Article

A complex mixture of diterpenic acids and their oxidation products from *Pinus sylvestris* resin comparatively analyzed by GC-MS and NMR

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Abstract: The wood of Pine trees can have significant amounts of diterpenic (resin) acids, particularly when the trees are stressed or injured. Resin acids are an important industrial raw material used in a myriad of applications. The analysis of complex resin acid mixtures have been done more commonly by GC-MS but also by NMR. Here we analyzed a resin acid sample from a *P. sylvestris* wood with an abnormally high resin acid content of 30%, both by GC-MS and NMR to compare the results. The GC-MS analysis showed a mixture of dominantly abietane-type resin acids, namely dehydroabietic acid (50%) and their hydroxylated forms (35%), and much smaller quantities of pimarane type resin acids (8%). The NMR analysis showed a higher proportion of abietic acid (42% to 3%) and pimarane type acids (95% to 15%) relative to dehydroabietic acid, comparatively to the GC-MS analysis. It is hypothesized that the conversion of abietadienoic acids (e.g. abietic) to dehydroabietic acid can be significant in high-temperature GC-MS and that pimarane (pimaric and isopimaric) acids are under-evaluated in GC-MS analysis relative to abietane acids comparatively to NMR analysis. It is concluded that the later can give more accurate results, minimizing resin acids degradation and avoiding GC-MS quantitation bias.

Keywords: resin; *Pinus sylvestris*; Scots pine; diterpenic acids; resin acids, dehydroabietic acid; GC-MS; NMR ..

1. Introduction

Pine (*Pinus* sp.) trees have an extensive network of resin canals, spreading both vertically and radially through its wood. Resin canals are defined and bordered by the so-called epithelial cells, where "oleoresin" (as the in vivo resin is called) is biosynthesized and accumulated. This resiniferous system is a defense mechanism of the living trees: upon any type of aggression, the tree exudes its resin, either sealing mechanical wounds or repelling biotic attacks. Furthermore, as a consequence of continued injury or stress, the pine trees will produce numerous resin canals in the affected areas. The later mechanism is exploited in the industrial extraction of resin from pine trees, where physical cuts and/or chemical attack are used to enhance resin production.

The oleoresin is a viscous solution made mainly of terpenic compounds: a mixture of monoterpenes makes the "solvent", and a mixture of diterpenic acids the "solute". Roughly, the more volatile monoterpenes account for a third of the oleoresin composition, and the non-volatile diterpenic (or resin) acids for the remaining two thirds. After separation by distillation, the volatile

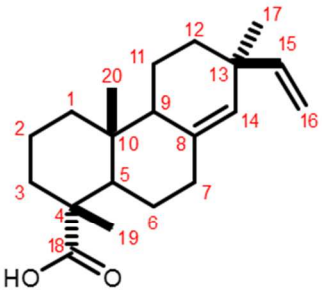
monoterpene fraction forms the "turpentine" (a colorless liquid), and the non-volatile fraction forms the "colophony" (or "rosin"), a yellow to brownish vitreous solid. Pines resin products and their derivatives have long been important materials and the list of their industrial applications is very long: monoterpenes are used in solvents, fragrances, insecticides, pharmaceutical products, etc.; resin acids and their derivatives are used in inks, lacquers, polymer additives, chewing gum, pharmaceutical products, synthetic rubber and many other niche uses. However, for a number of important wood-based products the presence of resin is a significant disadvantage, as is the case of pulp and paper and wood composites industries, where it can interfere with the manufacturing process or affect the performance of the end product.

From an analytical point of view, the separation and quantitation of resin components can be challenging, due to the complexity of the natural mixtures, and because of the structural similarities between some of its components. In the case of the diterpenic acids, which are our point of interest here, resin acids from to the same skeletal type, can differ only by the position of the double bonds within the rings system or the stereochemistry of asymmetric carbons (Figure 1). GC-MS is the standard technique for the analysis of resin diterpenic acids mixtures. GC can separate most of the individual resin acids and the EIMS spectra (together with the retention time) afford their safe identification. GC-MS is also used to quantify the resin acids, using the integrated areas of their peaks in the Total Ion Chromatograms (TIC). However, in the absence of costly and fastidious calibration, the TIC areas can be more or less erroneous in the quantitation of the resin acids, due to their known limitations: the peak areas are affected by the fragmentation pattern of each compound and the response to their relative quantity in the mixtures can be non-linear. Besides, because GC works at elevated temperatures, thermal degradation or isomerization of the resin acids is possible.

