



From plant to soil: Quantitative changes in pine and juniper extractive compounds at different transformation stages

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Abstract

Purpose The transformation of extractable plant compounds after their incorporation into soil was qualitatively and quantitatively studied in two forests under *Juniperus communis* L. and *Pinus sylvestris* L.

Methods Leaf, litter and soil samples were taken from representative pine and juniper forests in central Spain. The lipid fraction was extracted with dichloromethane, while methanol was used for polar compounds, which were then derivatized (silylation-oxidation). Extracts were analyzed by gas chromatography-mass spectrometry. van Krevelen's graphical-statistical method, enhanced as surface density

maps, was used to study changes in molecular assemblages during their transformation from plant to soil. Shannon Wiener diversity indices were also determined for the main groups of molecules to quantify the progressive removal or the appearance of new compounds throughout the transformation.

Results In the lipid fraction up to 126 compounds were identified, mainly alkanes (C₁₀–C₃₀ in pine forest and C₁₀–C₃₆ in juniper forest), fatty acids and cyclic compounds. In the polar extracts, up to 22 compounds were found, mainly sugars, polyols, cyclic acids and fatty acids.

Conclusion Comparing the successive stages of evolution of leaf extractive compounds, alkanolic acids and disaccharides tend to accumulate in the soil. On the other hand, the greatest molecular complexity was found in the intermediate stage (litter), and attributed to the coexistence of biogenic compounds with their transformation products, while the molecular complexity was simpler in soil extracts. This preliminary investigation could be extended to specific studies on the factors that determine the quality of soil organic matter under different environmental scenarios.

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Introduction

Soil organic matter consists of a complex mixture of very different compounds in terms of molecular weight, chemical structure and physical properties, which derive from the degradation of lignocellulosic plant biomass accumulated in the litter layer (Tu et al. 2017), or are produced from the heterotrophic activity of mesofauna, bacteria and fungi in soil and on its surface (de Nobili et al. 2020; Spaccini and Piccolo, 2007). Although free organic compounds of low molecular weight are minor components of soils (from 0.2 to 4% of total carbon), this fraction may exert an important influence on many environmental processes, including allelopathic interactions and the effect on soil physico-chemical properties (El-Ghorab et al. 2008).

The soil lipid fraction is a heterogeneous mixture that includes a wide variety of compounds soluble in organic solvents of low to medium polarity, including complex condensed non-volatile components and simpler functional classes such as hydrocarbons, fatty acids, wax esters, ketones, hydroxyl acids, terpenoids, steroids, acylglycerols, phospholipids, lipopolysaccharides, etc. (Dinel et al. 1990). These compounds fulfill crucial functions in the ecosystem, such as forming hydrophobic coatings on the surface of soil aggregates, turning them more hydrophobic and preventing their physical disruption and hindering the biodegradation of the encapsulated organic matter preserved within these soil microcompartments (Jambu et al. 1995). On the other hand, soil lipids could be considered a molecular record of diagnostic compounds that provide valuable biogeochemical information on the structure and dynamics of ecosystems (Aldana et al. 2020; Angst et al. 2016; Couvillion et al. 2020).

Other important low molecular weight compounds, which have been poorly studied and are found in soils associated with lipid molecules, are soluble low molecular weight carbohydrates (LMWC). Although plants represent the main source of these compounds in soils, microorganisms (fungi, bacteria, algae) and edaphic fauna also contribute to a lesser extent. In fact, these compounds also influence structure, chemical processes, plant nutrition and microbial activity of soils (Medeiros et al. 2006).

In any case, basic and applied research on the transformation processes of soil organic matter are

of crucial importance to understand the mechanisms of carbon sequestration in the soil, as well as for the early diagnosis of soil degradation and the evaluation of the environmental impact (Jiménez-González et al. 2020) and the benefits of organic matter for soil resilience and productivity (Cotrufo and Lavelle 2022).

However, the different processes involved in carbon stabilization have always been highly controversial, as no agreement has been reached to establish the relative importance of each of the different mechanisms (Schmidt et al. 2011; Lehmann and Kleber, 2015; Almendros et al. 2018; de Nobili et al. 2020). In this context, the analysis of molecular assemblages of soil components, including free lipids and soluble LMWC, can present very useful information, since it constitutes a biogeochemical record of biomarker compounds that provide information about organisms and processes involved in the transformation of organic matter (Tinoco et al. 2018). In particular, the study of the evolution of lipids and soluble LMWC from the plant to the soil would make it possible to assess to what extent the different types of soil present a different activity in terms of modifying the chemical composition of their biomass. This would constitute an objective molecular criterion to define the maturity of the humus.

Free lipids and LMWC can be extracted from the soil using appropriate organic solvents. In the case of lipids, hexane, ethyl ether, dichloromethane or its admixtures have been frequently used (Stevenson 1982), whereas polar solvents, such as methanol, ethanol or water are used for the extraction of carbohydrates (Mena-García et al. 2019). Obviously, the chemical nature of the extracted compounds will vary with the polarity of the solvent system and the physical conditions used for the extraction (Bull et al. 2000; White et al. 2009).

The analysis of compounds present in the free lipid and LMWC fractions has been carried out mainly by gas chromatography coupled to mass spectrometry (GC–MS) (Almendros et al. 1996; Bull et al. 2000; de Blas et al. 2013; Tinoco et al. 2018). This technique provides enough sensitivity and potential for structural identification in qualitative and quantitative analyses of these complex mixtures. However, the accurate identification of individual lipid and carbohydrate molecules is not straightforward. Regarding the quantitative analysis, previous exploratory studies have been

based on relative peak area integration values of each compound as regards the total chromatographic area (Almendros et al. 1996; de Blas et al. 2013; Tinoco et al. 2018), so a reliable quantitative analysis of these compounds has not yet been performed.

From a molecular point of view, the quality of the soil organic matter is defined by the extent to which the composition of the forest biomass turns into complex, humified organic matter by the action of biological activity, local and environmental factors resulting in the selective preservation of certain compounds, the transformation of biogenic precursor compounds and the incorporation of new products of microbial synthesis. Depending on the type of vegetation, the local factors and the successive stages of transformation, these processes would lead either to an increase in molecular diversity or to a simplification of its composition due to the preferential accumulation of specific structures that survive biodegradation. In any case, monitoring progressive changes over time in soil molecular assemblages would facilitate understanding the fate of biomass in different soil compartments. In fact, changes in the diversity patterns of the molecular assemblages of extractive compounds in soils have been an object of research for the recognition of source indicators and molecular tracers for environmental impacts on ecosystems (Eglington et al. 1962a, b).

Thus, the main objective of this work was to monitor the molecular transformations that occur in the soil during the processes of biodegradation and humification of organic matter in two Mediterranean forests in central Spain (*Juniperus communis* L. and *Pinus sylvestris* L.). These forests were selected taking into account their wide distribution throughout the world (Adams 2008; Eilmann et al. 2006) and the typical presence of slowly biodegradable terpene compounds, which retain the basic skeletal structures of their biological precursors and tend to accumulate in the soil as biomarker compounds. In particular, the study was oriented to the qualitative and quantitative GC–MS analysis of free lipids and LMWC in needles, litter and soils of these two forests for the establishment of molecular indicators to recognize the stage of evolution of the organic matter of soil and thus objectively define its quality and maturity.

Materials and methods

Samples

Samples (needles, litter and soils) were collected from forest ecosystems of *J. communis* L. (common juniper) and *P. sylvestris* L. (Scots pine) in El Espinar (Segovia, Central Spain) (N 40° 43.759', W 04° 11.099' at 1260 m and N 40° 43.527', W 04° 10.618' at 1330 m, respectively). Leafy twigs were cut from the plants, while soil samples were collected from the uppermost 6 cm after removing the litter layer. Sampling was carried out at three different positions of each forest, separated at least 100 m from each other. The subsamples were aggregated and homogenized to obtain representative pools of each species. All samples were air-dried before the extraction procedure. In the case of soil samples, the large aggregates were crushed with a wooden roller, the fragments of rocks and roots were removed by hand and the resulting soil material was sieved through a 2 mm mesh (fine earth).

Analytical standards

Analytical standards of phenolic compounds such as acenaphthenequinone, benzil, 1-phenyl-1-butanol, perylene and phenanthrene (used as internal standards for non-polar compounds) and phenyl- β -D-glucoside (used for polar compounds) were acquired from Sigma Chemical Co. (St. Louis, US). Carbohydrate standards of polyalcohols (*chiro*-inositol, mannitol, *muco*-inositol, *myo*-inositol and pinitol), monosaccharides (fructose and glucose) and disaccharides (sucrose and trehalose) were also obtained from Sigma Chemical Co. Sequoyitol was purchased from Extrasynthese (Genay, France) and quebrachitol from Acros (Geel, Belgium). Methyl-1-*muco*-inositol was obtained from a honey sample as indicated by Sanz et al. (2004).

Extraction procedure

Needles were ground with a grinder, while the soil and litter samples were ground in an agate mortar to obtain a fine and homogeneous powder. The samples were sieved through a 500 μ m mesh and stored in glass flasks under dry conditions and protected from direct light until analysis.

To obtain the non-polar extracts, 0.3 g of leaf powder and 2 g of litter and soil powder samples were suspended in 6 mL of dichloromethane; 20 μL of internal standard (acenaphthenequinone, benzil, 1-phenyl-1-butanol, perylene or phenanthrene) solution (1.6 mg mL^{-1}) were added to the sample solutions. Then, the suspensions were ultrasonicated for 30 min in a bath. The collected extracts were filtered using silanized glass wool and centrifuged at 4400 g for 5 min. The extracts were evaporated under vacuum (miVac Duo concentrator, Genevac TM, Ipswich, UK) at 37 °C until complete dryness and redissolved in 1 mL of dichloromethane.

The polar extracts were obtained from needle (0.3 g), litter and soil (2 g each) powder using methanol (6 mL) as extraction solvent. Ultrasound was applied for 30 min in order to enhance the extraction of the compounds. The samples were then allowed to stand for 30 min at room temperature. Extracts were filtered through Whatman No. 4 paper and centrifuged at 4400 g for 5 min. The extracts were evaporated under vacuum and redissolved in 2 mL of methanol.

Derivatization procedure

Prior to the GC–MS analysis of the polar extracts, a two-step derivatization was carried out as indicated by Ruiz-Matute et al. (2007). This two-step derivatization procedure (oximation+silylation) gave two peaks for reducing carbohydrates, corresponding to the *syn* (*E*) and *anti* (*Z*) forms, and a single peak for non-reducing sugars and inositols, corresponding to the *O*-persilylated derivative. Although it is a two step derivatization procedure, it is easy to be developed, resulting spectra give high structural information and derivatives are as volatile and much more stable than trimethylsilyl derivatives.

