forests

MDPI

Article

Abieta-7,13-Diene in Nematode-Infected Pinewood *Pinus pinaster* Branch Extracts: Isolation and the Elucidation and Characterization of Its Structure

Marisa C. Gaspar [1,2,*], Pedro F. Cruz [3], Rui M. M. Brito [3], Hermínio C. De Sousa [4] and Mara E. M. Braga [4,*]

[1] ESSLei—School of Health Sciences, Polytechnic Institute of Leiria, 2411-901 Leiria, Portugal
[2] ciTechCare—Center for Innovative Care and Health Technology, Polytechnic Institute of Leiria, 2414-016 Leiria, Portugal
[3] Chemistry Department, Nuclear Magnetic Resonance Facility (UC-NMR), Coimbra Chemistry Centre (CQC), University of Coimbra, Rua Larga, 3004-535 Coimbra, Portugal
[4] Department of Chemical Engineering, Chemical Engineering and Renewable Resources for Sustainability (CERES), University of Coimbra, 3030-790 Coimbra, Portugal
* Correspondence: marisa.gaspar@ipleiria.pt (M.C.G.); marabraga@eq.uc.pt (M.E.M.B.); Tel.: +351-244-845-050 (M.C.G.); +351-239-798-758 (M.E.M.B.)

Abstract: The oleoresin and volatile fraction produced by conifers, such as *Pinus pinaster*, play a crucial role in plant defence, acting as precursors to resin acids and adapting in response to environmental stress or pathogen attacks. Abietadiene (abieta-7,13-diene), the biosynthetic precursor to abietic acid, has been identified as the most abundant compound in extracts from pinewood nematode (PWN)-infected *P. pinaster* trees. As abietadiene is not commercially available, this study aimed to achieve, for the first time, its isolation, structure elucidation, and detailed characterization from readily available forestry residues. Abietadiene was successfully isolated using thin-layer chromatography (TLC), and its purity and identity were evaluated using multiple analytical techniques: gas chromatography (GC), liquid chromatography (LC), nuclear magnetic resonance (NMR), and Fourier-transform infrared spectroscopy (FTIR). GC analysis indicated a purity of over 70% for the isolated compound, while LC provided the higher purity value of 98%. The identity of abietadiene was unequivocally confirmed through LC, FTIR, and NMR analysis. This work represents the first isolation and comprehensive characterization of abietadiene from a natural source, making detailed chemical data on this compound available to the scientific community. These findings may be used for future studies on the biological interactions and ecological roles of abietadiene, particularly in the context of plant defence and pathogen resistance.

Keywords: abieta-7,13-diene; abietadiene; *Pinus pinaster*; isolation; branch extract; structure elucidation; plant defence; pine wilt disease

Academic Editor: Chunjian Zhao

Received: 22 November 2024
Revised: 18 December 2024
Accepted: 26 December 2024
Published: 1 January 2025

Citation: Gaspar, M.C.; Cruz, P.F.; Brito, R.M.M.; De Sousa, H.C.; Braga, M.E.M. Abieta-7,13-Diene in Nematode-Infected Pinewood *Pinus pinaster* Branch Extracts: Isolation and the Elucidation and Characterization of Its Structure. *Forests* **2025**, *16*, 61. https://doi.org/10.3390/f16010061

1. Introduction

Conifers produce a mixture of terpenoids, including monoterpenoids, diterpene acids, and sesquiterpenoids (in a minor concentration), through oleoresin secretions and volatile emissions, which are mainly responsible for their defence against herbivores and pathogens [1–3]. For the Pinaceae family, the usual insect pests are bark beetles and weevils [3], and for *Pinus pinaster*, the nematode *Bursaphelenchus xylophilus* is a common pest in Portugal [4], and it is transmitted by an insect vector.

The diversity of compounds present in conifer oleoresins and volatile fraction is due to the gene duplication and neofunctionalization of enzymes involved in the synthesis

and modification of terpenes [5]. Oleoresin may be classified as constitutive (primary) or induced (secondary). Constitutive oleoresin is continuously produced by the plant, whereas induced oleoresin is synthesized in response to injury [6] or as an adaptation to environmental stress. In the biosynthesis of resin acids in conifers, two types of diterpene synthases have been characterized: isopimaradiene synthase, which exclusively produces isopimaradiene and sandaracopimaradiene, and levopimaradiene/abietadiene synthase, which synthesizes four diterpenes—abietadiene (abieta-7,13-diene), levopimaradiene, neoabietadiene, and palustradiene (Figure 1) [5,7].

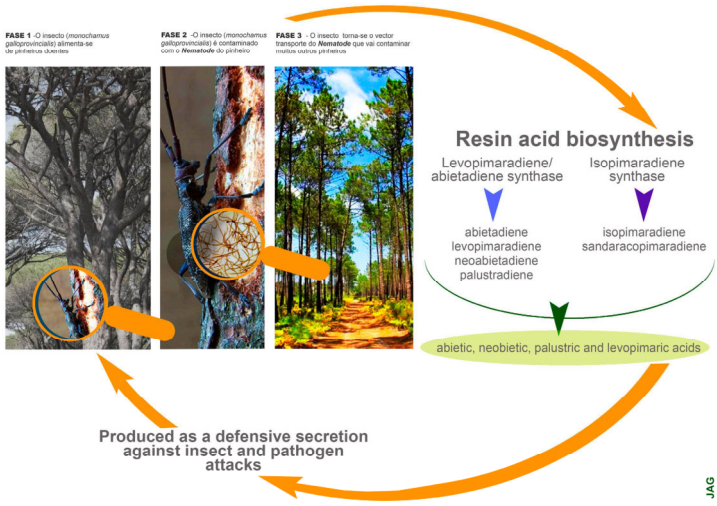

Figure 1. Schematic representation of pinewood nematode infection stages and resin acid defensive production in the tree.

These diterpenes may be, in part, oxidized by cytochrome P450; however, further characterization is needed to understand the diversity of diterpene acids present in conifer resin [8]. Furthermore, these four well-established diterpene products result from the dehydration of unstable alcohols, with their production being dependent on some conditions, such as the temperature, but their biosynthetic pathways are complex. Also, the in vivo production of diterpenes in metabolically engineered yeast was revealed to be affected by the lower pH of the yeast culture medium [7]. Slightly more abietadiene appears to be produced at a lower pH [5], and the engineering biosynthetic pathway may modulate yield production [9].

The four mentioned hydrocarbons are, in fact, rare, and a paucity of data exists. However, they are the intermediates in the biosynthesis of resin acids (abietic, neoabietic, palustric, and levopimaric acids), Figure 1, which are common and abundant on the market [10].

In the case of *P. pinaster*, a medium-sized coniferous tree native to the western Mediterranean Sea [11] and abundant in Portugal [12], the contents of needles' secretory system include resin acids and high amounts of diterpene hydrocarbons, mainly abietadiene (abieta-7,13-diene) [13]. This compound has also been identified and quantified (6.8%–34.2%) in needles of *P. pinaster* from Corsica [14]. The authors of this work have identified abietadiene in some extracts obtained by supercritical CO_2 from pine wilt disease (PWD)-symptomatic *P. pinaster* trees [15], which is probably associated with a defensive response in the tree due to pinewood nematode (PWN) infection. Recently, other authors have found an increase in 3-carene in *P. densiflora* species after PWN inoculation [16]. These findings are important in gaining a better understanding of the insect–nematode–tree interaction and, consequently, the ecological biochemistry.

