Comprehensive dissipation of azadirachtin in grape and tomato: effect of *Bacillus thuringiensis* and tentative identification of unknown metabolites

Jesús Marín-Sáez,[a*] Rosalía López-Ruiz,[a] Roberto Romero-Gonzalez,[a] Antonia Garrido Frenich[a]

[a]Department of Chemistry and Physics, Analytical Chemistry Area, University of Almería Research Centre for Agricultural Food Biotechnology (CIAIMBITAL), Agrifood Campus of International Excellence ceiA3, Carretera de Sacramento s/n, E-04120 Almería, Spain

ORCID CODES

Jesús Marín-Sáez: 0000-0002-4153-9788

Rosalía López-Ruiz: 0000-0003-0806-9013

Roberto Romero-González: 0000-0002-2505-2056

Antonia Garrido Frenich: 0000-0002-7904-7842

* Corresponding author at: University of Almería, Carretera de Sacramento s/n, E, 04120 Almería, Spain. E-mail address: jms485@ual.es (Jesús Marín Sáez).

Abstract

Neem oil is a biopesticide normally applied together with *Bacillus thuringiensis (Bt).* However, neither its dissipation nor the influence of *Bt* have been previously evaluated. In this study dissipation of neem oil was investigated when it was applied alone or together with Bt, at 3°C and 22°C. A methodology involving solid-liquid extraction and liquid chromatography-high resolution mass spectrometry was developed for that purpose. Method was validated obtaining recoveries from 87 to 103%, with relative standard deviations lower 19% and limit of quantifications from 5 to 10 µg/kg. Azadirachtin A (AzA) dissipation was fit to a single first order, being faster when neem oil was applied together with *Bt* and at 22°C (RL_{50}=12-21 days) than alone and at 3ºC (RL_{50}=14-25 days). Eight related compounds were found in real samples with similar dissipation curves than AzA, and 5 unknown metabolites were identified in degraded samples, with increasing concentrations during parent compound degradation.

Keywords: Biopesticides; HRMS; *Bacillus Thuringiensis*; azadirachtin; metabolites; neem oil

1. INTRODUCTION

Nowadays, the use of pesticides is necessary to support the high demand of a growing population. However, several adverse effects could be produced a cause of their use, including health effects as cancer or genetic diseases, and environmental effects as soil, air and water contamination.[1] Therefore, biopesticides, pesticides originated from a natural source as plant extract, essential oils or bacteria toxins, between others,[2] have been increasingly employed to control pests. These pesticides are normally more selective to specific pests than traditional ones, biodegradable and less toxic to human than synthetic pesticides.[3] The current global market for organic agricultural products (2021), including biopesticides, is valued at around US$ 103.36 billion with a growth rate of 8.4% per year.[4]

Tomato and grape crops are highly affected by pests as the lepidoptera, known as tomato and grape moth (*Tuta absoluta* and *Lobesia botrana*, respectively), being an important problem in countries as Spain, which is a big producer and exporter of these crops.[5] Although traditional pesticides had been used to control them (e.g. abamectin or clorantraniliprole), the use of neem oil, a biopesticide mainly composed of azadirachtin A (AzA) and in less proportion of azadirachtin B (AzB) and related compounds,[6] can be employed as a more environmentally friendly alternative. AzA is a limonoid obtained from the neem tree and sold as neem oil, whose concentrations oscillate between 0.1-2.6 %. It is a potent antifeedant to many insects, and it affects to insects in larval stage, by contact and ingestion, disturbing the hormonal balance of insects.[7] Besides, fungus is another important pests in tomato and grape cultivations, and bearing in mind that AzA has acaricide and fungicidal properties, the use of synthetic fungicides as triazole pesticides can be avoided when neem oil is applied.[8,9]

AzA acts upon insects in larval stage, but when the plague is established in the plants, including adult individuals, neem oil is not enough to eliminate it. For that reason,, neem is commonly applied together with another biopesticide, *Bacillus thuringiensis* (*Bt*). *Bt* was named like this because it was firstly isolated from flour moth in Thuringia, Germany, and it is a gram-positive

63 bacterium with insecticidal action.[10] That property is due to the formation of crystalline protein
64 inclusions during the sporulation phase thanks of their crystal protein genes (CRY). *Bt* protein
65 toxins interact with specific binding sites on the insect midgut epithelium producing their death
66 by digestion in basic conditions (there is no risk for animals, which have an acid digestion).[11] The
67 proteins with the highest pesticide potency are the CRY and vegetative insecticidal proteins
68 (VIP), which have a high specificity for lepidoptera species, highlighting the specificity of
69 biopesticides for which they are more recommended than synthetic pesticides.[12] The
70 combination of both biopesticides drastically reduces the negative effect to both leaves and
71 fruits affected by lepidoptera pests, being complementary between them.[13] Besides, when more
72 than one compound is applied to treat pests, it may allow a longer persistence of defences.[3]
73 On the other hand, *Bt* has pesticide degradation properties,[14–18] and this could promote AzA
74 degradation, restricting its insecticidal effect or producing known or unknown azadirachtin
75 metabolites. This last point is especially important since metabolites are in some cases more
76 toxic than the parent compounds, as it occurs with conventional,[19] or biopesticides,[20] being
77 necessary to investigate how Bt is able to affects pesticides as neem oil. Although some
78 metabolites of AzA have been previously identified (as 3-Deacetylazadirachtin or 22,23-
79 Dihydroazadirachtin (Table 1)), as far as we know, there is no study evaluating the effect of Bt
80 over neem oil components, even when these substances are normally applied together.[21,22]
81 Furthermore, pesticides with fungicidal properties as AzA can also alter and impact microbial
82 communities and thus *Bt* could be affected by them.[23]
83 Although biopesticides have less impact in human health and environment than traditional
84 pesticides, their analysis is also necessary to assure food safety. For example, nereistoxins as
85 thiocyclam, cartap or nereistoxin seem to be toxic at high concentrations and they are banned
86 in some countries,[20,24] or rotenone which, although it is allowed as pesticide, it has been
87 demonstrated to have some influence in diseases as Parkinson.[25] The detrimental effects caused
88 by some of them over environment and pollinators must also be taken into account.[26] Besides,

89 the fact that Bt could promote the emergence of azadirachtin metabolites with unknown food
90 safety risks, makes important to control them together. Indeed, the European Union (EU) has
91 established maximum residue levels (MRLs) for AzA (0.5 mg/kg in grape and 1 mg/kg in tomato),
92 which do not include its metabolites.[27]
93 For the extraction of AzA and related compounds, considered polar compounds, QuEChERS
94 method (quick, easy, cheap, effective, rugged, and safe) is normally applied.[7] However, for non-
95 targeted analysis the use of less specific methods as solid-liquid extraction (SLE) with acetonitrile
96 or methanol is necessary to ensure the extraction of compounds with a wider range of
97 physicochemical properties. Liquid chromatography coupled to mass spectrometry (LC-MS) is
98 considered the main analytical technique for these compounds.[7] Even though dissipation studies
99 are accomplished mostly by low resolution MS methods, recent development of high-resolution
100 mass spectrometry (HRMS) instruments has led to the evolution of more capable methodologies
101 to perform both, targeted and non-targeted studies.[28] In addition, new software platforms have
102 been developed in the field of unknown analysis, including statistical and data processing tools
103 (e.g. Compound Discoverer) and in-silico fragmentation software (e.g. MassFrontier).[28] Also
104 other software for metabolite searching, which simulate organic and inorganic reactions of a
105 parent molecule to elucidate them (e.g. MassChemSite),[29] can also be applied.
106 For all of that, this article aims to offer a complete and comprehensive overview of the fate of
107 AzA and related compounds and the influence of *Bt* on their dissipation under different storage
108 conditions (3°C and dark and 22°C and light). The analysis was carried out using an acetonitrile
109 SLE based method and ultrahigh performance liquid chromatography coupled to HRMS (UHPLC-
110 Q-Orbitrap-HRMS). Dissipation studies were accomplished in tomato and grape samples,
111 applying neem oil alone and mixed with *Bt*. The study was performed over 80 days. After
112 targeted and suspect analysis of parent and related compounds found in bibliography,
113 metabolites were elucidated employing different software: Compound Discoverer,
114 MassChemSite (for metabolites simulation) and MassFrontier (for in-silico fragmentation).