Samples were prepared by mixing methanol extracts (0.5 mL) with 0.1 mL of a 70% ethanol solution of phenyl- β -*D*-glucoside (1 mg mL^{-1}), which was used as internal standard. Samples were evaporated under vacuum and treated with 350 μL of 2.5% hydroxylamine chloride in pyridine at 75 °C for 30 min. Then, 350 μL of hexamethyldisilazane (HMDS) and 35 μL of trifluoroacetic acid (TFA) were added and kept at 45 °C for 30 min. The samples were centrifuged at 7000 g for 5 min at 5 °C,

and 1 μL of the supernatant was injected onto the GC column.

GC–MS analysis

Instrumental analysis of the extracts was carried out in a 6890A gas chromatograph coupled to a 5973 quadrupole mass detector (both from Agilent Technologies, Palo Alto, CA, USA), using He as carrier gas. A ZB-1MS (cross-linked methyl silicone) column (30 m \times 0.25 mm i.d.; 0.25 μm film thickness) from Phenomenex (Torrance, CA) was used.

For dichloromethane extracts, the oven was kept at 70 °C during 1.5 min, then heated to 290 °C at 6 °C min^{-1} and kept for 20 min (total analysis time of 58 min). Injections were carried out in splitless mode (1 min) at 275 °C. For the methanol extracts, the oven temperature was programmed from 120 °C to 300 °C at 5 °C min^{-1} and kept for 20 min (total analysis of 56 min). The injections were carried out in split mode (1:50) at 300 °C. The transfer line and the ionization source were thermostated at 280 and 230 °C, respectively. Mass spectra were recorded in electron impact (EI) mode at 70 eV within the mass range m/z 35–650. Data acquisition was performed using MSD ChemStation software (Agilent Technologies).

The identification of compounds in non-polar extracts was based on the comparison of experimental linear retention indices (I^T) and mass spectra with those reported on the literature (Adams 2007) and available databases (Wiley, NIST). Compounds of polar extracts were identified by comparing I^T and mass spectra with those of corresponding commercial standards described above. Compounds for which commercial standards were not available were tentatively identified based on their mass spectral information.

Quantitation was carried out by the internal standard method. Standard solutions of target compounds over the expected concentration range in the samples under study were prepared to calculate the response factor relative to internal standard. Phenyl- β -*D*-glucoside was selected as internal standard for polar extracts. The concentrations of compounds for which commercial standards were not available were estimated assuming a response factor equal to 1. Regarding non-polar compounds, several internal standards, chemically similar to the compounds to be analyzed but not expected to occur naturally in the samples

and eluting in different regions of the chromatogram, were evaluated: i.e., 1-phenyl-1-butanol, phenanthrene, benzil, acenaphthenequinone and perylene. Inter-day precision and the limit of detection (*LOD*) and the limit of quantitation (*LOQ*) were evaluated using these internal standards and considering specific compounds representative for each group of analytes investigated (*n*-nonacosane (C_{29}), hexadecanoic acid (C_{16}), α -thujene, caryophyllene and abietatriene). According to Foley and Dorsey (1984), the *LOD* was calculated as three times the signal to noise ratio (*S/N*), where *N* was five times the standard deviation of the noise, whereas the *LOQ* was considered ten times this ratio. Inter-day precision (relative standard deviation, *RSD*) for the above specific compounds was calculated from the results obtained for a leaf extract analyzed on five different days. All analyses were carried out in triplicate.

Data analysis and processing

To express the changes in the molecular complexity of the lipid compound assemblages, the Shannon–Wiener (*H'*) diversity indices were calculated for the different groups (alkanes, fatty acids, monoterpenes, sesquiterpenes and diterpenes). This index could illustrate the extent to which molecules are synthesized, transformed or degraded in the plant-soil system, and was calculated using the program Species Diversity & Richness (Seaby and Henderson 2006).

Van Krevelen plots

The representation of the quantitative data of the different molecules was carried out using a graphical-statistical approach based on the classic van Krevelen diagram (van Krevelen 1950). This procedure has proven useful in simplifying the interpretation of complex mixtures of compounds released by different analytical methods (Ikeya et al. 2015; Jiménez-Morillo et al. 2016; Ohno et al. 2014). The improved approach used consisted of plotting “surface density plots” constructed from the abundances of individual lipid compounds represented in the space defined by their *H/C* and *O/C* atomic ratios (Almendros et al. 2018). The scores for atomic *O/C* and *H/C* ratios of the molecules are plotted in the basal plane (*x,y* axes) and the vertical dimension (*z* axis) represents the normalized abundances (sum = 100) of the quantitative

data of individual compounds (Tables 1, 2, and 3). The resulting plot shows a series of 3D peaks or compound clusters, whose sizes are proportional to the collective abundances of the compounds with similar elemental composition.

Results

Analysis of non-polar extracts

Figure 1 shows the GC–MS profiles of dichloromethane extracts from needles, litter and soils from samples collected from of *J. communis* and *P. sylvestris* forests, respectively. In general, complex chromatographic profiles were observed, in particular for leaves and litter extracts. Prior to the identification and quantitation of lipids in the samples, a selection of appropriate internal standards was required. Taking into account the complexity of the samples, co-elutions of phenanthrene and acenaphthenequinone with compounds naturally present in the samples were observed; therefore, the use of these standards was discarded. Perylene eluted in a clean zone of the chromatogram, however, non-reproducible results were obtained when used as internal standard (*RSD*: 28–170%). Therefore, 1-phenyl-butanol and benzil were selected for the quantitative analysis of free lipids in the samples (*RSD* < 25%). *LOD* and *LOQ* on average were $1.3 \mu\text{g g}^{-1}$ and $4.8 \mu\text{g g}^{-1}$, respectively.

Table 1 shows the concentrations ($\mu\text{g g}^{-1}$ dry sample) of lipids found in the needles, litter and soil of pine and juniper forests. In these samples, up to 126 compounds were identified mainly to alkanes, fatty acids and cyclic compounds (terpenoids and steroids). Alkyl series, including alcohols, ketones, etc., were also detected, although in much lower concentrations, so these compounds were indicated in Table 1 as “other compounds”. Needle extracts showed the greatest lipid concentrations for both juniper (3.2 mg g^{-1}) and pine (5.6 mg g^{-1}). Only 28 free lipid compounds were found in pine soils; this sample also showed the lowest lipid concentration ($65 \mu\text{g g}^{-1}$). In general, among the different lipids detected, terpenoids were the most abundant in needles and litter of both juniper and pine, while alkanes were the predominant family in soils. Overall, monoterpenes were less abundant than sesquiterpenes and diterpenes.

Table 1 Concentration ($\mu\text{g g}^{-1}$) of lipids identified in dichloromethane extracts obtained from needles, litter and soils from forests of *Juniperus communis* and *Pinus sylvestris*

Analyte ^d		I^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>		
			Needles	Litter	Soil	Needles	Litter	Soil
Alkanes	Branched decane	998	16.2 (5.2) ^a	2.6 (2.3)	8.1 (1.3)	19.9 (21.6)	3.1 (0.5)	2.4 (0.5)
	Decane	1003	37.1 (25.6)	11.9 (0.8)	66.0 (53.4)	43.9 (40.1)	6.9 (1.5)	4.6 (1.0)
	Branched undecane	1093	17.6 (6.7)	4.5 (0.1)	6.7 (1.0)	20.1 (21.1)	3.4 (0.6)	2.9 (0.8)
	Undecane	1096	44.2 (32.3)	14.1 (0.1)	72.0 (52.5)	49.4 (42.8)	7.7 (1.9)	4.9 (1.2)
	Dodecane	1198	2.1 (1.0)	0.8 (0.1)	1.0 (0.3)	tr ^b	–	–
	Tridecane	1301	–	–	1.7 (0.1)	3.9 (0.1)	0.6 (0.1)	–
	Tetradecane	1403	–	–	–	15.1 (0.5)	0.41 (0.05)	–
	Pentadecane	1502	1.2 (0.5)	–	0.9 (0.2)	3.6 (2.9)	–	–
	Hexadecane	1600	–	–	1.3 (0.1)	3.1 (1.1)	–	–
	Heptadecane	1700	–	–	1.1 (0.1)	–	–	–
	Octadecane	1800	tr	–	1.0 (0.4)	–	0.4 (0.02)	–
	Nonadecane	1900	–	–	0.7 (0.3)	–	–	–
	Eicosane	2000	–	–	0.7 (0.3)	–	0.52 (0.01)	0.2 (0.1)
	Heneicosane	2101	3.8 (0.2)	3.2 (0.8)	0.71 (0.01)	–	2.6 (0.3)	0.5 (0.1)
	Docosane	2200	–	–	–	–	–	0.4 (0.1)
	Tricosane	2300	–	0.7 (0.1)	3.8 (1.6)	–	2.2 (0.2)	0.6 (0.1)
	Pentacosane	2501	–	1.2 (0.5)	2.3 (0.2)	4.6 (0.7)	1.9 (0.2)	1.3 (0.4)
	Hexacosane	2601	–	–	1.5 (0.1)	–	–	–
	Heptacosane	2700	–	2.1 (0.6)	4.32 (0.42)	7.8 (1.5)	3.8 (1.0)	2.7 (1.5)
	Octacosane	2799	–	–	1.6 (0.7)	tr	0.9 (0.1)	0.7 (0.3)
	Nonacosane	2897	11.4 (3.0)	4.2 (1.2)	13.8 (0.7)	6.8 (1.6)	10.7 (2.6)	11.1 (1.5)
	Triacontane	3001	–	0.8 (0.1)	1.10 (0.01)	tr	0.8 (0.1)	0.7 (0.6)
	Branched hentriacontane	3069	7.5 (1.8)	2.3 (0.4)	6.4 (4.2)	–	–	–
Hentriacontane	3099	52.6 (25.9)	14.6 (5.1)	10.4 (0.4)	238.0 (120.3)	15.2 (6.2)	13.1 (10.1)	
Dotriacontane	3199	13.8 (7.6)	3.6 (1.7)	6.3 (6.2)	15.3 (3.6)	3.0 (2.2)	1.0 (0.1)	
Tritriacontane	3301	447.3 (235.6)	81.75 (44.9)	22.2 (13.5)	9.8 (2.4)	11.9 (4.5)	4.8 (2.9)	
Tetracontane	3401	12.7 (7.7)	5.2 (3.2)	–	–	–	–	
Pentatriacontane	3499	59.5 (40.4)	8.3 (4.4)	3.35 (0.01)	–	–	–	
Hexatriacontane	3600	5.5 (3.5)	4.06 (0.02)	–	–	–	–	
Fatty acids	Hexanoic acid	961	8.9 (0.5)	–	–	176.0 (47.5)	–	–
	Oxononanoic acid	1452	–	–	–	52.9 (19.4)	–	–
	Dodecanoic acid	1545	16.3 (4.0)	–	–	59.8 (60.8)	–	–
	Tetradecanoic acid	1741	–	11.4 (0.6)	6.4 (3.5)	–	–	–
	Pentadecanoic acid	1866	12.5 (5.5)	12.6 (5.8)	0.61 (0.05)	–	–	–