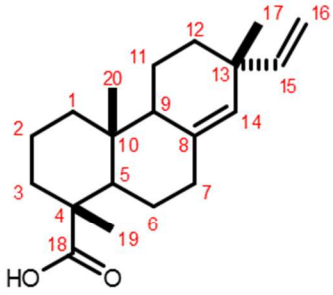
Another tool for the quantitative/qualitative analysis of diterpenic acid mixtures is NMR. ^{1}H signal peak areas are accurately proportional to the number of protons within a small error margin, when using appropriate NMR acquisition parameters, namely sufficiently long relaxation times between scans [1]. As long as one ^{1}H signal can be assigned to each of the resin acids, at least the relative quantitation the latter can be done by ^{1}H NMR. Thermal degradation can also be avoided this way, since most NMR manipulation and analysis can be carried out at ambient temperature. However when we are dealing with very complex mixtures (as the case here), overlapping of the ^{1}H NMR signals can prevent their assignment to a single compound and their use for quantitation. In the literature there are reports of *Pinus* sp. resin acids mixtures composition analysis both by ^{1}H and ^{13}C NMR [2-4]. Although ^{13}C decoupled peak lines from the different resin acids can be better resolved than ^{1}H ones, quantitation using 13C nuclei is much more demanding in the setup of the NMR experiment, for instance to get a good signal-to-noise ratio when dealing with minor components [1].

In the present work we analyzed the resin acids from the wood of a *Pinus sylvestris* (Scots pine) tree, both by GC-MS and NMR, and the results compared. The wood of this tree had an exceptionally high resin content, approximately 30% w/w, making it particularly interesting for the present analysis. Also, this resin sample was shown to be highly oxidized, a degradation process of resin acids due to its manipulation or contact with air and light. The resin diterpenic acids were identified by their EIMS spectra from the GC-MS analysis. This resin sample was analyzed in a high-field (800 MHz) NMR, and one-dimensional ^{1}H and ^{13}C APT, as well as two-dimensional homonuclear H-H J-resolved and COSY, and heteronucler C-H HSQC e HMBC spectra acquired. The NMR spectra were used to identify the structural moiety of the resin acids specific to each of them, and to select the ^{1}H s to be used for their quantitation. The results of the GC-MS and ^{1}H NMR quantitation are compared and the differences found discussed in the perspective of their use for the quantitation and composition analysis of resin acids mixtures and their degradation products.

Pimarane (skeletal type) diterpenic acids

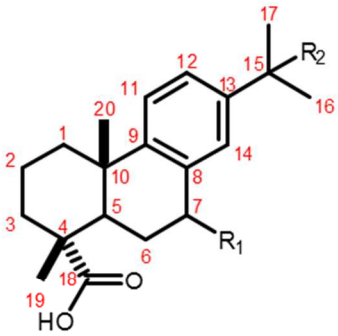


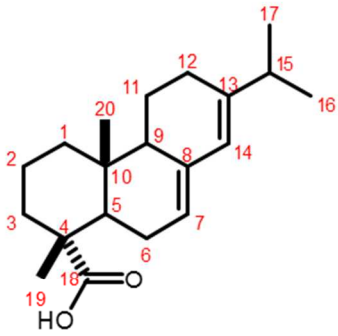
Pimaric acid



Isopimaric acid

Abietane (skeletal type) diterpenic acids





Abietic acid

	R_1	R_2
Dehydroabietic acid	H	H
15-Hydroxydehydroabietic acid	H	OH
7-Hydroxydehydroabietic acid	OH	H
7-Oxodehydroabietic acid	H	=O
7,15-Dihydroxydehydroabietic acid	OH	OH
15-Hydroxy-7-oxodehydroabietic acid	OH	=O

Figure 1. Structural formula, skeletal type and numbering of the diterpenic resin acids and oxidized derivatives identified in the GC-MS analysis of the *Pinus sylvestris* resin sample.