2 MATERIALS AND METHODS

2.1 Reagents and chemicals

AzA was obtained from Chengdu Biopurify PhytoChem (Chengdu, Sichuan, China). Two different standard solutions were prepared: A stock standard solution by weighing 10 mg of AzA (purity>99%) at 1000 mg/L in methanol (HPLC grade, Honeywell Riedel-de-Haën (Seelze, Germany)) and a working standard solution at 20 mg/L prepared from the stock standard solution.

Acetonitrile was purchased by Honeywell Riedel-de-Haën, water by J.T. Baker (Deventer, The Netherlands), acetic acid by Merck (St. Louis, MO, USA) and formic acid (>98% of purity) by Fisher Scientific (Erembodegem, Belgium).

ProteoMass LTQ/FT-hybrid ESI positive and ProteoMass LTQ/FT-HybridESI negative, from Thermo-Fisher Scientific (San Jose, CA, USA) were used for LC-QOrbitrap calibration.

2.2 Instrument and apparatus

Thermo Fisher Scientific Vanquish Flex Quaternary LC (Thermo Scientific Transcend™), with an Hypersil GOLD™ aQ UHPLC column (100 × 2.1 mm, 1.9 µm particle size) at 30°C was employed for the compound separation step. Injection volume of 10 µL was selected. Mobile phases were composed of methanol (Eluent A) and water 0.1% formic acid (Eluent B) at a flow rate of 0.3 mL/min. The chromatographic gradient employed was previously developed.[30] Briefly, from 95% of B (1 min), the percentage was decreased to 0% in 3 min and kept constant for 6 min. Then it was increased to 95% (0.5 min) and kept constant during 3.5 min. Run time was 14 min.

For the compound detection, a hybrid mass spectrometer Q-Exactive Orbitrap (Thermo Scientific QExactive™) was employed. The electrospray (ESI) parameters were: spray voltage, 4 kV; sheath gas (N_2, 95%), 35 (arbitrary units, au); auxiliary gas (N_2, 95%), 10 (au); S-lens RF level, 50 (au); heater temperature, 305°C; and capillary temperature, 300°C. The mass spectra were

acquired employing: (1) full MS, ESI+ and ESI-, without fragmentation, mass resolving power = 70,000 Full Width at Half Maximum (FWHM); AGC target = 10^6; mass range was set to *m/z* 60–900 (2) data independent mass spectrometry fragmentation (DIA-MS/MS), ESI+ (HCD on, collision energy = 30 eV), ESI- (HCD on, collision energy = -30 eV), mass resolving power = 35,000 FWHM; AGC target = 10^5; isolation window *m/z* 50. The chromatographic and spectrometric data is summarized in **Table 1**.

External calibration mode was used to acquire the results. For targeted and suspect screening, a home-made database was built. Xcalibur™ version 4.3.73.11 (Thermo Fisher Scientific, Les Ulis, France) and TraceFinder 5.1 (Thermo Fisher Scientific) were employed for targeted and suspect screening, whereas MassFrontier™v7.0, Compound Discoverer v3.2 (Thermo Fisher Scientific) and MassChemSite 3.1.0 (Molecular Discovery Ltd, Borehamwood, United Kingdom and Lead Molecular Design SL, Sant Cugat del Vallès, Barcelona) were used for unknown analysis.

2.3 Sample contamination and treatment

The commercial products applied to the blank samples were NeemAzal T/S (EC) (1%, *w/v* of AzA) and *Bacillus thuringiensis* kurstaki (WG, 32%, *w/w*). Ten kg of each sample were divided in two groups, one was sprayed with neem oil (group A) and the other with neem oil and *Bt* (group B), at the manufacturer recommend dose: 0.3% (*v/v*) for neem oil and 1 g/L for *Bt*. Then, each group was split in and stored at room temperature (22ºC ± 2ºC) (groups A.1 and B.1) in a lit-up place, and at refrigerated temperature (3°C ± 2ºC) (groups A.2 and B.2) in a dark place. The sample collection was performed at 2 h, 6 h, 1 day, 2 days, 5 days, 12 days, 20 days, 30 days, 40 days, 50 days, 60 days 70 days and 80 days (70 and 80 days were only analysed at 3°C). Around 60 days at room temperature and 80 days at refrigerated conditions, samples were not in good conditions to be consumed, so the study was not continued.

The water weight loss was monitored using two replicates of tomato and grape (≈100 g) stored at the same conditions as contaminated samples. Compound concentrations were calculated considering this loss of weight.

To assure an exhaustive extraction, a generic SLE with acetonitrile was employed.[31] In summary, 5 g of sample were extracted with 5 mL acetonitrile during 1 min in a vortex. After centrifugation (7500 rpm, 8170 rcf), 10 µL of the top layer was filtered and injected in the chromatographic system.

3 RESULTS AND DISCUSSION

3.1 Method optimization and validation

First MS characterization was performed for AzA, following the same procedure than in previous articles,[32] obtaining the precursor ion *m/z* 743.2522 and the product ions *m/z* 161.0592, 369.1157 and 567.1843 (**Figure 1)**. Then, different azadirachtins and related compounds (suspect compounds) were included in a homemade database (**Table 1**). The exact mass for precursor ions was calculated according to their molecular formula, and fragment ions for some compounds were obtained from bibliography. For those whose were not indicated in bibliography, in-silico fragmentation was performed using MassFrontier. According to the latest SANTE version,[33] characteristic and, at least one, fragment ion should be monitored with a mass error lower than 5 ppm and a variation of the isotopic pattern recognition lower than 30%.

Two SLE methods were evaluated for the extraction of the compounds from grape and tomato spiked at 5 and 100 µg/kg: a previously optimized SLE extraction with acidified methanol,[32] and a SLE with acetonitrile as extractant solvent. SLE performance (**Table 2**) show that both solvents were appropriated. However, acetonitrile extraction was slightly better than methanol extraction in terms of recoveries (87-103%) and matrix effect (-22 to -23%), calculated according to Lopez-Ruiz et al. study,[34] choosing it as the final method.

190 The method was validated in both matrices according to the SANTE guidelines (**Table 2**).[33]
191 Linearity was determined by comparison between a matrix-matched calibration and a solvent-
192 matched calibration, at concentrations from 2.5 to 500 µg/kg. Linear range was between 2.5 (5
193 µg/kg for tomato) and 250 µg/kg (R^2>0.9946) and working range between 5 (10 µg/kg for
194 tomato) and 100 µg/kg. Matrix effect was evaluated comparing matrix-matched calibration and
195 a solvent calibration at the working range concentrations, and it ranged from -22 for grape to -
196 23% for tomato, requiring the use of a matrix-matched calibration to quantify the samples. LOQs
197 were stablished according to SANTE guideline indications (minimum spiked concentrations of
198 target analytes for which signal to noise ratio (*s/n*)>10 for precursor and product ions,
199 withrecoveries between 70-120% and relative standard deviations (RDS) lower than 20%) and
200 they were set at 5 µg/kg for grape and 10 µg/kg for tomato. Finally, recoveries were tested at 5
201 or 10 µg/kg for grapes and tomato respectively, and 100 µg/kg (5 replicates for each
202 concentration), after spiking blank samples. Recoveries ranged from 87 to 103% with intraday
203 and interday precision, in terms of RSDs, lower than 14% and 19%, respectively.
204
205 **3.2 Suspect screening**
206 In addition to AzA, metabolites and/or related compounds were searched in neem oil through a
207 suspect screening, considering the detection parameters explained in *Section 3.1*. In neem oil,
208 there are a large number of compounds which have been isolated from *Azadirachta indica*
209 (neem tree) being some of them azadirachtin H (metabolite detected in soil), AzB, azadirachtin
210 I, azadirachtin J, azadiradione, nimbin or nimbinin (**Table 1**). The principal characteristic of these
211 compounds is that all of them have the polycyclic structure of AzA (**Figure 1**).[35,36]
212 For the searching of these suspect compounds, neem oil was analysed by direct injection, with
213 an appropriated dilution to avoid carry over contamination in the chromatographic or
214 spectrometric system. First a dilution 1:10,000,000 (*v/v*) with methanol was tested but, the
215 signal obtained for AzA was too low, choosing a dilution 1:100,000 (*v/v*) as the most appropriate.

Suspect compounds detected in the neem oil were shown in **Table 3** and their concentrations were estimated using an AzA calibration curve. Their fragments were obtained from bibliography or proposed by in-silico fragmentation (MassFrontier) (**Table 1**).

