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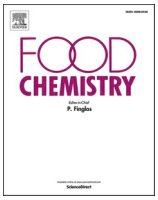

# Degradation study of the *trans*-cinnamaldehyde and limonene biopesticides and their metabolites in cucumber by GC and UHPLC-HRMS: Laboratory and greenhouse studies

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ABSTRACT

Degradation of *trans*-cinnamaldehyde and limonene in cucumber was evaluated under laboratory and greenhouse conditions. Two commercial biopesticides, one based on cinnamon extract and other from orange oil, were utilized. Compound degradation was monitored using gas chromatography (GC) and ultra-high-performance liquid chromatography (UHPLC) coupled to a quadrupole-high-resolution mass analyzer (Q-Orbitrap). In both studies, *trans*-cinnamaldehyde followed a second-order degradation kinetics, whereas limonene followed a first-order kinetics. The half-life values ($DT_{50}$ or $t_{1/2}$) for *trans*-cinnamaldehyde ranged from 2.02 to 2.49 h, while for limonene this value ranged from 0.49 to 6.17 h. Non-targeted analysis (suspect and unknown modes) allowed for the detection of *trans*-cinnamaldehyde and limonene metabolites. Benzyl alcohol, cinnamyl alcohol, cinnamic acid, *p*-tolylacetic acid and 4-hydoxycinnamic acid were tentatively identified as *trans*-cinnamaldehyde metabolites. While three limonene metabolites, carvone, limonene-1,2-epoxide, and perillyl alcohol, were tentatively identified. Greenhouse studies have not revealed any metabolites of these compounds because the parent compounds degrade more quickly.

## 1. Introduction

In recent years, biopesticides have been utilized in agriculture to reduce the use of synthetic pesticides that can harm the environment, as well as put human health at risk when ingested in food such as fruits or vegetables. Biopesticides are natural products found in plants, microorganisms or minerals (US EPA, 2022). They can be classified into various categories, including microbial pesticides (*Bacillus thuringiensis*), biochemical pesticides (insect pheromones, natural insect growth regulators, plant extracts and essential oils), and genetically modified organisms (GMOs). Algae- or cyanobacteria-based biopesticides, as well as RNA interference-based biopesticides and nanobiopesticides have also being used (Kumar et al., 2021). The last two classes have the advantage of being more selective and precise for targeted pests, as well as penetrating the plant more effectively.

Medicinal and insecticide products made from plant extracts and essential oils have been used for centuries. The plant's volatile compounds are the main ingredients in these extracts and oils. Most of these compounds are phenylpropanoic or terpenes, including monoterpenes and sesquiterpenes. Azadirachtin A (neem tree), pyrethrins (pyrethrum extract), or nicotine are the most commonly used commercial biopesticides that are based on plant extracts (Khursheed et al., 2022). Cinnamon extracts or orange oil are also being marketed as biopesticides, being *trans*-cinnamaldehyde (Wang et al., 2009) and limonene (Denkova-Kostova et al., 2021) the main compounds in these products, respectively. *trans*-Cinnamaldehyde is a phenylpropanoid that can be found in cinnamon bark (Lee et al., 2020). Limonene is a monoterpene present in many citrus fruits and essential oils, such as eucalyptus or lavender oil. Both compounds have antiviral, antibacterial, antifungal and antibiofilm properties. Thus, *trans*-cinnamaldehyde has been shown to be effective against bacteria, such as *Escherichia coli* (Denkova-Kostova et al., 2021; Mousavi et al., 2016) or *Agrobacterium tumefaciens* (Lee et al., 2020), nematodes (Jardim et al., 2018), fungi (Adfa et al., 2022) or insects, such as *Spodoptera littoralis* (Abdelgaleil et al., 2020) and *Tribolium castaneum* (Sujatha et al., 2019). Limonene is effective against *E. coli* (Denkova-Kostova et al., 2021), fungi (Leitão et al., 2020; Yu et al., 2022) and insects, such as cochineal (Bouharroud et al., 2018) or beetle (de Andrade Rodrigues et al., 2022). Due to this

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reason, they are widely used as a biopesticides and insect repellents (Gupta et al., 2021). There are several studies where the effectiveness of different biopesticides based on plant extracts has been evaluated, simulating semi-real conditions in banana (Cakmak et al., 2022) or tomato crops (Hafsi et al., 2012), observing that some, such as Prevam® (based on orange oil), had good insecticidal efficacy with a positive economic impact (Cakmak et al., 2022).

However, the effects of these biopesticides on environmental and food matrices have not been studied extensively. Until now, the degradation of *trans*-cinnamaldehyde and limonene has been evaluated in samples such as soil in laboratory studies (Huang et al., 2022; Reyes-Ávila et al., 2023a) and open field (Huang et al., 2022), whereas the degradation of limonene was evaluated in tomatoes (Huang et al., 2022). *trans*-Cinnamaldehyde has a half-life time values ($DT_{50}$ or $t_{1/2}$) of 0.16 to 0.28 days (Reyes-Ávila et al., 2023a), while limonene has a $DT_{50}$ of 0.08 to 2.84 days in soil (Huang et al., 2022; Reyes-Ávila et al., 2023a). In open field, after seven days of application, limonene was not detected in tomato (Huang et al., 2022). Degradation of *trans*-cinnamaldehyde in other types of matrices, such as the gastrointestinal tract of piglets (Michiels et al., 2008) or rat plasma (Zhao et al., 2014), has also been studied. In both cases, the degradation of *trans*-cinnamaldehyde occurred rapidly. In these studies, gas chromatography (GC) was used to evaluate the degradation of the compounds. Furthermore, they employed classical detection, such as flame ionization detector (Michiels et al., 2008) or low-resolution mass spectrometry analyzers as single quadrupole (Huang et al., 2022; Zhao et al., 2014). In some of these studies the presence of transformation products or metabolites were evaluated. Metabolites of *trans*-cinnamaldehyde such as cinnamic acid or cinnamyl alcohol (Reyes-Ávila et al., 2023a), and metabolites of limonene such as carveol, limonene epoxide or carvone (Huang et al., 2022) were detected.

