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Characterization of the volatile fraction emitted by *Pinus* spp. by one- and two-dimensional chromatographic techniques with mass spectrometric detection

E. Mateus[a,*], R.C. Barata[b], Jitka Zrostlíková[c], M.D.R. Gomes da Silva[b,*], M.R. Paiva[a]

[a] GUECKO, Departamento de Ciências e Engenharia do Ambiente, FCT, Universidade Nova de Lisboa, 2829-516 Campus de Caparica, Portugal
[b] REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal
[c] LECO Instrumente Pilsen – Application Laboratory Prague, Sokolovská 219, 190 00 Prague 9, Czech Republic

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ABSTRACT

The chemical composition of the needles of *P. pinea*, *P. pinaster*, *P. halepensis*, *P. nigra*, *P. brutia*, *P. patula*, *P. radiata*, *P. taeda*, *P. elliotti*, *P. kesiya*, *P. sylvestris* and *P. eldarica* was investigated. Headspace solid-phase microextraction and steam distillation extraction were used to collect the volatile fractions. Samples were analyzed using one-dimensional gas chromatography (1D-GC) and comprehensive two-dimensional gas chromatography (GC × GC) associated with a quadrupole and a time-of-flight mass detectors. Results showed that the analytical capabilities of 1D-GC are partially limited by the separation power of the columns. The higher sensibility and the absence of peak skewing of the time-of-flight mass analyzer, with the use of automated peak finding and deconvolution algorithms, allowed for the detection of trace components with qualitative full spectra and the extraction of true mass spectra from coeluting compounds, promoting their reliable identification and thus significantly improving results obtained by 1D-GC/MS, when using a quadrupole mass analyzer. The use of GC × GC resulted in enhanced separation efficiency and increased signal to noise ratio (sensitivity) of the analytes, maximizing mass spectra quality and improving compound detection and identification. This work shows the use of 1D-GC/ToFMS for the analysis of pine needles volatiles, achieving the detection of 177 compounds, that is more than twice the number previously identified by standard 1D-GC/MS. The analysis by GC × GC for the same sample allowed the detection of 212 compounds. The enantioselective GC × GC analysis performed for all the *Pinus* spp. under study achieved the detection of 422 different compounds. Cross-over phenomena according to operational conditions are highlighted and discussed.

1. Introduction

Plants produce a large variety of chemicals, some of which are essential to themselves, while others apparently have no direct function on plants. Plant chemicals can be divided into: (i) primary metabolites, if they are essential for a plant and are directly involved in plant growth, development and reproduction and (ii) secondary metabolites if no role has yet been found in plant growth, development, reproduction, or in any other "primary" functions. Plant volatiles are usually complex mixtures, comprising several hundred compounds [1–3]. Most of the common plant volatiles have 5–20 carbon atoms and include short-chain alcohols, aldehydes, esters, ketones, phenols, lactones, phenylpropanoids, and terpenoids [2–5].

Plant volatiles may be constitutively emitted by the plants or induced by insect feeding and oviposition [3,6,7]. These plant volatiles can play an important role in mediating interactions between plant and insect herbivores, between insect herbivores and their natural enemies and even between plants themselves [6–9]. Research studies have shown that plant volatiles can deter oviposition by insect herbivores [10], attract their natural enemies (parasitic and predatory insects) [8,11], and even induce defense and volatile emission in other plants, allowing them to respond faster to future herbivore attack [8]. The chemical identity of the volatile compounds and the composition of the blends vary with the plant species and with the herbivorous insect species [6,8,9,12]. In spite of the differences, there is a structural similarity among plant volatiles, which include mainly terpenes (homo-, mono- and sesqui-), aromatic compounds and green leaf volatiles [8,10]. This structural similarity suggests the activation of a common set of biosynthetic pathways, common to a wide range of plants [10]. Additionally, the ability of plants to differentiate between insect damage and mechanical wounding also

* Corresponding author. Tel.: +351 212948351; fax: +351 212948350.
E-mail addresses: epm@fct.unl.pt (E. Mateus), mdr@dq.fct.unl.pt (M.D.R. Gomes da Silva).

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suggests the presence of elicitors associated with insect feeding [10].

Plant emissions also contain chiral compounds, since many monoterpenes are produced in enantiomeric forms. α-Pinene, a common monoterpene, occurs as both (+)-α-pinene and (−)-α-pinene in *Pinus* spp. [13]. The importance of different enantiomers for the biological activity of a compound is well known [14]. Insects have olfactory receptor neurons that discriminate between enantiomers and can use them as fingerprints in insect host selection or avoidance [15], and plants for pollinator attraction [16]. They can be used as fingerprints in plant species chemotaxonomy studies [13].

Pines have established on a wide range of habitats, from sub artic regions to the tropics. In the northern hemisphere pines often constitute the main forest components. They are the dominant trees over large parts of the boreal forest landscapes, while over large areas of southern Europe, pines can form virtually monospecific forests [17–21]. In the Mediterranean region, pine forests comprise of 10 pine species and cover about 13 million hectares, which represents about 5% of the total regional area and about 25% of the total area forested. In the Mediterranean area, the most common species are *P. halepensis* and *P. brutia*, followed by *P. pinea*, scattered all over the region, and *P. pinaster* on the western part. *P. sylvestris* is also widespread in the region, but is more abundant in the northern regions of Europe [20,22]. Since prehistoric times, the distribution of pine forests has been strongly related to land use practices, especially those affecting fire regimes and reforestation to control erosion [21–23].

Presently GC/MS is the most frequently used hyphenate technique for the characterization and identification of volatile and semivolatile metabolites from plants. However, in spite of the continuous development of equipment, techniques and analytical methodologies, a total separation of all the sample components is still unachievable, due to the complexity of the sample and of the analysis (high number of components, structure similarities, isomers, and wide range of concentrations). High similarities are thus expectable between the retention times of several analytes, independently of the stationary phase used, that will result in coelutions. These coelutions are often impossible to detect and identify with some GC/MS instruments, in spite of the use of selective single ion monitoring (SIM) mode, or of complex deconvolution processes. An additional problem results from the wide range of analyte concentrations in their matrices. Consequently, the trace level analytes, that sometimes are the biologically active components in the matrix under study, may never be detected, if they are coeluting with high concentration compounds.

The high complexity of the chromatograms points out to new ways of chromatography, such as multidimensional systems (MD-GC), where the analytes are submitted to two or more independent separation steps, in order to achieve separation. One example came from the "heart-cut" systems, using flow switching devices, that allowed the isolation of selected peaks, or ranges, of the chromatogram by partial transference of the selected fraction, from a pre-column to a second column of different selectivity (often chiral), where only the target analytes are submitted to chromatographic separation, avoiding the potential coelution with the non-transferred compounds [24–27]. In spite of its efficiency, the MD-GC is a time consuming technique, with long analysis times, which does not fit with the demands of routine analysis. Additionally, it is technically difficult to carry out sequential transfers in a narrow window of retention times, since there is a likelihood of coelutions [27]. In the GC × GC system two columns with different selectivities are serially connected through a suitable interface, which usually is a thermal modulator [28–30]. At the GC × GC technique the entire sample separated on the first column is transferred to the second one, resulting in an enhanced chromatographic resolution into two independent dimensions, where the analytes are separated by two independent mechanisms (orthogonal separation) [31] and thus maximizing the column peak capacities and the separation power of the GC × GC system. Due to the orthogonal separation occurring in both columns, the chromatograms resulting from GC × GC are ordered, producing structured chromatograms, where the analytes have their spatial location, in the contour plot, based on their structures [28]. In the reconstructed 2D contour plots, characteristic patterns are obtained, in which the members of homological series with different volatilities are ordered along the first dimension axis, and the compounds are spread along the second dimension axis according to their polarity. This cluster representation of various subgroups of analytes in the GC × GC contour plots may be used as a tool for analyte class analysis and tentative identification of compounds [32,33]. Moreover, each compound is now obtained as a pure compound which constitutes a considerable advantage over 1D-GC when, in particular MS identification is performed [34].

