Development of an analytical method based on QuEChERS and GC-ECD for the determination of deltamethrin residue in pine needles and pine nuts

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Abstract

In recent years, recurrent droughts have weakened the stone pine (*Pinus pinea)* forests and favoured the emergence of harmful pests and diseases, including the *Leptoglossus occidentalis.* The stone pine's nuts production has declined over the past five years. To control this hemipteran pest, a synthetic pyrethroid insecticide called deltamethrin is being tested. However, it is necessary to estimate the residue left by these treatments in forest stands. Therefore, a fast and robust analytical procedure was developed based on QuEChERS clean-up extraction, followed by gas chromatography coupled with an electron capture detector. This optimized method can detect residual concentrations of deltamethrin in pine needles and pine nuts up to a few μg kg^{-1}. The results showed that two weeks after spraying, the deltamethrin content in the needles of stone pines decreased up to 75%, and after 9 months, its presence was like the nontreated trees.

Introduction

The stone pine, *Pinus pinea* L., is a woody species very common as a component of the Iberian forests on sandy soils. As scattered tree, member of the Mediterranean mixed forest, or forming to monospecific stands it occupies over 600,000 hectares in the Iberian Peninsula (Guàrdia et al., 2021; Mutke et al., 2012). However, the species are currently facing the consequences of emerging pests, whose expansion is mainly favoured by the recurrent droughts of recent years. One of the most damaging and concerning pests is the Western conifer seed bug (*Leptoglossus occidentalis* Heidemann), the which was identified in Catalonia - Spain in 2003 (Ribes and Escolà, 2003). These bedbugs suck vegetative and reproductive organs of pines and destroy cones of different ages, before they can mature. Pine nuts are nuts highly valued for their nutritional qualities (Evaristo et al., 2013; Guàrdia et al., 2021) but their production has dramatically decreased over the last five years due to the actions of these pests (Calama et al., 2020; de la Mata et al., 2019; Mutke et al., 2017). To control this pest, deltamethrin (DLT), a synthetic pyrethroid ester with broad-spectrum acaricidal and insecticidal activity, has been tested in the field with very good results. However, the use of pesticides raises concerns about their slow rates of degradation, toxicity, and the potential for accumulation in remote areas of the world (Araújo et al., 2012). They may enter the atmosphere through several processes: from pesticide formulations applied over farming areas and forested landscapes (Asman et al., 2005), volatilized from the surface of soil, water, and plants (Araújo et al., 2012), or the incineration of waste materials treated with pesticides (Breivik et al., 2004), among others. The World Health Organization (WHO) considers to be of utmost importance in global public hygiene, with pesticide and veterinary drug residues in foodstuffs posing potential hazards to food safety. In this context, the Food and Agriculture Organization (FAO)/WHO/Codex Alimentarius Commission (CAC) sets maximum residue limits (MRL) for more than 170 pesticide and veterinary drug residues in over 300 varieties of agricultural products and foods. In the case of pine nuts, the European commission has established a maximum allowed value of deltamethrin of 20 μg kg^{-1} (Reg. (EU) 2018/832) (The European Commission, 2015). However, there is no legislation regarding the concentration of this pesticide in *Pinus* stands. Therefore, to the best of our knowledge, no study has

reported a robust method capable of detecting and quantifying residue levels of deltamethrin in pine needles and pine nuts. Most of the projects that have studied pyrethroid residues have reported methodologies based on gas chromatography (GC) coupled to a mass spectrometry detector (MS) or electron capture detector (ECD) (Paramasivam, 2022; Saha et al., 2020). These substances have high molar mass, high boiling point, and are also very electronegative due to the action of Brom atoms in the molecules. Additionally, the complex matrix (pine needles and pine nuts) with many resins, wax (Araújo et al., 2012), lipids (nuts), and chlorophylls (needles) require a clean-up step to minimize the matrix effect in the analysis of the target analyte (Araújo et al., 2012; Hua et al., 2010; Paramasivam, 2022; Saha et al., 2020). Thus, the present study aims to develop and optimize a fast and robust analytical procedure capable of determining low residue levels of deltamethrin in pine needles and pine nuts through GC-ECD analysis. To check the efficiency of the method, a monitoring was carried out in a trial planned to evaluate the deltamethrin effectiveness to control Western conifer seed bug damages, which was designed in a productive stand of Stone pine.

Materials and methods

Reagents and chemicals

High purity standard of deltamethrin (DLT) was supplied by Merck (Darmstadt, Germany). Acetonitrile (ACN) was supplied by Scharlab (Barcelona, Spain), while Hexane (HX), Dichloromethane (DCM), and Acetone (CO), all of them Chromanorm® grade, were supplied by VWR Chemicals (Leuven, Belgium). Moreover, QuEChERS kits were purchased from Phenomenex (California, USA).

Stock solutions of DLT was prepared at 1,000 ng mL^{-1} in ACN, weighing 1.00 mg and diluting in 1 mL of solvent. This standard was kept at -20 ºC in amber vials to prevent degradation. Calibration standards were daily prepared in ACN.

