Q-Orbitrap-MS analysis

GC-Q-Orbitrap-MS was used to analyse volatile and non-polar co-formulants. The GC device used was a Trace 1310 GC equipped with a TriPlus RSH autosampler from Thermo Scientific™. The chromatographic column was a Varian VF-5ms (30 m × 0.25 mm, 0.25 μm) made of polydimethylsiloxane as a nonpolar stationary phase, acquired from Agilent Technologies (Santa Clara, CA, USA), and was attached to a Supelco precolumn (1.5 m × 0.25 mm). Furthermore, ultra-high purity helium (99.9999%) was used as the carrier gas, which was consistently pumped at a flow rate of 1 mL/min, in spitless mode. The initial column temperature remained at 35 °C for 10 min before gradually increasing to 75 °C at a rate of 5 °C/min, followed by a sharp increase to 300 °C at a rate of 100 °C/min, and then kept constant for a further 10 min. The total run time was 30.50 min. Analytes were extracted by headspace (HS)-SPME, using a PDMS fibre. The fibre was preconditioned at 250 °C for 30 min, and then subjected to incubation at 70 °C for 1 min. The extraction time was 30 min, with a depth of 30 mm. Analyte detection was achieved using a Q-Exactive Orbitrap high-resolution mass accuracy spectrometer. The method employed for ionization was positive electron ionization (EI) operating at 70 eV, along with a filament delay of 4 min, while the ion source temperature and the transfer line temperature were both set at 250 °C. Data acquisition was performed in Full Scan MS mode, covering a mass range of *m/z* 50 to 500, with a resolution value of 60,000 FWHM at *m/z* 200, and an AGC target value of 10^6.

2.7. Data treatment (kinetic analysis and suspect screening)

To obtain adjusted kinetic curves, the Excel Solver Add-In was utilized, by optimizing various parameters, such as initial concentration (C_0) or rate constant (k), using a least-squares adjustment, which then allowed for the calculation of dissipation half-lives ($t_{1/2}$).

Subsequently, a suspect screening was applied to determine difenoconazole and its metabolites in samples, using a home-made database that list up to 12 different metabolites of difenoconazole (Table S2), based on previous studies. This database was incorporated as an

Xcalibur 4.3 Quan Browser processing method to allow quick analysis of results. For raw data file processing, a mass error limit of 5 ppm was established, whereas either $[M+H]^+$ or $[M-H]^-$ adducts were searched in Full Scan MS mode.

Whenever any positive result matched any of the screened metabolite *m/z* values in all replicates, yielded acceptable peak shapes, but remained undetected in blanks, further assessment was carried out by examining its fragmentation patterns. In-silico fragments were predicted using Mass Frontier 7.0 software, from (Thermo Scientific™), and compared with experimental fragments resulting from DIA. In this way, the tentative identification of metabolites can achieve a higher level of confidence, because it relies on at least two coinciding fragments.

3. Results and discussion

3.1. Optimization of sample extraction and method validation

To find the best extraction conditions for difenoconazole, solid-liquid extraction (SLE) was tested using two different homogenization techniques, including Polytron® and vortex. The most appropriate method was chosen according to several SANTE/11312/2021 method validation parameters (SANTE/11312/2021, 2021). Polytron® technique involved the extraction of analytes by fast mechanical homogenization through microblades, for 1 min, and under an ice bath to avoid thermal degradation of the analytes, which could lead to better extraction of the analytes, while in vortex the extracts were agitated for 1 min. Regarding the validation parameters for difenoconazole, LODs, LOQs and sensitivity/linearity were assessed by means of solvent (acetonitrile) and matrix-matched calibration standards at 0.1, 0.35, 0.5, 1, 5, 10, 25, 50, 100, 150 and 250 μg/L (0.2, 0.7, 1, 2, 10, 20, 50, 100, 200, 300 and 500 μg/kg), whereas intra and inter-day precision and recovery values were determined at two different spiked levels (2 and 200 μg/kg). Selectivity was evaluated by reagent and sample blanks, as shown in Fig. S1, where the extracted ion chromatograms for difenoconazole are represented in a solvent/reagent blank and a matrix blank, along with the extracted ion chromatogram corresponding to a tomato fruit spiked at 2 μg/kg. As Table 1 shows, both homogenization techniques provided similar results. For instance, similar linearity (expressed as R^2) was offered with a value of 0.9981 for the Polytron technique and a value of 0.9992 for vortex. Interestingly, the obtained LOQ value (2 μg/kg), using the criteria indicated by the current SANTE/11312/2021 guidelines (SANTE/11312/2021, 2021). This value is lower than those described in the consulted literature, which ranged from 5 μg/kg to 100 μg/kg in tomato, and also other matrices (Hergueta-Castillo et al., 2022; Li et al., 2012; Man et al., 2021).

Table 1
Validation parameters for the extraction of difenoconazole in tomato: Polytron® and vortex techniques.