Retention time shifting depending on the chemical and physical characteristics of the stationary phase used, and among the same type of columns, between different purchasers [35,36] also introduces considerable difficulties in establishing unequivocal peak identity assignments which are of major importance when the goal is the full peak characterization of complex samples.

In order to characterize the volatile emissions of 11 pine species (*P. pinea*, *P. pinaster*, *P. halepensis*, *P. nigra*, *P. brutia, P. patula, P. radiata, P. taeda, P. elliotti, P. kesiya* and *P. sylvestris*) growing in Portugal, 54 samples (4–5 samples for each pine species) were randomly sampled from these 11 pine species in an experimental plot at Abrantes and from *P. eldarica* in a forest stand located 50 km north of Abrantes. In earlier studies [37–43] on average only 50–150 compounds were identified in *Pinus* spp. by 1D-GC/MS after SDE or SPME extraction procedures, and some identification inconsistencies can be observed when the literature was compared – mainly due to the fact that 1D-GC analysis is performed. In this study, the volatile compounds emitted by the needles were extracted by SDE and/or SPME and analyzed by GC-FID, and, as our knowledge concerns, for the first time by GC × GC/ToFMS and by enantiomeric 2D-GC (GC × eGC/ToFMS). Different gas chromatographic operational systems (variable column phases, film thicknesses, temperature program ramps, and hold temperature times) were performed in order to verify cross-over phenomena thus allowing for a more accurate peak assignment. The presence of the different compounds is indicated for each studied pine species and the pertinence to the use of 2D-GC/ToFMS for full matrix characterization is emphasized. The identification of assignments obtained after MS identification and using several columns of different polarity allowed to preclude erroneous identifications due to cross-over, and together with 2D separation system, pure MS spectra could be obtained and more accurate peak identifications could be performed.

2. Experimental

2.1. Samples

Needles were sampled from 11 different pine species planted in an experimental plot located in central Portugal, Abrantes region (N39°26′; W8°04′) in 1991. After collection, the samples were stored and transported under refrigerated conditions (4–5 °C) to the laboratory, in hermetic plastic bags. All samples were SPME extracted, within 48 h after collection.

2.2. Standards

All standards used were purchased from Aldrich (Deisenhofen, Germany), Fluka (Neu-Ulm, Germany) and Kasei (Tokyo, Japan). The hydrocarbon mixture (C9–C26) was purchased from AccuStandard (New Haven, USA) and Supelco (Supelco, Bellefont, PA, USA).

2.3. Sampling method

2.3.1. Simultaneous distillation–extraction (SDE)

100 g of needles from each pine species was mixed into a composite sample. Uncut needles from each pine species (10–20 g) were extracted by SDE according to a previous study [13].

2.3.2. Solid-phase microextraction SPME

Before extraction, needles from each pine species were mixed into a composite sample. Subsamples were cut, the pieces were mixed, and 0.2–0.7 g was placed inside a 7.0 mL vial (Supelco, Bellefonte, PA, USA). The vial was closed with a hole cap sealed with a PTFE/neoprene septa. The headspace inside the vial was sampled by solid-phase microextraction (SPME) using a 100 μm polydimethylsiloxane (PDMS) coated fibre purchased from Supelco (Supelco, Bellefonte, PA, USA). The headspace extraction was performed at room temperature according to a previous work [44] for 45 min and the trapped compounds desorbed at 250 °C in the chromatograph injection port, for 60 s. Before the analysis, the fiber was conditioned according to the manufacturer standard procedures.

2.4. 1D-GC analysis

2.4.1. GC/qMS

The first system consisted of a Trace GC 2000 Series gas chromatograph (Thermo Quest, Rodano, Italy) coupled to a Finnigan Trace MS quadrupole mass spectrometer (Thermo Quest, Manchester, UK) Electron Impact (EI). The separation and analysis of the pine needle volatiles were performed on three different columns:

Column 1: a DB-5 (5% phenyl-95% methylpolysiloxane) column with 30 m × 0.32 mm i.d. and 1.00 μm film thickness (d_f) (J&W Scientific, Folsom, USA).

Column 2: a DB-Wax (polyethylene glycol – PEG) column with 60 m × 0.25 mm i.d. and 1.0 μm d_f (J&W Scientific, Folsom, USA).

Column 3: a tailor made fused silica capillary column with 30 m × 0.25 mm i.d., coated with 0.25 μm film of 15% heptakis (2,3-di-O-methyl-6-O-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE52 (DiMe). This column was used for separation of enantiomeric monoterpenes.

Total ion chromatograms (TIC) were processed using the automated data processing software Xcalibur from Thermo Finnigan (ThermoFinnigan, Austin, TX, USA).

For column 1, the oven temperature program began at 50 °C, was held at that temperature for 2 min, raised to 125 °C at 4.0 °C min^{-1}, and then at 6.0 °C min^{-1} to 200 °C, held 10 min, and finally at 10.0 °C min^{-1} to 250 °C and held 1 min at this temperature. Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1} (in constant flow mode). The injection port was set at 250 °C and splitless injection was performed for 0.5 min. The MS detector operated in EI(+) mode at 70 eV. Source and interface temperature were set at 250 °C. The scan mass range used was 40–300 amu.

For column 2, the oven temperature program began at 50 °C, was held at that temperature for 2 min, raised to 125 °C at 4.0 °C min^{-1}, and then at 6.0 °C min^{-1} to 200 °C, held 10 min, and finally at 10.0 °C min^{-1} to 220 °C and held 1 min at this temperature. Helium was used as the carrier gas at a flow rate of 1.5 mL min^{-1} (in constant flow mode). The injection port was set at 250 °C and splitless injection was performed for 0.5 min. The MS detector operated as described for column 1.

For column 3, the oven temperature program began at 40 °C, was held at that temperature for 2 min, raised to 70 °C at 15.0 °C min^{-1}, held 25 min at this temperature, and then at 4.0 °C min^{-1} to 100 °C, held 1.0 min, and finally at 10.0 °C min^{-1} to 200 °C and held 10 min at this temperature. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} (in constant flow mode). The injection port was set at 250 °C and splitless injection was performed for 1.0 min. The MS detector operated as described for column 1.

Linear retention indices (LRI) values were calculated according to van den Dool and Kratz [45].

2.4.2. GC/ToFMS

The second system consists of a gas chromatograph GC System 6890N Series (Agilent Technologies, Palo Alto, CA, USA) coupled to a Pegasus III time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA). The separation was achieved on a DB-5ms (5% phenyl polysilphenylenesiloxane) column with 15 m × 0.25 mm i.d. and 0.25 μm d_f (J&W Scientific, Folsom, USA) and a Equity-5 (5% phenyl polysilphenylenesiloxane) column with 60 m × 0.25 mm i.d. and 1.0 μm d_f (Supelco, Bellefonte, USA).

The primary oven temperature program began at 35 °C, held 2 min at this temperature, then raised to 250 °C at 4.0 °C min^{-1}, and held 2 min at this temperature. The secondary oven temperature program began at 40 °C, held 2 min at this temperature, then raised to 255 °C at 4.0 °C min^{-1}, and held 2 min at this temperature. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} (in constant flow mode). The injection port was set at 300 °C and 1 μL of sample (when applied) injected with a split ratio of 1:50 was used. The MS detector operated in EI(+) mode at 70 eV. Source and interface temperature were set at 220 °C and 280 °C respectively. The scan mass range used was 45–600 amu. The detector voltage was set at −1750 V and the acquisition rate at 7 Hz. The *S*/*N* ratio for peak finding was set at 10.

Linear retention indices (LRI) values were calculated according to van den Dool and Kratz [45].

2.5. GC × GC/ToFMS analysis

The volatiles analysis was performed on a Pegasus 4D (LECO, St. Joseph, MI, USA) GC × GC–ToFMS system. This system comprised of a HP 6890 (Agilent Technologies, USA) gas chromatograph with a dual stage jet cryogenic modulator (licensed from Zoex) and a secondary oven connected to a Pegasus III time-of-flight mass spectrometer (LECO, St. Joseph, MI, USA).

Three sets of columns have been used to perform the separations of pine volatiles.