Some authors have already purified abietadiene synthase in *P. pinaster*, an enzyme responsible for catalyzing the cyclization of geranylgeranyl pyrophosphate to abietadiene [10,13,17]. However, the process of purification is not easy due to the high amounts of polyphenols and resin and because of the reduced secretory tissue development [18]. Nevertheless, some authors have already found abietadiene in *P. sylvestris* foliage [19] and others have isolated this compound from *Larix sibirica* rosin [20], *P. sibirica* resin [21], *Cedrus libani* cones [22], and *Cupressus arizonica* oil [23].

Abietadiene is the precursor to abietic acid, which has been already identified in *P. pinaster* resin samples [24], produced as a defensive secretion against insect and pathogen attacks [6]. The oxidation of abietadiene to abietic acid is achieved via membrane-bound CYP and soluble aldehyde dehydrogenase enzyme activities [25]. Moreover, the amount of abietic acid defines the tree's capacity to overcome attacks by mountain bark beetles [26].

Abietic acid has already shown anti-inflammatory effects, namely, in osteoarthritis [27], as well as antibacterial and antifungal activities. Other authors have evaluated some biological activities (e.g., antimycotic and antiviral) revealed by abietic acid derivatives, concluding that the addition of an aldehyde group improves those effects [28]. Antibacterial activity was also evaluated for a set of nine abietic and dehydroabietic acid derivatives [29]. On the other hand, dehydroabietinal may originate from abietadiene and may be responsible for activating a mechanism of systemic acquired resistance in pine trees [30]. Pine extracts rich in abietadiene, obtained in previous work, revealed possible insecticidal/nematicidal activity, based on in vitro studies with the acetylcholinesterase enzyme [15,31]. Such extracts may be incorporated in formulations to be used as traps to control pests such as the PWN.

Following that study, the aim of this work was to achieve the isolation, structure elucidation, and characterization of abietadiene from *P. pinaster* branches. Despite being previously identified in *P. pinaster* samples, to the best of our knowledge, this diterpene is not available commercially, and this is the first time that it has been isolated from *P. pinaster*. Opening up the possibility of isolating this compound from these samples is relevant, because it means that its potential biological activities can be assessed in further studies.

2. Materials and Methods

2.1. Raw Materials and Chemicals

P. pinaster tree branches were collected in pine forests in Oleiros (Centre region of Portugal), with the collaboration of the National Forest Services (Instituto de Conservação da Natureza e Florestas, ICNF), in August 2017. The extracts and fractionation were carried out during the same period as the collection of the raw materials.

Oleiros is part of the Centre region and "Pinhal Interior Sul" sub-region and belongs to the "Castelo Branco" district, Portugal. The landscape of the region is characterized by the boundary between the "Cordillera Central Iberica" and the "Meseta Meridional". The vegetation that is currently found in the municipality is mostly pine forests that occupy a large percentage of the forest area and other forest formations [32,33]. At the time of sample collection (August 2017), the mean air temperature value in the Oleiros region was in the 24–26 °C range, and the precipitation mean value was in the 10–25 mm range, according to IPMA (Instituto Português do Mar e da Atmosfera) reports.

The pine forest in Oleiros is composed of several species, including eucalyptus, pine, strawberry trees, and others, such as the chestnut tree. The *P. pinaster* area in Oleiros has decreased in recent years, mainly due to wildfires but also due to the PWD. Therefore, it is important to act in this zone to manage the *P. pinaster* forest area.

The selected samples were collected at −39°55′38.20″ N, 7°53′2.8″ W; −39°55′34.62″ N, 7°53′7.77″ W; −39°55′30.50″ N, 7°53′11.82″ W; and −39°55′23.75″ N, 7°53′11.61″ W, with approximately 531 m of elevation.

Samples were collected from trees around 25–30 years old, using the criteria of PWD-symptomatic (four samples) or non-symptomatic trees (three samples), according to the experience of the forest engineer José Bernardino Dias (Instituto da Conservação da Natureza e das Florestas—ICNF) and based on symptoms described in the literature [34], by nematologists, using techniques described previously [15]. Figure 2 presents some of the selected PWD-symptomatic *Pinus pinaster* trees (Figure 2A), an insect vector *Monochamus galloprovincialis* captured in a funnel trap in the region of sample collection (Figure 2B), pine branches that were cut (Figure 2C), and milled branches for volatile extraction (mainly composed of leaves) (Figure 2D).

Figure 2. Pine forest with pine wilt disease-symptomatic *Pinus pinaster* trees (**A**); insect vector *Monochamus galloprovincialis*, captured in a funnel trap in Oleiros (Portugal) close to pine wilt disease-symptomatic trees (**B**); pine branches that were cut before milling (**C**); milled branches for the extraction of bioactive compounds, such as abietadiene (**D**).

The raw materials were analyzed in terms of emitted volatiles by solid-phase microextraction and gas chromatography–mass spectrometry (SPME-GC/MS) and subjected to supercritical carbon dioxide ($scCO_2$) extraction for further GC analysis [15,31]. Different extraction conditions allowed the identification of all composition spectra, since the operational conditions may change the compounds' solubility in the used solvent. Nevertheless, abietadiene was the main compound in *P. pinaster* samples identified by gas chromatography in all pressure and temperature conditions applied in a previous work [31]. Moreover, both limonene (emitted as a volatile compound) and abietadiene (in extracts) were identified in branches of *P. pinaster* PWD-symptomatic trees. In the aforementioned previous work, these molecules were detected neither in $scCO_2$ extracts from non-symptomatic *P. pinaster* trees nor in *P. pinea* extracts. Nevertheless, limonene was identified in naturally emitted volatiles from all the studied *P. pinea* trees, as expected [15]. For this work, extracts containing abietadiene from PWD symptomatic *P. pinaster* trees (confirmed by PWN identification/quantification as evaluated in previous work [15]) were considered.

The chemicals and solvents used for the analyses were n-hexane (≥96%, HPLC-grade), ethyl acetate (≥99.9%, HPLC-grade), and acetonitrile (≥99.9%, HPLC PLUS-grade) from Carlo Erba and dimethylsulfoxide (DMSO) D6 (99.8% D, water < 0.02%) from Eurisotop (Saint-Aubin, France).

2.2. Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) experiments were performed by using Merck Silica gel GF 254 (Merck KGaA, Darmstadt, Germany) in 0.2 mm thick plates. The pine extract was applied to the silica gel TLC plates, which were then eluted by a mobile phase composed of hexane–ethyl acetate (80:20, *v*/*v*). The main band (containing the abietadiene) confirmed by GC-MS was recovered and then eluted in a second step by using a different proportion of hexane–ethylacetate (60:40), to obtain a fraction richer in abietadiene. It should be noted

that to increase the purity of the compound of interest, the same solvent was used three times to remove the residual bands.

The detection of the compounds in the TLC plates was carried out under UV light at 254 nm and 366 nm. For the confirmation and visualization of terpenes' presence, a solution of p-anisaldehyde–sulfuric acid was used, followed by heating on a hot plate [35]. The retention factor (Rf) value was calculated for abietadiene through at least ten applications of TLC. After the elution, the silica containing the Rf of abietadiene was removed from the plate and transferred to a tube, with ethyl acetate added for the desorption process. The solution was mixed and centrifuged. The supernatant, which contained the abietadiene-rich fraction, was collected and evaporated for further assays.