Therefore, to expand the knowledge about the degradation of these compounds and the appearance of their metabolites in other food matrices not studied so far, a study focused on their degradation in cucumber was carried out. In addition to a laboratory study, it has been decided to monitor the degradation of both compounds after their application in greenhouses. Additionally, in this study the appearance of metabolites has been studied to evaluate their possible toxicity. For that purpose, high-resolution mass spectrometry analyzer such as quadrupole (Q)-Orbitrap has been used to address all possible compounds with greater reliability due to high mass accuracy measurements, that allows the combination of targeted, suspect and unknown analyses. This is an advantage compared to previous studies that used a single quadrupole (Huang et al., 2022; Zhao et al., 2014). Furthermore, to cover a wider range of polarities, both GC and ultra-high-performance liquid chromatography (UHPLC) have been used. This allows for the detection of polar and non-polar metabolites. To identify possible metabolites in a new way, it has been decided to conduct a non-targeted analysis by combining the suspect (bibliographic search) and the unknown analyses.

## 2. Materials and methods

### 2.1. Materials

Two commercial biopesticides, Cinna (Hortalan; El Ejido, Spain) and Prevam® (ORO AGRI; Palmela, Portugal), based on cinnamon extracts and orange essential oil respectively, were acquired. A previous characterization was carried out to determinate their major compounds and concentration. Limonene concentration was 39.14 g/L in Prevam®, and *trans*-cinnamaldehyde concentration was 371.8 g/L in Cinna (Reyes-Ávila et al., 2023b).

Analytical standards used were *trans*-cinnamaldehyde (>98% purity) purchased by Dr. Ehrenstorfer (Augsburg, Germany), and (R)-(+)-limonene (98% purity) by Sigma Aldrich (Saint Louis, MO, USA). Internal standard (IS) was biphenyl (Dr. Ehrenstorfer). Magnesium sulfate ($MgSO_4$) and sodium chloride (NaCl) were provided by Scharlab (Barcelona, Spain).

Ethyl acetate (EtOAc, ≥99.7%) was acquired from Chem-Lab (Zedelgem, Belgium), and methanol (MeOH, ≥99.9%) and acetonitrile (ACN, ≥99.9%) from Honeywell (Charlotte, NC, United States). Formic acid (FA, 99.0%) and water ($H_2O$, LiChrosolv®) were from Merck (Darmstadt, Germany). All solvents were LC-MS grade.

Stock solutions were prepared at 1000 mg/L in EtOAc. Then, solutions at 10 and 1 mg/L in EtOAc were made from the stock solution. These solutions were stored at −18 °C.

The filters used were Econofltr nylon filter 0.2 μm, 13 mm provided by Agilent Technologies (Santa Clara, CA, US).

### 2.2. Chromatographic methods

The methods used for monitoring the degradation of limonene and *trans*-cinnamaldehyde have been previously optimized (Reyes-Ávila et al., 2023b).

The gas chromatographic equipment was a TRACE™ 1310 GC system with a TriPlus™ RSH autosampler (Thermo Fisher Scientific; Waltham, MA, US) and a non-polar column J&W DB-5ms (30 m × 0.25 mm × 0.25 μm; Agilent Technologies), coupled to a Q-Exactive Orbitrap (Thermo Fisher Scientific) mass spectrometer. The injector temperature was set at 250 °C. The injection volume was 1 μL. For chromatographic conditions, initial oven temperature was 60 °C (hold 2 min) and it was increased at 6 °C/min rate to 220 °C (hold 2 min). Finally, it was raised to 280 °C with a 20 °C/min rate (hold 4 min). The total running time was 37 min. For MS conditions, full scan in positive mode was used (30–450 *m/z* range) with a 70-eV positive electron ionization (EI). The resolution was 70,000 full width at half maximum (FWHM), and an AGC value was set at $10^6$. Helium was used as carrier gas with a constant flow rate of 1 mL/min.

The liquid chromatographic equipment was a Vanquish™ Flex Quaternary LC (Thermo Fisher Scientific) with a C18 Hypersil GOLD™ aQ column (2.1 × 100 mm, 1.9 μm) purchased by Agilent. Mass spectrometer was a Q-Exactive Orbitrap, provided by Thermo Fisher.

The acquisition mode used was full scan (74–1100 *m/z* range) with a resolution of 70,000 FWHM. The automatic gain control (AGC) value was equal to $10^6$. Data dependent acquisition (DDA), in negative and positive ionization modes, was used. DDA resolution was 35,000 FWHM, setting an AGC value at $10^5$. Minimum AGC target value was $8{\cdot}10^3$. The flow rate was 0.2 mL/min, the injection volume was set at 10 μL and the column temperature was 30 °C. The mobile phase consisted of MeOH as organic phase and an aqueous solution of formic acid (0.1%) as aqueous phase. The gradient mode started with a constant composition of 5 % MeOH during 2 min. Then, it was increased up to 100 % MeOH during 14 min, and this composition was kept constant from 16 min to 26 min. Finally, the composition decreased to 5 % MeOH in 1 min, and it was kept constant for 3 min to equilibrate the column. Total running time was 30 min. The electrospray interface (ESI) conditions were: auxiliary and sheath gas used, $N_2$ (95%); heater temperature, 305 °C; capillary temperature, 300 °C; spray voltage, 4 kV, and the S-lens radio frequency level was 50 (arbitrary units).