2. Results

2.1 GC-MS analysis

The structure of the resin diterpenic acids found in the present analysis, arranged according to their biosynthetic skeletal type, namely Abietane and Pimarane, are presented in Figure 1. The results of the GC-MS analysis of the *Pinus sylvestris* resin sample is presented in Figure 2, with the identified compounds pinpointed together with their relative peak areas. The compounds were identified based in their EIMS spectra, by comparison with library spectra (Nist11/Wiley9), published spectra [5] and

spectra of standards; the respective mass spectra (as TMS derivatives) are presented as Supplementary material, Figure S1 (*a-k*), with some of the more diagnostic fragment ions highlighted.

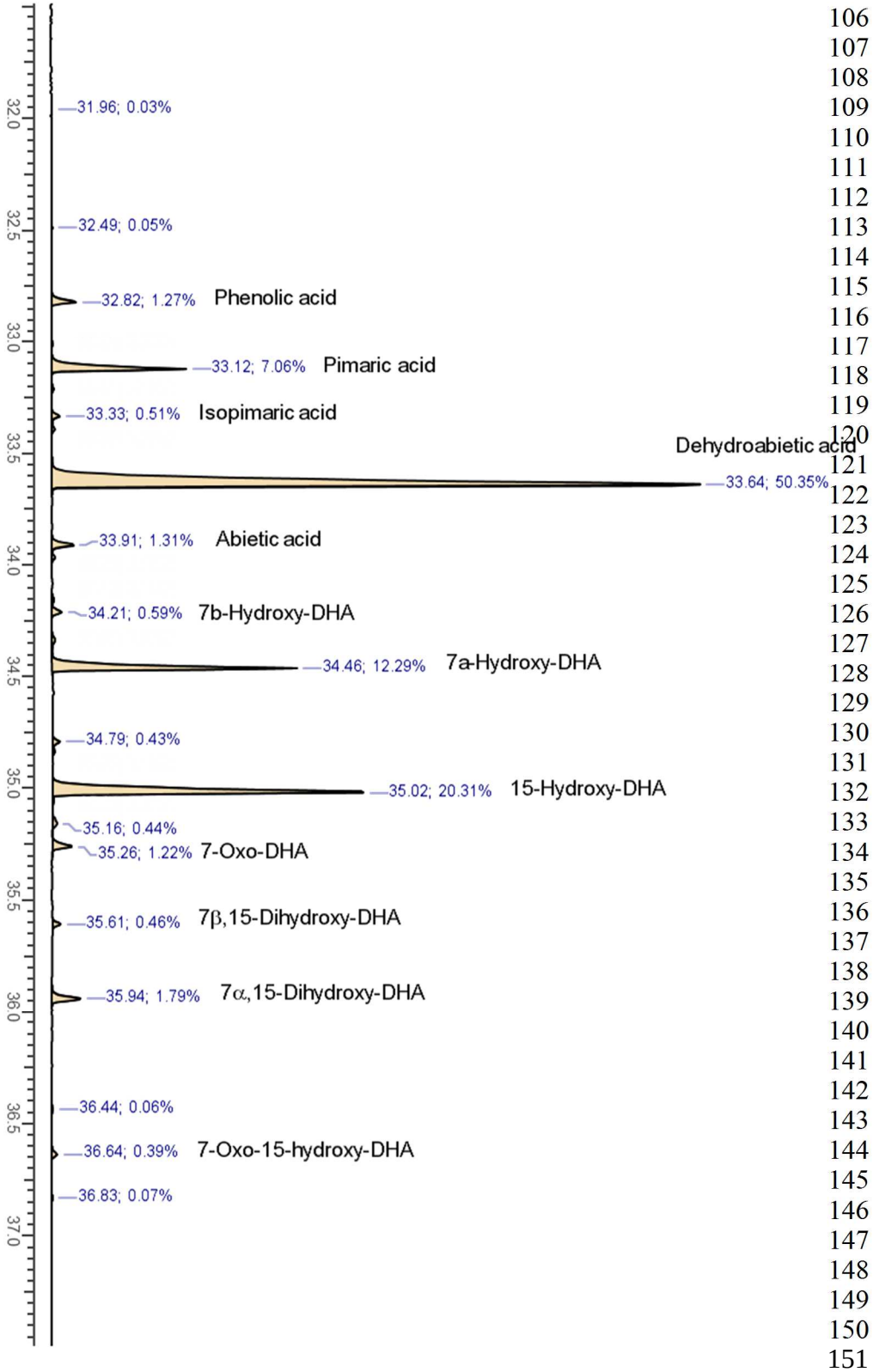
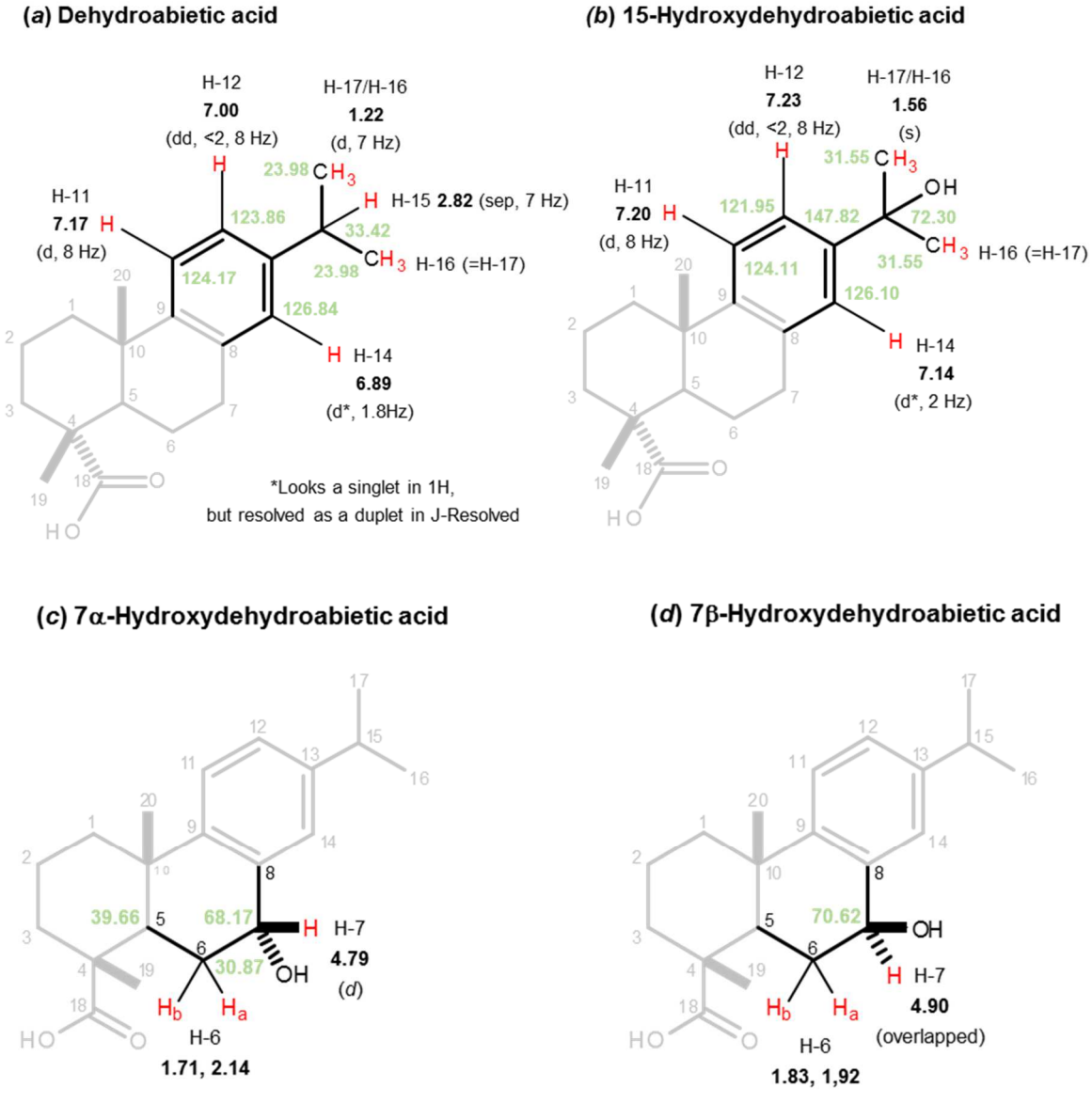


Figure 2. TIC chromatogram of the GC-MS analysis of the *Pinus sylvestris* resin sample.