Table 1 (continued)

Analyte ^d		I^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>					
			Needles	Litter	Soil	Needles	Litter	Soil			
Cyclic compounds	Monoterpenes	Hexadecanoic acid	1940	90.0 (16.9)	5.7 (1.0)	–	53.1 (10.8)	–	–		
		Heptadecanoic acid	2036	–	–	–	–	3.6 (1.8)	–		
		Octadecanoic acid	2139	19.1 (6.4)	–	–	–	–	–		
		Artemisiatriene	933	–	–	–	26.2 (4.3)	–	–		
		α -Thujene	942	39.6 (4.2)	66.1 (18.5)	3.81 (0.05)	466.2 (21.5)	0.56 (0.01)	0.53 (0.02)		
		α -Pinene	948	43.8 (21.9)	83.4 (20.9)	–	168.3 (5.0)	–	–		
		Camphene	952	–	–	–	67.1 (3.8)	–	–		
		Monoterpene (C ₁₀ H ₁₆)	971	6.0 (0.2)	1.9 (1.3)	–	–	–	–		
		Sabinene	976	12.9 (1.6)	27.7 (3.5)	–	24.8 (4.7)	–	–		
		β -Pinene	978	–	–	–	14.6 (3.6)	–	–		
		<i>p</i> -Cymene	1011	10.0 (0.9)	tr	6.2 (3.6)	–	–	–		
		Limonene	1020	15.0 (6.5)	14.0 (14.8)	–	–	–	–		
		Monoterpene (C ₁₀ H ₁₆)	1024	15.5 (1.8)	2.8 (2.9)	–	–	–	–		
		Monoterpene (C ₁₀ H ₁₆)	1050	10.9 (2.2)	19.6 (6.6)	–	–	–	–		
		Sabinene hydrate	1079	6.2 (0.8)	1.9 (2.3)	–	–	–	–		
		<i>p</i> -Cymenene	1107	–	–	7.86 (0.04)	–	–	–		
		α -Campholene aldehyde	1104	–	–	–	38.6 (8.5)	1.5 (0.8)	4.1 (0.7)		
		Verbenol	1124	13.5 (2.3)	17.5 (17.5)	–	50.7 (5.3)	11.2 (3.6)	–		
		α -Terpineol	1168	21.8 (14.7)	12.9 (7.6)	3.5 (0.1)	–	–	–		
		Verbenone	1176	–	–	–	73.9 (8.1)	10.6 (5.3)	–		
		Carvone	1213	9.7 (0.7)	2.0 (0.4)	–	–	–	–		
		Monoterpene (C ₁₀ H ₁₆)	1300	–	–	–	8.2 (2.4)	–	–		
		Limonene glycol	1306	18.7 (0.4)	3.3 (0.1)	–	–	–	–		
		Sesquiterpenes		α -Cubebene	1350	–	–	1.6 (0.3)	28.2 (1.8)	11.7 (5.1)	0.4 (0.2)
				Sesquiterpene (C ₁₅ H ₂₄)	1352	–	–	0.70 (0.01)	–	–	–
				α -Ylangene	1372	–	–	2.1 (0.2)	11.6 (3.1)	3.8 (0.3)	–
				α -Copaene	1377	–	–	1.4 (0.4)	33.0 (0.8)	5.4 (1.2)	–
				β -Bourbonene	1385	4.2 (0.3)	1.5 (0.6)	–	35.9 (6.7)	12.9 (2.4)	–
				Junipene + sesquiterpene (C ₁₅ H ₂₄)	1391	19.3 (1.6)	22.6 (9.7)	14.3 (0.6)	–	11.6 (2.9)	–
				Junipene isomer	1407	–	–	46.5 (0.1)	–	–	–
di- <i>epi</i> - α -Cedrene	1414			–	–	7.7 (0.6)	–	–	–		

Table 1 (continued)

Analyte ^d	<i>I</i> ^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>			
		Needles	Litter	Soil	Needles	Litter	Soil	
Caryophyllene	1418	30.4 (1.9)	82.4 (21.9)	8.4 (1.3)	269.7 (57.3)	20.2 (3.1)	–	
γ -Elemene	1429	17.8 (0.5)	13.5 (5.6)	6.5 (0.2)	–	–	–	
β -Copaene	1430	–	–	–	35.4 (18.7)	5.5 (0.5)	–	
Sesquiterpene (C ₁₅ H ₂₄)	1441	–	–	–	11.5 (0.7)	4.3 (0.07)	–	
Sesquiterpene (C ₁₅ H ₂₄)	1449	19.7 (1.7)	–	–	10.0 (6.4)	2.1 (0.2)	–	
α -Humulene	1454	–	–	–	52.9 (19.4)	2.9 (0.9)	–	
Muurola- 4(14),5-diene	1459	–	–	–	28.2 (7.3)	2.0 (0.3)	–	
Germacrene D	1471	9.4 (0.8)	9.3 (3.9)	1.0 (0.2)	144.4 (19.6)	31.0 (1.5)	–	
Germacrene isomer	1477	–	–	–	112.1 (30.7)	4.6 (0.2)	–	
β -Selinene	1483	9.0 (7.2)	22.5 (10.4)	2.2 (0.2)	29.3 (5.6)	10.2 (0.1)	–	
<i>trans</i> -Cadin- 1(6),4-diene	1488	–	–	–	41.70 (7.41)	9.3 (0.18)	–	
α -Muurolene	1495	7.7 (1.7)	–	–	64.8 (14.4)	18.4 (0.1)	–	
γ -Muurolene	1501	–	–	–	18.3 (9.3)	–	–	
Sesquiterpene alcohol	1504	–	–	–	128.5 (16.8)	4.8 (0.6)	–	
γ -Cadinene	1507	17.0 (3.2)	11.8 (4.3)	3.4 (0.4)	11.91 (0.01)	31.2 (1.1)	0.4 (0.2)	
δ -Cadinene	1516	–	–	–	119.5 (3.6)	18.1 (0.8)	0.27 (0.03)	
Sesquiterpene (C ₁₅ H ₂₄)	1560	–	–	3.2 (0.7)	–	–	–	
Spathulenol	1565	52.8 (8.8)	31.7 (16.6)	3.6 (0.6)	144.3 (16.3)	7.2 (0.5)	–	
Caryophyllene oxide	1571	644.0 (16.2)	173.7 (57.5)	3.7 (0.7)	370.1 (32.8)	22.4 (0.9)	–	
Sesquiterpene alcohol	1584	–	–	–	7.7 (1.4)	–	–	
Humulene epoxide	1595	328.9 (15.6)	103.5 (36.4)	–	91.3 (1.0)	5.4 (0.7)	–	
Caryophylla- 4,8-dien-5-ol	1621	22.9 (2.0)	9.2 (5.3)	2.3 (0.3)	8.9 (2.5)	–	–	
Sesquiterpene alcohol	1626	–	–	–	73.1 (8.1)	5.2 (1.1)	–	
γ -Costol	1637	–	9.1 (0.5)	–	–	–	–	
Sesquiterpene alcohol	1638	–	–	0.6 (0.1)	56.6 (7.7)	–	–	
Sesquiterpene alcohol	1641	26.3 (5.9)	7.3 (4.7)	–	–	–	–	
Sesquiterpene alcohol	1653	119.8 (17.9)	59.4 (19.1)	–	70.7 (2.7)	–	–	
Sesquiterpene alcohol	1674	6.6 (1.4)	2.6 (0.2)	5.4 (1.7)	–	–	–	
Oplopanone	1707	120.1 (7.8)	41.1(17.7)	–	107.3 (18.0)	4.0 (0.5)	–	
Spathulenol derivative	1791	11.5 (4.1)	1.9 (1.4)	–	–	–	–	
Diterpenes	Dehydroabietal	1960	–	–	–	13.0 (0.8)	4.7 (1.4)	–

Table 1 (continued)

Analyte ^d	\bar{I}^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>		
		Needles	Litter	Soil	Needles	Litter	Soil
Diterpene (C ₂₀ H ₃₂)	1976	–	–	–	42.7 (37.3)	4.1 (1.4)	0.2 (0.03)
Epimanoyl oxide	1981	12.3 (1.1)	10.0 (3.7)	2.4 (1.0)	11.2 (0.5)	2.9 (0.3)	–
Pimaradiene	1987	22.7 (2.3)	38.5 (14.3)	3.8 (0.4)	–	–	–
Abietatriene I	2034	6.8 (0.3)	10.9 (2.8)	5.0 (0.9)	55.9 (4.1)	6.7 (0.6)	0.3 (0.1)
Pimaradiene	2072	9.9 (1.9)	7.6 (1.9)	–	–	–	–
Diterpene (C ₂₀ H ₃₂)	2117	–	–	–	12.2 (3.1)	12.8 (6.1)	–
Abietatriene II	2176	–	–	–	tr	1.7 (0.2)	0.10 (0.01)
Diterpene (C ₂₀ H ₃₂)	2215	–	–	–	tr	0.90 (0.01)	–
Totarol	2250	–	–	10.1(4.5)	–	–	–
Ferruginol I	2275	–	7.3 (4.4)	40.8 (10.3)	–	–	–
Ferruginol II	2290	–	1.9 (1.3)	19.4 (18.8)	–	–	–
Dehydroabietic acid I	2303	–	–	–	354.4 (98.8)	71.0 (28.4)	0.47 (0.01)
Isopimaric acid	2331	74.8 (3.3)	100.9 (16.8)	50.1 (24.8)	128.4 (132.9)	10.4 (4.6)	–
Diterpene (C ₂₀ H ₃₂)	2371	–	–	–	318.3 (56.2)	72.5 (61.5)	0.99 (0.02)
Dehydroabietic acid II	2401	–	–	–	375.9 (84.4)	45.4 (17.8)	0.65 (0.05)
Diterpenoid (C ₂₀ H ₂₈ O ₂)	2562	12.7 (4.1)	12.8 (1.0)	2.3 (0.7)	–	–	–
Triterpenes	<i>D:A-Friedoolean-6-ene</i>	3331	–	–	–	7.4 (2.8)	–
	<i>α</i> -Amyrin acetate	3358	–	–	–	9.3 (4.8)	–
Steroids	Anthraergosta-tetraenol	2951	–	–	–	2.8 (1.6)	–
	Ergosta-4,22-dien-3-one	3235	–	–	–	6.2 (0.1)	–
	<i>β</i> -Sitosterol	3291	76.3 (20.1)	27.5 (0.1)	7.2 (3.3)	25.1 (3.3)	83.5 (31.4)
	Stigmast-4-en-3-one	3406	–	47.5 (5.4)	6.5 (3.7)	–	51.5 (24.6)
Other compounds	Benzoic acid	1135	–	–	–	31.6 (8.3)	–
	5,7-Octadien-4-one, 2,6,dimethyl	1151	9.1 (1.4)	–	–	–	–
	Benzaldehyde, 3,4,5-trimethoxy-	1554	1.6 (0.2)	–	–	–	–
	2-Pentadecanone, 6,10,14-trimethyl-	1828	35.0 (3.3)	20.9 (4.1)	–	82.8 (5.1)	–
	Heptadecanone	1829	39.9 (10.7)	–	48.2 (0.1)	–	–