Column set 1: the first dimension of the GC × GC column set was a DB-5ms (5% phenyl polysilphenylenesiloxane) column with 15 m × 0.25 mm i.d. and 0.25 μm d_f (J&W Scientific, Folsom, USA), with a BPX50 (50% phenyl polysilphenylenesiloxane) column as the second dimension with 1.0 m × 0.1 mm i.d. and 0.1 μm d_f (SGE International, Ringwood, Australia). The primary oven temperature program began at 35 °C, held 2 min at this temperature, then raised to 250 °C at 4.0 °C min^{-1}, and held 2 min at this temperature. The secondary oven temperature program began at 40 °C, held 2 min at this temperature, then raised to 255 °C at 4.0 °C min^{-1}, and held 2 min at this temperature. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} (in constant flow mode). The injection port was set at 300 °C and 1 μL of sample (when applied) injected with a split ratio of 1:50 was used. The MS detector operated in EI(+) mode at 70 eV. Source and interface temperature were set at 220 °C and 280 °C respectively. The scan mass range used was 45–600 amu. The detector voltage was set at −1750 V and the acquisition rate at 125 Hz. The modulation period was 3 s with a hot pulse of 0.6 s. The *S*/*N* ratio for peak finding was set at 10

Table 1
Operational conditions used for the different stationary phases.

Method	Temperature program	Constant flow (mL min^{-1})	Constant pressure (psi)	Carrier gas
M1	P1	–	10	H_2
M2	P1	1.5	–	H_2
M3	P2	–	10	H_2
M4	P3	–	6.5	H_2
M5	P3	1.5	–	H_2
M6	P3	1.0	–	He

Column set 2: the first dimension of the GC × GC column set was an Equity-5 (5% phenyl polysilphenylenesiloxane) column with 60 m × 0.25 mm i.d. and 1.0 μm df (Supelco, Bellefonte, USA), with a Supelcowax-10 (polyethylene glycol – PEG) column as the second dimension with 2.5 m × 0.1 mm i.d. and 0.1 μm df (Supelco, Bellefonte, USA). The operational conditions were the same as in the above column set 1

Column set 3: this column set was used for separation of enantiomeric monoterpenes. The first dimension of the GC × GC column set was a tailor made fused silica capillary column with 30 m × 0.25 mm i.d., coated with 0.25 μm film of 15% heptakis (2,3-di-O-methyl-6-O-*tert*-butyldimethylsilyl)-β-cyclodextrin in SE52 (2,3-DiMe) with a Supelcowax-10 (polyethylene glycol – PEG) column as the second dimension with 2.5 m × 0.1 mm i.d. and 0.1 μm d_f (Supelco, Bellefonte, USA). The primary oven temperature program began at 40 °C, held 1 min at this temperature, then raised to 200 °C at 2.0 °C min^{-1}, and held 2 min at this temperature. The secondary oven temperature program began at 50 °C, held 1 min at this temperature, then raised to 210 °C at 2.0 °C min^{-1}, and held 2 min at this temperature. Helium was used as the carrier gas at a flow rate of 1.0 mL min^{-1} (in constant flow mode). The injection port was set at 300 °C and 1 μL of sample (when applied) injected with a split ratio of 1:4 was used. The MS detector operated in EI(+) mode at 70 eV. Source and interface temperature were set at 220 °C and 250 °C respectively. The scan mass range used was 35–350 amu. The detector voltage was set at −1700 V and the acquisition rate at 100 Hz. The modulation period was 5 s with a hot pulse of 1.0 s. The *S*/*N* ratio for peak finding was set at 50.

Total ion chromatograms (TIC) were processed using the automated data processing software ChromaToF^{TM} from LECO Corp. (St. Joseph, MI, USA). Contour plots were used to evaluate the general quality of the separation and for manual peak identification.

Linear retention indices (LRI) values were calculated according to van den Dool and Kratz [45] considering the experimental first dimension column retention time.

2.6. Cross-over phenomena studies

2.6.1. Equipment

The systems used consisted of a GC-Trace 2000 (Thermo Quest Instruments), a Cambridge Scientific Instruments CSi 200 gas chromatograph, operating at constant column pressure of 10 psi, and a Hewlett-Packard HP 5890A, operating at constant column pressure of 6.5 psi, all with hydrogen as a carrier gas. The injections were performed with a split ratio of 1:20, the injector port temperature was set at 250 °C, and the detector temperature was set at 300 °C.

For mass spectrometric analysis a GC-Trace 2000 (Thermo Quest Instruments) equipped with a Thermo Quest Trace MS was used, operating in electron impact mode (70 eV) and using a mass range of 40–300 Da. Helium was used as carrier gas, at a constant flow rate of 1.0 mL min^{-1}. The injection port was set at 250 °C, and the ion source and interface were both set at 250 °C. The injected volume was 1 μL, with a split ratio of 1:20.

The Thermo Quest Instrument data was processed using Excalibur (Home Page version 1.2). The Cambridge Scientific Instruments CSi 200 data was processed with Chromatography Station for Windows 32 (CSW32). The Hewlett-Packard HP 5890A data was processed with a Merck-Hitachi D-2500 analog integrator.

2.6.2. Chromatographic analysis

Three different temperature programs were used. Program 1 (P1): the column was kept at 50 °C for 1 min, then heated at a rate of 3 °C min^{-1} to 230 °C, where it was kept for 5 min; Program 2 (P2): the column was kept at 50 °C, then heated at a rate of 4 °C min^{-1} until it reached 125 °C. The rate was then increased to 6 °C min^{-1} until the temperature reached 230 °C, where it was kept for 5 min. The column was finally heated, at a 10 °C min^{-1} rate, to 240 °C, where it was kept for 0.25 min; Program 3 (P3): the column was kept at 50 °C for 1 min, then heated at a rate of 4 °C min^{-1} until it reached 125 °C. The rate was then increased to 6 °C until the temperature reached 250 °C, where it was kept for 5 min. The column was finally heated, at a 10 °C min^{-1} rate, to 295 °C, where it was kept for 0.25 min. In this work, several methods were used. For clarity sake, they are presented in Table 1.

2.6.3. Chromatographic columns

Four columns with different stationary phases and/or column dimensions were used in this work: a DB-Wax (Col. 1 – polyethyleneglycol, 30 m × 0.32 mm × 0.50 μm, J&W Scientific, Folsom, USA), a Stabilwax (Col. 2 – polyethyleneglycol, 60 m × 0.32 mm × 1.0 μm, Restek Corp.), a DB-5 (Col. 3–5% phenyl in polydimethylsiloxilane, 30 m × 0.32 mm × 1.0 μm, J&W Scientific, Folsom, USA) and a ZB-5 (Col. 4–5% phenyl in polydimethylsiloxilane, 30 m × 0.25 mm × 0.25 μm, Zebron). For mass spectrometric analysis a ZB-5ms (Col. 5–5% phenyl in polydimethylsiloxilane, 30 m × 0.25 mm × 0.25 μm, Zebron) was used.

2.7. Statistical treatment

All retention times were determined in triplicate for each temperature program, under the different set of experimental variables. All the data was entered into a MS-Excel (Microsoft) datasheet and the linear retention indices (LRI) values were calculated, according to van den Dool and Kratz [45], the values were then averaged, thus obtaining the mean LRI value and the respective residuals.

3. Results and discussion

3.1. 1D-GC/qMS

When qMs was used, 94 compounds were identified, considering all studied species, whether compared with standards or tentatively identified using the NIST mass spectra library matching and retention index criteria (Table S1). The retention times and linear retention indices (LRI) reported were obtained with the DB-5 column. The compounds, 27–29 (hexyl acetate, hexan-1-ol, acetic acid respectively) and 91–94 (longipinene, α-ylangene, β-ylangene and ε-muurolene respectively) were only detected when the polar column was used, due to potential coelution in the apolar stationary phase. Their retention time in the polar column are thus presented. The experimental linear retention indices ($LRI_{calc.}$) show a slight shift, for the identified compounds, when compared

with the linear retention indices ($LRI_{lit.}$) reported by Adams [46]. In the cases where standard co-injection compounds were used the $LRI_{calc.}$ shifts compared to $LRI_{lit.}$ were, in average, less than 12 units, and when all compounds were considered, the $LRI_{calc.}$ shifts compared $LRI_{lit.}$ for the apolar column were less than 7 units. Moreover, the $LRI_{calc.}$ obtained for the *Pinus* spp. volatile compounds were plotted against the linear retention indices $LRI_{lit.}$ reported by Adams [46], as illustrated in Fig. S1. The $LRI_{calc.}$ presents a linear relationship when correlated with the $LRI_{ref.}$, described by a coefficient of determination (R^2) of 0.9996. Since not all standards were available, this linear correlation was used as a tool to support the identification task performed.