### 2.3. Degradation in cucumber studies

#### 2.3.1. Laboratory study

Cucumbers were provided for laboratory studies from a local market in Almería (Spain). Degradation studies were performed in the research group's laboratory. The experiments were carried under standard natural lighting conditions (8 h of light) and at room temperature (22 °C). The cucumbers with a weight about 300 g were chosen. A standard solution of *trans*-cinnamaldehyde or limonene was injected into each cucumber with a syringe. Cucumbers not injected with the standards were analyzed as blank samples. Two doses were administered for each compound: 200 and 1000 μg/kg. The volume of standard injected

depended on the weight of each cucumber, in order to have the same concentration, and it was injected equally throughout the cucumber. Sampling times were 0, 1, 2, 4, 8, 24 (1 day), 48 (2 days), and 72 h (3 days). The unwashed cucumber, including their peel (according to 90/642/ECC (Council of the European Union, 1990)), was crushed homogeneously before analysis at each sampling time. Three replicates were taken from each cucumber at each time.

#### 2.3.2. Greenhouse study

Cucumbers were grown in a greenhouse located in Níjar, Almería (southeastern Spain). The cucumbers had not been previously treated in this organic greenhouse and were close to the harvest. The study was carried out in May 2023, with a minimum temperature of 30 °C and maximum at 36 °C inside the greenhouse. Two commercial biopesticides were used at the normal recommended dose, being 8 L/Ha for Prevam® and 300 mL/hL for Cinna. A manual sprayer with a conical nozzle was used to spray the formulations, which were previously dissolved in 1.5 mL of water to obtain the recommended dose. Final *trans*-cinnamaldehyde concentration was 0.31 g/L, and limonene concentration was 1.12 g/L. Two rows with approximately 20 cucumber plants each, were treated with each biopesticide separately. Another two rows of cucumbers were not treated and were used as blanks. Different sampling times were selected: 0, 1, 2, 4, 24 (1 day), and 48 h (2 days). At every sampling time, a random sample of three cucumbers was taken from two rows for each biopesticide. The three unwashed cucumbers, including their peel, were crushed together homogeneously before analysis (90/642/ECC). Three replicates were analyzed at each time.

### 2.4. Extraction method

The extraction of biopesticides from cucumber was carried out using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) based extraction procedure. In 50 mL centrifuge tubes, 10 g of a previously crushed cucumber sample was weighed. After that, 100 μg/kg of biphenyl (IS) and 10 mL of EtOAc were added. The sample was vortexed for 1 min. Subsequently, 4 g of $MgSO_4$ and 1 g of NaCl were added. It was mixed for another 2 min in a vortex. The mixture was centrifuged for 5 min at 8170 *g*. The supernatant was filtered and analyzed by GC-Q-Orbitrap. Then, the samples were analyzed by UHPLC-Q-Orbitrap for the study of metabolites. Three replicates of each sample were made.

### 2.5. Method Validation

The method was validated according to SANTE 11312/2021 guide (Pihlström et al., 2022), calculating the matrix effect, linearity, limit of quantification (LOQ), trueness (% recovery), and precision (intra- and inter-day).

Matrix effect was measured by testing standards prepared in an extracted cucumber blank sample and in EtOAc (**Eq. (1)**, ranging from 1 to 100 μg/L.

$$Matrix\ effect(\%) = \left(\left(\frac{slope\ matrix}{slope\ standard}\right) - 1\right) \times 100 \quad (1)$$

Linearity was studied by calculating the determination coefficients ($R^2$) from the calibration curves, using peak area as analytical response. Recovery was studied by analyzing samples spiked at 2 and 100 μg/kg. Precision was determined by performing repeatability (intra-day) and reproducibility (inter-day) studies at 2 and 100 μg/kg, and results were expressed as relative standard deviation (% RSD) for each analyte. The LOQ was established as the lowest spike level tested that provided acceptable recovery (70–120%) and precision (RSD ≤ 20%) values when 5 replicates were injected.

For the quantification of the detected metabolites, a semi-quantification was carried out using the calibration curves of the respective parent compound from which they came from.

### 2.6. Data analysis

The data were processed using Xcalibur 3.0, including QualBrower and QuanBrowser. For the identification of metabolites, Compound Discoverer™ 3.3 program (Thermo Fisher Scientific) for GC and UHPLC raw data were employed. Moreover, NIST (National Institute of Standards and Technology) MS Search 2.2 library has also been utilized.

For untargeted analysis, the parameters chosen for Compound Discoverer were 5 ppm (mass tolerance), 50,000 (min peak intensity), 30% (intensity tolerance), 3 (S/N threshold), 0.1% (intensity threshold), and 0.1 min (retention time tolerance). The libraries selected in GC method were NIST library (mainlib, NISTDEMO and replib), GC-Orbitrap Contaminants Library, GC-Orbitrap Other Environments, GC-Orbitrap PCBs and GC-Orbitrap Pesticides. For UHPLC, the libraries were mzVault, mzCloud, Mass List such as EFS HRAM Compound Database, Lipid Maps Structure Database, Natural Products Atlas 2020_06 or LCMS Co-formulant PPP, and ChemSpider. The adducts were $[M-H]^-$, $[M+H]^+$, $[M-H+FA]^-$, $[M+Na]^+$ and $[M+H\text{-}H_2O]^+$.