Field experimental design

In a productive 10-year-old P. pinea orchard, located in Arenys d'Empordà, in the Northeast Catalonia (Spain), two areas were delimitated to set up the trial to evaluate the efficacy of the insecticide, DLT 2.5% w/v, in controlling the pest. One area, represented by green colour in Figure 1, was treated with the pesticide and labelled as treated pines (TR), while the other area, represented by red colour, was non-treated and labelled as untreated pines (NT). Each plot had a rectangular polygon shape and occupied at least two hectares to ensure representative sampling of pine needles and nuts from each treatment, as the farm is located in an area where persistent and strong wind blows (la 'tramontana') very often. All treatments were carried out in accordance with European and national regulations. Two treatments were applied in 2021 - one in May, after the peak of adult presence of the post-winter generation, and the other at the end of July, applied after the peak of the insect harmful stages (adults or nymphs 2+) presence at the first annual generation. The population dynamics of the Leptoglossus occidentalis was monitored weekly in both TR and NT plots to carefully guide the timing of treatments.

Two brands of deltamethrin-based product were applied, Deltaplant® and Decis®, both with a declared security period of 30 days.

Sampling

Pine needle samples, weighing 15-20 g, were randomly collected from three identified pines (25-30 needles per tree) in both the TR and NT during two consecutive crop seasons (2021 and 2022). Table 1 shows the number of samples, needles or pine nuts, established by each record. First year (2021), in TR area three samples of needles were collected at 7, 15, and 30 days after the last treatment and again at 9 months, already in 2022, which was a four sampling times per treatment (Table S1). The three NT samples were taken one in the centre of the plot and the other two at the edges, first and third rows, trying to assess the effect of product drift in this windy area such as Alt Empordà (Figure 1). In this NT area needles were removed only at 7 and 15 days and again at 9 months (Table S1). In the second crop season, 2022, the number of samples taken in the NT areas was five, three in the middle of plot and two in the edges (Figure 1); those from TR area were three, as in the previous crop season. The sampling times in NT areas were twice, at 7 and 15 days (Table S2), while in TR increased up to five in order to build a more detailed temporal decreasing pattern of DLT in pine needles; samples were collected at 7, 15, 30, 45, 60 days from the last treatment. Moreover, samples were collected after 9 months (Table S2). All needle samples were wrapped in aluminium foil in the field and stored in glass containers at a low temperature of 4 °C until GC-ECD analysis.

In the case of pine nuts, 20 g of pine nut kernels were extracted from harvested cones of both TR and NT areas in 2021 and 2022 crop seasons. Ripe cones were dried in an oven at 40ºC to get they open. Each sample of pine nut came from cones randomly collected on 10 trees per treatment and distributed among three samples for analysis (Table 1). Pine nuts were stored in glass containers at fridge temperature until analysis. In 2021, pine nut residues were only analysed once, at three months of harvest (February 2022), which was more than six months after the last treatment (6 analysis). In 2022, they were analysed twice at harvest and three months later as in the previous year (November 2022 & February 2023).

Table 1. Position and number of samples taken in the field at each sampling point (NT: non treated area, TR: treated area)

Crop season	Treatment	Needles			Pine nut	
		Id. Fig. 2,3.	Plot location	Samples	Id. Fig. 4	Samples
2021	**NT area**			**3**	NT1 NT2 NT3	**3**
		M1	First row	1		
		M2	Third row	1		
		M3	Centre of plot	1		
	TR area	M6 M7 M8	Centre of plot	**3**	TR1 TR2 TR3	**3**
2022	**NT area**			**5**	NT1 NT2 NT3	**3**
		M1	First row	1		
		M2	Third row	1		
		M3 M4 M5	Centre of plot	3		
	TR area	M6 M7 M8	Centre of plot	3	TR1 TR2 TR3	**3**

Deltamethrin extraction

Different extraction and clean-up procedures were tested to optimize the determination of residual levels of deltamethrin in pine needles and pine nuts. The extraction procedures tested were selected based on their different mechanisms of extraction and retention. Initially, sonication or ultrasound extraction was evaluated for its ability to extract analytes from the matrix using various solvents. Subsequently, different stationary phases with varying degrees of retention for the clean-up step were evaluated. Florisil (Strata®, Phenomenex), ENVI 18 (Supelclean®, Supelco Analytical), and HLB (OASIS®, Waters) were all used to retain pesticides. Additionally, QuEChERS, a matrix-dispersive mode of solid-phase extraction, was tested.