The main reason why only the $LRI_{calc.}$ obtained for the apolar column is presented in Table S1 is the fact that polar columns present much higher LRI shifts due to the chemical nature of the phase and column manufacturing process, namely different levels of cross-linkage, acid and basic treated PEGs, and PEGs with different average molecular weights [35]. This can promote variations of the calculated LRI of more than 60 units for a 95% confidence in the RI size. In order to solve this drawback, the samples were submitted to a 1D separation, using a ToFMS system, in order to allow spectral deconvolution, and thus more selectivity in the detection unit (see Fig. 1).

3.2. 1D-GC–ToFMS

When 1D-GC–ToFMS was applied using a LECO Pegasus GC/ToFMS, a system with a high mass spectral acquisition rate (up to 500 spectra s^{-1}), the high mass spectral acquisition rate property, together with a high degree of spectral continuity across the chromatographic peaks, achieved by ToF analysers, allowed the detection/identification of 177 compounds. This was possible because the accurate representation of the ion ratios for any particular analyte through the peak, allowed the use of automatic peak detection and deconvolution algorithms to resolve chromatographic coelutions and extract the spectrum of each analyte. This spectral resolution was used to detect (peak find) and identify the coeluting peaks in the *Pinus* spp. samples. After processing the spectral deconvolution, a number of peaks in this sample were found to be coeluting. One coelution example is presented in Fig. 1, where the TIC only shows one defined peak. In spite of the peak apparent symmetry, the use of deconvolution algorithms allowed for the detection of four peaks coeluting, and the extraction of the individual spectra for identification purposes (Fig. S2). This supports that the TIC may be an insufficient indicator of all peaks present in the sample, showing an overall profile, because some peaks due to coelution may appear as a single one. By extracting their "unique" masses (m/z 146, 71, 121 and 150) together with the TIC, the presence of the coeluting peaks under the baseline of the TIC became visually evident. The intensity of m/z 146, 76 and 150 was magnified, to allow their observation on a similar scale with m/z 121 from the major peak, and the TIC baseline. The similarity of the deconvoluted spectra, after comparison with the NIST/Wiley spectral library (2000), was 765 for the dichlorobenzene isomer (peak 1), 838 for the 1.4-cineol (peak 2) and 910 for α-terpinene (peak 3). For peak 4, no valid identification was achieved with the NIST/Wiley spectral library [47] and it was, therefore, assigned as unknown.

The tentative identifications were made by comparing the obtained spectra with the spectra of NIST/Wiley mass spectra database libraries, through the NIST MS Search Program, provided that the mass spectral similarity was higher than 800. In exceptional situations where the match was less than 800 and standards were available, the identification was considered positive. This match shifting can be probably due to the analyte presence in trace amounts, reference mass spectra recorded with different instruments under various experimental conditions or to the quadrupole spectra generation from the database library [48]. The database library reference spectra when compared with the experimental ToFMS generated spectra may produce lower matches, apparently due to some shifts in m/z fragments relative intensity, as already observed by others [34,48]. The identification assignments were supported by the compound retention indices calculated using the van den Dool and Kratz equation. A good relationship was obtained ($R^2 = 0.999$) between linear retention indices reported in the literature [46] and the calculated ones for the compounds identified in the 1D-GC/ToFMS analysis and thus supporting the tentative identifications proposed (Fig. S3).

A set of needles from 10 pine species were analysed by 1D-GC/ToFMS, after HS-SPME, in order to study their individual composition. A 60 m length column, with a 1.0 μm d_f was used to perform the analysis. Table S2 presents the peak tentative identification achieved for the 10 *Pinus* spp., after HS-SPME-1D-GC/ToFMS analysis, with calculated and literature retention data [46]. A total of 177 volatile compounds were detected. From these, 55 were not considered to be identified mainly due to their low mass similarities with the assigned spectra from the NIST/Wiley database. However the extracted spectra for some of the non-identified compounds present characteristics diagnostic ions that allow their comparison with published mass spectra and thus at least a chemical family assignment could be performed. About 76% of the non-identified compounds are located in the sesquiterpene retention time region. The identification of the sesquiterpene compounds is rather difficult due to their structural similarities and lack of available standards, which when existing are sometimes expensive. The analytical conditions provided by the 60 m length column allowed for a "theoretically" better resolution and detection of the volatile compounds under study, but also promoted the elution of the sesquiterpene compounds to higher retention times, and consequently to higher temperatures. Diagnostic ions allowed to identify the chromatogram region where the sesquiterpene compound class began to appear and those diagnostic m/z fragments were 204, 189, 161, 147, 93, 81, and 67. The elution temperature of the first sesquiterpene is approximately 228 °C (48 min), with a 60 m length non-polar column, in opposition to the 126 °C and 140 °C achieved with the used 15 m length and 30 m length non-polar columns. As a possible consequence one can observe in the chromatograms a thermal degradation during the migration process across the column of the sesquiterpenes (results not shown). It may also be possible that some of these compounds can experiment some degradation during the injection step, where the fibre is submitted to 250 °C. Nevertheless, the resulted sesquiterpene spots band, which can be a dynamic process, promotes the overlapping of the compounds thus producing erroneous peaks detections by the processing algorithm, and consequently incorrect mass spectra are obtained which lead to problems in peak identification. Another effect of the high elution temperatures can be observed, for example, for the pair myrcene/β-pinene, that suffers from retention cross-over, when compared with the smaller length columns as one can observe through dRI/dT value calculated for the 60 m versus 15 m column, as an example, for myrcene = 0.020 and β-pinene = 0.82, reflects the finer structural features of the solutes and the stationary phases [49]. The correlation between the column temperature (Tc) and the retention factor (k') of an analyte can be simply seen as $\ln k' \propto 1/Tc$ (known as an Antoine-like derive equation of the van't Hoff relationship) [50–52], where k' is the analyte retention or capacity factor, and Tc is the absolute column temperature. The expression indicates that the analyte retention decreases logarithmically with the increase of column temperature. The retention cross-over phenomenon has been reported in polar and non-polar stationary phases by others [53–55] for compounds with different carbon skeleton, molecule geometry and functional groups, as exemplified in Fig. 2 using two different oper-