Estimated concentration of AzA in neem oil should be 10000 mg/L (1%, *w/v*), but its concentration was 6880 mg/L (≈0.69%), as it can be observed in **Table 3**, which indicates that neem oil compounds start their degradation in the commercial product, as other authors indicated for the same plant protection product.[37] Besides, the compounds detected here are in concordance with those detected in previous studies, except for azadirachtin N, azadirachtin F and ohchinolide B, which were not detected in that study, and azadirachtin D, which was not detected in the current study. These differences may be caused because they used a previous liquid-liquid extraction,[37] while here neem oil was directly injected after dilution, so losses of compounds may have occurred.

3.3 Dissipation studies

Dissipation kinetics of AzA in tomato and grape was evaluated by plotting residue concentration against time. Several kinetics models were tested, such as single first order, biphasic, one-and-a-half-order and second order.[38] Single First-Order (SFO) was the fit selected, obtaining R^2 values higher than 0.98 in all cases. Other conventional pesticides shown the same tendency than AzA.[39,40] All the parameters for the tested kinetic models for AzA dissipation in tomato and grape samples are shown in **Table S1** and **S2**.

The residual concentration and half-life of AzA (residual lifetime, RL_{50}) was calculated using the SFO model applying **Equation 1** and **Equation 2**, where the terms C_0, k and C_T mean the initial concentration, the rate constant and the concentration at time t, respectively.

$$C_T = C_0 e^{-kt} \qquad \textbf{Equation 1}$$

$$RL_{50} = \frac{ln\,2}{k} \qquad \textbf{Equation 2}$$

241 In **Figure 2** (tomato) and **Figure S1** (grape) it can be observed that the concentration of AzA
242 decreased under all the tested conditions (*Bt* addition and storage conditions). It is important to
243 mention that the detected concentrations in grapes were higher than in tomato. This can be
244 explained because when the neem/neem+*Bt* solution was spread over both samples, grapes
245 keep a higher volume of the solutions due to its morphology, whereas for tomato the solution
246 slipped into the tray where the samples were disposed. The SFO kinetic model revealed that
247 values of rate constant (*k*) ranged from 0.029 $days^{-1}$ (neem in grape at 3ºC) to 0.057 $days^{-1}$ (neem
248 and *Bt* in tomato at 22ºC). Meanwhile RL_{50} was lower than 25 days and RL_{90} lower than 82 days
249 (**Table 4**). Differences between matrices were not observed, obtaining similar values of RL_{50} and
250 *k* in all cases, although a higher dissipation was achieved between storage conditions, observing
251 it was slow at refrigerated conditions, being RL_{50} between 21 days (grape and tomato) to 25 days
252 (tomato) at 3ºC and from 12 days (grape) to 20 days (tomato) at 22ºC (**Table 4**).
253 In relation to the differences between the treatment applied (neem or neem+*Bt*), although C_0
254 was similar when neem was applied together with *Bt* or alone, concentration decreased slightly
255 faster in the presence of *Bt*, obtaining values of RL_{50} from 12 days (tomato at 22ºC) to 21 days
256 (tomato and grape at 3ºC) whereas when neem oil was used alone, values ranged from 20 days
257 (tomato at 22ºC) to 25 days (tomato at 3ºC) (**Table 3**). A t-test was conducted to evaluate if the
258 data sets were significantly different when neem oil was applied mixed with *Bt* or not. *P*-values
259 (**Table 4**) were calculated and as it can be seen data sets were significantly different (a
260 significance level at 0.1 was set) only for grape data at 22°C (*p*-value = 0.076). Differences at
261 22°C were higher than at 3°C for both matrices, which could be due to the fact that *Bt* was at a
262 latent phase by the production of endospores due to the low temperatures. Therefore, it can be
263 concluded that the presence of microorganisms as *Bt* could produce a faster dissipation of AzA,
264 but its influence was limited.
265 Results obtained above indicated that persistence of AzA was low in tomato and grape samples,
266 when it was applied with or without *Bt* (lower than 25 days in all cases). According to previous

studies,[41] AzA, the main component of neem extract, degraded rapidly and less than 5% respect the AzA levels just after application was found at a preharvest intervals of 13 days in lettuce. Caboni et al.[42] obtained similar results, achieving RL_{50} values between 1.2 and 3 days, being the sunlight the main factor for the degradation of AzA (photodegradation). However, the remaining AzA content and its degradation is scarcely evaluated in post-harvest samples or in samples collected immediately after application (neem oil has not withdrawal period). Thus, in the study carried out by Tofel et al.,[43] AzA seems to be stable for more than 21 days in post-harvest treated maize and cowpea samples, remaining a high concentration in the samples. Besides, although in other samples it degrades faster, it is not clear the metabolites generated from AzA and if they may involve a health risk.

Once AzA dissipation was evaluated, the detected compounds in neem oil (*Section 3.2*) were searched in tomato and grape samples after application of neem oil. Whereas only AzB was found in tomato, in grape samples all of them were found, probably due to the higher concentrations of grape samples. Their initial concentrations were the same for all the storage and application conditions. Their behaviours were studied using AzA matrix matched calibration curves to calculate their concentrations (**Figure S2a** and **S2b**). The same trend than AzA was observed for all the suspect compounds, except for 11-hidroxyazadirachtin B. Its concentration slightly increased whereas AzB concentration decreased, which can be explained since one of the degradation products of AzB is 11-hidroxyazadirachtin B, produced by hydration.

3.4 Unknown analysis

Finally, in addition to the azadirachtin related compounds described above, MassChemSite 3.1.0 was used to search possible unknown metabolites in the treated samples. With this software, inorganic and organic reactions are simulated using the structure of the parent compound and the resulting compounds were searched in the samples based on their exact mass, excluding the background noise with the blank signals. Compound Discoverer was also used to search in the

293 samples possible metabolites from different databases as PubChem, m/z Cloud or ChemSpider.
294 Eighteen compounds were putatively identified (**Table 1**). To have a more precise identification,
295 different factors were considered, as retention times (metabolites must have similar retention
296 times than the parent compounds due to their similar structures) and isotopic and
297 fragmentation patterns, which should be like parent compounds, and they were determined
298 using in-silico fragmentation (MassFrontier™v7.0 software). Thirteen compounds were
299 discarded since they do not accomplish at least one of these factors, selecting 5 azadirachtin
300 metabolites by retention time and fragments ions (**Table 1**). In **Figures S3a**, **S3b**, **S3c**, **S3d** and
301 **S3e**, the extracted precursor and fragment ion chromatograms of these metabolites are shown.
302 The metabolites 3-azadirachtin and 8-azadirachtin were found at low concentrations, so no
303 fragment was assigned for 3-azadirachtin while only one was found for 8-azadirachtin. The other
304 metabolites had similar fragmentation pattern than AzA, being some fragments *m/z* 177.0552
305 (for 9 and 12-azadirachtin) or *m/z* 449.1448 (for 9 and 11-azadirachtin).
306 Finally, the main degradation routes for AzA and AzB involving the detected metabolites were
307 proposed and they are shown in **Figure 3**, comprising dehydration and demethylation. 1-
308 azadirachtin and 8-azadirachtin were only detected in tomato samples but at low concentrations
309 and only for a short period of time, while 3, 11 and 12-azadirachtin were only detected in grape
310 samples. 9-azadirachtin was detected in both matrices, but at higher concentrations in grape
311 than in tomato samples (about 10 times higher).
312 Behaviour of metabolites were also evaluated, and their concentrations were estimated as for
313 AzA related compounds. Their behaviour was different than for AzA and related compounds:
314 their concentrations increased, reaching the highest concentrations between days 5 and 40 and
315 then they fluctuate continuously (**Figures S4a** and **S4b**). Besides, concentrations increased faster
316 at 22°C than at 3ºC. This trend is explained by the progressive degradation of the parent
317 compounds into the metabolites and their own dissipation.

AzA toxicity has not been evaluated previously and no adverse effects were observed up to 32 mg/kg body weight (BW) per day.[44] However, due to the complexity of the neem oil extracts, neither the individual toxicity of each neem oil compound nor the toxicity of the metabolites generated from AzA dissipation have been studied. For that, the tool T.E.S.T. Version 5.1.1 (Environmental Protection Agency (EPA))[45] was employed to estimate the toxicity of neem compounds and metabolites individually. Using it, lethal doses in rats were calculated and added to **Table 1**. Estimated lethal doses for azadirachtin metabolites were lower than for AzA, which may indicated that these metabolites could be more toxic than the parent compound (except for 12-azadirachtin), highlighting the importance of their monitoring, although more studies are needed in this regard.