SANTE/11312/2021 Validation Parameter		Polytron®	Vortex
Intra-day Recovery[a]	2 μg/kg	112 (3)	103 (1)
	200 μg/kg	117 (2)	88 (9)
Inter-day Recovery[a]	2 μg/kg	115 (9)	109 (7)
	200 μg/kg	104 (10)	80 (15)
Matrix effect[b] (%)		−8	−5
Linearity (R^2)		0.9981	0.9992
Instrument LOQ (μg/L)		1	1
Method LOQ (μg/kg)		2	2
Instrument LOD (μg/L)		0.35	0.35
Method LOD (μg/kg)		0.7	0.7
Selectivity		No signal (Selective)	No signal (Selective)

[a] Precision values in parentheses (n = 5).

[b] Calculated using the following expression: $M.E.(\%) = 100 * \left[\left(m_m/m_s\right) - 1\right]$, where m_s and m_m represent the slopes of the solvent calibration curve and matrix-matched calibration curve, respectively.

Furthermore, each calibration point showed a deviation of back-calculated concentration from the real concentration greater than −20% but lower than +20%. The LOD value was 0.7 μg/kg employing the criterion that it must be the lowest concentration at which at least the precursor ion and one fragment ion can be observed with a mass error lower than 5 ppm. The matrix effect was lower than 20% in all cases; as consequence of it, matrix effect could be considered negligible and solvent calibration curves could be used instead of matrix-matched calibration curves to shorten the quantitation step.

Concerning recovery values, all of them fell within the 70–120% validation range, for both methods and spiked levels, either intra or inter-day recovery. For Polytron homogenization, recovery values ranged from 104% to 117% and for vortex, they ranged from 80% to 109 %.

Regarding precision, expressed as RSD (%), the values for the Polytron technique fluctuated from 2% to 10%, while the values for the vortex varied from 1% to 15%.

Taking all these results into account, both extraction methods were successfully validated, and could be applied equally for a satisfactory determination of difenoconazole in tomato samples. However, the Polytron® technique has severe disadvantages in terms of time consumption and automatization and complexation due to its need to introduce the sample into ice to avoid heating the sample and therefore, vortex strategy was selected. The evaluation of the extraction efficiency of field-incurred residues by radio-cross-validation was not performed by radio-cross-validation, which needs to be done in further studies for full SANTE compliance.

For HS-SPME-GC-QOrbitrap-MS, the method was previously validated for co-formulants as indicated by Marin-Saez et al. (Marín-Sáez et al., 2023) obtaining satisfactory results according to SANTE (SANTE/11312/2021, 2021).

3.2. Difenoconazole kinetic studies

Several kinetic models were evaluated to find the one that fits the best, including zero-order, single first-order (SFO), second-order or biphasic double first-order in parallel (DFOP) models, as depicted in Table S3.

3.2.1. Laboratory trials

Laboratory trials were conceived as a preliminary design study for greenhouse trials. Therefore, results are not decisive because of design features such as the application of a high concentration of difenoconazole, and different metabolomic processes occur in the plant (during growth) and others in the harvested fruit, which may lead to challenges. Concerning the dissipation of difenoconazole in laboratory studies, two main stages were observed during the trial monitoring: an initial increase in the concentration of difenoconazole, which suggests a pre-concentration of the pesticide (difenoconazole accumulates and its concentration increases gradually until it reaches a peak value), followed by a decrease in its concentration, which in this case represents the degradation of difenoconazole itself (after the peak concentration is reached, dissipation begins). Thus, DFOP adjustment was the only fitting model since all the other assessed models were unable to provide any kind of fitting to describe the experimental behavior of the compound. In fact, as Table 2 shows, the DFOP model showed an R^2 value as high as 0.99, which emphasises how satisfactory that fit was in all cases. This contrasts with studies in the literature that indicate an SFO for the dissipation of difenoconazole, although it is important to note that these studies were conducted in other matrices and under different conditions.

For single-dose trials, initial difenoconazole concentration (C_0) in tomatoes, estimated from the predicted DFOP model, was 456 μg/kg. On the other hand, the half-life of a single dose during the dissipation stage (decline of the difenoconazole concentration after the top concentration peak) was 16.64 h (0.69 days). As Fig. 1a shows, the concentration of

Table 2
Kinetic parameters (biphasic DFOP model) for the dissipation of difenoconazole dissipation in tomato: laboratory and greenhouse tests.

Parameter	Laboratory trials		Greenhouse trials
	Single dose	Double dose	Single dose
C_0 (μg/kg)	456	919	53
k_1 (h^{-1})	0.001419	0.0006012	0.003106
k_2 (h^{-1})	0.04164	0.04585	0.003176
R^2	0.9983	0.9986	0.9965
A	6.1057	3.7808	192.62
$t_{1/2}$ (h) for k_2	16.64	15.12	218.24

Abbreviations: C: concentration; C_0: initial concentration; k_1: rate constant (first stage); k_{12}: rate constant (second stage); R^2: coefficient of determination; a: fraction of C_0 applied to compartment 1; $t_{1/2}$: half-life.