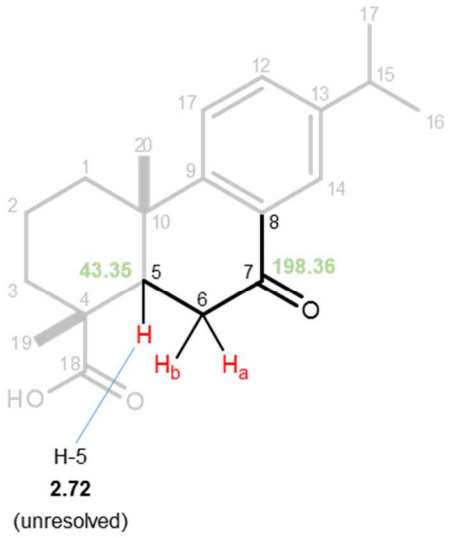
The composition of the present resin extract, as analyzed by GC-MS, was dominated by dehydroabietic acid (DHA), approximately 50%, and its oxidized forms, mostly as hydroxylated derivatives in positions 7 and 15, namely 15-Hydroxy-DHA (≈ 20%) and 7α-Hydroxy-DHA (≈ 12%). Abietic acid, which belong to the same structural family of DHA (Figure 1), was found in a minor quantity, ≈ 1%. Pimaric acid, ≈ 7%, and Isopimaric acid, <1%, representatives of the pimarane skeletal-type of diterpenic acids, were found in comparatively much lower percentages. A non-identified small "phenolic acid" represented a little more than 1% of the TIC integrated area, and other non-identified peaks totalized 2%.

2.2 NMR analysis

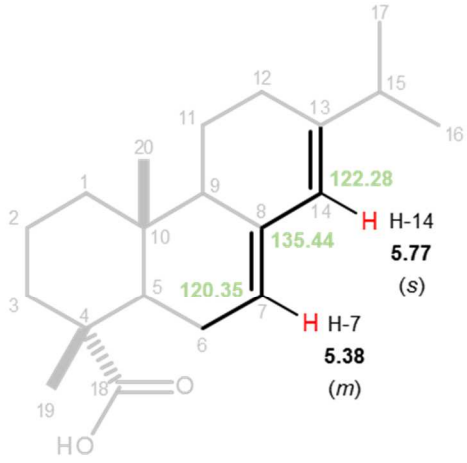
The assignment of the ^{1}H and ^{13}C chemical shifts of the more relevant and distinguishing structural moieties on the diterpenic acids found in the present analysis are presented in Figure 3 (*a-h*). The assignments were based on the interpretation of the NMR spectra, including one-dimensional ^{1}H and ^{13}C one-dimensional, and the two-dimensional H-H correlations (COSY), resolution of the H-H couplings (J-Resolved), C-H direct correlations (HSQC) and long range $^{2}J_{CH}$ and $^{3}J_{CH}$ correlations (HMBC), shown as Supplementary material, Figure S2 (*a-f*). Also, previous NMR analysis of some of the diterpenic acids were used to cross-check our assignments [4,7,9,12-22].