The high number of compounds present in the neem oil and all their possible metabolites may be the cause of the lack of knowledge concerning the degradation of azadirachtins. Besides, almost all the identified compounds have a poor fragmentation pattern, which hinder their study. Due to this complexity, the necessity of a deeper study of neem oil dissipation in samples is required, including the possible metabolites from *Bt* influence.

In addition to that, metabolite concentrations were very similar between both treatments (neem and neem+*Bt*) and, although AzA dissipation was slightly faster with *Bt*, this difference was not reflected in azadirachtin metabolites. This indicates that Bt does not affect AzA degradation routes or that if other azadirachtin metabolites are formed when Bt is used (probably including some biodegradation reactions as indicated by other authors as Garcia Birolli et al.),[17] but either the concentration of these theoretical compounds is too low, or the analytical methodology applied was not broad enough to detect them.

SUPPORTING INFORMATION

Supporting information associated with this article can be found in the online version. Here It is included the dissipation model adjustments (**Tables S1** and **S2**), the dissipation curves for target

compound (**Figures S1** and **S2**), the extracted characteristic and fragment ions for the unknown metabolites (**Figure S3**) and dissipation curves for unknown compounds (**Figure S4**). This material is available free of charge via the Internet at http://pubs.acs.org.

ACKNOWLEDGEMENTS

Authors gratefully acknowledge to the University of Almeria, the Andalusian Ministry of Economic Transformation, Industry, Knowledge and Universities and FEDER for financial support (project reference: UAL2020-FQM-B1943). RLR acknowledges to the Andalusian Ministry of Economic Transformation, Industry, Knowledge, and Universities for financial support from "Ayudas para Captación, Incorporación y Movilidad de Capital Humano de I+D+i (PAIDI 2020)".

CONFLICT OF INTEREST

The authors declare that there is not any conflict of interest

Table 1: UHPLC-QOrbitrap-HRMS parameters for targeted, suspect and unknown compounds

Compound	RTW (min)	Predicted oral rat LD_{50} (mg/kg)	Neutral exact mass	Neutral formula (M)	Precursor ion			Fragment ions†			Reference
					Theoretical exact mass (*m/z*)	Adduct	Mass error (ppm)	Theoretical exact mass (*m/z*)	$[M+H]^+$ molecular formula	Mass error (ppm)	
Targeted analysis											
Azadirachtin A	7.21-7.27	32.63	720.2629	$C_{35}H_{44}O_{16}$	743.2522	$[M+Na]^+$	-3.56	161.0592	$C_{10}H_9O_2$	-3.39	-
								369.1157	$C_{17}H_{21}O_9$	-4.95	
								567.1843	$C_{30}H_{31}O_{11}$	-3.21	
Suspect screening											
Azadirachtin B	7.34-7.38	39.27	662.2575	$C_{33}H_{42}O_{14}$	685.2467	$[M+Na]^+$	0.92	161.0592	$C_{10}H_9O_2$	-2.71	35
								469.2068	$C_{23}H_{33}O_{10}$	-4.25	
								567.2225	$C_{31}H_{35}O_{10}$	-4.72	
Azadirachtin I	7.15-7.19	24.12	618.2676	$C_{32}H_{42}O_{12}$	641.2568	$[M+Na]^+$	0.55	267.1233	$C_{14}H_{19}O_5$	-4.36	
								465.1913	$C_{27}H_{29}O_7$	3.72	
Azadirachtin H	-	-	662.2575	$C_{33}H_{42}O_{14}$	685.2467	$[M+Na]^+$	-	-	-	-	
Azadirachtin D	7.15-7.19	27.10	676.2731	$C_{34}H_{44}O_{14}$	699.2623	$[M+Na]^+$	-1.40	677.2809	$C_{34}H_{45}O_{14}$	4.61	
								523.1968	$C_{29}H_{31}O_9$	4.69	
Azadirachtin Q	-	-	664.2362	$C_{32}H_{40}O_{15}$	687.2259	$[M+Na]^+$	-	-	-	-	46
Azadirachtin M	-	-	634.2620	$C_{32}H_{42}O_{13}$	657.2518	$[M+Na]^+$	-	-	-	-	47
Azadirachtin N	7.36-7.41	25.47	680.2675	$C_{33}H_{44}O_{15}$	663.2647	$[M+H-H_2O]^+$	0.71	681.2759	$C_{33}H_{45}O_{15}$	-3.91	
								621.2178	$C_{30}H_{37}O_{14}$	2.23	
Azadirachtin F	7.44-7.47	-	664.2726	$C_{33}H_{44}O_{14}$	687.2623	$[M+Na]^+$	-2.14	665.2804	$C_{33}H_{45}O_{14}$	4.59	46
								611.2334	$C_{33}H_{39}O_{11}$	-0.55	
Azadirachtin L	-	-	704.2675	$C_{35}H_{44}O_{15}$	727.2572	$[M+Na]^+$	-	-	-	-	48
Azadiradione	-	-	450.2406	$C_{28}H_{34}O_5$	451.2479	$[M+H]^+$	-	-	-	-	41,49,50
Nimbin	-	-	540.2360	$C_{30}H_{36}O_9$	563.2257	$[M+Na]^+$	-	-	-	-	
6-desacetyl-nimbin	7.82-7.90	107.41	498.2248	$C_{28}H_{34}O_8$	521.2151	$[M+Na]^+$	-2.09	467.2064	$C_{27}H_{31}O_7$	-1.14	
Salannin	8.10-8.18	3.41	596.2985	$C_{34}H_{44}O_9$	619.2883	$[M+Na]^+$	-2.53	597.3064	$C_{34}H_{45}O_9$	-2.69	
3-desacetyl-salannin	8.07-8.11	3.95	554.2874	$C_{32}H_{42}O_8$	577.2777	$[M+Na]^+$	-1.75	555.2952	$C_{32}H_{43}O_8$	-2.12	
Ohchinolide B	7.75-7.80	-	624.2935	$C_{35}H_{44}O_{10}$	647.2832	$[M+Na]^+$	-2.67	625.3013	$C_{35}H_{45}O_{10}$	-2.25	
Nimbinin	-	-	466.2355	$C_{28}H_{34}O_6$	467.2428	$[M+H]^+$					3

Vilasinin	-	-	428.2557	$C_{26}H_{36}O_5$	429.2636	$[M+H]^+$					
Gedunin	-	-	482.2304	$C_{28}H_{34}O_7$	483.2377	$[M+H]^+$					
11-Hydroxyazadirachtin B			678.2518	$C_{33}H_{42}O_{15}$	701.2416	$[M+Na]^+$					[51]
Unknown analysis											
1-Azadirachtin	-	-	582.1949	$C_{27}H_{34}O_{14}$	605.1846	$[M+Na]^+$	-	-	-	-	Masschemsite/ Compound Discoverer
2-Azadirachtin	-	-	638.2211	$C_{30}H_{38}O_{15}$	661.2108	$[M+Na]^+$	-	-	-	-	
3-Azadirachtin	7.18-7.24	16.57	644.2468	$C_{33}H_{40}O_{13}$	667.2367	$[M+Na]^+$	0.13	-	-	-	
4-Azadirachtin	-	-	650.2211	$C_{31}H_{38}O_{15}$	673.2108	$[M+Na]^+$	-	-	-	-	
5-Azadirachtin	-	-	656.2316	$C_{30}H_{40}O_{16}$	679.2214	$[M+Na]^+$	-	-	-	-	
6-Azadirachtin	-	-	666.2312	$C_{35}H_{38}O_{13}$	689.2210	$[M+Na]^+$	-	-	-	-	
7-Azadirachtin	-	-	673.2133	$C_{33}H_{37}O_{15}$	696.2030	$[M+Na]^+$	-	-	-	-	
3-Deacetylazadirachtin	-	-	678.2524	$C_{33}H_{42}O_{15}$	701.2421	$[M+Na]^+$	-	-	-	-	
8-Azadirachtin	6.08-6.13	16.60	684.2418	$C_{35}H_{40}O_{14}$	707.2316	$[M+Na]^+$	0.80	305.1025	$C_{16}H_{17}O_6$	3.23	
9-Azadirachtin	7.18-7.25	23.95	688.2367	$C_{34}H_{40}O_{15}$	711.2265	$[M+Na]^+$	0.79	177.0552	$C_{10}H_9O_3$	-0.92	
								235.0970	$C_{13}H_{15}O_4$	-1.54	
								449.1448	$C_{22}H_{25}O_{10}$	2.84	
10-Azadirachtin	-	-	692.2316	$C_{33}H_{40}O_{16}$	715.2214	$[M+Na]^+$	-	-	-	-	
11-Azadirachtin	7.24-7.35	16.54	702.2524	$C_{35}H_{42}O_{15}$	725.2421	$[M+Na]^+$	0.70	449.1448	$C_{22}H_{25}O_{10}$	3.51	
								585.1972	$C_{30}H_{33}O_{12}$	-3.17	
12-Azadirachtin	7.08-7.19	31.06	706.2473	$C_{34}H_{42}O_{16}$	729.2371	$[M+Na]^+$	0.88	177.0552	$C_{10}H_9O_3$	0.45	
								179.0708	$C_{10}H_{11}O_3$	0.16	
								366.1679	$C_{19}H_{26}O_7$	-1.59	
13-Azadirachtin	-	-	708.2629	$C_{34}H_{44}O_{16}$	731.2527	$[M+Na]^+$	-	-	-	-	
22,23-Dihydroazadirachtin	-	-	722.2786	$C_{35}H_{46}O_{16}$	745.2684	$[M+Na]^+$	-	-	-	-	
14-Azadirachtin	-	-	724.2578	$C_{34}H_{44}O_{17}$	747.2476	$[M+Na]^+$	-	-	-	-	
15-Azadirachtin	-	-	736.2579	$C_{35}H_{44}O_{17}$	759.2476	$[M+Na]^+$	-	-	-	-	
16-Azadirachtin	-	-	794.2633	$C_{37}H_{46}O_{19}$	817.2531	$[M+Na]^+$	-	-	-	-	