## 3. Results and discussion

### 3.1. Optimization of the extraction method

To optimize the extraction method, different solvents were evaluated, including ACN and EtOAc. First, 10 g of blank cucumber was spiked with 100 μg/kg of *trans*-cinnamaldehyde, limonene and IS. Both solvents were utilized using the same extraction procedure described in Section 2.4. The compounds were extracted with 10 mL ACN for 3 min. Bearing in mind that this solvent can increase column bleeding (Cajka et al., 2008), which may complicate the identification of unknown metabolites, it was decided to evaporate the ACN with nitrogen to eliminate it, and after that, the residue was dissolved with EtOAc. The recoveries obtained for *trans*-cinnamaldehyde and limonene were not within the acceptable values (70–120%), being 134.2% and 11.9%, respectively. Therefore, *trans*-cinnamaldehyde and limonene were extracted with EtOAc. In this case, the recoveries for both compounds improved when EtOAc was used as an extraction solvent, being 86.9% and 108.4%, within the acceptable range. For this reason, EtOAc was selected as extraction solvent.

### 3.2. GC-Q-Orbitrap method validation

The GC method was validated by evaluating the various parameters mentioned in Section 2.5. **Eq. (1)** was utilized to calculate the matrix effect. The results were −3.4% for *trans*-cinnamaldehyde and −5.7% for limonene. Due to the acceptable range of the matrix signal (between −20% to 20%), the matrix effect was considered negligible in both cases. This can be explained because the large amount of water (Mukherjee et al., 2013) in cucumber makes it a simple matrix that does not have a high matrix effect. As can be seen in **Table S1**, the linearity of the calibration curves was $R^2 > 0.993$. *trans*-Cinnamaldehyde and limonene had LOQ values of 2 μg/kg. The recovery for 2 μg/kg was 79.4% for *trans*-cinnamaldehyde and 110.5% for limonene. For 100 μg/kg, the recoveries were 110.4% (*trans*-cinnamaldehyde) and 107.6% (limonene). Intra-day precision values ranged from 1.2 to 10.0% for *trans*-cinnamaldehyde and from 2.1 to 5.6% for limonene, while inter-day values varied from 2.0 to 2.8% (*trans*-cinnamaldehyde) and 4.0–7.5% (limonene).

### 3.3. Degradation study

To determine the degradation kinetics followed by *trans*-cinnamaldehyde and limonene, different kinetic models (first- and second-order) were evaluated. The degradation of *trans*-cinnamaldehyde was better adjusted to second-order kinetics (**Eq. (2)**. The reason for this was that the $R^2$ values in the second-order kinetics (0.970–0.993, Table 1)

**Table 1**
Kinetic parameters of *trans*-cinnamaldehyde and limonene degradation[a].

| Kinetic parameter | *trans*-Cinnamaldehyde | | | Limonene | | |
|---|---|---|---|---|---|---|
| | Second-order | | | First-order | | |
| | Laboratory | | Greenhouse | Laboratory | | Greenhouse |
| | 200 μg/kg | 1000 μg/kg | ND | 200 μg/kg | 1000 μg/kg | ND |
| $DT_{50}$ (hours) | 2.02 | 2.48 | 2.49 | 1.35 | 6.17 | 0.49 |
| $DT_{90}$ (hours) | 18.20 | 22.33 | 22.41 | 4.48 | 20.49 | 1.64 |
| k (hours$^{-1}$) | 0.01 | 0.003 | 0.06 | 0.51 | 0.11 | 1.41 |
| $R^2$ | 0.970 | 0.980 | 0.993 | 0.997 | 0.998 | 0.993 |

[a] Abbreviation: $DT_{50}$: half-life time; $DT_{90}$: 90% dissipation time; k: rate constant; ND: normal recommended doses; $R^2$: coefficients of determination.

were higher than those in first-order kinetics (0.924–0.975, **Table S2**).

$$C_t = \frac{C_0}{1 + ktC_0} \quad (2)$$

where: $C_0$: concentration at time 0, $C_t$: concentration at a certain time, $k$: rate constant, and $t$: time (hours).

This differs from its behavior in different soil types where it degraded following first-order kinetics (Reyes-Ávila et al., 2023a). This behavior is observed in both studies (greenhouse and laboratory) as shown in **Fig. S1**. After 72 h in laboratory studies, *trans*-cinnamaldehyde was not detected, while in the greenhouse one this happened after 48 h. The $DT_{50}$ and 90% of the dissipation time ($DT_{90}$) values, were calculated by **Eqs. (3)** and **(4)**. $DT_{50}$ at 200 μg/kg has been 2.02 h, and 2.48 h at 1000 μg/kg in laboratory study. In the greenhouse study, $DT_{50}$ was 2.49 h as shown in Table 1. $DT_{90}$ values were 18.20 and 22.33 h at 200 and 1000 μg/kg, respectively, in laboratory study, while $DT_{90}$ at greenhouse study was 22.41 h.

$$DT_{50} = \frac{1}{kC_0} \quad (3)$$

$$DT_{90} = \frac{9}{kC_0} \quad (4)$$

The degradation of *trans*-cinnamaldehyde has a similar trend in both laboratory and greenhouse experiments, and at different concentrations (200 and 1000 μg/kg), as evidenced by the similar $DT_{50}$ values obtained. In previous soil experiments (Reyes-Ávila et al., 2023a), *trans*-cinnamaldehyde was degraded in a similar manner in different soil types used. Regardless of the soil used, their $DT_{50}$ values (0.16–0.28 days) were very similar. According to this, the degradation of *trans*-cinnamaldehyde is only influenced by the matrix change, but it behaves similarly when varying the dose within the same matrix.