Table 1 (continued)

Analyte ^d	I^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>		
		Needles	Litter	Soil	Needles	Litter	Soil
Xanthotoxin	2180	–	–	6.1 (2.2)	–	–	–
Phenolic compound	2525	–	–	–	138.9 (21.6)	25.7 (7.8)	–
Octacosanol	3092	347.7 (72.3)	48.7 (34.7)	4.0 (0.2)	227.9 (92.9)	7.0 (3.4)	1.4 (0.2)

^a Standard deviation in parentheses

^b tr: Traces

^c Roman numbers indicate different isomers

^d IUPAC names and formula of these compounds are shown in Table S1

Figure 2 shows the chemical structures of the lipids identified in the different extracts analyzed from juniper and pine forests (i.e., needles, litter and soil). These compounds could be useful biomarkers of these samples. Specific results of the different groups can be found below.

Shannon (H') diversity indices obtained for the main groups (alkanes, monoterpenes, sesquiterpenes and diterpenes) for juniper and pine needles, litter and soils are shown in Fig. 3. Similar trends were observed for the H' values of alkanes, sesquiterpenes and diterpenes in extracts from *J. communis*; diversity increased from needles ($H' = 1.37$ for alkanes, $H' = 1.83$ for sesquiterpenes and $H' = 1.40$ for diterpenes) to soil ($H' = 1.91$ for alkanes, $H' = 2.17$ for sesquiterpenes and $H' = 1.56$ for diterpenes). However, H' values of alkanes and diterpenes reached a maximum in pine litter ($H' = 2.26$ for alkanes, $H' = 2.90$ for sesquiterpenes and $H' = 1.71$ for diterpenes), decreasing in pine soil ($H' = 2.05$ for alkanes, $H' = 0$ for sesquiterpenes and $H' = 0.69$ for diterpenes). Regarding monoterpenes, the maximum values of H' were observed in both juniper and pine needles ($H' = 2.38$ for juniper and $H' = 1.64$ for pine), decreasing these values in litter and soil ($H' = 1.87$ and 1.34 for juniper and $H' = 1.05$ and 0.50 for pine, respectively).

n-Alkanes

Series of *n*-alkanes ranging from *n*-decane (C_{10}) to *n*-tritriacontane (C_{33}) were detected in pine forest and up to *n*-hexatriacontane (C_{36}) in juniper forest. Some branched alkanes were also identified with I^T values

slightly lower than those of the corresponding linear alkanes, such as branched *n*-decane (C_{10}), *n*-undecane (C_{11}) and *n*-hentriacontane (C_{31}). It should be noted that the highest concentration of *n*-alkanes was found in juniper leaves ($732.5 \mu\text{g g}^{-1}$), followed by pine needles ($441.3 \mu\text{g g}^{-1}$). In addition, juniper forests also contained high concentrations of alkanes in the litter and soil (165.9 and $238.9 \mu\text{g g}^{-1}$, respectively), while the pine soil showed a relatively simpler alkanes profile and a lower concentration of these compounds ($51.9 \mu\text{g g}^{-1}$).

Regarding juniper forests, and from a qualitative point of view, the alkane profile of soil showed a great similarity to that of litter and needles; only some alkanes such as *n*-heptadecane (C_{17}), *n*-nonadecane (C_{19}) and *n*-hexacosane (C_{26}) are soil specific (Fig. 2). However, quantitative differences were observed among these groups. While juniper needles and litter were characterized by the predominance of long-chain ($> C_{20}$) alkanes (83% and 80% of the total alkanes, respectively), juniper soil showed comparatively higher proportion of short-chain ($< C_{20}$) alkanes (78% of the total alkanes).

The predominance of individual alkanes for each sample is shown in Fig. 4. Tritriacontane (C_{33}), characteristic of *J. communis* (Maffei et al. 2004) and other plants from the Cupressaceae family (del Castillo et al. 1967), was the predominant alkane found in juniper needles and litter with a concentration of 447.3 and $81.7 \mu\text{g g}^{-1}$, respectively (Table 1). On the contrary, high values of *n*-undecane (C_{11} ; $72 \mu\text{g g}^{-1}$) and *n*-decane (C_{10} ; $66 \mu\text{g g}^{-1}$) were found in the juniper soil.

Pine forest was characterized by the predominance of *n*-hentriacontane (C_{31}) in all samples

Table 2 Concentration of LMWC (mg g⁻¹) identified in polar extracts obtained from needles, litter and soils from forests of *Juniperus communis* and *Pinus sylvestris*

Compound ^c	<i>I</i> ^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>		
		Needles	Litter	Soil	Needles	Litter	Soil
Pentitol	1768	–	0.036 (0.004) ^a	–	0.45 (0.05)	0.016 (0.001)	–
1-Methyl- <i>muco</i> -inositol	1867	57.7 (9.9)	0.054 (0.002)	0.0016 (0.0008)	0.0092 (0.0003)	–	–
Pinitol	1886	10.4 (2.1)	0.006 (0.001)	–	108.5 (27.5)	0.0049 (0.0002)	–
Quebrachitol	1914	0.053 (0.006)	–	–	0.013 (0.001)	–	–
Inositol	1960	0.022 (0.006)	–	–	0.019 (0.005)	–	–
<i>muco</i> -Inositol	1976	0.030 (0.001)	–	–	–	–	–
<i>D</i> -Mannitol	1994	0.25 (0.03)	0.234 (0.001)	0.027 (0.001)	0.44 (0.02)	0.116 (0.003)	–
Fructose	2004/2013	8.7 (0.5)	0.0057 (0.0001)	0.0015 (0.0001)	1.6 (0.3)	–	–
Sequoiitol + <i>chiro</i> -inositol	2018	1.43 (0.3)	0.0011 (0.0001)	–	0.08 (0.02)	–	–
Glucose	2061/2080	17.3 (0.8)	0.0128 (0.0001)	0.0031 (0.0001)	2.2 (0.3)	0.0048 (0.0004)	–
<i>myo</i> -Inositol	2152	0.064	0.0006 (0.0001)	–	0.33 (0.05)	–	–
Sucrose	2736	15.4 (1.4)	0.022 (0.001)	0.0020 (0.0002)	2.20 (0.07)	0.009 (0.001)	–
Trehalose	2840	t ^b	0.24 (0.03)	0.15 (0.02)	0.32 (0.01)	0.234 (0.004)	0.094 (0.003)
Total LMWC		111.05	0.61	0.19	116.28	0.38	0.09

^a Standard deviation in parentheses^b tr: Traces^c IUPAC names and formula of these compounds are shown in Table S2**Table 3** Concentration of acids (mg g⁻¹) in polar extracts obtained from needles, litter and soils from forests of *Juniperus communis* and *Pinus sylvestris*

Compound ^b	<i>I</i> ^T	<i>Juniperus communis</i>			<i>Pinus sylvestris</i>		
		Needles	Litter	Soil	Needles	Litter	Soil
Shikimic acid	1829	10.7 (1.4) ^a	–	–	0.29 (0.02)	–	–
Tetradecanoic acid	1844	–	–	–	0.08 (0.01)	–	–
Quinic acid	1907	10.6 (2.4)	0.012 (0.001)	–	0.29 (0.01)	–	–
Hexadecanoic acid	2042	0.92 (0.03)	0.092 (0.002)	0.11 (0.01)	0.97 (0.05)	0.11 (0.01)	0.09 (0.01)
Octadecanoic acid	2239	0.12 (0.07)	0.093 (0.003)	0.081 (0.001)	0.76 (0.01)	0.10 (0.01)	0.06 (0.01)
Pimaric acid	2311	0.16 (0.01)	0.051 (0.002)	–	0.11 (0.01)	0.012 (0.001)	–
Isopimaric acid	2321	0.63 (0.08)	0.12 (0.01)	–	0.102 (0.002)	–	–
Eicosanoic acid	2437	–	–	–	–	0.012 (0.002)	–
Docosanoic acid	2637	–	0.015 (0.002)	–	–	0.010 (0.005)	–

^a Standard deviation in parentheses^b IUPAC names and formula of these compounds are shown in Table S3

(concentrations of 238.0, 15.2 and 13.1 µg g⁻¹ in needles, litter and soil, respectively) (Table 1). In general, total concentration of the alkanes with an odd number of C atoms was higher than that of alkanes with even number of C atoms (Fig. 4).