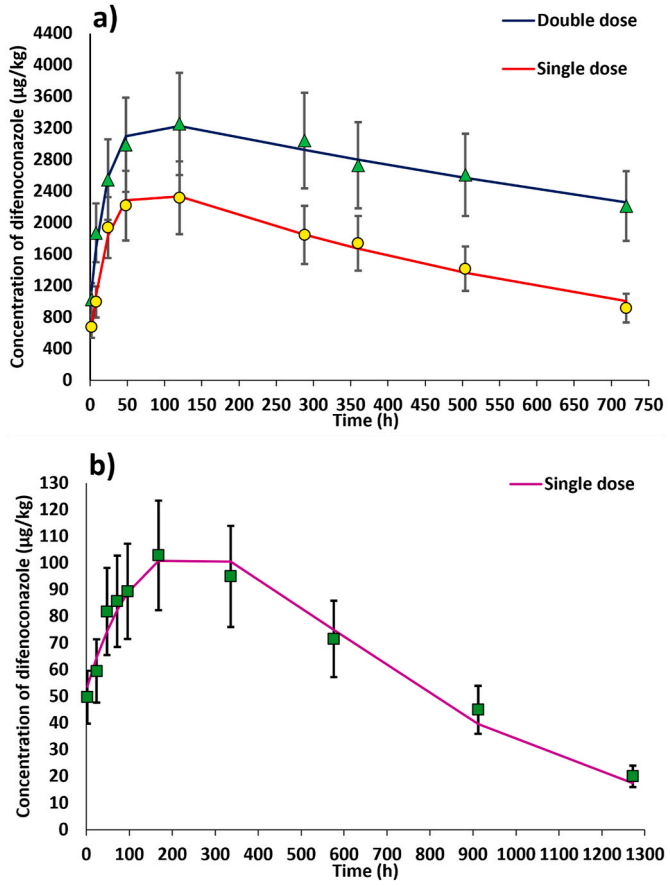

Fig. 1. Biphasic DFOP dissipation kinetic adjustment for difenoconazole in: (a) laboratory trials at double dose and single dose, and (b) greenhouse trials at single dose. Number of replicates: 3.

difenoconazole gradually increased from 674 μg/kg at 2 h (0 days) to 2315 μg/kg at 120 h (5 days), the time at which the highest concentration of difenoconazole was reached. In general, this represents an increase in concentration of 13.9 μg/kg per hour. Subsequently, the concentration dropped until it reached a value of 913 μg/kg at 720 h (30 days), when difenoconazole was last monitored. Therefore, this accounts for a decrease rate of 2.3 μg/kg per hour. As can be observed, dissipation of difenoconazole occurred at a considerably slower pace than in the initial concentration stage of the pesticide. These data indicate that difenoconazole had not fully dissipated at the end of the trials, even though as much as 30 days had passed by. In fact, its final concentration (913 μg/kg) was twice the initial concentration (456 μg/kg).

Regarding double dose trials, the initial difenoconazole concentration (C_0), estimated from the predicted DFOP model, was 919 μg/kg, which is exactly double the initial difenoconazole concentration in single dose experiments (456 μg/kg). This fact suggests that Ceremonia was correctly and homogeneously sprayed in all tomato samples. In this case, the half-life of double dose dissipation during the dissipation stage was 15.12 h (0.63 days), less than the half-life value for single dose dissipation, implying that difenoconazole suffered a slightly faster dissipation in double dose trials, as it reached half-life concentration 1.62 h before difenoconazole in single dose trials. However, this tiny variability could also be attributed to experimental errors.

It can be seen in Fig. 1a that concentration of difenoconazole increased from 1025 μg/kg at 2 h (0 days) to 3252 μg/kg at 120 h (5 days), when the maximum concentration of difenoconazole was reached, similarly to single dose trials. This implies an increase in concentration of 18.87 μg/kg per hour. Subsequently, the concentration of difenoconazole decreased steadily until its value was 2210 μg/kg at 720 h (30 days). Once again, the final concentration of difenoconazole was higher than its initial concentration. Taking these results into account, dissipation for single dose experiments showed a slightly higher dissipation rate (2.3 μg/kg/h vs 1.74 μg/kg/h). However, these results are contradictory to the half-life findings and these differences can be attributed to experimental error and variability. Most previous studies for difenoconazole focused on this topic determined first-order dissipation kinetics in soils or vegetables, in which the pesticide was applied exclusively in crop fields (Wang et al., 2012; Xu et al., 2019). However, it is very important to note that difenoconazole is a systemic pesticide and, as such, DFOP models such as those determined in the present study could best describe the initial preconcentration of difenoconazole, and its later release and dissipation (Marín-Sáez et al., 2022). Regarding other pesticides as myclobutanil (Marín-Sáez et al., 2022) penconazole or flutriafol (Hergueta-Castillo et al., 2023a, 2023b) similar behaviour was observed during laboratory trials, observing a DFOP kinetic model, with a high increase at the beginning of the trial to a later decrease. There are various reasons that could explain the divergent kinetic behaviour observed. These include the use of a solid EC formulation in our research, unlike other studies that used difenoconazole as an analytical standard or used a different type of formulation (such as a soluble concentrate (SC)). Furthermore, the type of tomato used, as well as variations in applied doses or experimental settings, could also have contributed to the differences. Furthermore, some studies reveal that pesticides such as chlorpyrifos or fluopyram (Mekonnen et al., 2019), can interact with various components within the fruit, such as sugars, organic acids, and proteins. These interactions can influence the rate of degradation by protecting or stabilising pesticide molecules and for this reason during monitoring studies, the pesticide was detected in less quantity, as in this study during the first days (Ohkawa et al., 2007).