2.3. Gas Chromatography–Mass Spectrometry/Flame Ionization Detection Analyses (GC-MS/FID)

The $scCO_2$ extracts and TLC fractions were dissolved in ethyl acetate (1 mg/mL and 2 mg/mL, respectively), and the respective composition was determined, in duplicate, by coupled gas chromatography–mass spectrometry (GC/MS 7890A, 5975 C inert MSD with triple-axis detector, from Agilent Technologies, Santa Clara, CA, USA). The injection volume was 0.2 µL, and the separation was achieved on a DB5-MS fused silica capillary column (30 m × 0.25 mm i.d. × 0.25 µm, from Agilent J & W Scientific, Santa Clara, CA, USA), using helium as the carrier gas, at a flow rate of 1 mL/min. The MS system was operated in scan mode (30–550 u) with an ionization potential of 70 eV and an MS quadrupole temperature of 150 °C. The temperature programme included an isothermal hold at 50 °C for 5 min, followed by a temperature ramp of 10 °C/min up to 270 °C, where it was held for 5 min [36].

The identification of volatile compounds was based on a comparison of their mass spectra with those of mass spectrum libraries (NIST and Flavors and Fragrances of Natural and Synthetic Compounds, FFNSC2.L). Semi-quantitative analysis based on the peak relative areas of the identified compounds was performed, as previously described [15], and the abietadiene purity (%) was assessed.

The TLC fraction richest in abietadiene was also analyzed by GC-flame ionization detection (GC-FID) with the same equipment, column and temperature programme. Similarly to the GC-MS analysis, the TLC fraction was dissolved in ethyl acetate (2 mg/mL), sequential dilutions were performed, and eight concentrations were injected (0.2 µL), at least in triplicate, to infer method linearity and the possibility of abietadiene quantification. The system's suitability was evaluated by analyzing the peak areas and retention time between experiments and its selectivity was assessed by the ability of the method to differentiate the compound of interest from other components of the samples. The limit of quantification (LOQ) was also inferred based on the signal-to-noise ratio (S/N), which should be greater than 10 [37].

2.4. Liquid Chromatography–Photodiode Array–Electrospray Ionization–Mass Spectrometry (LC-PDA-ESI-MS)

The abietadiene from the richest TLC fraction was also identified by liquid chromatography–photodiode array–electrospray ionization–mass spectrometry (LC-PDA-ESI-MS). A Linear Ion Trap (LIT-MS) (LTQ XL, Thermo Scientific, Waltham, MA, USA) coupled to a Liquid Chromatograph of High Performance (Surveyor, Finnigan, Thermo, Waltham, MA, USA) and a PDA Plus Detector (Surveyor, Finnigan, Thermo) was used. The sample (1 mg/mL in acetonitrile) was injected (20 µL) on a Brisa LC2 C18 column (250 mm × 4.6 mm i.d., 5 µm particle size, Teknokroma Analytical S.A., Sant Cugat del Vallès, Spain) at 30 °C. The mobile phase was prepared with water (A) and acetonitrile (B) in isocratic mode (15% A and 85% B) at a flow rate of 0.5 mL/min. PDA detection was recorded at a wavelength range of 200–600 nm, followed by detection in the mass spectrom-

eter. Mass spectra were acquired in positive-ion mode. The mass spectrometer performed two consecutive scans: full mass (m/z 272–274) and MS2 of m/z 273. The source and capillary voltages were 5.0 kV and 40 V, respectively. The capillary temperature was 275 °C. Nitrogen was used as a sheath and auxiliary gas at 40 and 5 Finnigan arbitrary units, respectively, and helium was used as a collision gas with a normalized energy of 35%. Data treatment was carried out with XCALIBUR 4.2 software (Thermo Scientific, Waltham, MA, USA).

2.5. Ultra-Fast Liquid Chromatography–Diode Array Detector (UFLC-DAD)

The suitability, selectivity, and linearity parameters were also evaluated for abietadiene quantification by ultra-fast liquid chromatography (UFLC), with the system equipped with the same column used in the LC-MS assay, a diode array detector (SPD-M20A), and an HPLC pump (LC-20AD) from Shimadzu (Kyoto, Japan). The mobile phase was also prepared with (A) water (15%, v/v) and (B) acetonitrile (85%, v/v) in isocratic mode. The column temperature was 30 °C, the flow rate was 0.5 mL/min, and detection was performed at 240 nm. The extract was diluted in acetonitrile at a concentration of 2 mg/mL, and distinct volumes were injected. A standard calibration curve was used to verify the linearity of abietadiene quantification, and this analysis was performed at least in duplicate.

2.6. Attenuated Total Reflection–Fourier-Transform Infrared Spectroscopy (ATR-FTIR)

An FTIR/NIR spectrometer (Perkin Elmer, Beaconsfield, UK) equipped with an ATR accessory was used to perform the IR analysis for the extract and the isolated compound (abietadiene richest TLC fraction), with 128 scans between 4000 and 550 cm^{-1} for each spectrum (4 cm^{-1} resolution). Spectra were compared to infer the differences between the functional chemical groups of the complex extract and the isolated compound.

2.7. Nuclear Magnetic Resonance (NMR)

NMR analysis was performed to confirm the identity of abietadiene. The purified TLC fraction with abietadiene was dissolved in DMSO d6 at a concentration of 20 mg/mL. Lower concentrations were tested first, but it was difficult to perform the assignment correctly. NMR spectra were obtained on a Bruker Avance III 400 spectrometer (Billerica, MA, USA) operating at 400.13 (1H) and 100.61 (13C) MHz. The 1H and 13C NMR spectra were assigned using two-dimensional COSY (correlated spectroscopy), 1H–13C multiplicity-edited HSQC (heteronuclear single-quantum correlation spectroscopy), and 1H–13C HMBC (heteronuclear multiple-bond correlation spectroscopy) methodologies. The chemical shifts are reported in ppm relative to the methyl 1H (δ = 2.50 ppm) and 13C (δ = 39.51 ppm) resonances of the dimethyl sulfoxide solvent used as an external reference. The following abbreviations are used: singlet (s), doublet (d), and multiplet (m). All NMR data were processed and analyzed using the programme MestReNova V9.1.5.

3. Results and Discussion

TLC experiments with a mobile phase composed of hexane–ethylacetate (80:20) revealed the possibility of abietadiene isolation (Figure S1A), present in a dark yellow/brown band with an Rf of 0.79 $\pm$ 0.02 and a purity of 60% (achieved by GC/MS). To improve its separation from the other components of the pine extract, a second TLC step was performed with a mobile phase (60:40), hexane–ethylacetate, allowing the better separation of abietadiene and other fractions (Figure S1B). While abietadiene eluted close to the top of the plate, the other fractions were observed on the plate at a lower distance from the application point. All these fractions were analyzed by GC/MS. After successive elutions for cleaning, the abietadiene reached a purity of 73.31 $\pm$ 2.34%, as indicated in the next subsection.

The results of GC/MS showed that the other bands (observed at 254 or 366 nm) were not selective for any specific compound, presenting some compounds already identified in the $scCO_2$ pine extracts, such as sandaracopimarinol and sandaracopimarinal; dehydroabietol; dehydroabietic acid; dehydroabietane; β-caryophyllene and caryophyllene oxide; Δ-cadinene; abietol; abietal, and α-cubebene. Some of these compounds were present in more than one band.

In addition to its abundance in the highlighted band (Figure S1), abietadiene was present in another band (immediately below) and only at a relative percentage of 5%.