Fatty acids

Up to 8 major fatty acids were detected in the dichloromethane extracts (Table 1). The highest concentration of fatty acids was found in pine

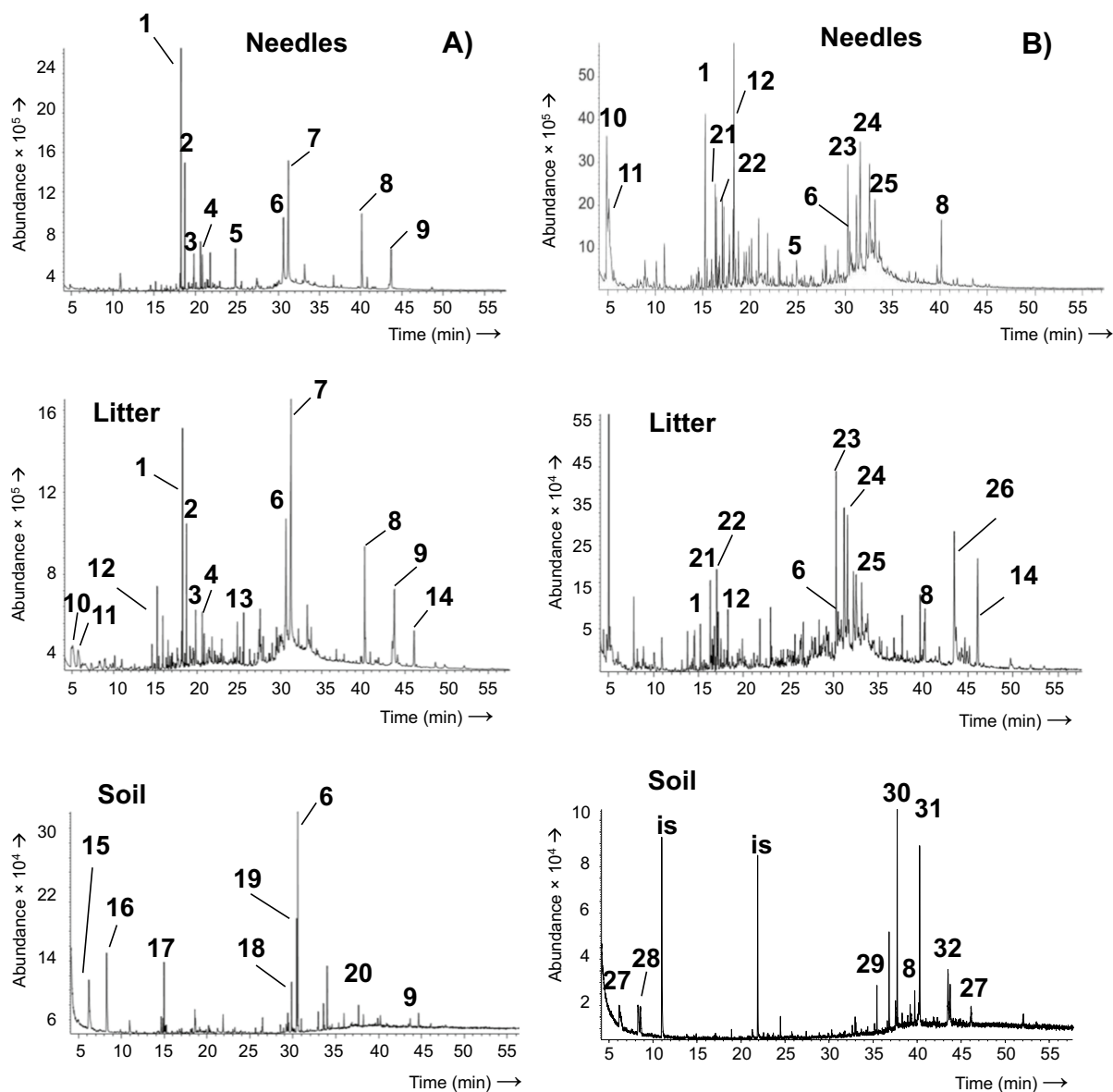


Fig. 1 GC–MS profiles of dichloromethane extracts from needles, litter and soils from forests of *Juniperus communis* (A) and *Pinus sylvestris* (B). 1- Caryophyllene oxide; 2- Humulene epoxide; 3- Sesquiterpene alcohol; 4- Oplopanone; 5- Hexadecanoic acid; 6- Isopimaric acid; 7- Unknown; 8- Octacosanol; 9- Tritriacontane; 10- α -Thujene; 11- α -Pinene; 12- Caryophyllene; 13- Pimaradiene; 14- Stigmast-4-en-3-one; 15- Decane;

16- Undecane; 17- Junipene isomer; 18- Ferruginol I; 19- Ferruginol II; 20- Nonacosane; 21- Germacrene D; 22- Spathulenol; 23- Dehydroabietic acid I; 24- Dehydroabietic acid II; 25- Phenolic compound; 26- β -Sitosterol; 27- Decane; 28- α -Campholene aldehyde; 29- Heptacosane; 30- Nonacosane; 31- Hentriacontane; 32- Tritriacontane; is- Internal standard

needles ($341.8 \mu\text{g g}^{-1}$), followed by juniper needles ($158.2 \mu\text{g g}^{-1}$). However, a higher variety of fatty acids was observed in the needles of juniper forests with the predominance ($90.0 \mu\text{g g}^{-1}$) of *n*-hexadecanoic acid (C_{16}). On the contrary,

only *n*-pentadecanoic acid (C_{15} , $0.6 \mu\text{g g}^{-1}$) could be detected in juniper soil and no fatty acids were found in pine soil.

The presence of *n*-tetradecanoic (C_{14}), *n*-pentadecanoic (C_{15}) and *n*-hexadecanoic (C_{16}) acids

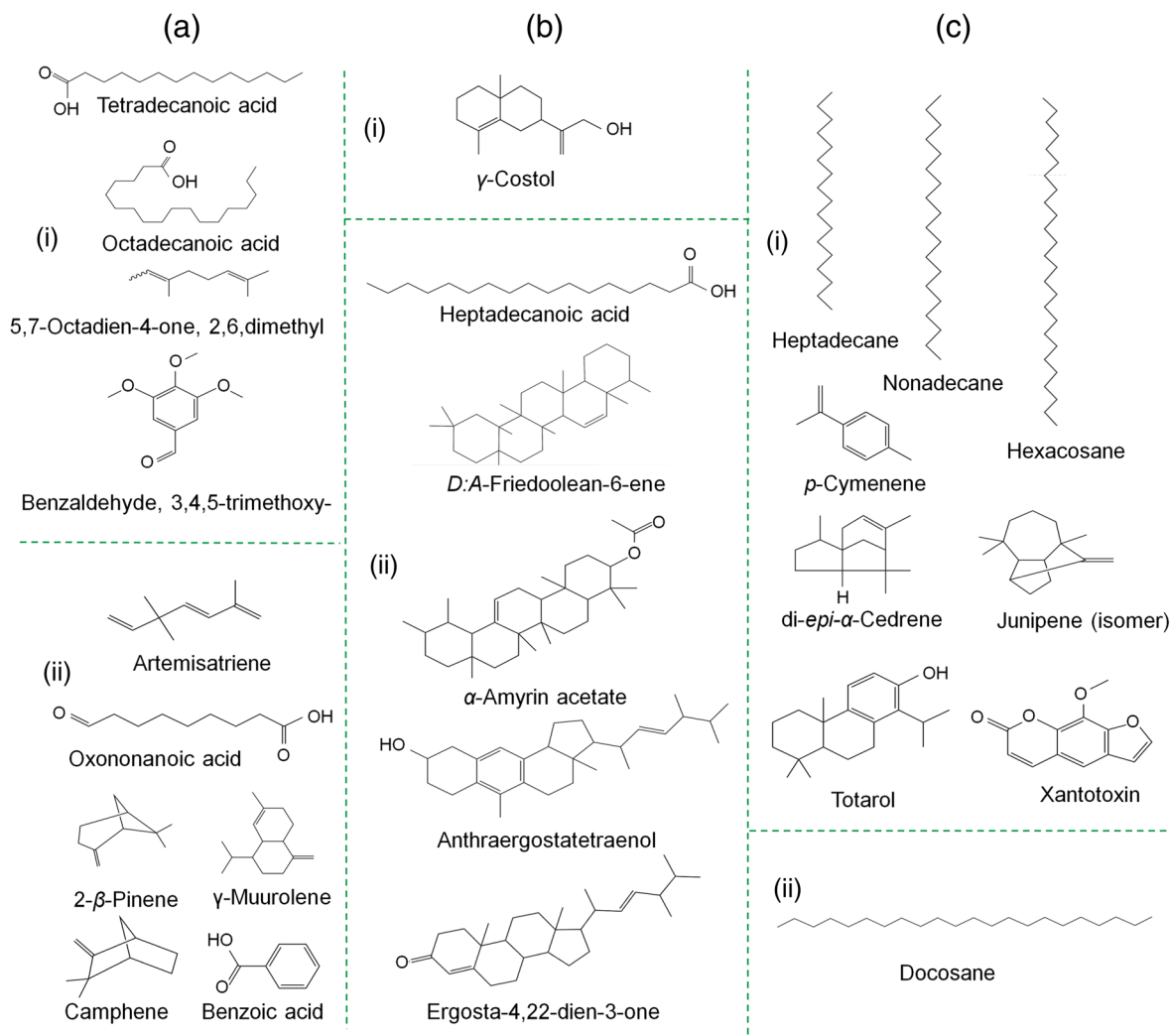


Fig. 2 Classification of the chemical structures of specific lipids found in juniper and pine forest. Columns from left to right are compounds (a) only present in needles, (b) only pre-

sent in litter and (c) only present in soil. Cell in columns from top to bottom represent compounds (i) only present in juniper and (ii) only present in pine

was also found in juniper litter, in general, in lower concentrations than in the needles. Similar to the behavior of alkanes, a higher concentration of short chain fatty acids was found in pine needles than in juniper leaves; however, unexpectedly, pine litter did not follow this distribution and only *n*-heptadecanoic (C₁₇) acid was detected (Fig. 2).