3.2.2. Greenhouse trials

Regarding greenhouse trials, they were carried out exclusively at single dose, since day-to-day application of difenoconazole PPPs involves the exclusive utilisation of a single dose, and therefore in this case, the double dose would not provide realistic insight into the dissipation of difenoconazole under greenhouse conditions. The DFOP model was also found to be the sole fitting model, as Fig. 1b shows. A R^2 value of 0.9965 was obtained, so the fit is deemed highly satisfactory. The initial concentration of difenoconazole was found to be 53 μg/kg, which is significantly lower than the initial concentration of difenoconazole determined in single dose laboratory studies (456 μg/kg), and this can be explained by the fact that while in the laboratory study the PPP was applied directly to the tomato, in the greenhouse study, the dose was applied to the entire plant. Furthermore, half-life during the dissipation was 218.24 h (9.09 days). The difenoconazole concentration started at 50 μg/kg at 2 h (0 days) and reached its peak at 103 μg/kg after 168 h (7 days). In this case, the increase in concentration was 0.32 μg/kg per hour. Subsequently, the concentration decreased to 20 μg/kg at 1272 h (53 days), when difenoconazole was analysed for the last time. Noticeably, and unlike laboratory trials, the final monitored concentration was

lower than the initial concentration, mainly because greenhouse trials were extended over a longer period of time (23 days more). In light of these results, difenoconazole did not exceed its MRL value for tomato samples, which is set at 2 mg/kg.

To compare dissipation of laboratory and greenhouse trials, difenoconazole concentration values of difenoconazole in laboratory studies were extrapolated at 53 days using the optimised kinetic equation. This provided a result of 458 μg/kg, which is considerably higher than the final concentration value in greenhouse trials (20 μg/kg), and nearly the same value as the initial concentration of difenoconazole right after Ceremonia application (456 μg/kg). The total percentage of dissipation from the peak to the final concentration at 1272 h was 80% for laboratory studies, while it was also 80% for greenhouse studies. This finding shows that higher concentration values in laboratory trials are misleading, and the dissipation rate was virtually the same for both types of trials.

3.3. Metabolites

After kinetic studies, difenoconazole metabolites were searched and determined by suspect screening, using a home-made database containing 12 different difenoconazole metabolites. In total, 3 different difenoconazole metabolites were tentatively identified in tomato samples from laboratory trials, as described in Table 3. Therefore, this represents a positive result of up to 25% of all listed metabolites. The tentatively identified metabolites were CGA-205374, CGA-205375 (also known as difenoconazole alcohol) and hydroxy-difenoconazole, whose chemical structures can be seen in Fig. 2. CGA 205374 and CGA 205375 were described in vegetables in the official EFSA registration documents for the approval of difenoconazole, while hydroxy-difenoconazole was not detected in EFSA studies. However, these differences are expected as a result of the different environmental conditions. Metabolite CGA-205374 is generated by loss of the cyclic diether present in difenoconazole, whereas difenoconazole alcohol is generated because of the substitution of this cyclic diether for a hydroxyl molecule. Hydroxy-difenoconazole is generated by hydroxylation of the outer phenyl ring of difenoconazole.

CGA-205375 and difenoconazole showed similar retention times (8.27 and 8.65 min, respectively). To provide a more reliable tentative identification, the chlorine isotopic pattern was evaluated, as can be seen in Fig. 3, which tested positive for the *m/z* value suspected of belonging to CGA-205375, as two different characteristic ions could be observed (350.04504 for the ^{35}Cl isotopologue and 352.04201 for the ^{37}Cl isotopologue). Furthermore, two different low-mass DIA fragments were found *m/z* 119.08554 and 70.04105, the latter being a characteristic fragment for the triazole family, which corresponds to the compound 1,2,4-triazole, and with a mass error lower than 5 ppm, which reinforced the identification of **CGA-205375**.