Both pine needles and pine nuts samples were ground in a grinder with a steel propeller before weighing 5 g of the sample and placing it in a 50 mL Falcon tube. Two different methods were tested: ultrasonic extraction (Method A) and QuEChERS (Method B). Four different organic solvents, namely hexane (HX) (Method A1), acetonitrile (ACN) (Method A2), HX:ACN 50:50 (Method A3), HX:DCM 50:50 (Method A4), and HX:Acetone (CO) 50:50 (Method A5), were employed for ultrasonic extraction. Twenty-five millilitres of each solvent was placed in the Falcon tube containing the sample, and the mixture was sonicated in an ultrasonic bath for 30 minutes. After this period, the solvent was replaced and filtered through a cellulose filter by gravity into a round-bottom flask. This process was repeated thrice, and the final volume was 75 mL, with a total extraction time of 90 minutes. The round-bottom flask containing the solvent was then placed in a rotavap to reduce the solvent to almost dryness (about 2 mL). A clean-up step was then implemented to minimize interferences from the matrix, with three different cartridges containing different solid extractants (HLB, Florisil, and C18) tested.

First, the cartridges were conditioned with 10 mL of the extraction solvent. Then, the remaining volume (≈ 2 mL) was then charged into the cartridge and were dried under vacuum for 10 minutes. The elution step was carried out using 10 mL of solvent (2 x 5 mL). The final extract was almost dried using a stream of N_2 in a Sample Concentrator (Techne®, Staffordshire, UK) and transferred to a 1.8 mL chromatographic vial. Finally, the samples were evaporated to dryness and reconstituted with 1 mL of ACN.

On the other hand, in Method B, a kit containing 4 g $MgSO_4$, 1 g NaCl, 1 g SCTD (sodium citrate tribasic dihydrate), and 0.5 g SCDS (sodium citrate dibasic sesquihydrate) was placed in each Falcon tube containing the sample. Then, 10 mL of ACN were added to the mixture and shaken vigorously for 1 minute. The tubes were centrifuged at 4,000 rpm for 5 min in an OrtoAlresa (Madrid, Spain) centrifuge. Afterwards, 5 mL of the supernatant was transferred to a 25 mL Falcon tube containing 0.9 g $MgSO_4$ and 150 mg PSA (primary and secondary amine exchange material). The mixture was shaken manually for 30 seconds and centrifuged at 4,000 rpm for 5 min. Finally, 1 mL of the supernatant was transferred to a chromatographic vial for the GC-ECD analysis. Figure SI1 displays the summary of all the extraction methods tested.

GC-ECD analysis

Deltamethrin analysis was performed by GC-ECD, using an Agilent 6890N instrument coupled to an electron capture detector (ECD). The carrier gas was Helium (He) at a constant flow rate of 51 mL min^{-1}. The injector temperature was set at 250 °C and 2 µL were injected, in splitless mode with a purge flow of 40 mL min^{-1}. The GC column was HP-5MS (30m x 0.32 cm x 0.25 mm) and the elution was performed using a gradient program. First, the column oven was set at 120 °C and held for 3 min. Then, temperature increased at a rate of 20 °C min^{-1} to 310 °C, and held for 8 min, resulting in a total run time of 20 minutes. The pressure was 21.76 psi. Regarding detector parameters, it was set at 350 °C and the make-up flow was fixed at 70 mL min^{-1}.

Quality assurance

The analytical method was evaluated in terms of quality regarding different parameters such as linearity, limit of detection, limit of quantification, inter- and intra- day precision and recoveries. Regarding the linearity of the instrument methodology, five standards of pure DLT were prepared in ACN in the range between 10 and 500 µg L^{-1}. Instrumental detection limit (IDL) was assessed using the signal-noise ratio (S/N) of the less concentrated standard. Precision was determined by analysing the same standard three times at the same day (intra-day precision) and the same standard three consecutive days (inter-day precision).

Furthermore, for each extraction methodology assay, four replicates of quality controls (QCs) were prepared at two different levels (0.02 and 1 mg kg^{-1}) with non-treated samples of both matrix (pine needles and pine nuts). Analysis of QCs allowed to determine the other quality parameters. Methodological detection limit (MDL) and limit of quantification (LOQ) were evaluated using the S/N of

QCs. Recoveries, expressed in percentage, were determined comparing the concentration spiked with the experimental concentration. Moreover, blank (no sample and non-spiked), solvent blank (no sample but spiked), and procedural blanks (with sample but non-spiked) were studied in order to check that there was no crossed-contamination. Finally, matrix effect (ME) was assessed by comparing the peak area of DLT from the spiked sample with the peak area of the analyte from the standard solution used in calibration curve. This parameter is an indicator of detection suppression or enhancement of the analytes.

Results and discussions

Optimization of the extraction procedure

The efficiency of the analytical procedure was calculated through the recovery method. Low recoveries were obtained in all the procedures based on ultrasounds extraction (Method A). Figure 2A displays a standard of DLT at 100 μg L^{-1} (RT = 12.6 min) in comparison with a spiked sample (QC) of a pine needle (Fig. 2B) using Method A. In the case of the spiked pine needle a lot of chromatographic peaks are observed due to matrix interferences. However, in the spiked pine nuts samples (QCs), no interferences were observed and the peak of DLT was clearly distinguished. With all of these results, it can be assumed that the peaks observed in pine needles samples could be due to the large number of chlorophylls present in the tree foliage. These molecules have four nitrogen atoms in its structure giving higher response in ECD due to its high electronegative character. In pine nuts samples, no interfering peaks were observed since chlorophylls are not typically found in these samples. Moreover, solvent blanks (without sample but spiked) presented good recoveries above 70% (with all the tested cartridges), indicating that no matrix effect was occurring.