Cyclic compounds

Among the cyclic compounds, terpenoids were the most abundant family found in pine and juniper

forests (leaves, litter and soil). Different monoterpenes, sesquiterpenes, diterpenes and triterpenes including terpenic alcohols, acids, oxides and acetates were found in all analyzed samples (Table 1). In this work, all these compounds are referred to as terpenes. The identification of these compounds was carried out mainly by their I^T values, but taking into account also that each terpene family showed diagnostic mass spectral features (e.g., the characteristic ions at m/z 93 and 136 of monoterpenes, at m/z 161 and 204 of sesquiterpenes, and at m/z 161 and 272 of diterpenes). Those compounds whose

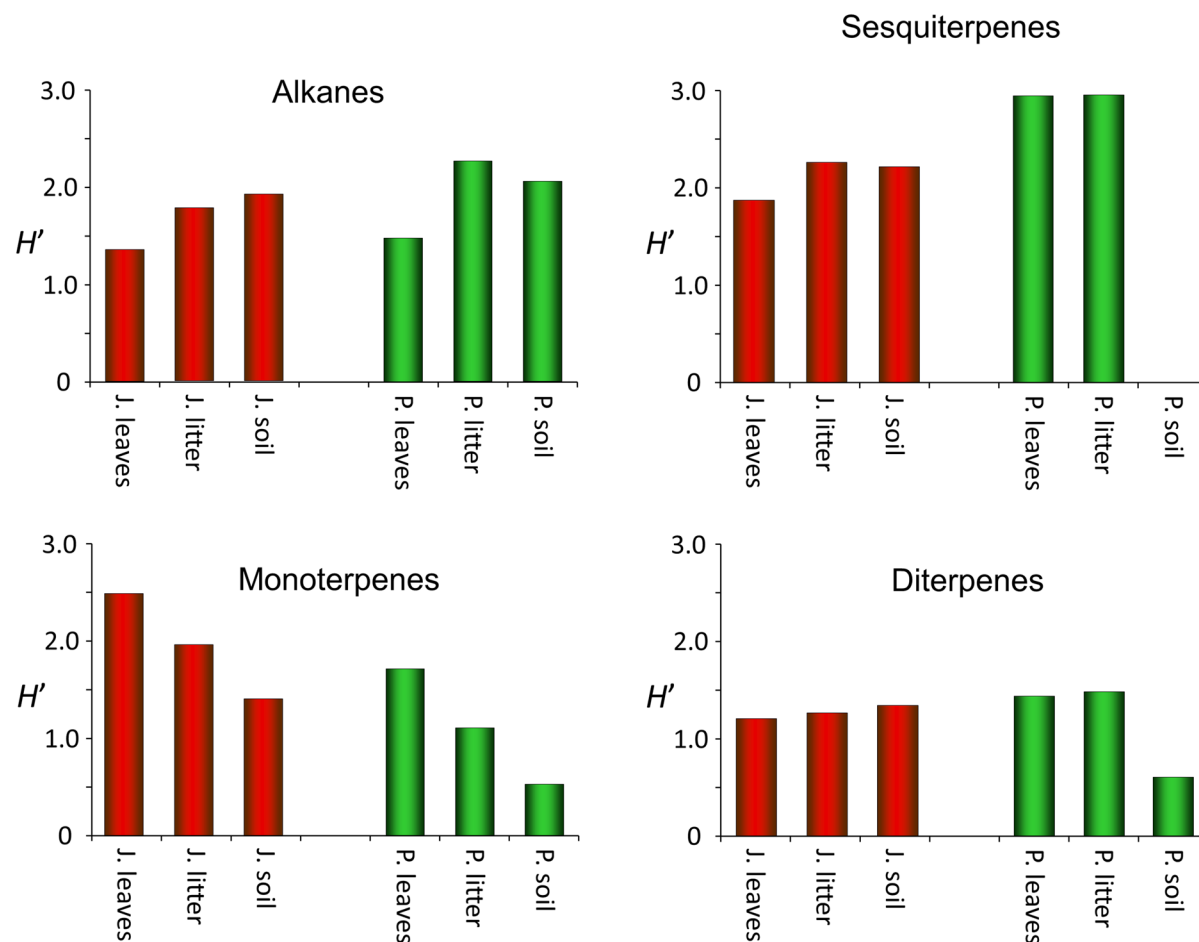


Fig. 3 Shannon's (H') diversity indices obtained for alkanes, monoterpenes, sesquiterpenes and diterpenes in juniper (J) and pine (P) leaves, litter and soils

identification was not possible have been assigned as monoterpenes, diterpenes or sesquiterpenes, considering their typical mass spectra and their retention index.

Figure 1S shows the total concentration ($\mu\text{g g}^{-1}$) of monoterpenes, sesquiterpenes, diterpenes and triterpenes of needles, litter and soil of both juniper and pine forests. Sesquiterpenes were the most abundant terpenes in all samples except soil samples. Pine needles showed the highest concentration of monoterpenes ($938.6 \mu\text{g g}^{-1}$), sesquiterpenes (2.1 mg g^{-1}) and diterpenes (1.3 mg g^{-1}). Triterpenes were only detected in low amounts in pine litter ($16.7 \mu\text{g g}^{-1}$). The lowest concentrations of terpenes were found in soil samples of both juniper ($269.9 \mu\text{g g}^{-1}$) and pine ($8.4 \mu\text{g g}^{-1}$).

Regarding monoterpenes, up to 20 compounds were detected and characterized based on their R^T values and mass spectra. Pine needles showed the greatest concentrations of these compounds; however, their concentrations in pine litter and soil were particularly low, even lower than those found in juniper litter and soil. In particular, α -thujene was the most abundant monoterpene of pine needles ($466.2 \mu\text{g g}^{-1}$), followed by α -pinene ($168.3 \mu\text{g g}^{-1}$). Monoterpenoids such as limonene, sabinene, *p*-cymenene, carvone and terpineol were only found in juniper forest, while artemisiatriene, camphene, α -campholene, aldehyde and 2- β -pinene were only found in pine forest.

Pine needles showed the highest concentration of sesquiterpenes, while very low amounts of these compounds were found in pine litter and soil. On the

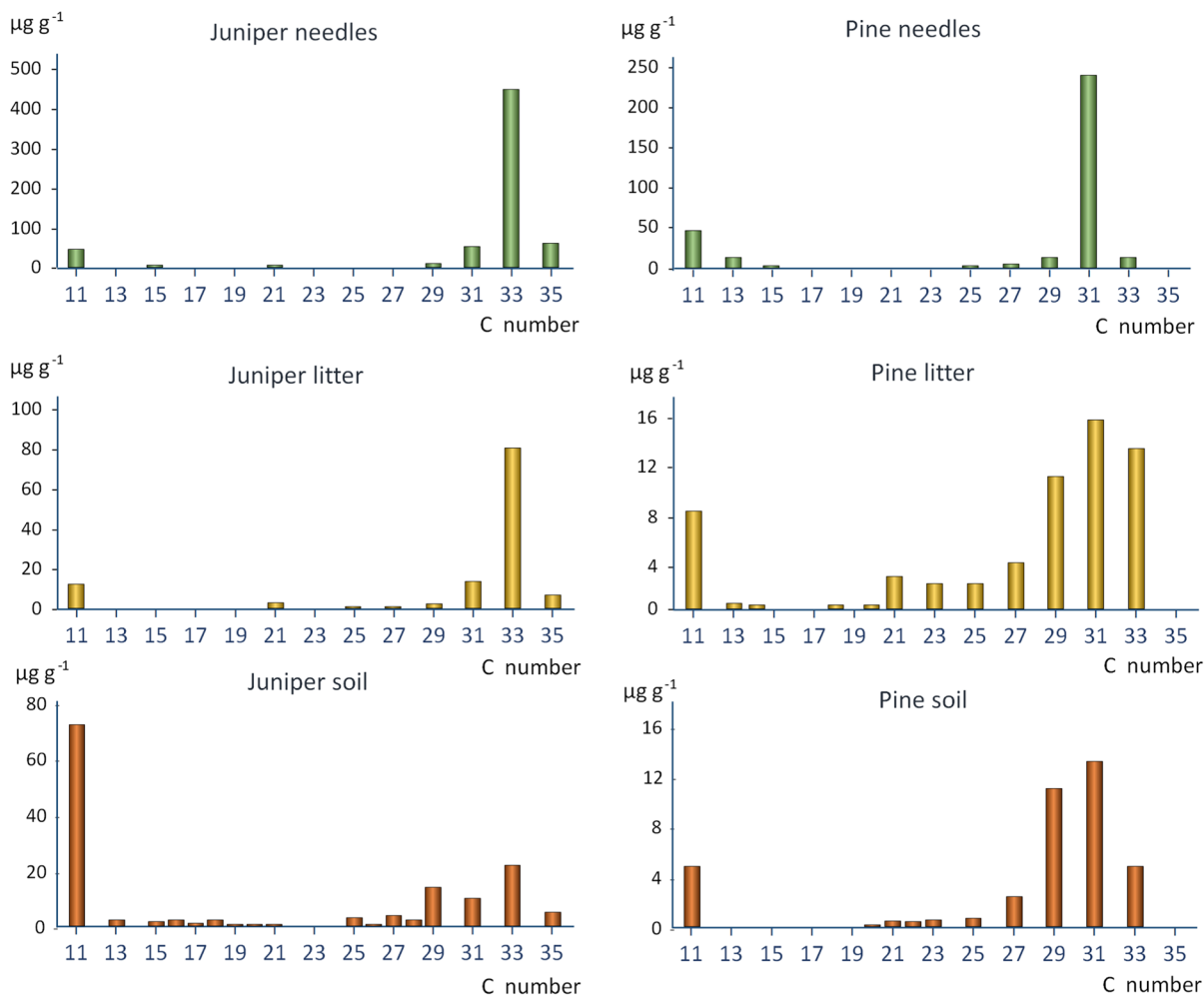


Fig. 4 Homologous series showing the concentration ($\mu\text{g g}^{-1}$) of individual alkanes of needles, litter and soils from juniper and pine forests

contrary, although the concentrations of sesquiterpenes were lower in juniper leaves, their abundance in juniper soil was higher than those in pine soil. Sesquiterpenes showed a tendency to accumulate in juniper soil, whereas small amounts were found in pine soil. Caryophyllene oxide was the most abundant sesquiterpene in pine needles ($370.1 \mu\text{g g}^{-1}$), followed by caryophyllene ($269.7 \mu\text{g g}^{-1}$). Regarding pine litter, γ -cadinene, germacrene D, caryophyllene oxide and caryophyllene were the most abundant. In pine soils only γ -cadinene, δ -cadinene and α -cubebene were detected.

The highest concentration of sesquiterpenes in juniper needles was found for caryophyllene oxide ($644.0 \mu\text{g g}^{-1}$) and for humulene epoxide

($328.9 \mu\text{g g}^{-1}$). Juniper litter showed greater concentrations of sesquiterpenes than pine litter; caryophyllene oxide ($173.7 \mu\text{g g}^{-1}$), humulene epoxide ($103.5 \mu\text{g g}^{-1}$) and caryophyllene ($82.4 \mu\text{g g}^{-1}$) were also the most abundant. Other sesquiterpenes such as spathulenol, β -selinene, γ -elemene, β -bourbene, and γ -cadinene among others, were also detected in juniper needles and litter, while junipene isomers and cedrene were found in juniper soils.