In relation to limonene, as can see in **Fig. S1** and Table 1, its degradation follows first-order kinetics (**Eq. (5)**.

$$C_t = C_0 e^{-kt} \quad (5)$$

where: $C_0$: concentration at time 0, $C_t$: concentration at a certain time, $k$: rate constant, and $t$: time (hours).

The $R^2$ values were better for first-order kinetics (Table 1) than those obtained for second-order kinetics (**Table S2**). There are previous studies of limonene in different types of soil where it also follows first-order kinetics (Huang et al., 2022; Reyes-Ávila et al., 2023a). In our study, limonene was no longer detected in the cucumber 8 h after its application in the greenhouse and after applying 200 μg/kg in the laboratory. On the other hand, when applying a higher dose of 1000 μg/kg, the presence of limonene was detected up to 24 h. The equations used to calculate $DT_{50}$ and $DT_{90}$ were **Eqs. (6)** and **(7)**.

$$DT_{50} = \frac{ln\,2}{k} \quad (6)$$

$$DT_{90} = \frac{ln\,10}{k} \quad (7)$$

Limonene $DT_{50}$ values were 1.35 at 200 μg/kg (laboratory), 6.17 at 1000 μg/kg (laboratory), and 0.49 h in greenhouse. Similar to $DT_{50}$ values did, the limonene $DT_{90}$ values differ significantly between experiments, with a range of 1.64 to 20.49 h. The $DT_{50}$ of limonene significantly increased as the applied dose increased in the laboratory study. Furthermore, in the greenhouse study limonene had a very short half-life time. The high temperature inside the greenhouse (36 °C), which was much higher than the laboratory (22 °C), may be the reason for this rapid dissipation. In contrast to the *trans*-cinnamaldehyde behavior, the degradation of limonene in cucumber was affected by the experimental conditions and the dose used. Something similar occurred when its degradation has been studied in different soil types (Huang et al., 2022; Reyes-Ávila et al., 2023a). The kinetics of limonene was greatly influenced by the soil's characteristics and the dose used.

### 3.4. Metabolite study

The use of HRMS has allowed the search for potential metabolites derived from *trans*-cinnamaldehyde and limonene. Firstly, a suspect analysis was carried out to search for metabolites described in literature (Huang et al., 2022; Ma et al., 2011; van der Werf et al., 1999). In total, six metabolites from the literature were searched for *trans*-cinnamaldehyde and eleven for limonene, showing in **Table S3**, the suspected metabolites. Three metabolites have been identified for *trans*-cinnamaldehyde in the laboratory study, cinnamyl alcohol, 4-hydroxycinnamic acid (*p*-coumaric acid) and cinnamic acid (Fig. 1). These metabolites were not detected in the greenhouse study. It is plausible that the concentration of these metabolites was below the detection limit, hence remaining undetectable in the greenhouse samples. The presence of these metabolites had previously been described in matrices such as soil, being 4-hydroxycinnamic acid detected for the first time as a metabolite (Reyes-Ávila et al., 2023a), or cinnamyl alcohol in rats (Zhao et al., 2014). The metabolite cinnamyl alcohol was tentatively identified by GC-Q-Orbitrap (**Table S4**) and 4-hydroxycinnamic acid by UHPLC-Q-Orbitrap. Moreover, cinnamic acid was detected by both techniques. As can be seen in Fig. 2, the mass spectrum obtained at 17.23 min for cinnamic acid coincided with the mass spectrum recorded in the NIST library. The presence of cinnamic acid could be confirmed since it can be detected by both methods. The adduct of the detected metabolites by UHPLC was $[M-H]^-$, and their retention times were 12.15 (4-hydroxycinnamic acid) and 12.43 min (cinnamic acid). Several investigations have reported the transformation of *trans*-cinnamaldehyde into cinnamic acid and cinnamyl alcohol (Gottardi et al., 2017; Ma et al., 2011; Pennacchio et al., 2013; Yamanaka et al., 2015). This transformation of *trans*-cinnamaldehyde is usually caused by microbial action (Ma et al., 2011).

Semi-quantification was carried out to determine the concentration of these metabolites based on the calibration curves of *trans*-cinnamaldehyde. Cinnamyl alcohol was the metabolite that has been