On the other hand, for the spiked pine needles and pine nuts samples using Method B, recoveries were above the 80% for both matrices. This methodology represented an improvement in the efficiency of the method, but also faster, cheaper and easier to perform than the other methodologies tested. Moreover, this methodology was also more environmentally friendly since it used lower amount of solvent than the used in Method A. For all these reasons, Method B was selected to perform all the analysis in real samples.

Quality parameters

Great correlation was obtained (R^2 = 0.998) for the range between 10 to 500 μg L^{-1} of DLT in ACN. Moreover, the instrumental detection limit was 3 pg, while the MDLs for pine needles and pine nuts were 6 μg kg^{-1} and 0.1 μg kg^{-1}, respectively. Limits of quantification were assessed at 20 μg kg^{-1} (pine needles) and 0.4 μg kg^{-1} (pine nuts). On the other hand, precision was calculated as intra-day and inter-day precision obtaining results of 0.9% and 9.8%, respectively.

As it can be observed in Table 2, for pine needles the average recovery was 84.9% for the lower level and 83.8% for the higher level, while for pine nuts the mean recovery at low level was 101.7%, and 87.3% for

the higher level. Finally, no matrix effect was observed in either pine needles or pine nuts ($ME_{pine\ needle}$ = 83±2%, $ME_{pine\ nuts}$ = 80±1%).

Table 2
Quality parameters of the optimized methodology for determining deltamethrin by GC-ECD

Quality parameters		Pine needles	Pine nuts
Limit of detection	Instrumental	3 pg	
	Methodological	6 µg kg^{-1}	0.1 µg kg^{-1}
Limit of quantification		20 µg kg^{-1}	0.4 µg kg^{-1}
Precision	Inter-day	0.9%	
	Intra-day	9.8%	
Recovery	Low level	84.9%	101.7%
	High level	83.8%	87.3%
ME		83±2%	80±1%

Residues of deltamethrin in pine needles and pine nuts

Results allowed a clear differentiation between the two treatments, treated by the insecticide (TR) and non-treated (NT), in both crop seasons. Throughout the first growing season, from August 2021 to April 2022, all residue contains were under the limit of quantification (LOQ) of the method. During this period, the TR pines (needles), which started 7 days after application with a DLT content of about 200 µg kg^{-1}, lost more than 65% of the insecticide after 30 days (Fig. 3A).

However, this concentration was still above 20 µg kg^{-1}, the limit established for pine nut consumption by the European Pesticide Data Base (2021) and which was considered in this work since there is no such limit for pine needles. On the other hand, after nine months of insecticide application needles did not show differences between both TR and NT treatments, and the residue found was lower than 20 µg kg^{-1}. Only M8, from the TR zone, showed slightly higher levels than the samples taken 30 days after insecticide application. All concentration levels observed in the first crop season are summarized in Table S1.

Results issued from the second crop season (2022) followed similar pattern to those of 2021 for both treatments (Fig. 3B). In the TR zone the reduction in DLT content in needles was progressive over time, but there was a discordant value at 60 days (M6 in Table S2). However, after nine months in field conditions, needles did not show differences between both TR and NT treatments, and in both cases the

amount found was lower than 20 μg kg^{-1}. The results of the second campaign are summarized in Table S2.

In the analyses of pine nuts performed, both three months after harvest (2021 and 2022) and just after harvest (2022), only traces of DLT were found, with values well below the permitted limit for pine nut consumption (European Pesticide Data Base, 2021) (Fig. 4).

Deltamethrin residue showed similar evolution pattern by the two growing seasons for both pine nuts and needles. However, the concentration in the nut matrix was much lower than in the needles. The application of DLT in the control of *Leptoglossus occidentalis* does not imply any limitation for the consumption of pine nuts, in all the determinations carried out the residue level was below the limits established by the relevant European control administration. The difference between the results on nuts at harvest and those at three months was minimal and often below the limit of determination of the method (2 μg kg^{-1}). In addition, the harvest of this species is not marketed until well into the spring of the following year, which separates the application of the product until its consumption by more than 9 months.

For needles, it has been verified in 2021 that 9 months after application on trees residues in the field have dropped to very low levels like those of NT areas. Data of 2022 showed that at 60 days the residue of DLT was like those after 9 months in 2021, and a considerable decrease was observed after 9 months. It should be borne in mind that DLT is a broad-spectrum insecticide and widely used in the production of many vegetables and fruits and its transport could be expected by the wind, particularly in windy areas like Alt Empordà (Girona-Spain).