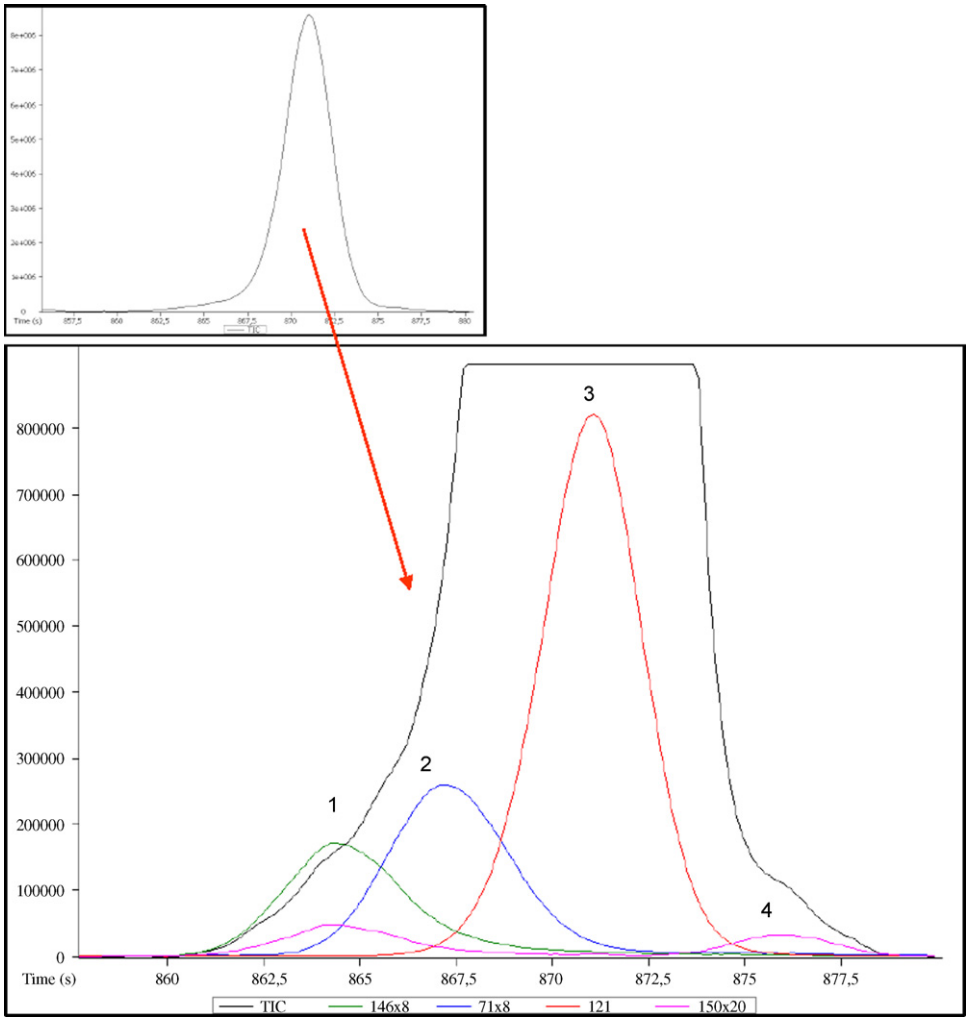

Fig. 1. Expanded view from an 18 s window of the *Pinus* spp. composite sample TIC, showing the deconvolution of four coeluting peaks. Peak identification: (1) dichlorobenzene isomer; (2) 1,4-cineol; (3) α-terpinene; (4) disulfite isopropyl. Analytical conditions see text.

ational conditions for column 1 (polar column) (under Section 2.6.3).

The shifts in the peak elution order on the apolar 60 m length column lead to difficulties in the assignment of the relationships between the experimental and the reference retention indices such as those published by Adams [46], which were obtained using a "standard" 30 m length column. Considering this, a different polarity columns (polyethylene glycol or 5% phenyl in polydimethylsiloxane column) were used in order to build 2D index retention maps with density probabilities aiming to assign probability regions or probability spots, within an interval of confidence <95% for compound identifications, taking into consideration the variations in the operational GC system, such as column characteristics, oven temperature gradients, flow conditions and column manufacture. A similar strategy helped to solve identifications problems as recently described by others for apolar and medium polarity stationary phases [56]. For the standards assayed, this allowed to define a probability region where a particular peak might be found, independently of the chromatographic system (polyethylene glycol or 5% phenyl in polydimethylsiloxane column) and operational conditions used. Fig. 3 shows the probability spots for some standard compounds. Having this in mind, the identification task can be enhanced since wrong identification assignments can be more effectively avoided. Nevertheless, a careful mass interpretation of the obtained mass spectra cannot be precluded.

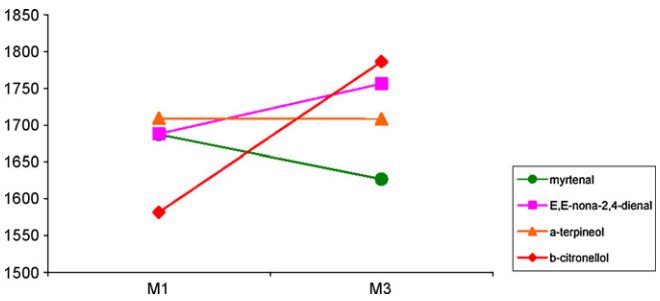

Fig. 2. Cross-over phenomenon for a polyethyleneglycol, 30 m × 0.32 mm × 0.50 μm, under two different operational conditions. See analytical conditions under Table 1.

3.3. GC × GC–ToFMS

3.3.1. *Pinus* spp. composite sample

The *Pinus* spp. composite sample was analyzed by comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry (GC × GC/ToFMS), and the data processed with a signal to noise (*S*/*N*) threshold value of 10, to maximize the detection of trace compounds. For data processing it was assumed that no spectral skew is verified during the acquisition of the mass spectra

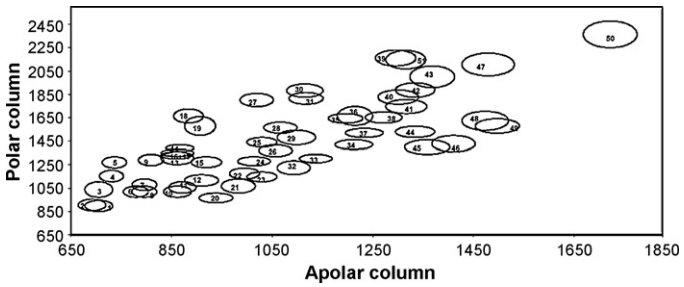

Fig. 3. Probability retention index regions for a mixture of compounds in polyethyleneglycol, and 5% phenyl in polydimethylsiloxilane columns under different analytical conditions. See details under Table 1. The peak identities are as follow: (1) 2-ethylfuran; (2) pentan-3-one; (3) pentan-2-ol: (4) 2-methylbutan-1-ol; (5) methyl lactate; (6) hexan-2-one; (7) 4-methylpent-3-en-2-one; (8) hexanal; (9) ethyl lactate; (10) 2-methyl-1-propyl propionate; (11) 2-methyl-1-buthyl acetate; (12) heptanal; (13) hexan-1-ol; (14) (Z)-hex-2-en-1-ol; (15) anisole; (16) (Z)-hex-3-en-1-ol; (17) (E)-hex-2-en-1-ol; (18) pentan-2,4-diol; (19) γ-butyrolactone; (20) α-pinene; (21) sabinene; (22) ethyl hexanoate; (23) limonene; (24) (E)-2-hexenyl acetate; (25) 2-ethylhexan-1-ol; (26) (E)-oct-2-enal; (27) hexanoic acid; (28) phenylethyl acetate; (29) nonan-2-ol; (30) heptanoic acid; (31) 1-phenylethanol; (32) terpinolene; (33) 2-octyl acetate; (34) 1-octyl acetate; (35) α-terpineol; (36) (E,E)-nona-2,4-dienal; (37) β-citronellol; (38) citral, E (geranial); (39) carvacrol; (40) whisky lactone (isomer 1); (41) (E,E)-deca-2,4-dienal; (42) whisky lactone (isomer 2); (43) γ-nonalactone; (44) methyl decanoate; (45) α-cubebene; (46) α-copaene; (47) γ-decanolactone; (48) α-humulene; (49) γ-gurjumene; (50) γ-dodecalactone; (51) γ-ethoxycarbonyl-γ-butyrolactone.

[34,57]. The column set used was a DB-5 versus BPX-50, according to the indicated on materials, with a modulation period of 5 s. The true peak finding, mass spectral deconvolution of coeluted analytes and library searching of the "true" mass spectra of individual peaks, against mass spectral database libraries, was done automatically by the ChromaTof software. The first results of the processed raw data resulted in the detection of over 2000 peaks. A preliminary analysis of the results showed that the majority of the peaks detected were baseline noise components, such as siloxanes/silanols from column phase bleeding, and artifact peaks from the modulation of the solvent peak tailing, together with hydrocarbons that probably come from the sample solvent. However, these interferences, due to the resolving power of the technique, were totally separated from the potential components of the essential oil, as already observed elsewhere for other matrices [34,57,58].

A reprocess of the sample at *S*/*N* of 50 resulted in the detection of 500 peaks. An additional processing was conducted in order to remove the siloxanes/silanols and the hydrocarbons that were still detected. The analysis of the remaining peaks led to the assignment of 200 analytes that were tentatively identified, including monoterpenes, oxygenated monoterpenes, sesquiterpenes, oxygenated sesquiterpenes, diterpenes, aldehydes, ketones and esters.