Pine needles showed the highest concentrations of diterpenes (1.3 mg g^{-1}), followed by pine litter ($233.1 \mu\text{g g}^{-1}$). Only low concentrations of diterpenes were found in pine soil (Fig. 1S). Although total diterpenes accounted for 12% of the terpenoids of juniper needles and 18% in juniper litter, this

percentage was higher in juniper soil (50%). Juniper diterpenes belonged mainly to the chemical families of abietanes and pimaranes (Fig. 2S), the latter being the most abundant in needles and litter, while abietanes predominated in soils. Isopimaric acid, pimaradiene isomers and epimanoyl oxide were the pimaranes found in juniper needles, litter and soil; the first one was the most abundant pimarane found in these samples (74.8, 100.9 and 50.1 $\mu\text{g g}^{-1}$, respectively). Regarding the abietanes, abietatriene was detected in the three juniper samples; however, feruginol was only detected in litter and soil samples. Meanwhile, totarol was only found in soil samples. Abietane diterpenes were abundant in pine needles; among them, isomers of dehydroabietic acid showed the highest concentrations (375.9 and 354.4 $\mu\text{g g}^{-1}$), while a noticeable reduction in the concentration of these compounds was observed in the litter (45.4 and 71.0 $\mu\text{g g}^{-1}$).

Other cyclic compounds

Two triterpenes (friedoolean-6-ene and α -amyrin acetate) were only detected in pine litter at relatively low concentrations (7.4 and 9.3 $\mu\text{g g}^{-1}$, respectively).

Regarding steroids, β -sitosterol was the most abundant, and was detected in all samples, except in pine soil. Stigmast-4-en-3-one was also detected at relatively high concentrations in both the litter and soil of juniper and pine.

Analysis of polar extracts

Figure 5 shows the GC–MS profiles of the derivatized methanol extract of needles, litter and soil from (A) juniper and (B) pine. Several peaks with characteristic mass spectra compatible with LMWC structures such as monosaccharides, disaccharides and polyols were identified in these samples. In addition, cyclic acids (such as shikimic, quinic, pimaric and isopimaric acids) and fatty acids (such as *n*-hexadecanoic (C_{16}) and *n*-octadecanoic (C_{18}) acids) were also found.

Regarding LMWC, glucose, fructose and sucrose were detected in all extracts, except from those of pine soil. The presence of trehalose in these extracts was also confirmed by comparison with the corresponding standard. It should be noted that this compound was the only LMWC detected in pine soil.

Linear polyols, such as mannitol, were also detected in these samples. Furthermore, different free inositols such as *myo*-inositol, and methyl-inositols such as pinitol (3-*O*-methyl-*D*-*chiro*-inositol), 1-methyl-*muco*-inositol, sequoyitol (5-*O*-methyl-*myo*-inositol) and quebrachitol (2-*O*-methyl-*L*-*chiro*-inositol), were detected in both pine and juniper leaves; *muco*-inositol was also found in leaves of the latter species. Different small peaks with mass spectra compatible with LMWC, mainly inositol derivatives, were also found in pine and juniper needles and litter. However, due to coelution problems, it was not possible to identify them.

Table 2 shows the I^T values and concentrations (mg g^{-1}) of the LMWC and acids quantified in the extracts studied. The highest concentration of total LMWC was accounted by pine needles (116.3 mg g^{-1}), followed by juniper needles (111.3 mg g^{-1}). The concentration of these compounds was much smaller in the litter and soils of both forest species. 1-Methyl-*muco*-inositol was the most abundant LMWC detected in juniper needles, followed by glucose and sucrose and pinitol was also found in relatively high concentrations in this particular extract (10.4 mg g^{-1}). Mannitol and trehalose were the most abundant LMWC of juniper litter (0.23 and 0.24 mg g^{-1} , respectively) and soil (0.03 and 0.15 mg g^{-1} , respectively). Regarding the pine forest samples, pinitol was the most abundant LMWC of pine needles, its concentration (108.5 mg g^{-1}) notably higher than that observed in juniper needles. As for juniper litter, trehalose and mannitol (0.23 and 0.12 mg g^{-1} , respectively) were the most abundant LMWCs found in pine litter and, as previously commented, only low concentrations of trehalose (0.09 mg g^{-1}) were detected in pine soil.

The concentrations (mg g^{-1}) of cyclic and fatty acids found in juniper and pine needles, litter and soil are shown in Table 3. The highest amounts of these compounds were found in the juniper needles (23.13 mg g^{-1}), mainly due to the high contribution of shikimic (10.7 mg g^{-1}) and quinic (10.6 $\mu\text{g g}^{-1}$) acids. On the other hand, *n*-hexadecanoic (C_{16}) and *n*-octadecanoic (C_{18}) acids were dominant in the soil samples. This trend was also previously observed for fatty acids extracted using the non-polar solvent.

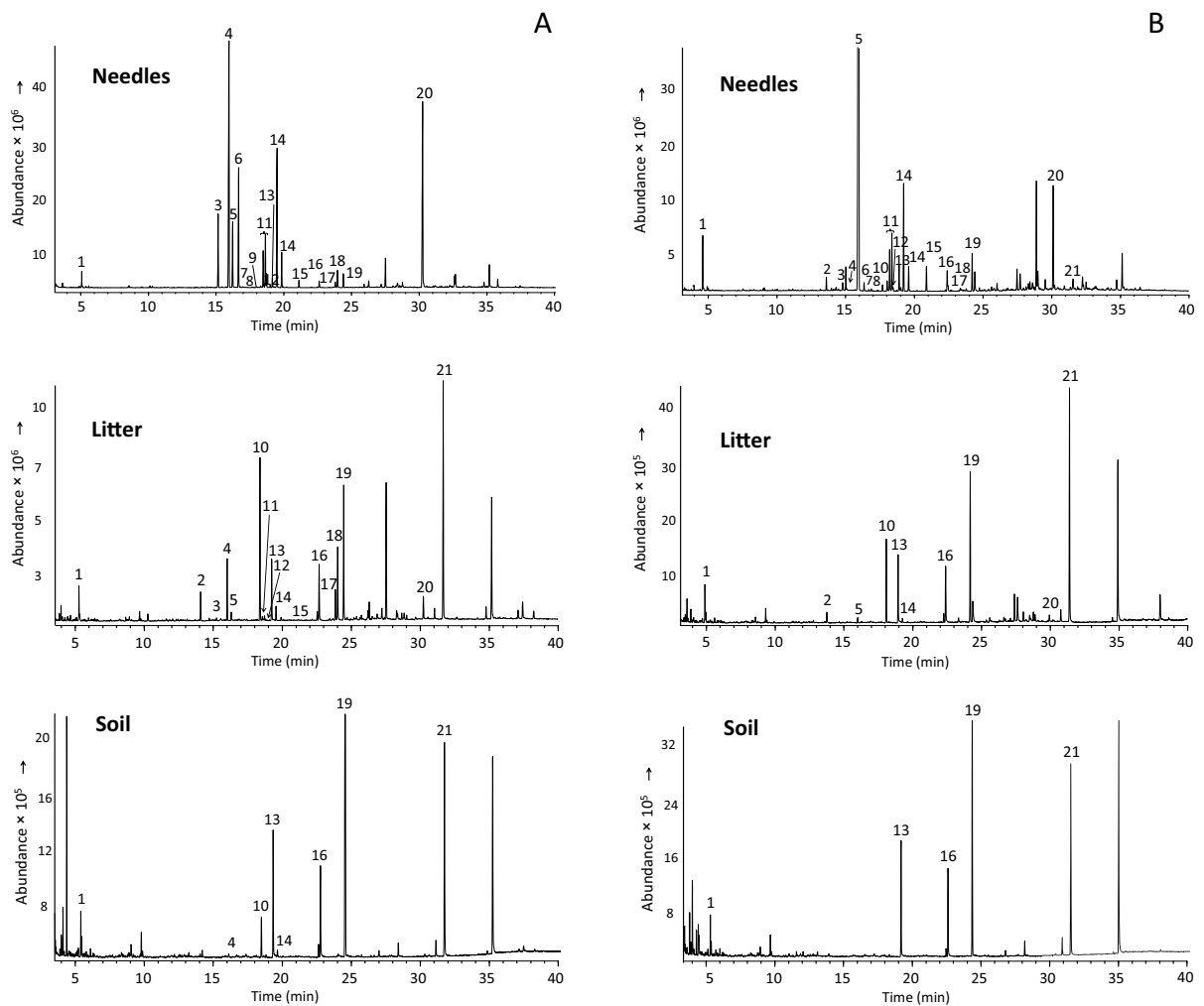


Fig. 5 GC–MS profile of methanol extracts of needles, litter and soil in juniper (A) and pine (B) forests. Peak identification-1- glycerol; 2- pentitol; 3- shikimic acid; 4- 1-methyl-*muco*-inositol; 5- pinitol; 6- quinic acid; 7- quebrachitol; 8- inositol; 9- *muco*-inositol; 10- mannitol; 11- fructose 1 and 2; 12- *chiro*-

inositol + sequoyitol; 13- hexadecanoic acid; 14- glucose *E* and *Z*; 15- *myo*-inositol; 16- octadecanoic acid; 17- pimaric acid; 18- isopimaric acid; 19- phenyl- β -*D*-glucoside (internal standard); 20- sucrose; 21- trehalose

Evolution of free lipid and polar compounds in van Krevelen diagrams

To monitor the molecular transformations during the different stages of biodegradation and humification of soil organic matter, van Krevelen diagrams were used. An interesting aspect of this graphical representation procedure is that it is possible to quickly compare samples using subtracted density maps that represent differences between the normalized concentrations of each of the compounds in different samples (quantitative data from Tables 1, 2, and 3 in this

study). In these diagrams, with positive and negative values resulting from the subtraction, the differences between the successive stages (leaves \rightarrow litter \rightarrow soil) can immediately be interpreted in the form of concentration or selective loss of the different types of compounds. Since the calculation is made by subtracting the concentration values of the leaves from those of the litter or the soil, the positive values (represented in green) indicate the compounds formed by the leaf or predominant in the leaf, whereas negative values (in red) indicate new compounds that appeared in the litter or soil during the transformation, or leaf

compounds that increased their concentration during this process.

Free lipid compounds

Free lipid compounds quantified in samples from the pine and juniper forest were plotted in the space defined by their H/C and O/C atomic ratios on a van Krevelen diagram (Fig. 3S A of Supplementary Material shows the average concentration values of all the samples studied). The coincidence of isomers is noted, but also of compounds of similar elementary composition that are grouped in more or less sharp clusters corresponding to alkanes, low molecular weight terpenes (mono- and sesquiterpenoids), mid-oxidation terpenoids (alcohols, oxides and acetates) and highly oxidized terpenoids (resin acids, terpenones...). Other less abundant compounds also tend to cluster as independent groups, mainly steroids, alcohols and aliphatic ketones.