Residues of deltamethrin in other food matrices

In the last years, several studies have reported higher levels of deltamethrin residues in vegetables than the established MRLs. In 2021, Elqueta et al. in a study published about the analysis of multi-pesticide residues in fresh tomatoes, determined that 61% of the tomatoes cultivated in greenhouses in the Mediterranean region of Turkey contained many active substances, among which DLT was found (Elgueta et al., 2021). Moreover, Salghi et al. reported DLT residues in tomatoes from greenhouses in Morocco higher than MRL (0.3 mg kg^{-1}). In this case, 10% of the samples where above MRL, while the other samples were between 0.003 and 0.25 mg kg^{-1} (Salghi et al., 2012). Furthermore, Jallow et al. performed an analysis to monitor pesticide residues in commonly consumed fruits and vegetables in Kuwait, and they reported residues of DLT exceeding the MRLs in watermelons, apples, and grapes. Interestingly, deltamethrin was the second most detected pesticide (11% of the analyzed samples contained DLT) (Jallow et al., 2017). However, regarding studies performed in Spain, determined values below MRLs for deltamethrin in lettuce, spinaches and Swiss chard (González-Rodríguez et al., 2008). Anyhow, previously reported values in other matrices such as vegetables or fruits, are higher than the reported values in this study for pine nuts.

However, it's worth noting that the detection of DLT residues above the MRLs does not necessarily mean that the vegetables are unsafe for consumption. MRLs are set based on scientific data and risk assessments, and exceeding them does not always imply a health risk. However, it does indicate that the levels of DLT in those vegetables are higher than what is deemed acceptable by the regulatory authorities. In such cases, appropriate measures are taken to prevent further contamination, and the vegetables are withdrawn from the market.

Conclusions

A comprehensive optimization of a QuEChERS method followed by GC-ECD was performed for the unequivocal identification and quantification of deltamethrin in pine needles and pine nuts. The analytical performance of the GC-ECD method provides high selectivity and sensitivity and enhanced identification capabilities. After the analysis of samples, the study successfully differentiated between the two treatments: insecticide-treated (TR) and non-treated (NT) in both crop seasons. During the first growing season (August 2021 to April 2022), all residue concentrations for NT areas were below the limit of quantification (LOQ) of the method used. Initially, the TR pine needles had a DLT content of approximately 200 μg kg^{-1}, but after 30 days, they lost more than 65% of the insecticide. After nine months of insecticide application, there were no significant differences between the TR and NT treatments in terms of residue levels in the pine needles, and the amount found was below 20 μg kg^{-1}, for all samples except one of them. In the second crop season (2022), the results followed a similar pattern to the previous season for both treatments (TR and NT). Therefore, it can be concluded that even though deltamethrin is considered a persistent pollutant, the residue in pine needles after a “short” period since the treatment were below the limit that European administration established for pine nuts.

Finally, in the case of pine nuts, the application of deltamethrin in the control of *Leptoglossus occidentalis* implies a limitation of DLT residues for the consumption of pine nuts. In all the determinations carried out, the residue levels were below the limits established by the relevant European control administration.

Declarations

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Ethical Approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

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Competing interest

The authors declare no competing interests.

Author contribution

M. Bellot: Conceptualization, Methodology, Writing – original draft preparation.

A. Teixidó: Methodology, Field data curation.

N. Aletà: Conceptualization, Field experiments design, Data processing, writing Review, Funding acquisition.

A. Torrell: Conceptualization, Field data curation, Funding acquisition.

C. Gómez: Conceptualization**,** Data processing, Writing review, Project administration, Supervision, Funding acquisition.