The detailed view of the terpene bands allow the visualization on the second dimension (separation upon polarity) of compounds that were coeluting in the first dimension (separation upon volatility). Fig. 4A presents, as an example, a 18 s section of the contour plot (TIC already shown in Fig. 1), showing one defined peak that hides a coelution of 4 compounds. On GC × GC (Fig. 4B), due to the different polarities of the analytes, they were separated on the second dimension with a higher chromatographic resolution than in 1D-GC.

Regarding peak 4, in Fig. 4A, it was not considered identified when the 1D-GC system was used, due to a low spectral match given by the library database search. In GC × GC, the peak 4 from the 1D-GC analysis, was shown to be composed of three individual compounds, peaks 4, 5 and 6, which are now separated on the second dimension. Compounds 4 and 5, on GC × GC, partially coelute on the first dimension, but are successfully deconvoluted and tentatively identified under the experimental conditions. Peak 5 is present at low concentration, but due to the improvement of its *S*/*N* ratio, a consequence of its re-focusing by the modulator, it achieved then a better separation from the system noise than on the 1D-GC, and a mass spectra record, which allowed its tentative identification. The similarities achieved by library database search were 956 for peak 1, 871 for peak 2, 895 for peak 3, 817 for peak 4, 856 for peak 6 and 430 for peak 5. A survey on the Adams data base

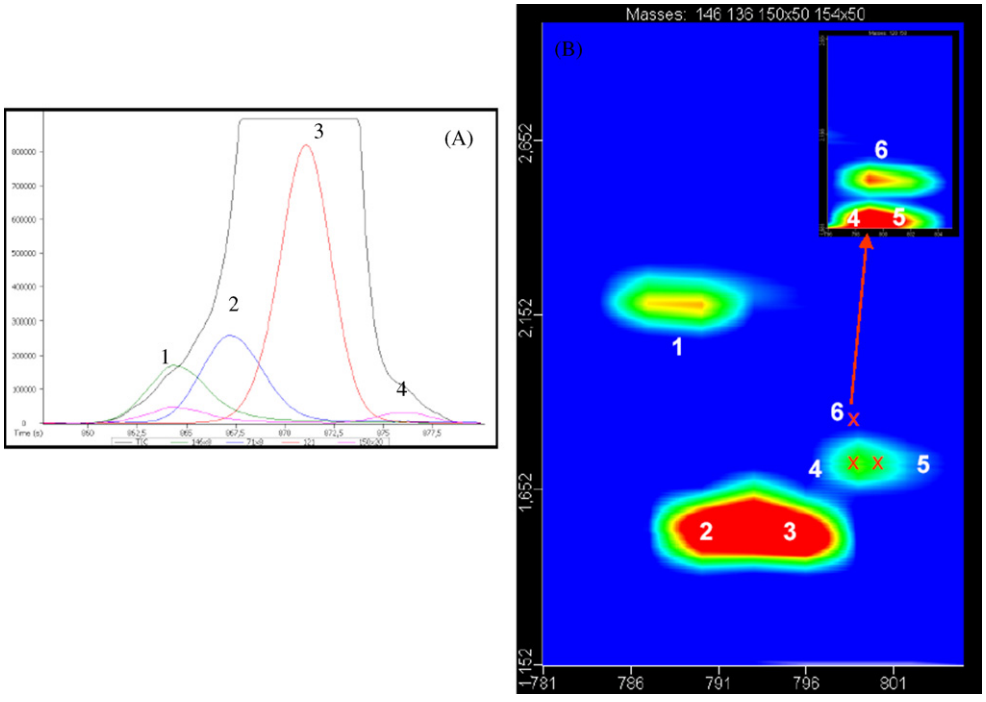

Fig. 4. Comparison of separation and detection of selected analytes from the *Pinus* spp. composite sample in two GC systems: (A) 1D-GC/ToFMS system and (B) GC × GC/ToFMS system. Marked compounds on (A): (1) dichlorobenzene isomer; (2) 1,4-cineol; (3) α-terpinene; (4) unknown. Marked compounds on (B): (1) dichlorobenzene isomer; (2) 1,4-cineol; (3) α-terpinene; (4) disulfite isopropyl; (5) *o*-cimene and (6) trimethylbenzene (pseudocumene).

[46] allowed the assignment of compounds identification with their retention index (RI): 1,4-dichlorobenzene (RI = 1014), 1,4-cineol (RI = 1015), α-terpinene (RI = 1017), cymene isomer (RI = 1025) and trimethylbenzene (RI = 1026). The isopropyl disulfite was not registered at Adams database [46]. Concerning peak 5, the hypothesis of a cymene isomer presence can be assigned, based on the Adams data [46] and the existence of cymene *m*/*z* 119 and 134 on the deconvoluted mass spectra, in spite of the low spectral similarity achieved for Wiley spectral database [59]. Another consequence of GC × GC is the increase of the signal to noise ratio (*S*/*N*) for all the analyte peaks. The increased *S*/*N*, together with the enhanced resolving power of GC × GC, maximizes the purity of the obtained mass spectra, allowing for a more accurate identification of the sample compounds. Fig. S4 presents the good correlation obtained (R^2 = 0.99965) between linear retention indices reported by Adams [46] and calculated retention index data for the compounds identified in the GC × GC/ToFMS analysis of *Pinus* spp. composite sample, in spite the fact that GC × GC LRI data are compared with 1D-GC data reported by Adams [46].

3.3.2. Enantioselective GC × GC–ToFMS

The enantiomers of chiral terpenes present in the volatile blends emitted by *Pinus* spp. should analytically be considered as individual components, due to their inherent potential role in insect–host plant relationships [60,61], and in the chemical discrimination of pine species [13]. The separation of enantiomeric monoterpenes by GC × GC analysis was performed with a tailor made fused silica capillary column 2,3-DiMe on the first dimension. The column was coupled to a polar column (Supelco-Wax). This chiral/polar column set was chosen taken in consideration the fact that an isothermal separation occurs in the second dimension column and poor selectivity might result if the second column was the enantioselective one, as discussed elsewhere [62].

In order to collect information and evaluate the set performance in GC × GC, a solution with a mixture of 43 standards (Table 2) was analysed before the *Pinus* samples.

Fig. 5 shows the reconstructed first dimension TIC and the correspondent enantioselective GC × GC/ToFMS contour plot obtained for the test mixture. The first view of the elution patterns from the test mixture components, on the two-dimensional plot, shows

Table 2
Chemical standards used in the evaluation of the column set used for enantiomeric GC × GC/ToFMS analysis.

Peak no.	Standard	Peak no.	Standard
1	Isoamyl alcohol	23	(+)-β-Phellandrene
2	*trans*-2-Hexenal	24	γ-Terpinene
3	(−)-α-Pinene	25	Terpinolene
4	(+)-α-Pinene	26	Octan-1-ol
5	Hexan-1-ol	27	Myrtenal
6	*cis*-3-Hexen-1-ol (leaf alcohol)	28	(−)-Verbenone
7	(−)-Camphene	29	(+)-Verbenone
8	(+)-Camphene	30	(−)-α-Terpineol
9	Myrcene	31	γ-Terpineol
10	Sabinene	32	(+)-α-Terpineol
11	(+)-β-Pinene	39	(+)-Calarene
12	*cis*-Hex-2-en-1-ol	40	*trans*-Caryophyllene
13	(−)-β-Pinene	41	α-Caryophyllene
14	α-Phellandrene	42	(+)-Aromadendrene
15	3-Carene	43	(+)-γ-Gurjunene
16	α-Terpinene	33	Citronellol
17	Hex-2-en-1-ol acetate	34	Geraniol
18	1,8-Cineol (Eucalyptol)	35	(−)-α-Cubebene
19	*p*-Cymene	36	Terpinyl acetate
20	(−)-Limonene	37	(−)-α-Copaene
21	(−)-β-Phellandrene	38	(−)-α-Cedrene
22	(+)-Limonene		

that they are separated according to their volatility and chiral discrimination on the first dimension, and according to their polarity on the second dimension. Coelutions of some compounds are still observed on the first dimension.