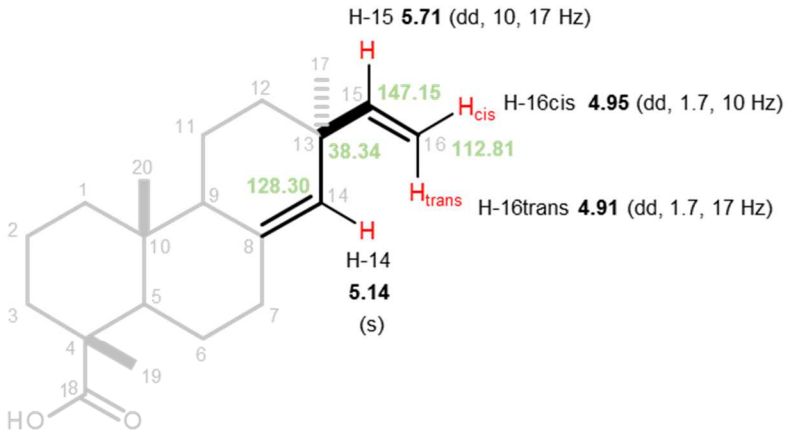
(e) 7-Oxodehydroabietic acid



(f) Abietic acid



(g) Pimaric acid



(h) Isopimaric acid

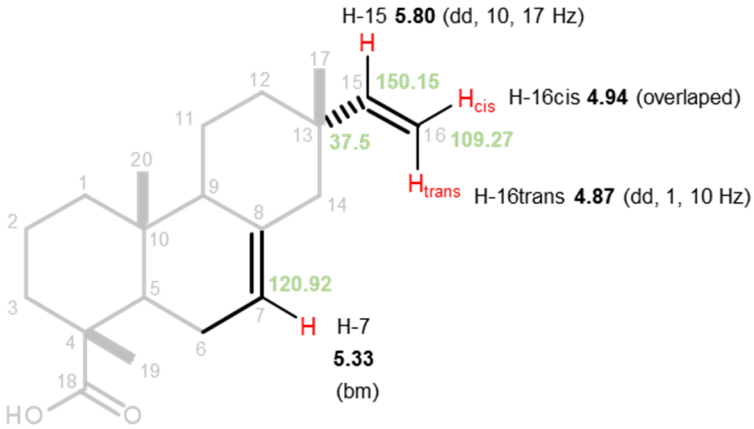


Figure 3. [Precedent pages] *a-h*. 1H and 13C NMR assignments of the structurally relevant (and discriminating) moieties of the diterpenic acids identified in the analysis of the *Pinus sylvestris* resin.

The main component of the resin acid mixture, dehydroabietic acid (Figure 4*a*), could be singled out by the three aromatic protons and carbons of its aromatic ring, together with the proton at position 15 in the propyl branch, which gave rise to a mostly clean septet at 2.82 ppm, due to the coupling to the two equivalent methyl groups at positions 16 and 17. The 15-Hydroxy-DHA acid (Figure 4*b*) could be distinguished from DHA by the slightly deshielded aromatic protons and the two methyl groups in the propyl branch (positions 16 and 17), whose comparatively deshielded ^{1}H's due to the vicinity of the 15-OH, gave rise to a prominent singlet at 1.56 ppm. The 7α-Hydroxy-DHA and 7β-Hydroxy-DHA (Figures 4*c* and *d*) could be identified and discriminated by their downfield oxygenated methyne proton in position 7, with different chemical shifts due to the α or β stereochemistry, at 4.79 ppm for the former and 4.90 ppm for the later. The 7-Oxo-DHA (Figure 4*e*), could be identified by its 7-keto carbon at ≈198 ppm, and some its long range correlations in the HMBC spectrum. Abietic acid and Pimaric acid could be distinguished by their vinyl single protons in the ring system, H-14 in abietic acid at 5.77 ppm (Figure 4*f*) and at the same H-14 position in Pimaric acid at 5.14 ppm (Figure 4*g*). Pimaric and Isopimaric acids could be distinguished by the H-7 proton in the later (Figure 4*h*), due to the different position of the double-bond in the ring system, but also by the differences in the vinyl protons in positions 15, 16 and 17.

3. Discussion

Resin acid content. The analyzed resin sample was extracted from the wood of a *Pinus sylvestris* tree located in a mountainous (Serra do Gerês) region in north Portugal; *P. sylvestris* spreads for most of northern and central Eurasia, and the tree analyzed is located in the most southwestern point of its distribution area. The wood sample analyzed had an exceptionally high resin content, 31.3% w/w, as assessed by its dichloromethane extract. Much lower values had been found elsewhere: in north Sweden, the resin acid content of *P.sylvestris* averaged 1.5% in the heartwood, less than 0.3% in the sapwood, with a mean of 1% for the total wood [6]; in Central Norway the vales for resin acid content in sapwood/heartwood were 0.3%-1.1%/1.4-4.4% [7]. The reasons behind the exceptionally high resin content of the wood of the *P. sylvestris* tree sampled, either genetic, environmental or result of an eventual injury, were not determined.