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Figures

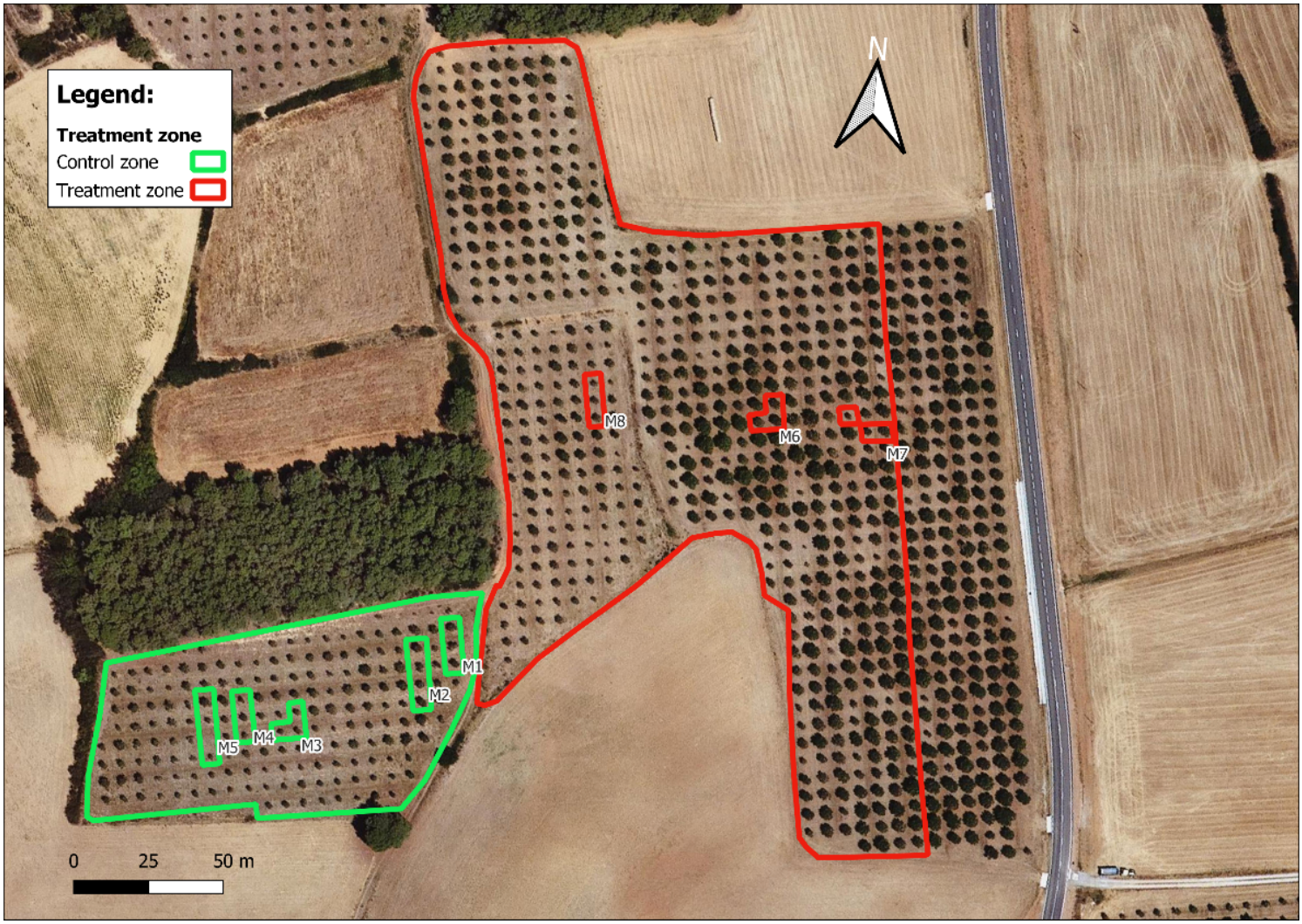

Figure 1

Location of the two zones of study. Treated pines (TR, red), and untreated pines (NT, green). In 2021's crop season NT samples were collected from M1, M2 and M3, while in 2022's crop season NT samples were collected from all pointed samples (M1 – M5).