A mixture of about 30–40 volatile compounds was observed in the GC/MS chromatograms for the $scCO_2$ extracts from *P. pinaster* branches, essentially corresponding to diterpenes and oxygenated terpenes. Abietadiene was the main volatile for all extraction conditions [31] and in most PWD-symptomatic trees [15]. Additional compounds structurally related to abietadiene were identified in considerable amounts in the $scCO_2$ extracts: abietic acid, dehydroabietane, abieta-(8(14), 13(15)-diene), abietal, and abietol, as recently reported by the authors [15,31]. The obtained $scCO_2$ extracts were separated by TLC experiments, as already discussed, to isolate the abietadiene (abieta-7,13-diene). After TLC experiments, the purity of this compound, which was eluted at 23.6 min, was improved, attaining a relative mean area of 73.31%, identified here by compound number 7 (Figure 2A and Table 1). Only one compound (abietatriene) had a relative mean area higher than 4%. All other identified compounds presented lower areas, close to 1%–3% (Table 1).

Table 1. Volatile compounds identified (GC/MS) in the TLC band (with abietadiene) and relative areas, as a percentage, of each compound.

Compound Number	Retention Time (min) *	Identified Compound	Relative Area (%)
1	17.44	Δ-cadinene	3.64 ± 2.30
2	18.20	β-calacorene	2.59 ± 1.38
3	20.44	octadec-1-ene	0.90 ± 0.24
4	20.89	neophytadiene	3.81 ± 0.61
5	22.99	levopimaradiene	1.09 ± 0.06
6	23.28	abietatriene	11.93 ± 0.04
7	23.63	abietadiene	73.31 ± 2.34
8	24.19	abieta-(8(14)13(15)diene)	2.45 ± 0.69
	Total identified (%)		99.70 ± 7.66

* Rt: variability range from 0.001 to 0.01 min.

The results of the GC/FID analysis revealed that abietadiene eluted at 24.2 min (Figure 3A), independently of the concentration, which was close to the retention time observed in the GC/MS assays. This slight difference may be related to the distinct detectors that were used, since the method, column, and equipment were the same. The relative area observed for abietadiene was close to 75% for the highest concentrations (2, 1, and 0.5 mg/mL) that were injected. However, its purity increased up to 100% with the subsequent decrease in the injected concentrations (the lowest tested concentration in the calibration curve was 0.00624 mg/mL). This is explained by the fact that at very low concentrations, the only compound quantified by GC/FID with similar integration parameters is abietadiene. These parameters were as follows: initial area reject = 0; initial peak width = 0.05; initial threshold = 15.5.

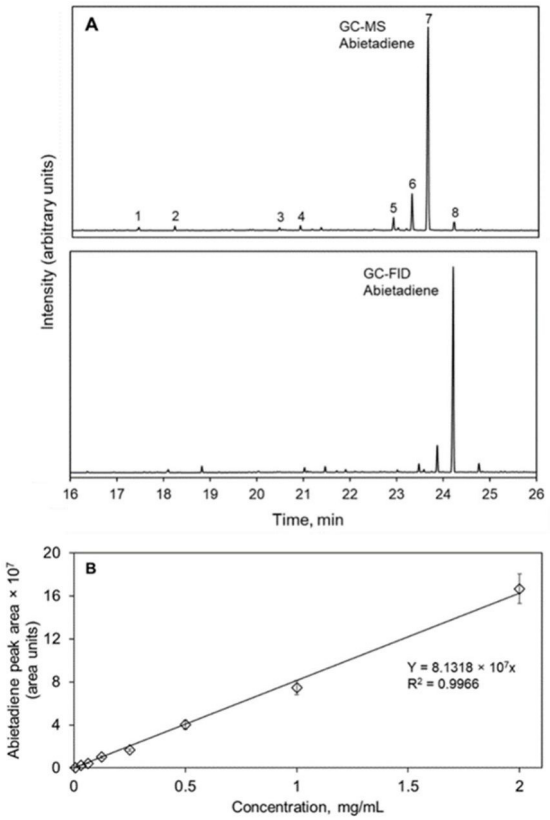

Figure 3. GC-MS/FID chromatograms of the TLC fraction richest in abietadiene (**A**). Peak identification from the GC/MS analysis: 1. Δ-cadinene; 2. β-calacorene; 3. octadec-1-ene; 4. neophytadiene; 5. levopimaradiene; 6. abietatriene; 7. abietadiene; 8. abieta-(8(14)13(15)diene). Purity of ~73% by GC/MS. Abietadiene standard curve obtained by GC-FID based on the fraction richest in abietadiene (**B**).

The method was revealed to be suitable since a well-defined and symmetrical peak was observed for abietadiene and similar retention times and peak areas (among replicas) were verified, confirming the reproducibility of the method. Furthermore, the linearity of the method (between 0.00624 and 2 mg/mL, 0.2 µL injection) was observed, with an $R^2 > 0.99$ (Figure 3B). Regarding the LOQ, it was revealed to be 0.00624 mg/mL (1.25 ng of injected mass), with value also included in the calibration curve. This is the value that corresponds to the abietadiene peak height at least 10 times higher than the noise height (Figure S2).

Abietadiene was the major compound detected in the UFLC-DAD analysis, and its identity was confirmed by LC-PDA-ESI-MS analysis (Figures 4A and S3). The retention time for abietadiene was observed at 110 min in the UFLC analysis and at about 130 min in the LC-MS analysis. This difference may be due to the different equipment that was used and/or the distinct detectors, considering that the column was the same for both LC analyses. However, in both chromatographic methods, this compound had a similar absorption UV band at 240 nm, confirming that the peak corresponds to the same molecule (Figure S4).

For the LC-MS analysis, the identification of the main compound that eluted at 130 min was achieved, as well as the determination of its molecular structure, by using mass spectrum analysis. The molecular ion peak has an m/z of 273.26 (Figure 4C) and therefore corresponds to abietadiene, which has a molecular weight of 272.47. Furthermore, the main fragments obtained in the MS2 spectrum corroborate this information, since they correspond to the molecular weight of the abietadiene fragments. The complete data regarding the chromatograms of PDA, UV at 240 nm, and the MS spectrum are included in

Figure S3. UV spectra for wavelengths from 215 to 265 nm, also confirming the spectrum profile for abietadiene with higher intensity at 240 nm, are included in Figure S5A.