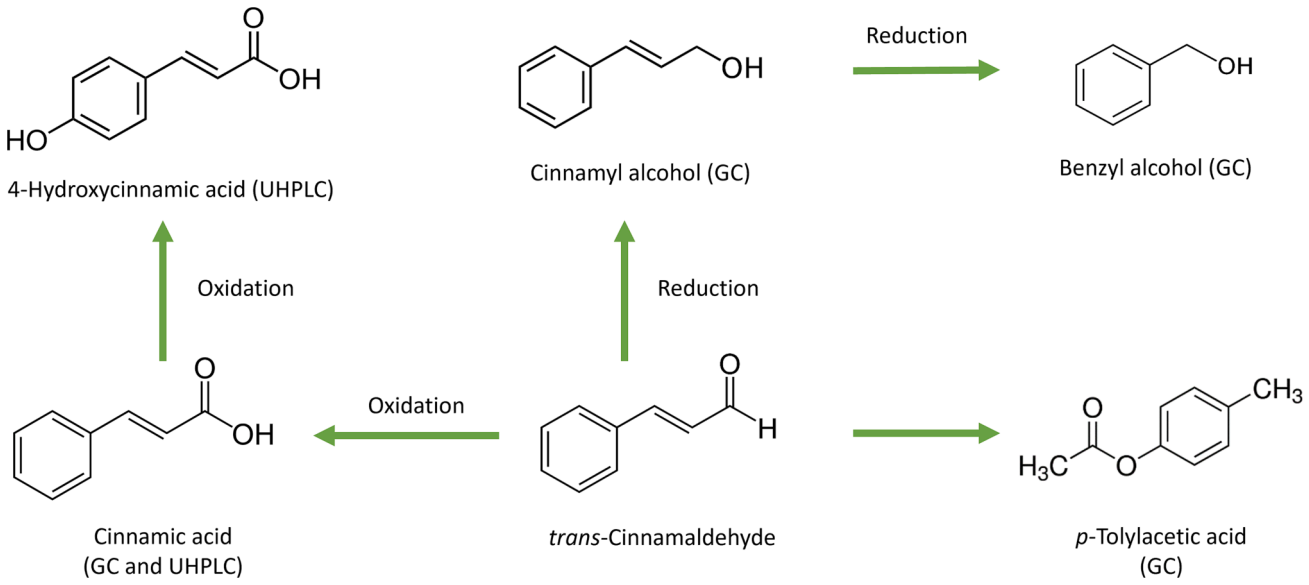


**Fig. 1.** *trans*-Cinnamaldehyde metabolites tentatively detected by GC and UHPLC-Q-Orbitrap.

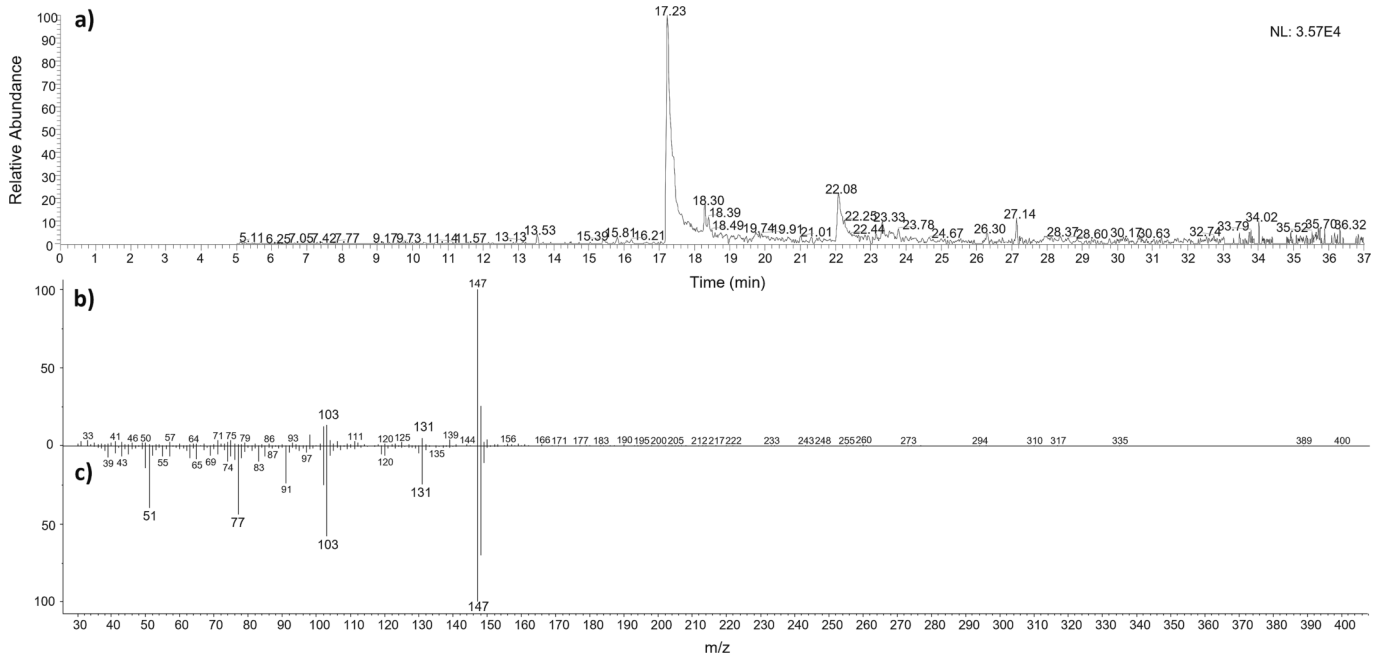


**Fig. 2.** a) GC-Q-orbitrap chromatogram for ion *m/z* 147.04398, b) experimental MS spectrum, and c) NIST MS spectrum of cinnamic acid.

tentatively detected at the highest concentration, presenting values between 19.9 and 94.5 μg/kg at 200 μg/kg, and between 28.1 and 392.0 μg/kg at 1000 μg/kg (Table 2). The concentration of this compound changed from high to low concentration over time, rising the maximum concentration at 4 h and then decreased. On the other hand, three limonene metabolites have been tentatively identified by GC-Q-Orbitrap. Carvone, limonene-1,2-epoxide, and perillyl alcohol were found in the laboratory study (Fig. 3). No metabolite could be identified in the greenhouse study. Carvone and limonene-1,2-epoxide were previously detected in soil samples (Huang et al., 2022). It was not possible to perform a semi-quantification for these metabolites due to their low concentration that was outside the quantification limits. **Table S4** shown the retention times and the fragments of these possible metabolites. In this case, limonene has undergone an oxidation process to form these metabolites because it is a volatile compound that tends to oxidize very easily and can even autoxidize in the presence of oxygen (van der Werf et al., 1999).