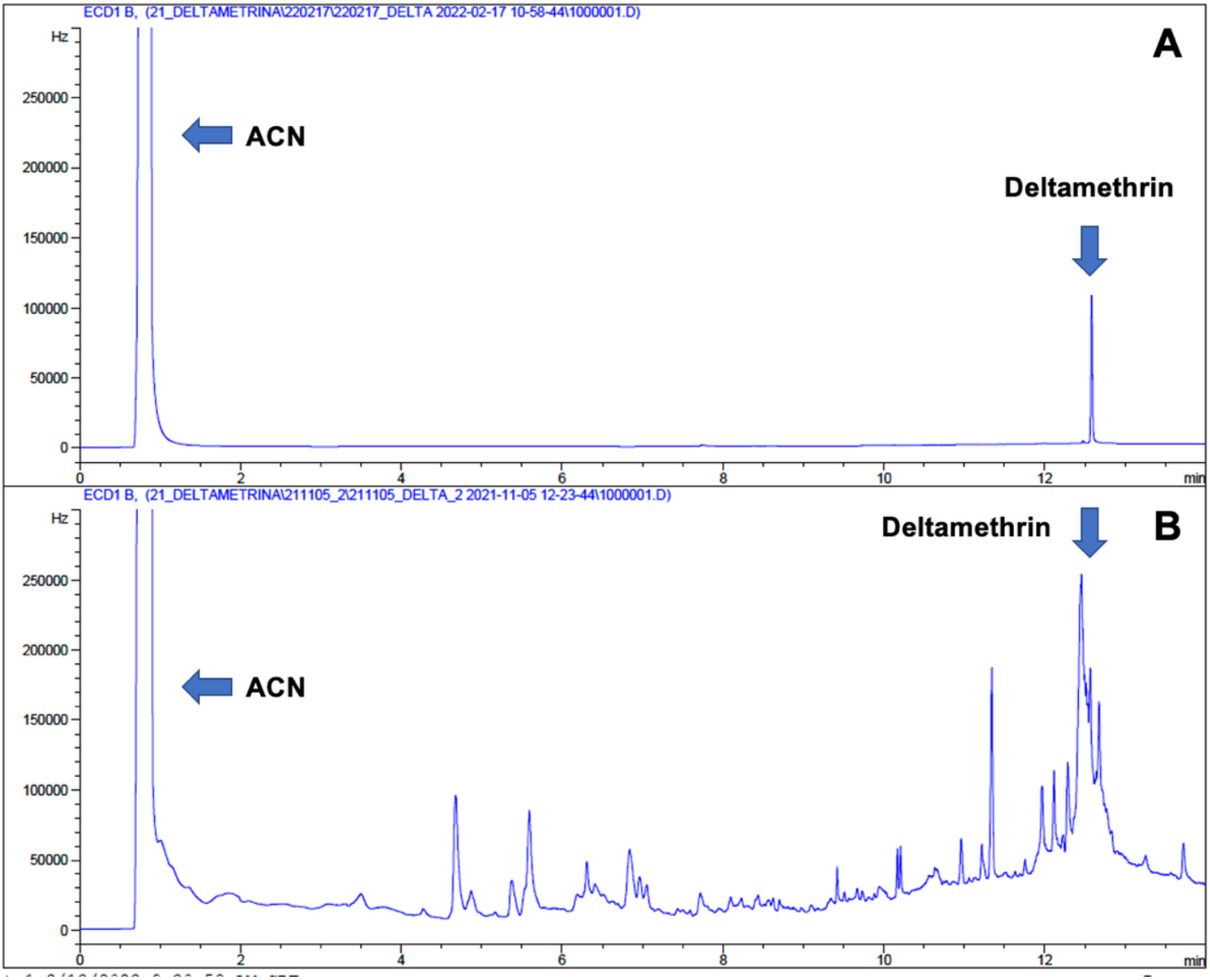

Figure 2

Chromatogram of deltamethrin the standard (A), where it can be observed elution peak of solvent (ACN) and deltamethrin. Chromatogram of a QC of pine needles analysis using Method A (B).