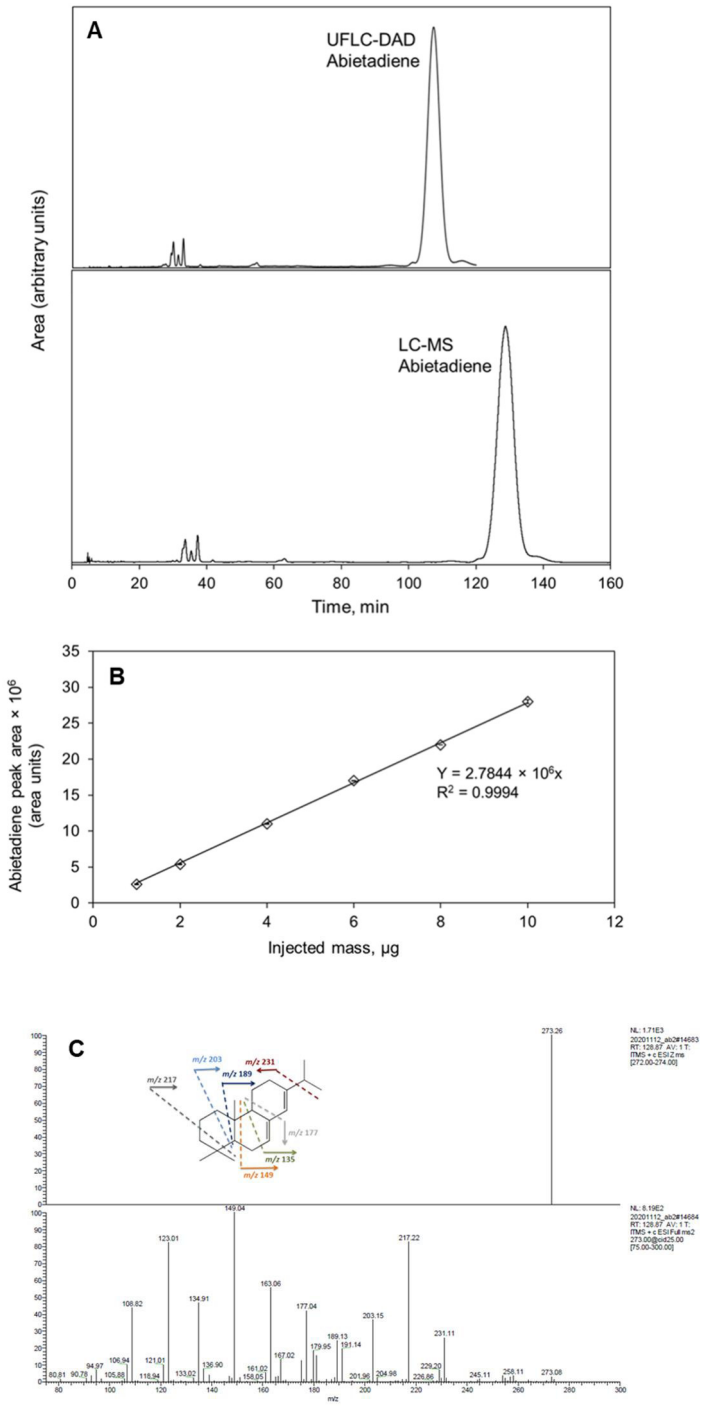

Figure 4. Chromatography for abietadiene identification (UV at 240 nm) by UFLC-DAD and LC-MS (**A**); abietadiene standard curve obtained by UFLC-DAD based on the fraction richest in abietadiene (purity of ~98% by UFLC) (**B**); and spectrum MS-MS2 and MS2 fragmentation of abietadiene (**C**).

The purity of abietadiene was also evaluated by UFLC, as the average, of its relative proportion in thirteen chromatograms, with injections of different volumes, presenting a value of 97.7 ± 0.5%. This is a fraction with very high purity, and Figure S5B confirms this high purity, presenting the peak purity index of 0.999999.

For UFLC analysis, it was possible to verify the linearity of the method in relation to the possibility of abietadiene quantification (Figure 4B). An R^2 value of 0.9994 was achieved by injecting six different amounts (1–10 μg) of the isolated fraction in duplicate.

The results from ATR-FTIR experiments denote the composition differences between the $scCO_2$ extract and the isolated compound (the richest TLC fraction). Despite being the main compound in the pine extracts, the abietadiene fraction isolated from the TLC experiments presents a distinct FTIR spectrum (Figure 5). There is no OH stretching, which usually occurs at 3000–3500 cm^{-1} [38]. This agrees with the absence of OH groups in the abietadiene molecule. However, for the $scCO_2$ extract, a broad band between 3000 and 3600 cm^{-1} is present, probably due to the OH stretching of oxygenated terpenes present in the extract, such as manool and abietol [31].

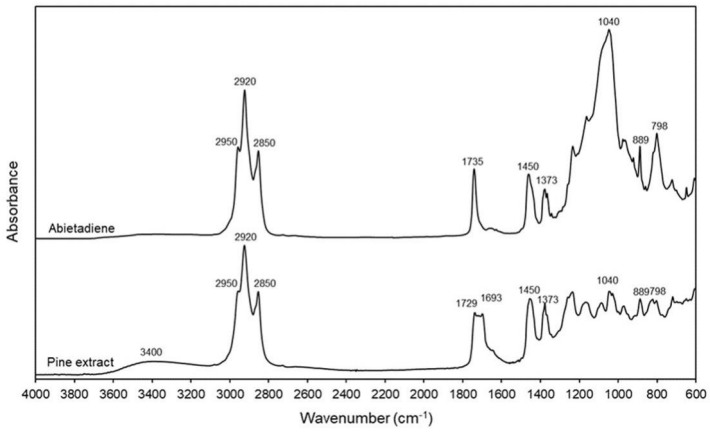

Figure 5. FTIR spectra of $scCO_2$ pine extract and the TLC fraction richest in abietadiene (~73% purity by GC and ~98% by UFLC).

The C-H stretching of CH_3-CH_2 groups usually has bands between 2700 and 3000 cm^{-1}, and this can be observed in both the extract and abietadiene FTIR spectra (2850, 2920, and 2950 cm^{-1}) [38,39]. The band at 1735 cm^{-1} may correspond to C=C stretching [40], which is present in the cyclic structures of the abietadiene molecule. Close to this wavelength, in the pine extract spectrum, another band (1693 cm^{-1}) appears to exist and, despite its low intensity, may be ascribed to the stretching vibrations of C=O bonds [39] that are present in oxygenated compounds from the extract, such as the abietal [31]. The band that may correspond to C=C stretching is slightly different from the one present in the purified compound, and its peak is at 1729 cm^{-1}. The bands between 1350 and 1500 cm^{-1} are attributed to C-H deformation vibrations [39]. At 1040 cm^{-1}, there is a strong/intense peak in the abietadiene spectrum, probably attributed to CH_2 wagging, and this kind of vibration also occurs at 800–900 for the C–H bond [40]. Another possibility is an out-of-plane C–H bending, usually characterized by a strong band. Other less intense peaks observed at wavelengths lower than 1000 cm^{-1} may be eventually attributed to other functional groups or vibration types.

The ^1H and ^{13}C NMR resonances (Table 2 and Figure S6) were assigned using COSY, NOESY, HSQC-ed, and HMBC. H–H coupling analysis, in conjunction with integral values, represented the first approach to assigning the methyl groups, as well the vinyl protons; in the second step, using HSQC-ed, it was possible to assign the corresponding 13C resonances to these protons. To better discriminate the methyl groups, we took into consideration the COSY/NOESY cross-peak patterns, in particular, the assignment of geminal protons 1, 2, and 3, because of their low chemical shift dispersion in the proton spectrum. Using HMBC data, it was possible to complement and finish all protons and carbons, specifically, quaternary carbon assignments, to confirm unequivocally the structure of abietadiene.

Table 2. $^{1}H/^{13}C$ NMR (400/100 MHz, DMSO-d6) data and assignments for abietadiene. Number attribution for the abietadiene structure can be seen in Figure 6.

Position	^{1}H (ppm)	^{13}C (ppm)
1α	1.00 (m)	38.61
1β	1.80 (m)	
2α	1.42 (m)	18.97
2β	1.55–1.46 (m)	
3α	1.15 (m)	41.87
3β	1.39 (m)	
4	-	32.06
5	1.17 (m)	49.74
6α	2.05 (m)	23.47
6β	1.90 (m)	
7	5.38 (m)	120.98
8	-	134.71
9	1.78 (m)	50.27
10	-	35.18
11	1.09 (m)	22.13
12	2.01–2.07 (m)	23.89
13	-	144.06
14	5.72 (s)	122.50
15	2.19 (septet, 6.8)	23.19
16	0.96 (d, 7.1)	21.33
17	0.98 (d, 6.7)	20.73
18	0.85 (s)	33.08
19	0.89 (s)	21.68
20	0.73 (s)	13.48

Chemical shifts (multiplicity, observed coupling(s) in Hertz).