To broaden the search for metabolites, a search was carried out through an unknown analysis. For that, the Compound Discoverer software was used. Two more possible metabolites of *trans*-cinnamaldehyde have been tentatively identified by GC-Q-Orbitrap, benzyl alcohol and *p*-tolylacetic acid (Fig. 1). It is the first time that they have been identified as metabolites of this compound. Their chromatographic and MS parameters were collected in **Table S4**. *trans*-Cinnamaldehyde calibration curve was used for semi-quantification to calculate the concentration of these new metabolites. Both have been found at low concentrations as can be seen in Table 2. Similar to previous metabolites, they have only been detected in the laboratory but not in the greenhouse study. On the other hand, through the unknown mode analysis, it was not possible to determine any metabolite of limonene.

**Table 2**
Concentration (μg/kg) of *trans*-cinnamaldehyde metabolites (semi-quantification approach) obtained in the laboratory study by GC and UHPLC-Q-Orbitrap[a].

| **GC-Q-Exactive Orbitrap** | | | | | | | |
|---|---|---|---|---|---|---|---|
| **Metabolites** | **Doses (μg/kg)** | **0 h** | **1 h** | **2 h** | **4 h** | **8 h** | **24 h** |
| Benzyl alcohol | **200** | 15.7 | 25.3 | 21.6 | 12.7 | 144.7 | 26.1 |
| | **1000** | 12.6 | 14.6 | 20.5 | 18.4 | 13.9 | 14.1 |
| Cinnamyl alcohol | **200** | 94.5 | 64.3 | 70.7 | 19.9 | 28.7 | 22.8 |
| | **1000** | 392.0 | 297.1 | 62.3 | 224.9 | 86.4 | 28.1 |
| *p*-Tolylacetic acid | **200** | 13.1 | 13.8 | 16.0 | 15.4 | 11.7 | 11.7 |
| | **1000** | 13.3 | 14.6 | 17.4 | 15.8 | 11.8 | 9.2 |
| Cinnamic acid | **200** | 21.6 | 18.7 | 22.1 | 10.5 | 6.0 | 11.1 |
| | **1000** | 28.5 | 35.3 | 22.1 | 46.1 | 7.3 | ND |
| **UHPLC-Q-Exactive Orbitrap** | | | | | | | |
| 4-Hydroxycinnamic acid | **200** | 59.4 | 104.8 | 775.3 | 371.1 | 398.4 | 136.4 |
| | **1000** | ND | 95.7 | 21.6 | 202.5 | 205.7 | ND |
| Cinnamic acid | **200** | 63.5 | 62.4 | 538.9 | 355.9 | 114.6 | 84.5 |
| | **1000** | 63.8 | 246.5 | 188.4 | 167.5 | 346.2 | 339.9 |

[a] Abbreviation: ND: not detected.