†Fragment ions were obtained from in-silico fragmentation employing MassFrontier™v7.0

Table 2: Validation parameters obtained for the evaluated extraction methods in tomato and grapes

Matrix	Linearity (R^2)	Trueness (%)		Intra/Interday precision (%)†		LOQ (µg/kg)	Matrix effect
		5 µg/kg	100 µg/kg	5 µg/kg	100 µg/kg		
Acetonitrile extraction							
Grape	0.9998	103	87	1/19	14/12	5	-22
Tomato	0.9946	97	93	3/11	3/2	10	-23
Methanol 0.5% acetic acid							
Grape	0.9956	117	103	9/18	3/14	5	-56
Tomato	0.9963	126	121	7/11	6/10	10	-47

†Number of replicates=5

Table 3: Concentration of targeted and suspect compounds in neem oil

Compounds	Concentration (mg/L) †
Azadirachtin A	6880.20
Azadirachtin B	1156.75
Azadirachtin I	170.53
Azadirachtin H	ND
Azadirachtin D	273.39
Azadirachtin Q	ND
Azadirachtin M	ND
Azadirachtin N	66.79
Azadirachtin F	367.43
Azadirachtin L	ND
Azadiradione	ND
Nimbin	ND
6-desacetyl-nimbin	244.08
Salannin	331.96
3-desacetyl-salannin	362.90
Ohchinolide B	168.17
Nimbinin (epoxyazadiradione)	ND
Vilasinin	ND
Gedunin	ND
11-Hydroxyazadirachtin B	ND

†Estimated using AzA calibration curve in methanol; Abbreviations: ND: Non detected

REFERENCES

(1) Kotsonis, K. N.; Burdock, G. A. Chapter 31: Food Toxicology. In *Casarett & Doull's Essentials of Toxicology*; 2013; pp 1305–1356.

(2) Kumar, J.; Ramlal, A.; Mallick, D.; Mishra, V. An Overview of Some Biopesticides and Their Importance in Plant Protection for Commercial Acceptance. *Plants* **2021**, *10* (6), 1185. https://doi.org/10.3390/plants10061185.

(3) Rossi, M.; Fernandes da Silva, M. F. das G.; Batista, J. Secondary Metabolism as a Measurement of Efficacy of Botanical Extracts: The Use of Azadirachta Indica (Neem) as a Model. In *Insecticides - Advances in Integrated Pest Management*; Perveen, F., Ed.; 2012; pp 367–390. https://doi.org/10.5772/27961.

(4) BusinessWire. *Organic Farming Global Market Report 2021*. https://www.businesswire.com/news/home/20210628005637/en/Organic-Farming-Global-Market-Report-2021-COVID-19-Growth-and-Change-to-2030---ResearchAndMarkets.com (accessed 2022-04-11).

(5) Food and Agriculture Organization of the United Nations (FAO). *FAOSTAT*. http://www.fao.org/faostat/es/#data/QCL (accessed 2022-04-11).

(6) Forim, M. R.; Fernandes Da Silva, M. F. D. G.; Cass, Q. B.; Fernandes, J. B.; Vieira, P. C. Simultaneous Quantification of Azadirachtin and 3-Tigloylazadirachtol in Brazilian Seeds and Oil of Azadirachta Indica: Application to Quality Control and Marketing. *Analytical Methods* **2010**, *2* (7), 860–869. https://doi.org/10.1039/c0ay00008f.

(7) Prestes, O. D.; Padilla-Sánchez, J. A.; Romero-González, R.; Grio, S. L.; Frenich, A. G.; Martínez-Vidal, J. L. Comparison of Several Extraction Procedures for the Determination of Biopesticides in Soil Samples by Ultrahigh Pressure LC-MS/MS. *J Sep Sci* **2012**, *35* (7), 861–868. https://doi.org/10.1002/jssc.201101057.

(8) University of Hertfordshire. *PPDB: Pesticide Properties DataBase*. http://sitem.herts.ac.uk/aeru/ppdb/ (accessed 2022-04-01).

(9) Chai, Y.; Liu, R.; Du, X.; Yuan, L. Dissipation and Residue of Metalaxyl-M and Azoxystrobin in Scallions and Cumulative Risk Assessment of Dietary Exposure to Hepatotoxicity. *Molecules* **2022**, *27* (18). https://doi.org/10.3390/molecules27185822.

(10) Roh, J. Y.; Choi, J. Y.; Li, M. S.; Jin, B. R.; Je, Y. H. Bacillus Thuringiensis as a Specific, Safe, and Effective Tool for Insect Pest Control. *J Microbiol Biotechnol* **2007**, *17* (4), 547–559.

(11) Hofte, H.; Whiteley, H. R. Insecticidal Crystal Proteins of Bacilllus. *Microbiol Rev* **1989**, *53* (2), 242–255.

(12) de Escudero, I. R.; Estela, A.; Porcar, M.; Martínez, C.; Oguiza, J. A.; Escriche, B.; Ferré, J.; Caballero, P. Molecular and Insecticidal Characterization of a Cry1I Protein Toxic to Insects of the Families Noctuidae, Tortricidae, Plutellidae, and Chrysomelidae. *Appl Environ Microbiol* **2006**, *72* (7), 4796–4804. https://doi.org/10.1128/AEM.02861-05.

(13) Sarr, O. M.; Bal, A. B.; Fossati-Gaschignard, O.; Gauthier, N. Effectiveness of Two Biopesticides against the Invasive Tomato Pest Tuta Absoluta. *Entomol Exp Appl* **2021**, *169* (7), 674–685. https://doi.org/10.1111/eea.13047.

(14) Ambreen, S.; Yasmin, A.; Aziz, S. Isolation and Characterization of Organophosphorus Phosphatases from Bacillus Thuringiensis MB497 Capable of Degrading Chlorpyrifos, Triazophos and Dimethoate. *Heliyon* **2020**, *6* (7), e04221. https://doi.org/10.1016/j.heliyon.2020.e04221.

(15) Gangireddygari, V. S. R.; Kalva, P. K.; Ntushelo, K.; Bangeppagari, M.; Djami Tchatchou, A.; Bontha, R. R. Influence of Environmental Factors on Biodegradation of Quinalphos by Bacillus Thuringiensis. *Environ Sci Eur* **2017**, *29* (11), 1–10. https://doi.org/10.1186/s12302-017-0109-x.

(16) Mandal, K.; Singh, B.; Jariyal, M.; Gupta, V. K. Microbial Degradation of Fipronil by Bacillus Thuringiensis. *Ecotoxicol Environ Saf* **2013**, *93*, 87–92. https://doi.org/10.1016/j.ecoenv.2013.04.001.

(17) Birolli, W. G.; dos Santos, A.; Pilau, E.; Rodrigues-Filho, E. New Role for a Commercially Available Bioinsecticide: Bacillus Thuringiensis Berliner Biodegrades the Pyrethroid Cypermethrin. *Environ Sci Technol* **2021**, *55* (8), 4792–4803. https://doi.org/10.1021/acs.est.0c06907.