Resin diterpenic acid composition. Compared with literature results for the *P. sylvestris* wood resin composition, the present results are fundamentally different: previous analyses showed that abietadienoic resin acids – abietic, neoabietic, palustric and levopimaric – are largely dominant, when compared with dehydroabietic acid; the oxidized forms of the later are, as a rule absent or found as minor components [4,7-9]. The main difference between abietadienoic acids and dehydroabietic acid, both of the abietane structural type, is that the former have two conjugated double-bonds (the difference between them being their location within the ring system), and the three double bonds of the later, making the aromatic ring (Figure 1). Dehydroabietic acid thus is a dehydrated form of the abietadienoic acids. This means that the sample we have analyzed was a highly dehydrated and oxidized resin acid fraction of the original *P. sylvestris* wood resin. The dehydration-oxidation of abietadienoic acids to dehydroabietic acid, and the later to its oxidized and hydroxylated forms is a known phenomenon: after exposure to air and light, abietadienoic acids degrade to the more stable form of dehydroabietic acid [10]. Also, oxidation happens in the long run, and is found in historical and archaeological materials where resin acid products were originally used [11]. However, thermal degradation of abietadienoic acids to dehydroabietic acid and their derivatives, can also occur due to the analytical methodologies used, as discussed below when comparing GC-MS and NMR results.

GC-MS vs NMR quantitative analysis. The comparison of the relative quantities of the different diterpenic resin acids in the *Pinus sylvestris* resin fraction, as analyzed by GC-MS and NMR is presented in Table 1. Several issues are to be considered. First, the choice of the protons to be used in the relative NMR quantitation of the resin acids. The selection of the proton signals followed two main criteria: first to be unequivocally be part of the targeted resin acid (see Results); and second, to be in a "clean" part of the ^{1}H spectrum, with no overlapping with other signals. These criteria could be mostly met. However, some of the "distinguishing" signals used for the main resin acids could also come from some of the minor ones, structurally related to the former. This means that some of the areas accounted for the main resin acids, probably also include some of those minor resin acid constituents. This situations are listed in the notes of Table 1.

Table 1. Relative composition of resin diterpenic acids in *Pinus sylvestris* wood, as quantified by GC-MS and ^{1}H NMR peak areas integration*

		^{1}H NMR			**GC-MS**
	H-position	ppm	Split	Area	Area
Pimaric acid	H-14	5.14	s	0.54	0.14
Isopimaric acid	H-7	5.33	bm	0.41	0.01
Dehydroabietic acid	H-14	6.88	d	1.00	1.00
Abietic acid	H-14	5.77	s	0.42	0.03
7α-Hydroxy-DHA[1]	H-7	4.80	d	0.44	0.24
7β-Hydroxy-DHA[1]					0.01
?-Hydroxy-DHA					0.01
15-Hydroxy-DHA	H-11	7.20	d	0.53	0.40
7-Oxo-DHA[1]					0.02
7α,15-Dihydroxy-DHA[2]					0.04
7β,15-Dihydroxy-DHA[2]					0.01
15-Hydroxy-7-oxo-DHA[2]					0.01

* "Areas" are relative to the biggest compound detected in the GC-MS and NMR analysis, in both cases the dehydroabietic acid peak area, assigned as 1.00; the NMR areas were calculated based in the molar proportion of the selected protons for quantitation and corrected for the different molecular masses of the diterpenic acids.