In general, it can be observed that CGA-205374 was generated as soon as 2 h after the application of Ceremonia and lasted up to 21 days, not being detected after 30 days. Regarding difenoconazole-alcohol, it was undetectable until the second day, but unlike difenoconazole, its presence lasted throughout the remaining trials. Finally, hydroxy-difenoconazole was only detected starting from the 12th day after PPP application, and again, it could be detected until the last day of the study. This time period makes much sense considering the metabolic pathway of difenoconazole, described in literature (Ministerio de Sanidad, 2020), in which CGA-205374 is generated directly from difenoconazole, while difenoconazole alcohol is generated as a result of the hydrolysis of the ketone present in CGA-205374. This could explain why CGA-205374 was first detected as early as 2 h, while difenoconazole alcohol could not be detected until the second day.

Considering that difenoconazole and its metabolites share essential structural similarities, as they steam from the parent pesticide, a semi-quantification was carried out by using a difenoconazole analytical standard. This allowed an estimation of the concentration of its metabolites. It is important to note that the same matrix instrumental dilution factor applies to difenoconazole and its metabolites (2 μg/kg in tomato fruit per 1 μg/L in injected sample).

For all 3 identified difenoconazole metabolites, the concentration values were discarded for all days, except day 30, as they were above the LOD (0.7 μg/kg), but well below the method LOQ for difenoconazole (2 μg/kg), usually 10 times lower, and therefore were not reported. On the 30th day of the trials, samples sprayed with a double dose of Ceremonia showed concentration values of CGA-205375 and hydroxy-difenoconazole barely above LOQ. Thus, CGA-205375 was detected at 3 μg/kg, whereas hydroxy-difenoconazole was detected at 5 μg/kg, as shown in Table 3. This finding makes sense, since the concentration of metabolites is expected to gradually increase as the parent pesticide dissipates, to the point that it may eventually surpass the LOQ value after enough time has passed since the beginning of the dissipation. However, this does not imply that these metabolites, despite being present in a low concentration, cannot pose toxicological risks.

Related to this problem, there is limited literature available on the toxicological properties of the tentatively identified metabolites. As a result, the toxicity of these metabolites was evaluated using the Toxicity Estimation Software Tool (TEST), an open-access program created by the U.S. Environmental Protection Agency (EPA), which predicts various toxicological parameters based on the chemical structure of the metabolite.

CGA-205374 was negative for mutagenicity, and had a developmental toxicity value of 0.38, and is then classified as a developmental nontoxicant. Additionally, difenoconazole alcohol also tested negative for mutagenicity, but had a predicted developmental toxicity value of 0.56, therefore it was deemed to be a developmental toxicant. On the other hand, difenoconazole was tested negative for mutagenicity, as well as its metabolites, but had a predicted developmental toxicity value of 0.41, as therefore it was classified as a developmental non-toxicant. Therefore, according to these predictions, difenoconazole-alcohol could be considered to be more toxic than difenoconazole itself.

Concerning greenhouse trials, no metabolite was detected, probably due to the very low concentrations in which they could be present, which may fall below the method LOD, unlike in laboratory trials. This finding ensures that the use of Ceremonia at a single dose, as it is meant to be applied according to the legislation in force, does not pose a direct risk.

3.4. Co-formulants analysis

The presence of co-formulants was qualitatively investigated in

Table 3
Detection and semi-quantification of difenoconazole metabolites in tomato in laboratory trials.

Metabolite	Formula	Adduct *m/z*	2 h	8 h	Day 1	Day 2	Day 5	Day 12	Day 15	Day 21	Day 30
CGA-205374	$C_{16}H_{11}Cl_2N_3O_2$	348.03011 (+)	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	**ND**
CGA-205375/Difenoconazole-alcohol	$C_{16}H_{13}Cl_2N_3O_2$	350.04576 (+)	**ND**	**ND**	**ND**	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	**3 μg/kg**[a]
Hydroxy-difenoconazole	$C_{19}H_{17}Cl_2O_4N_3$	422.06689 (+)	**ND**	**ND**	**ND**	**ND**	**ND**	< LOQ	< LOQ	< LOQ	**5 μg/kg**[a]

Abbreviations: ND: not detected; < LOQ: Detected, but its concentration is lower than the limit of quantification of difenoconazole.
[a] Concentration values for double dose trials.