Figure 6 presents the chemical structure of abietadiene, which follows the literature. Furthermore, the IUPAC name for this molecule is given as (4aS,4bR,10aS)-1,1,4a-trimethyl-7-propan-2-yl-2,3,4,4b,5,6,10,10a-octahydrophenanthrene, with the CAS number of 35241-40-8 [41].

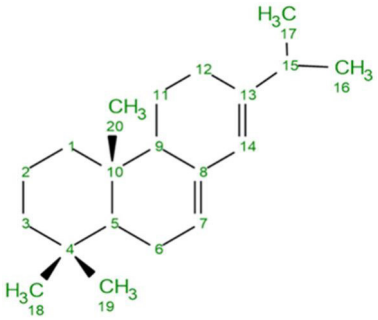

Figure 6. Chemical structure of the diterpene abietadiene (MestReNova V9.1.5 software). The numbers are related to the NMR attribution, which is detailed in Table 2.

The PWD is a worldwide threat to forests, especially to some *Pinus* species. Multidisciplinary efforts have been made to understand why some species are resistant while others are susceptible to PWN infection. Many strategies to stop the PWD spreading have been researched, but insect–PWN–tree interactions are complex and dependent on the species and environmental conditions, among other factors [34].

This work represents a contribution to this complex interaction, since a compound, abietadiene, potentially produced as a defensive action by the tree against PWN infection, was identified. However, this is just a hypothesis, since a more complete study would

be needed to explore this possibility. The volatile profile of *Pinus* spp. is not easy to understand because of the chemical variability between species. Different chemotypes are present simultaneously, and no site is exclusive to a single chemotype [42]. Moreover, the *P. pinaster* volatile profile usually changes during *M. galloprovincialis* feeding, and this behaviour is also distinct between different chemotypes. In general, an increase in some compounds is observed, but there is no response pattern. For example, an increase in β–caryophyllene and germacrene D was observed for chemotype 1 (C1), while for C2 trees, α-pinene and β-pinene presented the highest increase. Interestingly, these two terpenes together with abietic acid were identified in the insects after they had fed on *P. pinaster* [43]. The authors found abietadiene, the precursor of abietic acid (Lafever et al., 1994) [6], in PWD-symptomatic *P. pinaster* trees, and other authors have also identified this compound in volatile oils obtained from pine wood, cones, and needles [44]. Other researchers have found an increase in 3-carene in *P. densiflora* species after pinewood nematode (PWN) inoculation [16]. However, this is an area still under research, with more studies needed to help us understand such interactions.

The mechanisms implicated in the resistance of *P. pinaster* to PWN are complex. Some authors have identified that the resistance to PWN may be mediated by the induction of the jasmonic acid defence pathway, secondary metabolism pathways, and resistance genes, among others [45]. Moreover, other factors such as the region and edaphoclimatic conditions may influence the type and content of emitted volatiles [46,47].

The isolation and characterization of the abietadiene molecule were investigated in *P. pinaster* extracts. The *P. pinaster* trees selected for branch collection presented as approximately 25–30 years old. These age values were inferred, based on the size of trees, by forest engineers. Therefore, the analyzed pines were mature trees, and the observed differences in terms of volatiles, and the presence or absence of abietadiene in pine extracts, may be related to different chemotypes or geographical conditions, and a more extensive and detailed study would be needed to draw additional conclusions.

Regarding the characterization of the abietadiene molecule, the results of TLC confirm that the applied method was able to separate abietadiene from the other extract components. The isolated fraction, rich in abietadiene, was further characterized by other techniques, including GC-MS/FID, but also by LC-PDA-ESI-MS and UFLC-DAD, and FTIR and NMR methods. For both GC-MS and GC-FID, abietadiene was eluted close to 24 min with high purity in the isolated fraction. Furthermore, the FID method was shown to be suitable and linear, meaning that it can be used to quantify abietadiene in further studies.

The identification of abietadiene and its molecular structure was also achieved by LC-PDA-ESI-MS. Further assays with another column (Spherisorb ODS-2) with a Spherisorb ODS-2 guard cartridge at a flow rate of 0.2 mL/min were carried out and revealed that it is possible to obtain the abietadiene elution around 20 min. The UFLC-DAD system suitability and respective reproducibility were confirmed due to the well-defined and symmetrical peak that was observed and the similar peak areas and retention times among experiments. The analytical method was also selective and specific since it was able to differentiate abietadiene from the other components.

FTIR analysis confirmed the identity of abietadiene, as well as the NMR assay, which showed that all assignments for abietadiene were in agreement with those in the literature [10,48].

4. Conclusions

In conclusion, abietadiene was isolated from $scCO_2$-fractionated pine extract by using TLC, and it was further characterized. A purity of ~73% by GC/MS and ~98% by UFLC-DAD analysis was achieved for abietadiene. The FTIR results confirmed the difference

between the extract and the isolated fraction, namely, the absence of OH and CO vibrations. The confirmation of abietadiene identity was carried out by LC-PDA-ESI-MS, based on their MS fragments, and by NMR, which also confirmed that the isolated molecule was abietadiene (abieta-7,13-diene), based on 1H and 13C assignments.

The quantification of abietadiene can be achieved by UV and FID detectors, and the standard curves presented very good linearity. Moreover, these two analytical methods were revealed to be suitable, linear, specific, and reproducible. Despite all techniques having complementarity, UFLC-DAD offers high sensitivity, rapid analysis, and ease of use, making it particularly suitable for routine purity assessments in quality control and large-scale screening applications. The results included in this work related to abietadiene isolation, identification, and characterization could form the basis for other biological studies related to PWN-infected trees.

Therefore, isolated abietadiene or an extract rich in abietadiene could probably be used in formulations to manage PWD dissemination.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/f16010061/s1, Figure S1: TLC of $scCO_2$ extracts, observed under UV at 366 nm and 254 nm, and by using the anisaldehyde reagent, appropriate for terpenes detection using hexane:ethylacetate (80:20) as eluent (A); TLC of yellow band isolated from the $scCO_2$ extracts, observed under UV light, using hexane:ethylacetate (60:40) as eluent (B); Figure S2: GC-FID chromatogram of the abietadiene richest TLC fraction, at the lowest concentration of 0.00624 mg/mL (0.2 µL injection volume), which corresponds to the LOQ, based on the signal to noise ratio (S/N), which should be greater than 10; Figure S3: Chromatograms PDA-Scan, UV (240 nm), and MSn-TIC (180 min); Figure S4: Abietadiene UV spectrum from LC-PDA detector for a sample at 20 µg (A) and UFLC-DAD detector for a sample at 1 µg (B); Figure S5: Spectrum UV-DAD detector for wavelengths from 215 up to 265 nm (A) and purity curve (B) for a sample at 1 µg with the purity of ~98 % by UFLC; Figure S6: 1H NMR, 13C NMR, COSY/NOESY, HSCQ, and HMBC spectra of abietadiene in DMSO-d6 at 25 °C. See numbers attribution in abietadiene structure (Figure 6).