[1] Eventually included in the NMR "Dehydroabietic acid" area (similar signal for H-14)

[2] Eventually included in the NMR "15-Hydroxy-DHA" area (similar signal for H-11)

In spite of this limitation, the results in Table 1 clearly show that both abietic acid and the pimarane-type pimaric and isopimaric acids were under-evaluated in the GC-MS analysis, assuming that the NMR approach is more accurate. In the case of abietic acid, the relation to DHA is 3% in the GC-MS analysis, and 42% in the ^{1}H NMR quantitation. This means that abietic was probably present in the resin extract in a higher relative quantity to DHA than the "1%" seen in the GC-MS analysis. This would be the expected, since the *P. sylvestris* resin acid composition from several other sources typically shows high amounts of abietic acid (and other abietadienoic acids) in relation to DHA. This would mean that the dehydration-oxidation of abietic (or other abitadienoic acids eventually present originally in resin acid before analysis) occurred during the GC-MS analysis experimental setup or the high temperature conditions used.

Besides the degradation of the abietadienoic acids to DHA and its oxidized forms due to the GC-MS methodology, the other possibility is that the GC-MS analysis indeed gives biased intensities

for different compounds. This is illustrated by the fact that both pimaric and isopimaric, which are not supposed to be converted to DHA, also showed under-evaluated in the GC-MS analysis as compared to the NMR quantitation approach, respectably by a factor o 4x and 40x, respectively. Also, some error might exist in the peak are integration both in the GC-MS and NMR analysis, when peaks are small (as in these two later resin acids). However the results clearly indicate that both pimaric and isopimaric acids were probably under-evaluated in the GC-MS analysis as compared to DHA.

Together this show that in the analysis of resin acid mixtures, the history of the sample manipulation, experimental setup and methodology choices can have a profound impact in the results obtained. GC-MS analysis of resin acids showed to be prone to discrimination due to structure – pimarene type acids could be under-evaluated, and thermal conversation of abietadienoic acids like abietic acid to dehydroabietic acid can occur. In this sense, NMR would a preferable tool to the composition analysis of resin diterpenic acids mixtures.

4. Materials and Methods

Wood material. A wood sample from a *Pinus sylvestris* (commonly named Scots pine) tree located in Serra do Gerês, Braga, Portugal (Project Reference Sample 49) was taken with an increment (Pressler) borer at 1.30 height. The wood section was taken to include all the radial growth, from the pith to the cambial zone. The wood sample was air dried and ground in a Retsch SM 2000 mill to pass a 6 mm sieve, and further ground in a Retsch ZM 100 mill to pass a 0.5 mm reverse sieve.

Resin extraction. An aliquot of the wood ground material (1.5 g) was extracted with the dichloromethane (6 hours) in a Soxhlet apparatus. The extractives content was assessed by the weight loss after the extraction.

GC-MS analysis. Aliquots of the dried extracts (1 mg) were derivatized with 20 uL of Pyridine and 20 uL of BSTFA, and kept at 60ºc for 30 min before injection. GC-MS were run in an Agilent Technologies 78990A/5975C MSD, with the following GC conditions: injector 320ºC; Initial temperature 80ºC (5 min), 5ºC/min up to 110ºC, 20ºC/min up to 250ºC, and 8ºC/min up to 320ºC (15 min).

NMR analysis. For the NMR spectroscopy analyses the dried resin extract, 5 mg, was dissolved in 1 mL of perdeuterated chloroform. All spectra were acquired on a Bruker AVANCE III 800 spectrometer (Bruker, Rheinstetten, Germany) working at a proton operating frequency of 800.33 MHz, equipped with a four channel 5 mm inverse detection probe head with pulse-field gradients along the Z axis.

Spectra were run at 25ºC using standard Bruker pulse programs. ^{1}H and ^{13}C chemical shifts are referenced to tetra methyl sylane. ^{13}C spectra were recorded at 201.24 MHz using the APT (attached proton test) sequence. The modulation of peak sign, to distinguish methyl and methyne from methylene signals, was achieved using a delay of 6.89 ms for the evolution of $^{1}J_{CH}$. Proton decoupling was applied during the acquisition stage using the WALTZ-16 sequence.In the two-dimensional ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra, a delay of 3.45 ms was used for evolution of $^{1}J_{CH}$, while in the heteronuclear multiple bond connectivity (HMBC) spectra a delay of 73.5 ms was used for evolution of long range couplings. In the HSQC, proton decoupling was achieved using the GARP4 sequence.

Supplementary Materials: The following are available online at www.mdpi.com/link, Figure S1: GC-MS spectra Figure S2: NMR spectra.

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

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