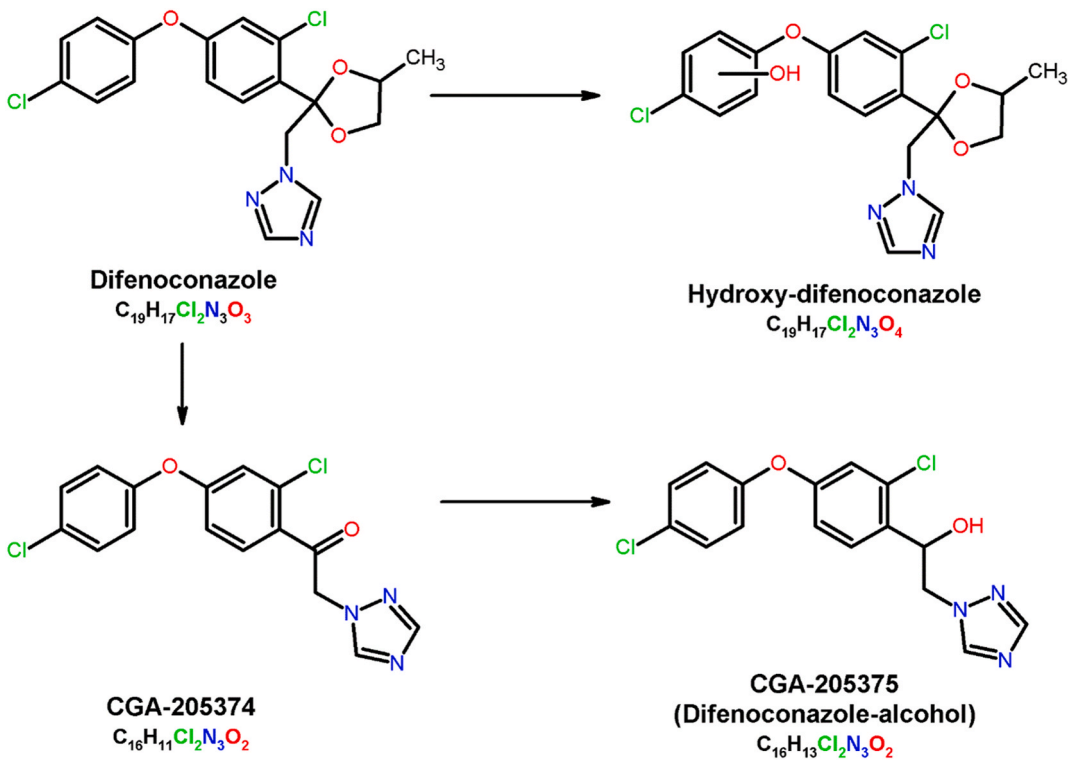

Fig. 2. Dissipation pathway of difenoconazole: TPs hydroxy-difenoconazole, CGA-205374 and CGA-205375.

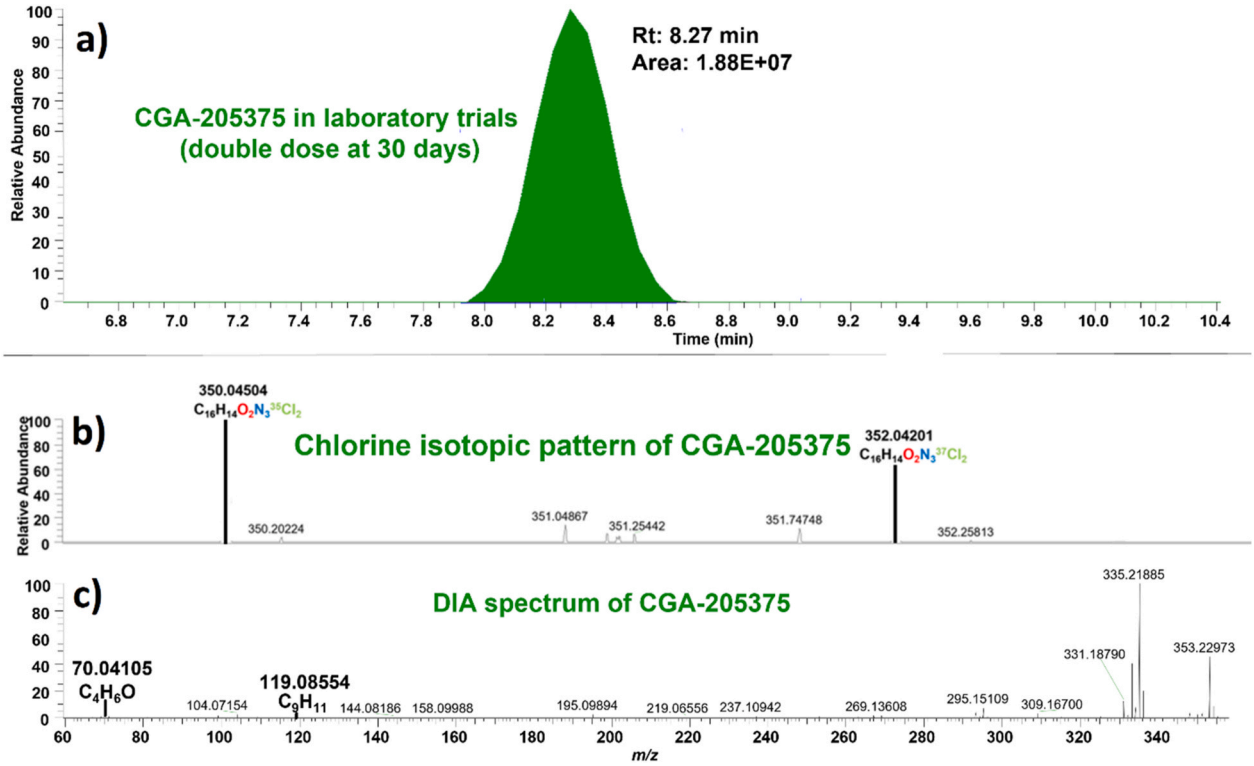

Fig. 3. Determination of CGA-205375 in laboratory trials: a) double dose at 30 days, b) verification of the isotopic pattern, and c) DIA fragmentation pattern of CGA-205375.