(18) Ferreira, L.; Rosales, E.; Danko, A. S.; Sanromán, M. A.; Pazos, M. M. Bacillus Thuringiensis a Promising Bacterium for Degrading Emerging Pollutants. *Process Safety and Environmental Protection* **2016**, *101*, 19–26. https://doi.org/10.1016/j.psep.2015.05.003.

(19) López-Ruiz, R.; Romero-González, R.; Martínez Vidal, J. L.; Garrido Frenich, A. Determination of Flonicamid and Its Metabolites in Bell Pepper Using Ultra-High-Performance Liquid Chromatography Coupled to High-Resolution Mass Spectrometry (Orbitrap). *Food Additives and Contaminants - Part A* **2016**, *33* (11), 1685–1692. https://doi.org/10.1080/19440049.2016.1232865.

(20) López-Ruiz, R.; Belmonte-Sánchez, E.; Romero-González, R.; Martínez Vidal, J. L.; Ramírez-Cassinello, J. J.; Garrido Frenich, A. A Laboratory Study on Dissipation and Risk Assessment of the Proinsecticide Thiocyclam and Its Metabolite Nereistoxin in Tomato Using Liquid Chromatography High Resolution Mass Spectrometry. *Food Chem* **2021**, *344*. https://doi.org/10.1016/j.foodchem.2020.128729.

(21) Buragohain, P.; Saikia, D. K.; Sotelo-Cardona, P.; Srinivasan, R. Evaluation of Bio-Pesticides against the South American Tomato Leaf Miner, Tuta Absoluta Meyrick (Lepidoptera: Gelechiidae) in India. *Horticulturae* **2021**, *7* (9). https://doi.org/10.3390/horticulturae7090325.

(22) Turanli, F.; Gümüş, E.; Güzel, B. Studies on the Efficacies of Combinations of Bacillus Thuringiensis with Neem Extracts. *Turkish Journal of Entomology* **2012**, *36* (3), 433–439.

(23) White, P. M.; Potter, T. L.; Culbreath, A. K. Fungicide Dissipation and Impact on Metolachlor Aerobic Soil Degradation and Soil Microbial Dynamics. *Science of the Total Environment* **2010**, *408* (6), 1393–1402. https://doi.org/10.1016/j.scitotenv.2009.11.012.

(24) Yang, S.-H.; Choi, H. Simultaneous Determination of Nereistoxin Insecticides in Foods of Animal Origins by Combining PH-Dependent Reversible Partitioning with Hydrophilic

Interaction Chromatography-Mass Spectrometry. *Sci Rep* **2022**, *12* (1). https://doi.org/10.1038/s41598-022-14520-3.

(25) Khairnar, A.; Ruda-Kucerova, J.; Arab, A.; Hadjistyllis, C.; Sejnoha Minsterova, A.; Shang, Q.; Chovsepian, A.; Drazanova, E.; Szabó, N.; Starcuk, Z.; Rektorova, I.; Pan-Montojo, F. Diffusion Kurtosis Imaging Detects the Time-Dependent Progress of Pathological Changes in the Oral Rotenone Mouse Model of Parkinson's Disease. *J Neurochem* **2021**, *158* (3), 779–797. https://doi.org/10.1111/jnc.15449.

(26) Cappa, F.; Baracchi, D.; Cervo, R. Biopesticides and Insect Pollinators: Detrimental Effects, Outdated Guidelines, and Future Directions. *Science of the Total Environment* **2022**, *837*. https://doi.org/10.1016/j.scitotenv.2022.155714.

(27) European Food Safety Authority. *Maximum Residue Levels*. https://ec.europa.eu/food/plants/pesticides/maximum-residue-levels_en (accessed 2021-08-31).

(28) López-Ruiz, R.; Romero-González, R.; Garrido-Frenich, A. Ultrahigh-Pressure Liquid Chromatography-Mass Spectrometry: An Overview of the Last Decade. *TrAC Trends in Analytical Chemistry* **2019**, *118*, 170–181. https://doi.org/10.1016/j.trac.2019.05.044.

(29) López-Ruiz, R.; Romero-González, R.; Ortega-Carrasco, E.; Garrido Frenich, A. Dissipation Studies of Famoxadone in Vegetables under Greenhouse Conditions Using Liquid Chromatography Coupled to High-Resolution Mass Spectrometry: Putative Elucidation of a New Metabolite. *J Sci Food Agric* **2019**, *99* (12), 5368–5376. https://doi.org/10.1002/jsfa.9794.

(30) Prata, R.; López-Ruiz, R.; Henrique Petrarca, M.; Teixeira Godoy, H.; Garrido Frenich, A.; Romero-González, R. Targeted and Non-Targeted Analysis of Pesticides and Mycotoxins in Baby Foods ByUHPLC-Q-Orbitrap-MS. *Food Control* **2022**, *139*, 109072. https://doi.org/https://doi.org/10.1016/j.foodcont.2022.109072.

(31) Hergueta-Castillo, M. E.; López-Rodríguez, E.; López-Ruiz, R.; Romero-González, R.; Garrido Frenich, A. Targeted and Untargeted Analysis of Triazole Fungicides and Their Metabolites in Fruits and Vegetables by UHPLC-Orbitrap-MS2. *Food Chem* **2022**, *368*, 130860. https://doi.org/10.1016/j.foodchem.2021.130860.

(32) Marín-Sáez, J.; Romero-González, R.; Garrido Frenich, A. Multi-Analysis Determination of Tropane Alkaloids in Cereals and Solanaceaes Seeds by Liquid Chromatography Coupled to Single Stage Exactive-Orbitrap. *J Chromatogr A* **2017**, *1518*, 46–58. https://doi.org/10.1016/j.chroma.2017.08.052.

(33) Pihlström, T.; Fernández-Alba, A. R.; Ferrer Amate, C.; Erecius Poulsen, M.; Lippold, R.; Carrasco Cabrera, L.; Pelosi, P.; Valverde, A.; Mol, H.; Jezussek, M.; Malato, O.; Štěpán, R. Analytical Quality Control and Method Validation Procedures for Pesticide Residues Analysis in Food and Feed SANTE 11312/2021. **2021**, 1–57.

(34) López-Ruiz, R.; Romero-González, R.; Garrido Frenich, A. Residues and Dissipation Kinetics of Famoxadone and Its Metabolites in Environmental Water and Soil Samples under Different Conditions. *Environmental Pollution* **2019**, *252*, 163–170. https://doi.org/10.1016/j.envpol.2019.05.123.

(35) Song, L.; Wang, J.; Gao, Q.; Ma, X.; Wang, Y.; Zhang, Y.; Xun, H.; Yao, X.; Tang, F. Simultaneous Determination of Five Azadirachtins in the Seed and Leaf Extracts of Azadirachta Indica by Automated Online Solid-Phase Extraction Coupled with LC–Q-TOF–MS. *Chem Cent J* **2018**, *12* (85), 1–9. https://doi.org/10.1186/s13065-018-0453-y.

(36) European Food Safety Authority. Conclusion on the Peer Review of the Pesticide Risk Assessment of the Active Substance Myclobutanil. *EFSA Journal* **2010**, *8* (10), 1–83. https://doi.org/10.2903/j.efsa.2010.1783.

(37) Barrek, S.; Paisse, O.; Grenier-Loustalot, M. F. Analysis of Neem Oils by LC-MS and Degradation Kinetics of Azadirachtin-A in a Controlled Environment : Characterization of Degradation Products by HPLC-MS-MS. *Anal Bioanal Chem* **2004**, *378* (3), 753–763. https://doi.org/10.1007/s00216-003-2377-0.

(38) Fantke, P.; Juraske, R. Variability of Pesticide Dissipation Half-Lives in Plants. *Environ Sci Technol* **2013**, *47* (8), 3548–3562. https://doi.org/10.1021/es303525x.

(39) Zhao, P.; Chai, Y.; Liu, R.; Yuan, L. Dissipation, Residue, and Dietary Risk Assessment of Bifenthrin, Bifenazate, and Its Metabolite Bifenazate-Diazene in Apples Based on Deterministic and Probabilistic Methods. *J Agric Food Chem* **2021**, *69* (47), 14302–14310. https://doi.org/10.1021/acs.jafc.1c05847.

(40) Chai, Y.; Liu, R.; He, W.; Xu, F.; Chen, Z.; Li, L.; Li, W.; Yuan, L. Dissipation Behavior, Residue, and Risk Assessment of Benziothiazolinone in Apples. *Int J Environ Res Public Health* **2021**, *18* (9). https://doi.org/10.3390/ijerph18094478.