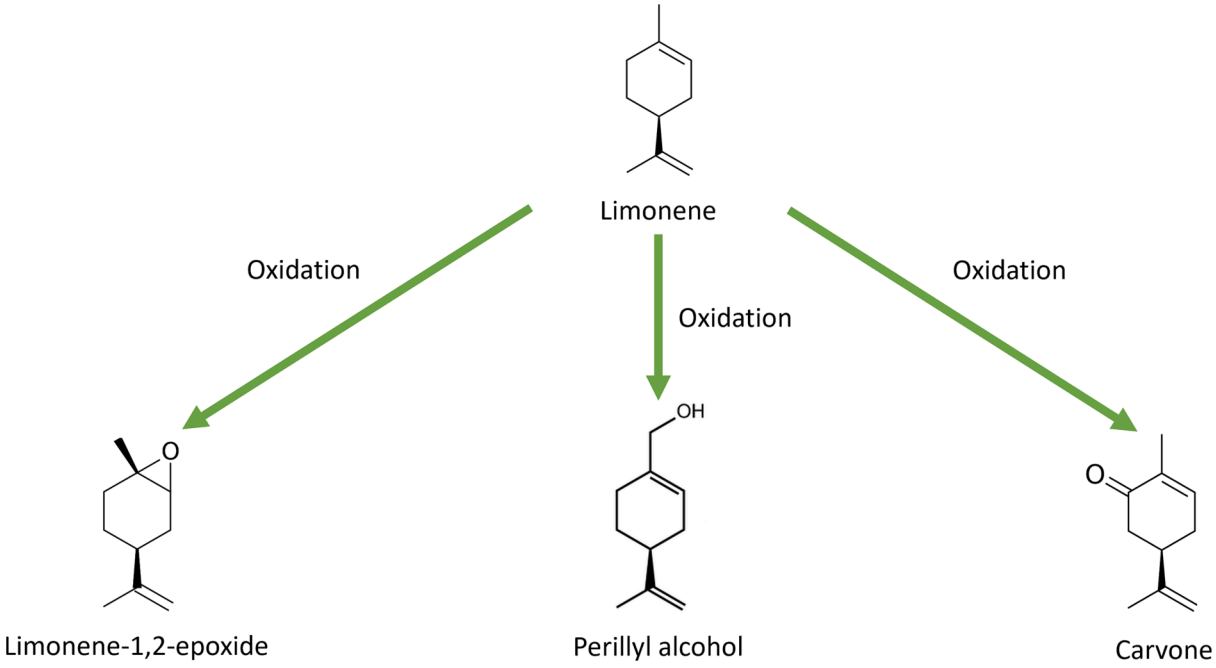


**Fig. 3.** Limonene metabolites tentatively identified by GC-Q-Orbitrap.

### 3.5. Toxicity study

Finally, the toxicity of the metabolites found has been studied to determine if they are more toxic than the precursor compounds. The Toxicity Estimation Software Tool (TEST) software provided by the EPA was used for this purpose (US EPA, 2023). This program calculated the predicted value of the median lethal oral dose ($LD_{50}$) in rats for each compound, and it also shows experimental $LD_{50}$ values for some compounds. The predicted values have been extrapolated to obtain the $LD_{50}$ in humans. To do this, the dose obtained in rats has been divided by 6.2 (Noga et al., 2023). The values of the $LD_{50}$ (predicted and experimental) for rats and those converted to humans are shown in Table 3. *trans*-Cinnamaldehyde has an $LD_{50}$ in human of 0.38 g/kg and its metabolites presented a similar toxicity (0.34–0.45 g/kg), except for benzyl alcohol, which has the highest toxicity with a $LD_{50}$ of 0.07 g/kg. However, limonene metabolites presented a more variable toxicity compared to limonene (0.78 g/kg), with carvone being the metabolite with the highest toxicity ($LD_{50}$ = 0.26 g/kg). According to these values, concentrations between 0.07 and 0.88 g/kg would need to be exceeded for

**Table 3**
$LD_{50}$ values of *trans*-cinnamaldehyde, limonene, and their tentative metabolites[a].

| **Compound** | **Oral $LD_{50}$ (g/kg)** | | |
|---|---|---|---|
| | **Rats** | | **Human[b]** |
| | **Predicted** | **Experimental** | **Predicted** |
| ***trans*-Cinnamaldehyde** | 2.36 | – | 0.38 |
| 4-Hydroxycinnamc acid | 2.81 | – | 0.45 |
| Cinnamyl alcohol | 2.53 | 2.00 | 0.41 |
| Cinnamic acid | 2.29 | 2.50 | 0.37 |
| *p*-Tolylacetic acid | 2.10 | – | 0.34 |
| Benzyl alcohol | 0.46 | 1.23 | 0.07 |
| **Limonene** | 4.84 | 5.30 | 0.78 |
| Limonene-1,2-epoxide | 5.47 | – | 0.88 |
| Perillyl alcohol | 2.27 | 2.10 | 0.37 |
| Carvone | 1.64 | 1.64 | 0.26 |

[a] Abbreviation: $LD_{50}$: median lethal dose.
[b] Calculated by dividing the predicted $LD_{50}$ by 6.2.

these compounds to be considered toxic in humans. Considering that these compounds have a low toxicity and are present in cucumber at concentrations lower than 1 mg/kg, they do not pose a high health risk.

## 4. Conclusion

It was the first time that the degradation of *trans*-cinnamaldehyde and limonene in cucumbers has been studied, including two different study conditions such as laboratory and greenhouse. A new extraction method has been optimized and validated for GC-Q-Orbitrap. Furthermore, UHPLC-Q-Orbitrap has been utilized as a complementary way to GC-Q-Orbitrap to increase the number of possible metabolites detected during the degradation of these compounds. The use of these two techniques has allowed an exhaustive study of the metabolites of *trans*-cinnamaldehyde and limonene. Five *trans*-cinnamaldehyde metabolites and three limonene metabolites have been tentatively identified. The detection of two of them was done by UHPLC and five by GC. Furthermore, one was detected by both methods. Some metabolites of *trans*-cinnamaldehyde were detected for the first time by unknown analysis. In the greenhouse study, the presence of metabolites could not be corroborated. The low toxicity of the metabolites found for these biopesticides allows a lower risk for human consumption unlike when chemical pesticides are used. In addition, its persistence in vegetables is much lower than conventional pesticides. Therefore, applying biopesticides for crop protection could have less negative impact than the synthetic pesticides widely used until now.

## CRediT authorship contribution statement

**Alba Reyes-Ávila:** Data curation, Formal analysis, Investigation, Software, Writing – original draft. **Roberto Romero-González:** Conceptualization, Funding acquisition, Methodology, Supervision, Visualization, Writing – review & editing. **Antonia Garrido Frenich:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

## Declaration of Competing Interest

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

## 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.2024.138443. Validation parameters obtained for *trans*-cinnamaldehyde and limonene (Table S1); Other kinetic parameters evaluated for *trans*-cinnamaldehyde and limonene degradation (Table S2); Characteristic chromatographic-MS parameters of possible metabolites of *trans*-cinnamaldehyde and limonene (Table S3); Characteristic chromatographic-MS parameters of *trans*-cinnamaldehyde and limonene metabolites tentatively identified by GC-Q-Orbitrap (Table S4); Degradation curves in studies for: *trans*-cinnamaldehyde in a) greenhouse, b) 200 μg/kg in laboratory and c) 1000 μg/kg laboratory, and limonene in d) greenhouse, e) 200 μg/kg in laboratory and f) 1000 μg/kg laboratory. Error bars: standard deviation (number replicates = 3) (Fig. S1).

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