Author Contributions: Conceptualization: M.E.M.B. and M.C.G.; Methodology: M.C.G., M.E.M.B. and P.F.C.; Formal analysis and investigation: M.C.G., M.E.M.B. and P.F.C.; Writing—original draft preparation: M.C.G.; Writing—review and editing: M.E.M.B., P.F.C., H.C.D.S. and R.M.M.B. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by COMPETE 2020, Fundação para a Ciência e Tecnologia (FCT, Portugal), through the Project ECOVECTOR—POCI-01-0145-FEDER-016820, CERES (PEst-C/EQB/UI0102/2019, UIDB/00102/2020, and UIDP/00102/2020), and ciTechCare (UIDB/05704/2020 and UIDP/05704/2020). M. C. Gaspar thanks FCT for financial support (CEECINST/00060/2021). NMR data were collected at the UC-NMR facility, which is supported in part by FEDER—European Regional Development Fund through COMPETE; FCT (RECI/QEQ-QFI/0168/2012, CENTRO-07-CT62-FEDER-002012); Rede Nacional de Ressonância Magnética Nuclear (RNRMN); and Coimbra Chemistry Centre (UID/QUI/00313/2019).

Data Availability Statement: The original contributions presented in the study are included in the article and Supplementary Materials; further inquiries can be directed to the corresponding authors.

Acknowledgments: The authors are grateful to Fátima Isabel Antunes Nunes from the Laboratory of Mass Spectrometry (LEM) integrated in the National Mass Spectrometry Network (RNEM) of Portugal for performing the LC-MS analyses and to forest engineer José Bernardino Dias from National Forest Services (Instituto de Conservação da Natureza e Florestas, ICNF) for the identification of the PWD-symptomatic trees and the collection of branch samples for this study. The authors are also grateful to designer José Gomes for his work in the preparation of Figure 1.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Keeling, C.I.; Bohlmann, J. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol.* **2006**, *170*, 657–675. [CrossRef] [PubMed]
2. Trapp, S.; Croteau, R. Defensive resin biosynthesis in conifers. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **2001**, *52*, 689–724. [CrossRef]
3. Zulak, K.G.; Dullat, H.K.; Keeling, C.I.; Lippert, D.; Bohlmann, J. Immunofluorescence localization of levopimaradiene/abietadiene synthase in methyl jasmonate treated stems of *Sitka spruce* (*Picea sitchensis*) shows activation of diterpenoid biosynthesis in cortical and developing traumatic resin ducts. *Phytochemistry* **2010**, *71*, 1695–1699. [CrossRef]
4. Vicente, C.; Espada, M.; Vieira, P.; Mota, M. Pine Wilt Disease: A threat to European forestry. *Eur. J. Plant Pathol.* **2012**, *133*, 89–99, Erratum in *Eur. J. Plant Pathol.* **2012**, *133*, 497. [CrossRef]
5. Keeling, C.I.; Weisshaar, S.; Lin, R.P.C.; Bohlmann, J. Functional plasticity of paralogous diterpene synthases involved in conifer defense. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 1085–1090. [CrossRef] [PubMed]
6. Lafever, R.; Vogel, B.; Croteau, R. Diterpenoid resin acid biosynthesis in conifers: Enzymatic cyclization of geranylgeranyl pyrophosphate to abietadiene, the precursor of abietic acid. *Arch. Biochem. Biophys.* **1994**, *313*, 139–149. [CrossRef] [PubMed]
7. Keeling, C.I.; Madilao, L.L.; Zerbe, P.; Dullat, H.K.; Bohlmann, J. The primary diterpene synthase products of *Picea abies* levopimaradiene/abietadiene synthase (palas) are epimers of a thermally unstable diterpenol. *J. Biol. Chem.* **2011**, *286*, 21145–21153. [CrossRef]
8. Keeling, C.I.; Bohlmann, J. Diterpene resin acids in conifers. *Phytochemistry* **2006**, *67*, 2415–2423. [CrossRef] [PubMed]
9. Morrone, D.; Lowry, L.; Determan, M.K.; Hershey, D.M.; Xu, M.; Peters, R.J. Increasing diterpene yield with a modular metabolic engineering system in *E. coli*: Comparison of MEV and MEP isoprenoid precursor pathway engineering. *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1893–1906. [CrossRef] [PubMed]
10. Lee, H.-J.; Ravn, M.M.; Coates, R.M. Synthesis and characterization of abietadiene, levopimaradiene, palustradiene, and neoabietadiene: Hydrocarbon precursors of the abietane diterpene resin acids. *Tetrahedron* **2001**, *57*, 6155–6167. [CrossRef]
11. European Forest Institute. *Pinus pinaster*. European Forest Genetic Resources Programme. 2019. Available online: http://www.euforgen.org/species/pinus-pinaster/ (accessed on 30 December 2024).
12. ICNF. *6° Inventário Florestal Nacional, Áreas dos Usos do Solo e das Espécies Florestais de Portugal continental. Resultados preliminaries*; Instituto da Conservação da Natureza e das Florestas: Algés, Portugal, 2013; p. 34.
13. Walter, J.; Laprébande, B.; Laferriere, A.; Saint-Guily, A.H. Purification and characterization of abietadiene cyclase of maritime pine (Pinus pinaster Ait.). In *Plant Lipid Metabolism*; Kader, J.-C., Mazliak, P., Eds.; Springer: Dordrecht, The Netherlands, 1995; pp. 356–358. [CrossRef]
14. Ottavioli, J.; Bighelli, A.; Casanova, J. Diterpene-rich needle oil of *Pinus pinaster* Ait. from Corsica. *Flavour Fragr. J.* **2008**, *23*, 121–125. [CrossRef]
15. Gaspar, M.; Agostinho, B.; Fonseca, L.; Abrantes, I.; de Sousa, H.; Braga, M. Impact of the pinewood nematode on naturally-emitted volatiles and $scCO_2$ extracts from *Pinus pinaster* branches: A comparison with *P. pinea*. *J. Supercrit. Fluids* **2020**, *159*, 104784. [CrossRef]
16. Hwang, H.-S.; Han, J.-Y.; Choi, Y.-E. Enhanced Emission of Monoterpene 3-Carene in *Pinus densiflora* Infected by Pine Wood Nematode and Characterization of 3-Carene Synthase. *Forests* **2021**, *12*, 514. [CrossRef]
17. Peters, R.J.; Ravn, M.M.; Coates, R.M.; Croteau, R.B. Bifunctional abietadiene synthase: Free diffusive transfer of the (+)-copalyl diphosphate intermediate between two distinct active sites. *J. Am. Chem. Soc.* **2001**, *123*, 8974–8978. [CrossRef] [PubMed]
18. Zhang, H.; Wang, Z.; Liu, O. Development and validation of a GC–FID method for quantitative analysis of oleic acid and related fatty acids. *J. Pharm. Anal.* **2015**, *5*, 223–230. [CrossRef] [PubMed]
19. Kännaste, A.; Laanisto, L.; Pazouki, L.; Copolovici, L.; Suhorutšenko, M.; Azeem, M.; Toom, L.; Borg-Karlson, A.-K.; Niinemets, Ü. Diterpenoid fingerprints in pine foliage across an environmental and chemotypic matrix: Isoabienol content is a key trait differentiating chemotypes. *Phytochemistry* **2018**, *147*, 80–88. [CrossRef] [PubMed]
20. Lisina, A.I.; Pentegova, V.A. Abietadiene from the rosin of the Siberian larch (*Larix sibirica*). *Sib. Khim. Zh. Izv. Sib. Otd. Akad. Nauk SSR Ser. Khim Nauk.* **1965**, *2*, 96–100.
21. Pentegova, V.A.; Kashtanova, N.K. Diterpene hydrocarbons of the resin of Pinus sibirica R. *Mayr. Chem. Nat. Compd.* **1965**, *1*, 171–172. [CrossRef]
22. Norin, T.; Winell, B. Diterpenoids of cones from two Cedrus species. *Phytochemistry* **1971**, *10*, 2818–2821. [CrossRef]
23. Carman, R. Sutherland M.D. Cupressene and other diterpenes of *Cupressus* species. *Aust. J. Chem.* **1979**, *32*, 1131–1142. [CrossRef]
24. Ottavioli, J.; Paoli, M.; Casanova, J.; Tomi, F.; Bighelli, A. Identification and Quantitative Determination of Resin Acids from Corsican *Pinus pinaster* Aiton Oleoresin Using 13C-NMR Spectroscopy. *Chem. Biodivers.* **2019**, *16*, e1800482. [CrossRef] [PubMed]
25. Steinberg, C. *Stress Ecology—Environmental Stress as Ecological Driving Force and Key Player in Evolution*; Springer: Dordrecht, The Netherlands, 2012; p. 480.