(41) European Food Safety Authority. Peer Review of the Pesticide Risk Assessment of the Active Substance Azadirachtin (Margosa Extract). *EFSA Journal* **2018**, *16* (9), 1–31. https://doi.org/10.2903/j.efsa.2018.5234.

(42) Caboni, P.; Sarais, G.; Angioni, A.; Lai, F.; Dedola, F.; Cabras, P. Fate of Azadirachtin A and Related Azadirachtoids on Tomatoes after Greenhouse Treatment. *J Environ Sci Health B* **2009**, *44* (6), 598–605. https://doi.org/10.1080/03601230903000669.

(43) Tofel, K. H.; Nukenine, E. N.; Stähler, M.; Adler, C. Degradation of Azadirachtin A on Treated Maize and Cowpea and the Persistence of Azadirachta Indica Seed Oil on Callosobruchus Maculatus and Sitophilus Zeamais. *J Stored Prod Res* **2016**, *69*, 207–212. https://doi.org/10.1016/j.jspr.2016.08.011.

(44) Arena, M.; Auteri, D.; Barmaz, S.; Brancato, A.; Brocca, D.; Bura, L.; Carrasco Cabrera, L.; Chiusolo, A.; Court Marques, D.; Crivellente, F.; de Lentdecker, C.; Egsmose, M.; Fait, G.; Ferreira, L.; Goumenou, M.; Greco, L.; Ippolito, A.; Istace, F.; Jarrah, S.; Kardassi, D.; Leuschner, R.; Lythgo, C.; Magrans, J. O.; Medina, P.; Miron, I.; Molnar, T.; Padovani, L.; Parra Morte, J. M.; Pedersen, R.; Reich, H.; Sacchi, A.; Santos, M.; Serafimova, R.; Sharp, R.; Stanek, A.; Streissl, F.; Sturma, J.; Szentes, C.; Tarazona, J.; Terron, A.; Theobald, A.; Vagenende, B.; Villamar-Bouza, L. Peer Review of the Pesticide Risk Assessment of the Active Substance Azadirachtin (Margosa Extract). *EFSA Journal* **2018**, *16* (9), 1–31. https://doi.org/10.2903/j.efsa.2018.5234.

(45) U.S. Environmental Protection Agency. *Toxicity Estimation Software Tool (TEST)*. https://www.epa.gov/chemical-research/toxicity-estimation-software-tool-test (accessed 2022-06-17).

(46) PubChem. *PubChem*. https://pubchem.ncbi.nlm.nih.gov/ (accessed 2021-12-01).

(47) Luo, X.; Ma, Y.; Wu, S.; Wu, D. Two Novel Azadirachtin Derivatives from Azadirachta Indica. *J Nat Prod* **1999**, *62* (7), 1022–1024. https://doi.org/10.1021/np980452d.

(48) Kanokmedhakul, S.; Kanokmedhakul, K.; Prajuabsuk, T.; Panichajakul, S.; Panyamee, P.; Prabpai, S.; Kongsaeree, P. Azadirachtin Derivatives from Seed Kernels of Azadirachta Excelsa. *J Nat Prod* **2005**, *68* (7), 1047–1050. https://doi.org/10.1021/np050064t.

(49) Schaaf, O.; Jarvis, A. P.; Van Der Esch, S. A.; Giagnacovo, G.; Oldham, N. J. Rapid and Sensitive Analysis of Azadirachtin and Related Triterpenoids from Neem (Azadirachta Indica) by High-Performance Liquid Chromatography-Atmospheric Pressure Chemical Ionization Mass Spectrometry. *J Chromatogr A* **2000**, *886* (1–2), 89–97. https://doi.org/10.1016/S0021-9673(00)00492-1.

(50) Kuravadi, N. A.; Yenagi, V.; Rangiah, K.; Mahesh, H. B.; Rajamani, A.; Shirke, M. D.; Russiachand, H.; Loganathan, R. M.; Lingu, C. S.; Siddappa, S.; Ramamurthy, A.; Sathyanarayana, B. N.; Gowda, M. Comprehensive Analyses of Genomes, Transcriptomes and Metabolites of Neem Tree. *PeerJ* **2015**, *2015* (8), 1–25. https://doi.org/10.7717/peerj.1066.

(51) Kumar, C. S. S. R.; Srinivas, M.; Yakkundi, S. Limonoids from the Seeds of Azadirachta Indica. *Phytochemistry* **1996**, *43* (2), 451–455. https://doi.org/10.1016/0031-9422(96)00226-9.

FIGURE CAPTION

Figure 1. Extracted ion chromatograms of AzA in tomato at the LOQ (10 µg/kg): precursor and fragments extracted ions (A); experimental (B) and theoretical (C) mass spectra

Figure 2. Concentration of the parent compound (adjusted to kinetic model "Single first order'') for tomato samples: A=Azadirachtin at 22°C; B=Azadirachtin+*Bt* at 22°C; C=Azadirachtin at 3°C; D=Azadirachtin+*Bt* at 3°C

Figure 3. Azadirachtin metabolites detected in real samples and their formation route

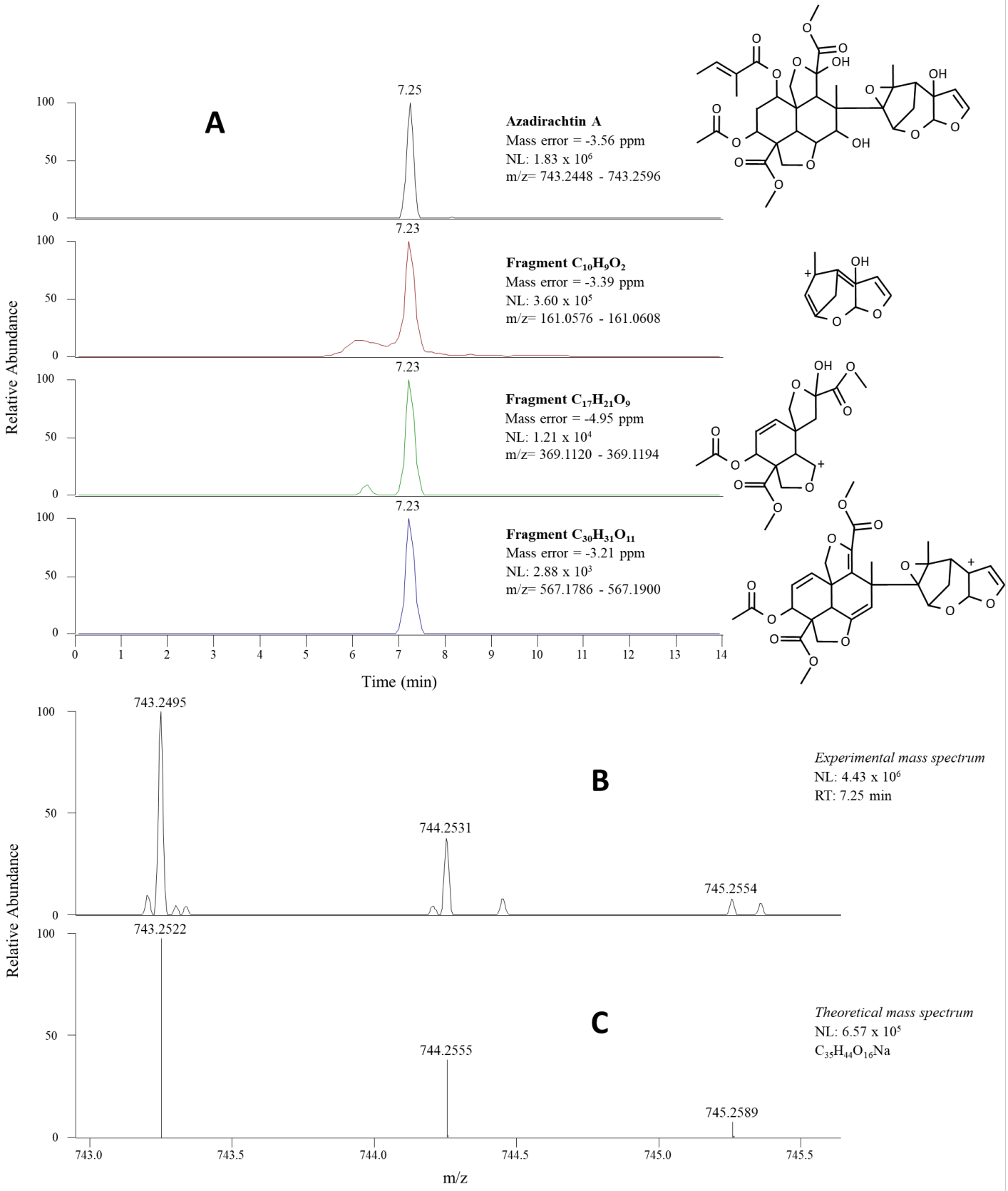

Figure 1. Extracted ion chromatograms of AzA in tomato at the LOQ (10 µg/kg): precursor and fragments extracted ions (A); experimental (B) and theoretical (C) mass spectra