Monoterpenes (zone I) and sesquiterpenes (peaks 35 and 37–43) form clusters, and the same happens for the alcohols and aldehydes (peaks 1, 2, 5, 6 and 12) on the top of second dimension. The oxygenated monoterpenes are scattered in the second dimension with the verbenone enantiomers, citronellol and geraniol eluting on the second dimension after the modulation period and thus suffering wrap-around effect (Fig. 5). On the bottom right corner of Fig. 5, an expanded view from zone II is represented, showing the separation of α-terpineol enantiomers (peaks 30 and 31). The enantiomers of α-terpineol, with identical polarity, did not have the same second dimension retention time. The last eluted enantiomer shows a lower retention time on the second dimension. As the chromato-

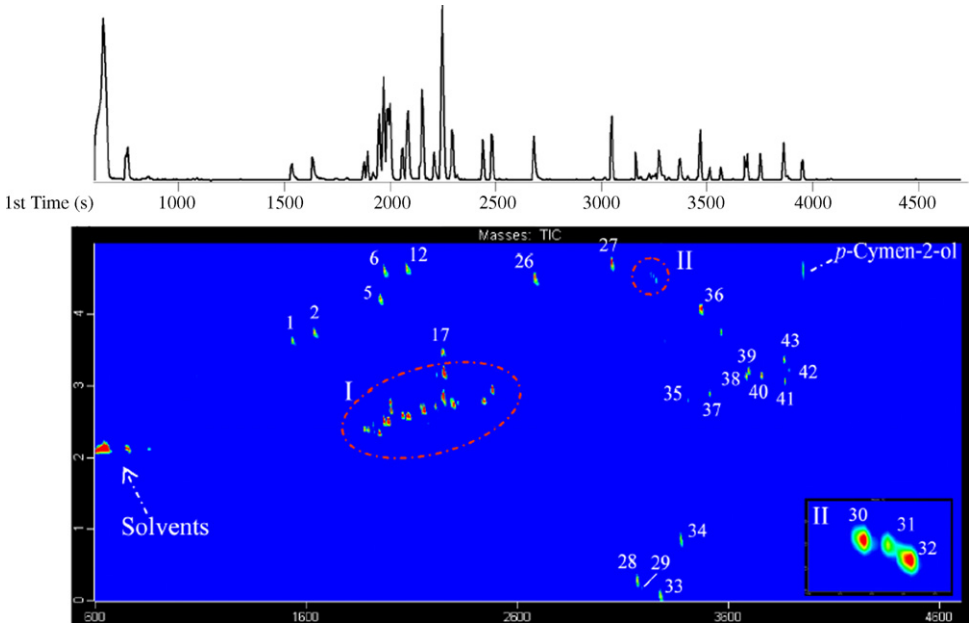

Fig. 5. Reconstructed first dimension TIC and the correspondent enantioselective GC × GC/ToFMS contour plot obtained for the test mixture of Table 2. Zone II was magnified on the left bottom corner of the contour plot.

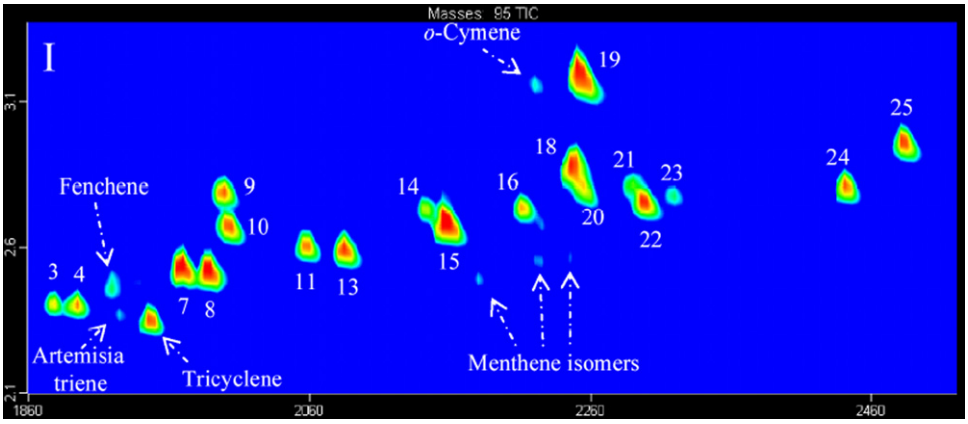

Fig. 6. Expanded enantioselective GC × GC/ToFMS contour plot of zone I of Fig. 5.

graphic analysis was performed with a temperature programmed ramp, the second enantiomer will elute at a slightly higher elution temperature and thus its second dimension retention time will be lower than that of its pair. This observation is expected to be observed in all enantiomeric pairs and consequently it will be used as support for enantiomer pair detection.

Fig. 6 shows an expanded view of zone I of Fig. 5, which comprises the monoterpenes.

In Fig. 6, the peak of tricyclene comes from the standard of camphene which has 10% of tricyclene. The fenchene, artemisia triene and the menthenes are impurities of the standards. The enantiomeric pairs of α-pinene, camphene, β-pinene, limonene and β-phellandrene are effectively separated on the first dimension. However, coelutions can be observed on the first dimension. One of the enantiomer of sabinene (peak 10) is coeluting with myrcene (peak 9) making not possible their evaluation in the 1D-analysis. Other coelutions or partial coelutions on the first dimension can be observed for (−)-limonene (peak 20), *p*-cymene (peak 19) and 1,8-cineol (peak 18). Those coelutions are resolved on the second dimension. Fig. 7 shows an expansion of the plot area that comprises the limonene enantiomers. The peaks 17–20 and one of the menthene isomers are coeluting on the first dimension. By GC × GC these compounds are separated on the second dimension, by their polarity differences. As expected, the second enantiomer from the enantiomeric pairs of limonene (peaks 20 and 22), and β-phellandrene (peaks 21 and 23), are eluting at a lower retention time on the second dimension. Additionally, the increase of sensitivity, due to modulation, also allows for the detection of *trans*-ocimene and 1,3,4-trimethylbenzene, that is also coeluting with the peaks 17–20.

Fig. S5B shows the 3D plot of the expanded area of Fig. 7, where the reconstructed first dimension chromatogram only presents two peaks in opposition to the 2D and 3D plots. A section of the modulated chromatogram with the two peaks is presented on Fig. S5A, where different colours represent the distribution of the individual extracted ions and consequently of the coeluting compounds. Fig. S5C presents the second dimension chromatogram obtained for the major modulated peak from peak 1 ($^1t_R = 2250$ s) showing its separation into five individual peaks.

The results of the analysis with the 43 standards show that even samples with a small number of components can present coelutions, which may be difficult to solve using conventional one-dimensional chromatography. In spite of the first enantiomeric column be the key tool for the proposed analysis, its assemblage with the polar column added more resolving power to the analysis, promoting the separation of overlapped compounds and thus making the used set suitable for the characterization of the enantiomeric volatile components of *Pinus* spp.

3.4. Characterization of the volatile fraction of Pinus spp.

A total of 10 pine species collected from Abrantes experimental plot, in the summer months, were analysed using enantioselective GC × GC–ToF/MS.