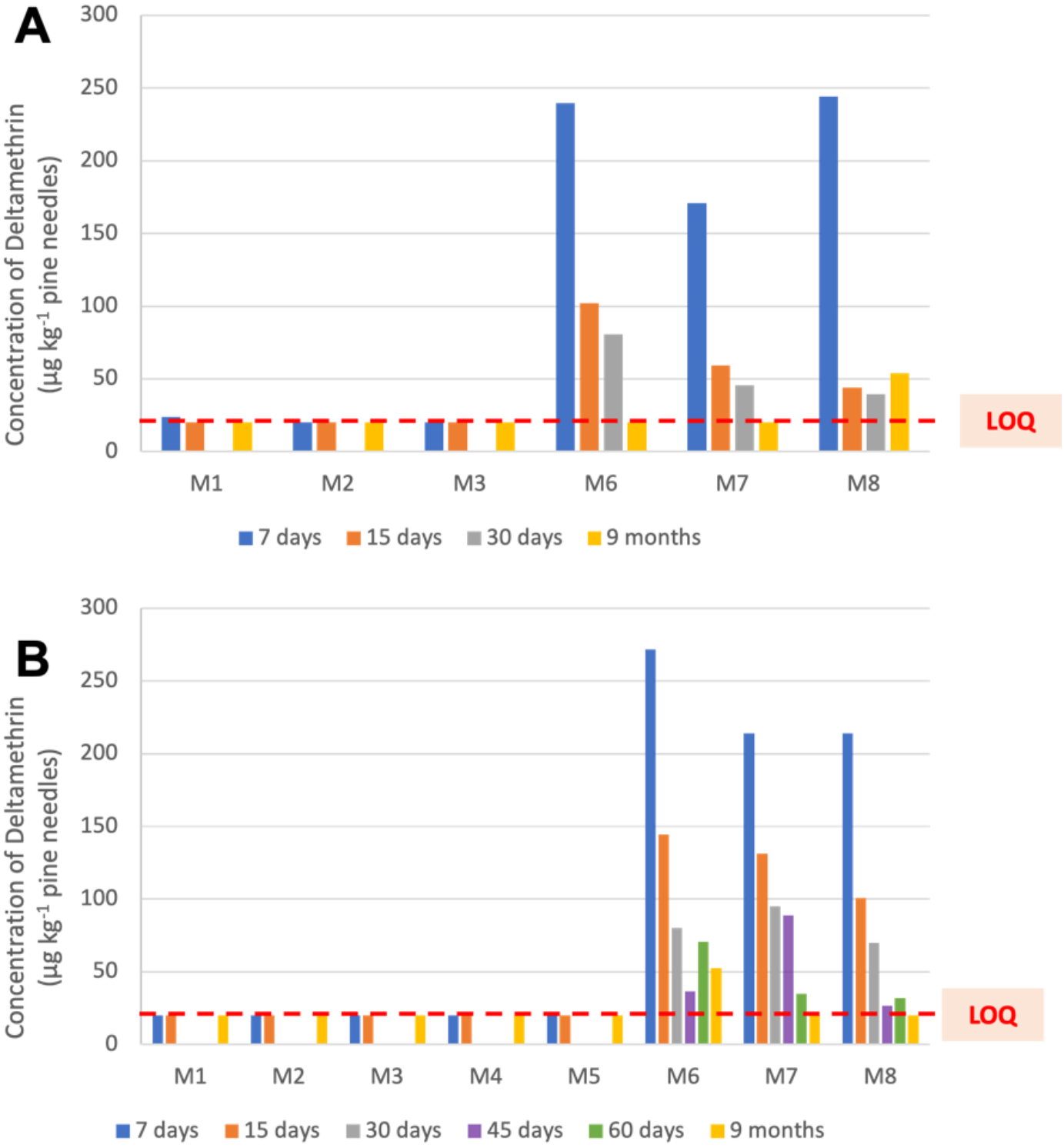

Figure 3

Deltamethrin concentration observed in pine needles in 2021's crop season (A) and 2022's (B). Red discontinuous line indicates values below the limit of quantification (20 µg kg^{-1}).

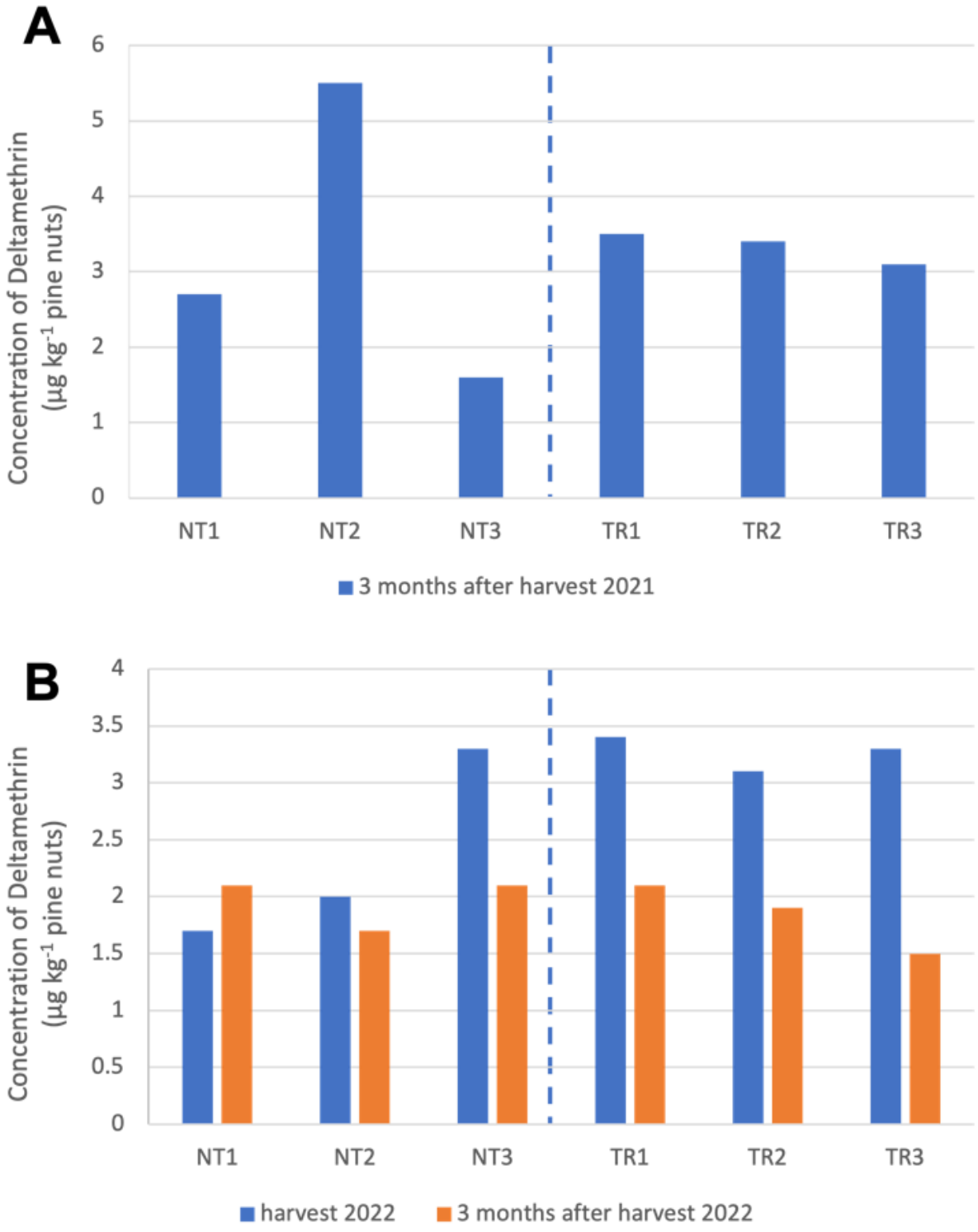

Figure 4

Deltamethrin concentration in pine nuts of harvest 2021 (A) and 2022 (B). In the left part, samples from non-treated areas (NT), and in the right part samples from treated areas (TR).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SuppMaterialDeltamethrin.docx