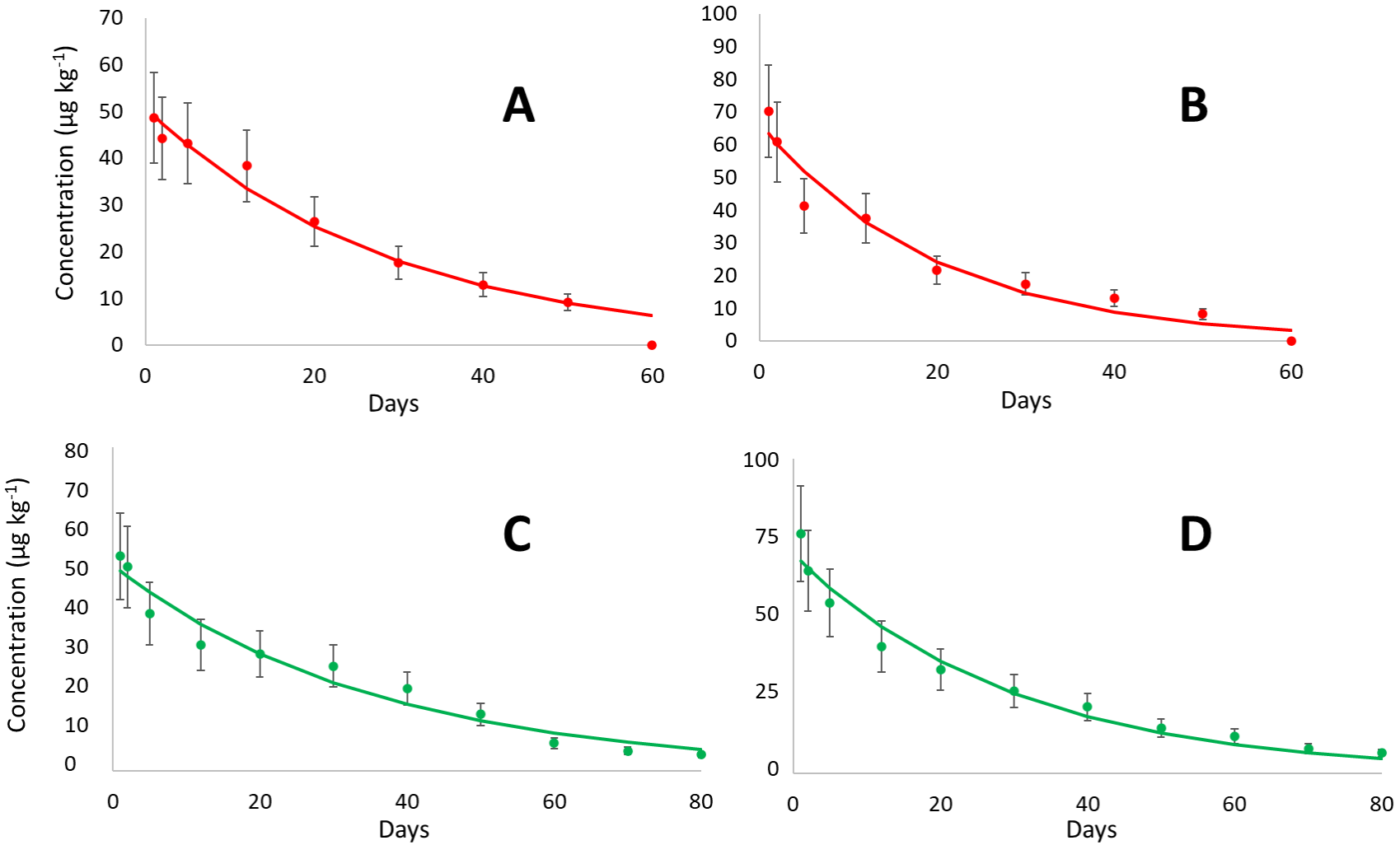

Figure 2. Concentration of the parent compound (adjusted to kinetic model "Single first order'') for tomato samples: A=Azadirachtin at 22°C; B=Azadirachtin+*Bt* at 22°C; C=Azadirachtin at 3°C; D=Azadirachtin+*Bt* at 3°C

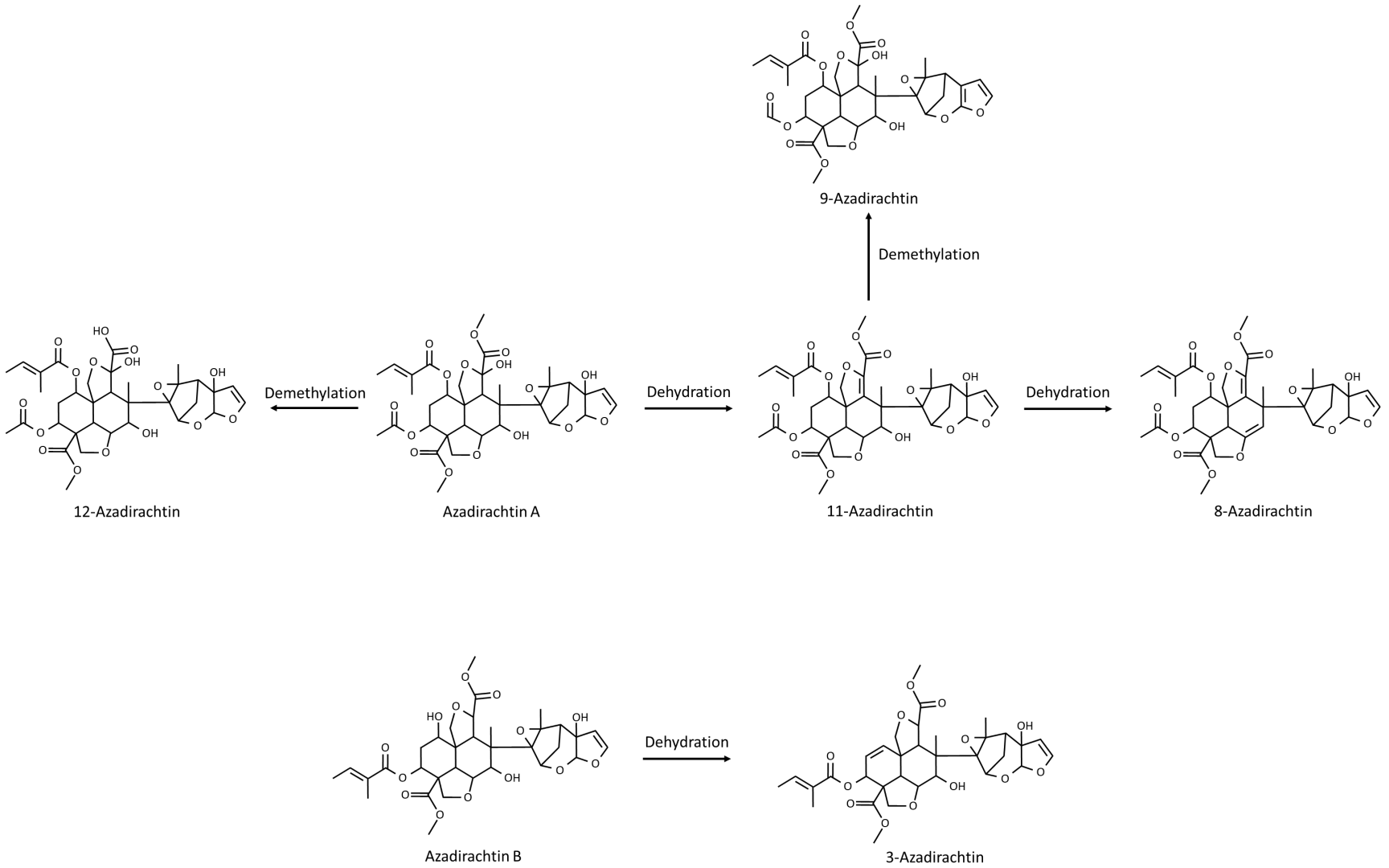

Figure 3. Azadirachtin metabolites detected in real samples and their formation route

Table of content