Fig. 8 shows, as an example, the 2D plot of an enantio-GC × GC/ToFMS analysis obtained from a *P. pinaster* sample. The signals in the contour plot are magnified in order to visualize the minor components present in the sample. Each spot on the images

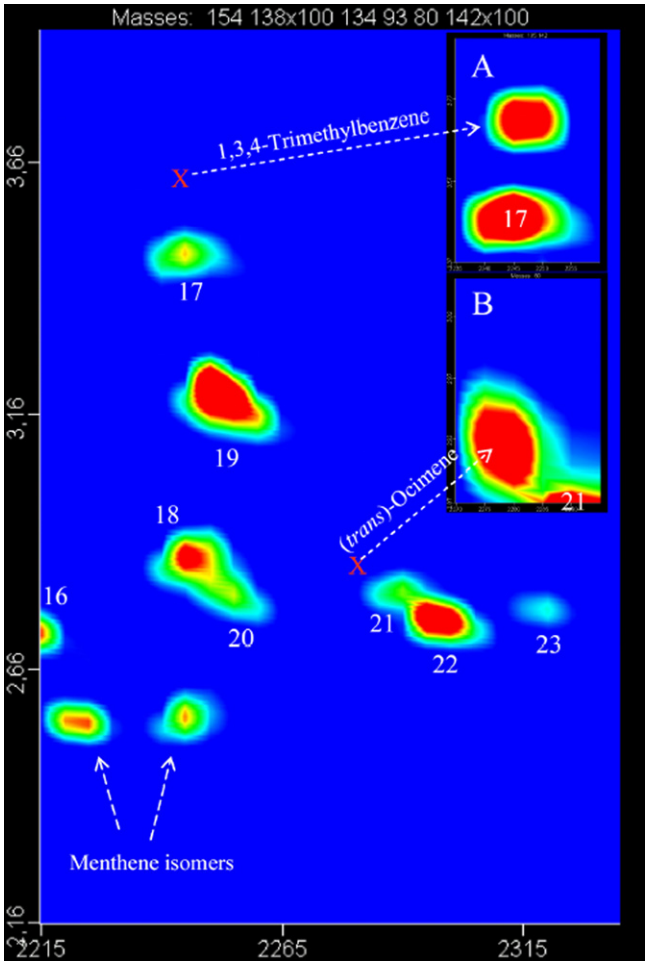

Fig. 7. Expanded enantioselective GC × GC/ToFMS contour plot of Fig. 6 showing the range of the limonene enantiomers. (A) Expanded view of the area of peak 17 and 1,2,4-trimethylbenzene; (B) expanded view of the area of peak 21 and *trans*-ocimene.

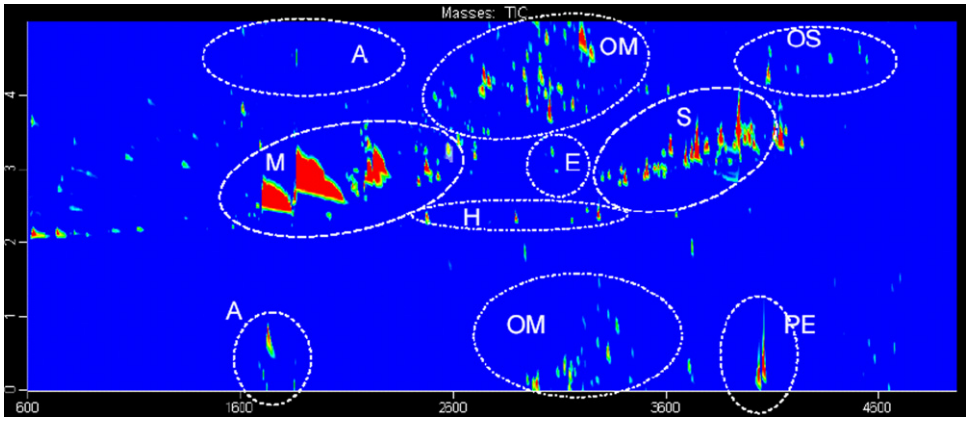

Fig. 8. Enantio-GC × GC/ToFMS total ion current chromatogram (TIC) data contour plot from a *P. Pinaster* sample, showing the distribution of classes of compounds in different regions of the chromatographic space: (A) linear alcohols; (M) monoterpenes; (OM) oxygenated monoterpenes; (H) hydrocarbons; (S) sesquiterpenes; (OS) oxygenated sesquiterpenes; (PE) phenyls esters; (E) esters. The signal is magnified in order to allow visualization of the minor compounds.

represents an individual compound, for which a full mass spectrum was available. The contour plot from *P. pinaster* is rather complex due to the number of components present. However, the cluster of volatile compounds classes of can be observed, distributed through the plot producing a structured chromatogram. It is also observable the higher retention times of the oxygenated compounds on the second dimension, due to their interaction with the polar column. Oxygenated monoterpenes (alcohols and aldehydes) (OM) and linear alcohols were divided in two regions due to wrap-around effect.

The phenyl esters (PE), that have a high polarity, appear in a lower retention time of the second dimension, due to wrap-around effect (Fig. 8). This effect may promote a decrease in the chromatographic resolution, since overlap of the analytes may occur. However, in this work, the observed wrap-around represents just a visual "inconvenience", since it did not affect the separation and identification of the compounds. The more strongly retained components, responsible for the wrap-around, did not overlap peaks that were weakly retained in the subsequent modulation, and thus the only consequence was the maximization of the separation space with the filling of the lower region of the 2D plot.

Fig. S6 shows an expansion of the central area of the contour plot, where the oxygenated monoterpenes are located, together with its reconstructed first dimension chromatogram, in order to illustrate the complexity of the sample under analysis, and the number of potential coelutions that occur on the first dimension. Fig. S7 shows a 3D expanded view of the highlighted section of the contour plot shown in Fig. S6, illustrating the good chromatographic resolution obtained, where it is possible to observe the occupation of the chromatographic space by the analytes, and thus allowing for high-quality spectral data to be produced. For sake of clarity, the peaks are presented according to Table S3 (column set 3, operational conditions described in Section 2.5).

Table S3 presents the results obtained for all pine species, with the compound retention times in both dimensions, and retention indices calculated according to the van den Dool and Kratz equation. As the first dimension column is a 5%-phenyl-methylpolysiloxane, similar to the DB-5 columns used by Adams and others, with a β-cyclodextrin phase imbibed on, the retention index from Adams and the literature are also presented. These literature retention indexes were used as an indicative of the elution order of the volatile compounds present in the samples. The enantio-GC × GC analysis resulted in 422 volatile compounds detected in the 10 pine species. 54 compounds were not identified, comprising 23 sesquiterpenes, 16 oxi-terpenes, 6 diterpenes, 2 monoterpene acetates, 1 phenylethyl ester and 6 unknowns. With the exception of the unknowns, all the others presented library matches, that present mass spectra similarities higher than 800 (e.g. non-identified sesquiterpenes) with similar compounds. Most of the 368 peak identifications reported are tentative identifications. However, all peaks indicated in more than one pine species were simultaneously found in the samples. The highest similarity value found for a compound, among the pine species, is also registered in Table S3.

4. Conclusions

The GC × GC showed to be a powerful technique for the analysis of the chemical components of the needles of the pine species, considering both the volatile and non-volatile fractions. The use of GC × GC resulted in enhanced separation efficiency and in an increased signal to noise ratio (*S*/*N*) from the analyte peaks, which maximizes the purity of the mass spectra obtained, making possible a more accurate tentative identification of the pine needles components. The analysis of the mass spectra data, together with the analytes retention times and their positioning in the 2D plot, allowed for the tentative identification of more than 70% of the detected compounds. The mass spectra obtained by GC × GC showed a better quality than those obtained by 1D-GC/ToFMS, providing higher match library factors with the NIST/Wiley searchable libraries, and thus a stronger identification of the compounds present in the samples than by one-dimensional GC analysis. In spite of the higher mass spectral quality achieved, the mass spectra of several chromatographic peaks did not match either any of the reference spectra, or similar spectra which were not consistent with their retention index. These findings show the limitation of the spectral libraries and databases presently available, and highlight the need for the establishment of more comprehensive mass spectral libraries and databases, that may allow for complete identifications, especially in view of new technologies like GC × GC, leading to a new level of analytical information. Those libraries should also include retention index probability regions for, at least, two different polarity columns in order to correlate the spectral match result and literature retention index independent of the operational system used.

For the volatile analysis, the less robust results were achieved when using the non-polar column with 60 m, due to analyte retentions cross-over and to the thermal degradation of some sesquiterpenes, which promoted the finding of some false positive peaks by the software on 1D-GC/ToFMS. However, on GC × GC using the same column in a set with a polar column, the degradation

bands were separated from the analytes, reinforcing the separation power of this technique.

Finally, the results obtained constitute a first contribution to the detailed knowledge of the chemical composition of the needles of the important *Pinus* spp. Comparing with previous literature, the GC × GC/ToFMS allow to confirm, in a single run, the identity of previously detected compounds [37–43] together with new compounds, comparing to 1D-GC/MS systems. This may be an important achievement in chemical ecology, where insect host specialization probably allows for the detection of host volatile compounds at trace level. Further research in this topic in ongoing.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2010.01.045.

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