tomato samples treated with Ceremonia in a single dose under greenhouse conditions, to shed light on other unwanted substances in vegetables, from the first application (2 h), to the last day of sample collection (53 days). As a result of the applied suspect screening, a total of 13 co-formulants were tentatively identified. Most of these co-formulants had already been identified in previous studies aiming to characterise the co-formulant composition of PPPs, including Ceremonia (Maldonado-Reina et al., 2021), and consist of naphthalene and

benzene, parabens, dioxolanes or terpenes. However, it must be considered that there may be different co-formulant isomers, and analytical standards were not available for confirmation purposes, so one or more co-formulant names were allocated for every positive suspect screening m/z value.

As Table 4 shows, no co-formulant could be detected beyond 7 days after Ceremonia was applied. On the other hand, trimethylbenzene was already undetectable at the 3rd day, whereas pentamethylbenzene was already undetectable at the 2nd day, which indicated that these co-formulants either volatilized or dissipated before the other identified co-formulants. Interestingly, while all co-formulants were detectable right after the first application of Ceremonia, 4-(4-hydroxyphenyl) butan-2-one/4-Methyl-2-phenyl-1,3-dioxolane could not be detected until the 2nd day. In conclusion, these types of substances do not remain in the samples for a long period of time.

Previously, Marín-Sáez et al. (2022) identified 7 different volatile co-formulants in tomato samples treated, in laboratory settings, with Mitrus, a myclobutanil PPP.

Similarly, four of these co-formulants (or their isomers) were also detected in the present study, specifically 1,2,4-trimethylbenzene, 2-methylbiphenyl, pentamethylbenzene and tert-butylbenzene. Although it cannot be confirmed due to the lack of analytical standard confirmation, it is suspected that 1,2,4-trimethylbenzene, 2-methylbiphenyl, and tert-butylbenzene were among the co-formulants present in tomato samples treated with Ceremonia, based on all possible isomers described in Table 4. The co-formulants were monitored at six different time points after Mitrus application, specifically 2 h, 6 h, 1 day, 2 days, 5 days, and 12 days. On analysis, tert-butylbenzene and 2-methylbiphenyl were found to have dissipation times to the present study, as they were still detectable on day 12. In contrast, 1,2,4-trimethylbenzene and pentamethylbenzene had a longer dissipation time compared to the observations of the present study, as both remained detectable on day at 3 °C or 22 °C.

In summary, the analytical techniques employed allow the identification and evaluation of the kinetic dissipation of the insecticide difenoconazole in tomato. In addition, its metabolites, and accompanying co-formulants are found in tomato samples that have been treated with a difenoconazole PPP. It is worth noting that a DFOP kinetic model fit was observed in all instances, which contrasts with the majority of previous studies that reported an SFO kinetic model fit for tomato. Furthermore, this work introduces a significant novelty by providing valuable data on the simultaneous detection and identification of difenoconzole metabolites and co-formulants present in difenoconazole-based PPPs in tomato samples, which was not addressed in previous research, identifying a total of 13 volatile coformulants, and three metabolites (CGA-205374, CGA-205375, and hydroxy-difenoconazol). These degradation products exhibited persistence, since their total dissipation was not achieved under any of the conditions performed, regardless of the length of the study. Furthermore, the study presents the introduction of high-resolution mass spectrometry (HRMS) as a more reliable means of identifying metabolites, thus offering a promising outlook for future research into the presence of metabolites for a wider range of matrices. In conclusion, this study has successfully explored the analytical assessment of difenoconazole, as well as other associated components, in tomato, including metabolites and co-formulants. These findings have crucial implications for food safety and provide a basis for future research aimed at monitoring any chemical substances that may be derived directly or indirectly from the application of PPPs, not only in vegetables, but also in other matrices.

4. Conclusions

In summary, this paper provides relevant information on the dissipation of difenoconazole, and the identification of several possible contaminants associated with the use of PPPs, such as co-formulants or metabolites. Laboratory trials were designed as a preliminary study which would help make an optimal design of greenhouse trials (such as a different number and frequency of the harvesting dates), and therefore, they should not replace greenhouse studies, but complement them. Additionally, laboratory studies were intended to evaluate the behaviour and fate of difenoconazole in harvested tomatoes, and at a high concentration, although the loss of water during lab trials can affect the fate of the compounds. Despite these differences, difenoconazole followed a biphasic double first-order in parallel (DFOP) dissipation in greenhouse and laboratory trials, with virtually the same dissipation rate for both settings. Regarding its metabolites, CGA-205374, CGA-205375 (difenoconazole-alcohol) and hydroxy-difenoconazole were identified and semi-quantified in laboratory tomato samples, with CGA-205374 remaining below the LOQ from the first day to the third week, while CGA-205375 remained detectable from day up, and could be quantified on day 30 (3 μg/kg), similarly to hydroxy-difenoconazole, which was detectable from day 12 and quantifiable on day 30th as well (5 μg/kg). No metabolites were detected in greenhouse tomatoes. Concerning co-formulants, 13 volatile co-formulants were identified and monitored in greenhouse studies, most of which could still be detected even 7 days after the initial application of the PPP, indicating that this type of substances must be analysed along with pesticides in crops.