26. Boone, C.K.; Keefover-Ring, K.; Mapes, A.C.; Adams, A.S.; Bohlmann, J.; Raffa, K.F. Bacteria associated with a tree-killing insect reduce concentrations of plant defense compounds. *J. Chem. Ecol.* **2013**, *39*, 1003–1006. [CrossRef] [PubMed]
27. Kang, S.; Zhang, J.; Yuan, Y. Abietic acid attenuates IL-1β-induced inflammation in human osteoarthritis chondrocytes. *Int. Immunopharmacol.* **2018**, *64*, 110–115. [CrossRef]
28. González, M.A.; Correa-Royero, J.; Agudelo, L.; Mesa, A.; Betancur-Galvis, L. Synthesis and biological evaluation of abietic acid derivatives. *Eur. J. Med. Chem.* **2009**, *44*, 2468–2472. [CrossRef] [PubMed]
29. Helfenstein, A.; Vahermo, M.; Nawrot, D.A.; Demirci, F.; İşcan, G.; Krogerus, S.; Yli-Kauhaluoma, J.; Moreira, V.M.; Tammela, P. Antibacterial profiling of abietane-type diterpenoids. *Bioorganic Med. Chem.* **2017**, *25*, 132–137. [CrossRef]
30. Chaturvedi, R.; Venables, B.; Petros, R.A.; Nalam, V.; Li, M.; Wang, X.; Takemoto, L.J.; Shah, J. An abietane diterpenoid is a potent activator of systemic acquired resistance. *Plant J.* **2012**, *71*, 161–172. [CrossRef] [PubMed]
31. Gaspar, M.C.; de Sousa, H.C.; Seabra, I.J.; Braga, M.E. Environmentally-safe scCO$_2$ *P. pinaster* branches extracts: Composition and properties. *J. CO$_2$ Util.* **2020**, *37*, 74–84. [CrossRef]
32. ICNF. *6° Inventário Florestal Nacional 2015 Relatório Final*; Instituto da Conservação da Natureza e das Florestas: Lisbon, Portugal, 2015.
33. Geographical situation. Municipality of Oleiros. Available online: https://cm-oleiros.pt/situacao-geografica/ (accessed on 23 February 2022).
34. Sousa, E.; Vale, F.; Abrantes, I. *Pine Wilt Disease in Europe: Biological Interactions and Integrated Management, 1st ed*; Federação Nacional das Associações de Proprietários Florestais: Lisbon, Portugal, 2015; p. 328, ISBN 978-989-99365-2-2.
35. Wagner, H.; Bladt, S. *Plant Drug Analysis—A Thin Layer Chromatography Atlas*; Springer: Berlin/Heidelberg, Germany, 1996; p. 384.
36. Szmigielski, R.; Cieslak, M.; Rudziński, K.J.; Maciejewska, B. Identification of volatiles from *Pinus silvestris* attractive for *Monochamus galloprovincialis* using a SPME-GC/MS platform. *Environ. Sci. Pollut. Res.* **2012**, *19*, 2860–2869. [CrossRef] [PubMed]
37. De Bièvre, P.; Günzler, H. *Validation in Chemical Measurement*; Springer: Berlin, Germany, 2005; p. 168.
38. Chaturvedula, V.S.P.; Mubarak, C.; Prakash, I. IR spectral analysis of diterpene glycosides isolated from *Stevia rebaudiana*. *Food Nutr. Sci.* **2012**, *3*, 1467–1471. [CrossRef]
39. Bui, N.Q.; Fongarland, P.; Rataboul, F.; Dartiguelongue, C.; Charon, N.; Vallée, C.; Essayem, N. FTIR as a simple tool to quantify unconverted lignin from chars in biomass liquefaction process: Application to SC ethanol liquefaction of pine wood. *Fuel Process. Technol.* **2015**, *134*, 378–386. [CrossRef]
40. Schulz, H.; Baranska, M. Identification and quantification of valuable plant substances by IR and Raman spectroscopy. *Vib. Spectrosc.* **2006**, *43*, 13–25. [CrossRef]
41. PubChem. Compound Summary for CID 443470, Abieta-7,13-Diene. National Center for Biotechnology Information. 2020. Available online: https://pubchem.ncbi.nlm.nih.gov/compound/Abieta-7_13-diene (accessed on 6 October 2021).
42. Rodrigues, A.M.; Mendes, M.D.; Lima, A.S.; Barbosa, P.M.; Ascensão, L.; Barroso, J.G.; Pedro, L.G.; Mota, M.M.; Figueiredo, A.C. *Pinus halepensis*, *Pinus pinaster*, *Pinus pinea* and *Pinus sylvestris* essential oils chemotypes and monoterpene hydrocarbon enantiomers, before and after inoculation with the pinewood nematode *Bursaphelenchus xylophilus*. *Chem. Biodivers.* **2016**, *14*, e1600153. [CrossRef]
43. Gonçalves, E.; Figueiredo, A.C.; Barroso, J.G.; Henriques, J.; Sousa, E.; Bonifácio, L. Effect of *Monochamus galloprovincialis* feeding on *Pinus pinaster* and *Pinus pinea*, oleoresin and insect volatiles. *Phytochemistry* **2020**, *169*, 112159. [CrossRef]
44. Tümen, İ.; Akkol, E.K.; Taştan, H.; Süntar, I.; Kurtça, M. Research on the antioxidant, wound healing, and anti-inflammatory activities and the phytochemical composition of maritime pine (*Pinus pinaster* Ait). *J. Ethnopharmacol.* **2018**, *211*, 235–246. [CrossRef] [PubMed]
45. Modesto, I.; Sterck, L.; Arbona, V.; Gómez-Cadenas, A.; Carrasquinho, I.; Van de Peer, Y.; Miguel, C.M. Insights into the mechanisms implicated in *Pinus pinaster* resistance to pinewood nematode. *Front. Plant Sci.* **2021**, *12*, 690857. [CrossRef] [PubMed]
46. Menéndez-Gutiérrez, M.; Alonso, M.; Toval, G.; Díaz, R. Variation in pinewood nematode susceptibility among *Pinus pinaster* Ait. provenances from the Iberian Peninsula and France. *Ann. For. Sci.* **2017**, *74*, 76. [CrossRef]
47. Street, R.; Owen, S.; Duckham, S.; Boissard, C.; Hewitt, C. Effect of habitat and age on variations in volatile organic compound (VOC) emissions from *Quercus ilex* and *Pinus pinea*. *Atmos. Environ.* **1997**, *31*, 89–100. [CrossRef]
48. Feliciano, A.S.; del Corral, J.M.M.; Gordaliza, M.; Salinero, M.A. 13C NMR data for abieta-7,13-diene diterpenoids. *Magn. Reson. Chem.* **1993**, *31*, 841–844. [CrossRef]