Table 4
Detection of Ceremonia co-formulants in tomato in greenhouse trials.

Co-formulant(s)	Formula	Exact mass	1st app.	2nd app.	Day 1	Day 2	Day 3	Day 4	Day 7	Day 14
1-(2-propenyl)naphthalene/2-Methyl-1,1′-biphenyl/1-(2-propenyl)naphthalene/ Diphenylmethane	$C_{13}H_{12}$	168.09390	YES	YES	YES	YES	YES	YES	YES	**ND**
4-(4-hydroxyphenyl)butan-2-one/4-Methyl-2-phenyl-1,3-dioxolane	$C_{10}H_{12}O_2$	164.08318	**ND**	**ND**	**ND**	YES	YES	YES	YES	**ND**
1-(1,1-Dimethylethyl)-3,5-dimethylbezene/4-Tertbutyl-o-xylene1-(1,1-Dimethylethyl)-3,5-dimethylbezene/4-Tertbutyl-o-xylene/1,3-Diisopropylbenzene	$C_{12}H_{18}$	162.14085	YES	YES	YES	YES	YES	YES	YES	**ND**
1,3-Dimethylnaphthalene	$C_{12}H_{12}$	156.09390	YES	YES	YES	YES	YES	YES	YES	**ND**
Methylparaben	$C_8H_8O_3$	153.05462	YES	YES	YES	YES	YES	YES	YES	**ND**
Pentamethylbenzene	$C_{11}H_{16}$	148.12520	YES	YES	YES	**ND**	**ND**	**ND**	**ND**	**ND**
2,3-Dihydro-1,2-dimethyl-1H-indene	$C_{11}H_{14}$	146.10955	YES	YES	YES	YES	YES	YES	YES	**ND**
2-Ethenyl-1,3,5-trimethylbenzene	$C_{11}H_{12}$	144.09335	YES	YES	YES	YES	YES	YES	YES	**ND**
1-Methylnaphthalene	$C_{11}H_{10}$	142.07825	YES	YES	YES	YES	YES	YES	YES	**ND**
D-Limonene	$C_{10}H_{16}$	136.12520	YES	YES	YES	YES	YES	YES	YES	**ND**
1,2,3,4-Tetramethylbenzene/1,4-Diethylbenzene/1-Methyl-3-propylbenzene/4-Ethyl-m-xylene/Propyltoluene/tert-Butylbenzene	$C_{10}H_{14}$	134.10955	YES	YES	YES	YES	YES	YES	**ND**	**ND**
Trimethylbenzene (1,2,4-Trimethylbenzene/mesitylene/1,2,3-trimethylbenzene)	C_9H_{12}	120.09390	YES	YES	YES	YES	**ND**	**ND**	**ND**	**ND**
Ethylbenzene	C_8H_{10}	106.07825	YES	YES	YES	YES	YES	YES	**ND**	**ND**

Abbreviations: app.: application; ND: not detected; Note: some co-formulants have various names due to being isomers, and reliable identification is only possible through analytical standards.

CRediT authorship contribution statement

Antonio Jesús Maldonado-Reina: Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Rosalía López-Ruiz:** Writing – review & editing, Supervision, Software, Investigation, Formal analysis, Data curation. **Jesús Marín Sáez:** Writing – review & editing, Software, Investigation, Formal analysis, Data curation. **Roberto Romero-González:** Software, Investigation, Formal analysis, Data curation, Writing – review & editing. **Antonia Garrido Frenich:** Writing – review & editing, Supervision, Software, Data curation, Formal analysis, Funding acquisition, Investigation, 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.

Acknowledgements

Project, project ref. PID2019-106201RB-I00) funded by MICIU/AEI/10.13039/501100011033. RLR acknowledges to the Andalusian Ministry of Economic Transformation, Industry, Knowledge and Universities for financial support from "Ayudas para Captacion, Incorporacion y Movilidad de Capital Humano de I + D + I (PAIDI 2020)". AJMR acknowledges the Ministry of Universities of Spain for financial support from "Ayudas para la Formación del Profesorado Universitario (FPU)", ref. FPU19/04260. JMS thanks the University of Almería for his "Convocatoria de Recualificación do Sistema Universitario Español-Margarita Salas" postdoc grant under the "Plan de Recuperación Transformación" program funded by the Spanish Ministry of Universities with European Union's NextGenerationEU funds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2024.123924.

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