Polar compounds

Fig. 3S B illustrates the average composition of polar compounds found in all the samples (needles, litter and soil) from the pine and juniper forest (the quantitative values corresponded to the normalized average concentration of all the compounds found in the samples studied). The major compounds correspond to the three large groups of cyclitols, disaccharides and alkanolic acids. The remaining minor compounds are grouped into four main clusters, referred to as linear polyols (pentitol and mannitol), monosaccharides, diterpene resin acids and cyclohexanecarboxylic acids

Discussion

Evaluation of non-polar compounds in pine and juniper forests

Shannon (H') diversity indices, calculated for alkanes, monoterpenes, sesquiterpenes and diterpenes in juniper and pine leaves, litter and soils (Fig. 3), traditionally have been used in ecology to describe the composition of populations defined by the number of individuals of different species. Also, they have been related to the degree of maturity or

evolution of plant associations, as they are progressively colonized for new species. However, the H' index can also be very useful to define the molecular populations of the soil as a whole, the complexity of which also increases during humus formation processes. In particular, the greatest diversity is related to emergent properties related to the biogeochemical activity of the soil. Such is the case of resilience or resistance to biodegradation, related to the potential carbon sequestration of soils (Jiménez-González et al. 2018), and could allow an early evaluation of the humification quality, defined by the physicochemical activity of the soil humus i.e., the enhancement of its interactions with organic compounds and with mineral surfaces. Indeed, the effectiveness of these interactions depends on the accumulation in soil of organic matter with chaotic structures that progressively differs from the precursor biogenic macromolecules of plant and microbial biomass, where the increase in molecular diversity paralleling humus maturity would be explained by the structural alteration of the precursor compounds in addition to the incorporation of new microbial metabolites (Almendros et al. 1996).

The increase observed in this work of H' values of alkanes, sesquiterpenes and diterpenes from needles to soil could be due to the contribution of microbial products in soils and/or to molecular rearrangements, leading to new lipid molecules. On the other hand, the decrease in the diversity of monoterpenes, indicating a simplification in the molecular composition of the apolar fraction, points to their selective biodegradation, their condensation or fixation into macromolecular structures, or both, with a corresponding decrease in the concentration of these molecules as free extractive compounds (Almendros et al. 1996).

Regarding alkanes (Fig. 4), the predominance of long-chain ($>C_{20}$) alkanes in juniper needles and litter and of short-chain ($<C_{20}$) alkanes in juniper soil indicated that most of the alkanes in the litter are inherited from the plant rather than derived from microbial metabolism (Simoneit and Mazurek 1982). The high predominance of short-chain alkanes in juniper soils could be attributed to the degradation of long chain alkanes from needles and litter due to active microbial metabolism in the soil (Fustec et al. 1985; Moucawi et al. 1981). In general, in pine samples, the higher total concentration of the alkanes with an odd number of C atoms than that of even

number of C atoms pointed to a biogenic signature of alkanes inherited from epicuticular waxes of higher plants (Eglinton et al. (1962a, b).

Regarding monoterpenes, the low concentration of these compounds in pine litter and soil compared to pine needles, could be due to the fact that most of these monoterpenes could be physically entrapped into macromolecular lipid substances or in the organomineral matrix of the soil (Almendros and Sanz 1991; Almendros et al. 2001). On the other hand, sesquiterpenes are also major biogenic compounds that allow recognizing the different stages of transformation of the soil organic matter. In fact, cadinene-related molecules are ubiquitous in plants and are the main odor constituents of some conifer woods and leaves, but can also be metabolites of various fungi (Rowe 1989). Sesquiterpenes such as spathulenol, β -selinene, γ -cadinene and junipene, among others, had been previously reported in juniper essential oils (Adams 2008; El-Sawi et al. 2007; Kosalec et al. 2005; Orav et al. 2010; Rezvani 2010).

Diterpenes found in juniper samples, such as abietanes, abietatriene, ferruginol and totarol are considered as diagnostic molecules of this forest ecosystems (Almendros et al. 1996). Abietane diterpenes have already been described as oxidation and thermal rearrangement products of methyl levopimarate (Rowe 1989) and have also been identified in pine soils affected by fires (Almendros et al. 1996).

Evaluation of polar compounds in pine and juniper forests

As expected, glucose, fructose and sucrose were present in all extracts, except from those of pine soil. These carbohydrates are ubiquitous in plants and have previously been found in pine needles (Kltšeiko 2006) and in juniper berries (Türkoglu et al. 2008).

The presence of pinitol, sequoyitol and *myo*-inositol in pine needles has been previously reported in the literature (Duquesnoy et al. 2008). Moreover, it is known that 1-methyl-*muco*-inositol is ubiquitous in gymnosperms (Dittrich and Kandler, 1972) and probably comes from the epimerization of pinitol. However, Dittrich and Kandler (1972) did not find this compound in the Pinaceae family, and suggested the absence of the enzyme that converts pinitol into methyl-*muco*-inositol in these plants. In the present work, 1-methyl-*muco*-inositol was found in pine

needles at very low levels. Methyl-inositols are usually secondary plant metabolites and are not directly involved in their normal growth, but these compounds play an important role the defense against stressful environmental conditions (Al-Suod et al. 2017).

Evolution of free lipid and polar compounds in van Krevelen diagrams

Free lipid compounds

Figure 6 shows surface density maps displaying the cumulative abundances of different groups of compounds present in lipid fractions extracted from needles, litter and soil samples collected in pine and juniper forests. Contour density maps showing the differences between the normalized values of the proportions of the different lipid compounds in the course of their transformation are shown in Fig. 4S. In the case of the pine forest samples, moderate differences are observed when comparing the leaf samples with respect to the litter (Fig. 4S A), where most of the transformations correspond to the relative decrease in the proportion of medium-oxidation products (both aliphatic alcohols, alkanones and fatty acids), as well as aromatic compounds (mainly alcohols, triterpenoidal acetates or oxides). In contrast, steroids, alkanes and diterpene resin acids tend to increase in relative terms in litter. Higher differences were observed when comparing the lipids of the leaves with those of the soil (Fig. 4S C). In the pine soil, a large relative enrichment in long-chain aliphatic compounds was observed, mainly long-chain alkanes (presumably derived from epicuticular waxes), while the relative concentration of most aromatic compounds decreased, especially in the case of hydrocarbons (mono-, sesqui- and diterpenes) compared to compounds with a high oxidation degree. The most persistent compounds correspond to steroids and sesquiterpene alcohols.

Compared to pine, juniper leaves showed a different molecular composition where the predominant fraction consists mainly of terpenoidal alcohols and oxides, whereas the proportions of hydrocarbons, both alkanes and mono- and sesquiterpenes were comparatively low (Fig. 6). In the course of lipid evolution from juniper needles to litter (Fig. 5S A) a large accumulation of terpenes, but also of highly oxidized terpenoids, i.e., acetates, ketones and resin acids, was observed. This is in agreement with the typical

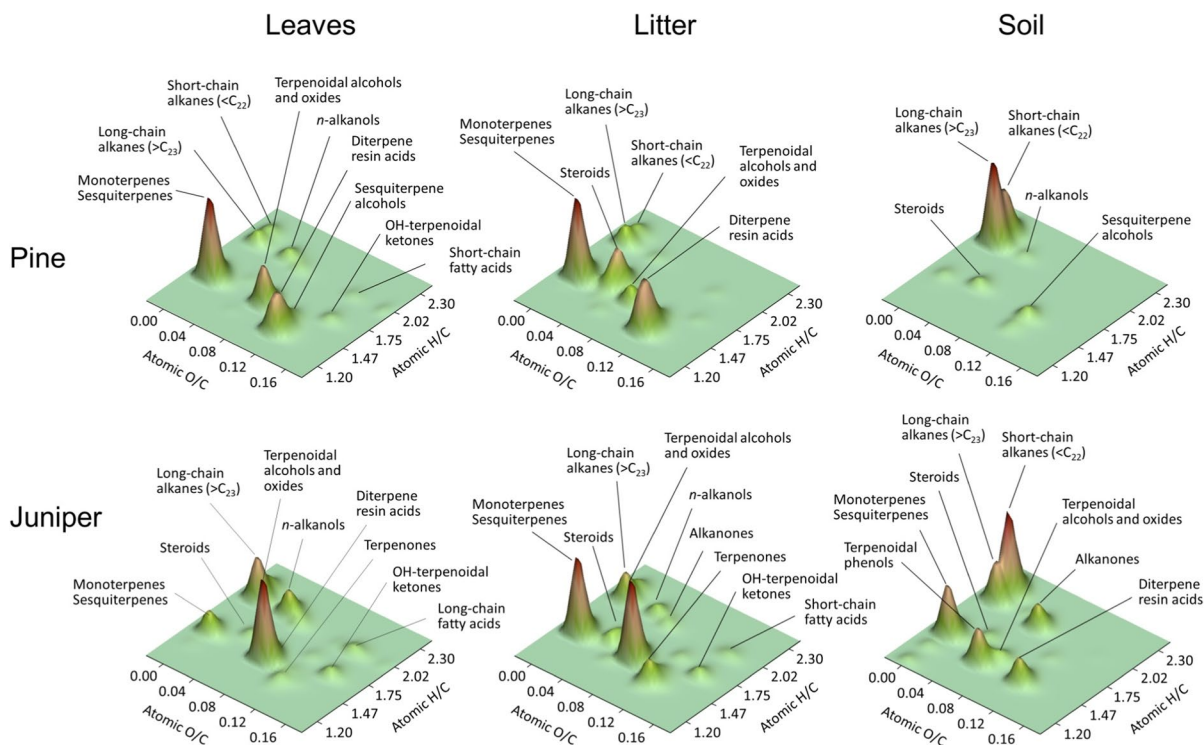


Fig. 6 Surface density maps displaying cumulative abundances of different groups of compounds present in lipid fractions extracted from needles, litter and soil samples collected in pine and juniper forests

trends described for the progressive transformation of terpenoids in soil, accompanied by progressive aromatization and oxidation (Simoneit and Mazurek 1982). However, there was a decrease in the proportion of intermediate oxidation degree terpenoids, mainly alcohols, as well as all aliphatic products, such as alkanes and alkanols, which is also expected with the tendency of selective degradation of compounds traditionally considered more easily biodegradable. In the case of soil lipid compounds (Fig. 5S B and C), there was a relative enrichment in alkanes (mainly of short chain) and terpenoidal phenols while a depletion of aromatic compounds in oxidized forms, such as alcohols, oxides and triterpenoidal acetates, was observed. Also in accordance with the above-indicated trends during the evolution of terpenoids, these changes would be characteristic of the advanced stages of transformation, in which defunctionalized hydrocarbons tend to accumulate preferentially, predominating over the oxygen-containing compounds that would characterize the intermediate stages.

It seems clear that there were two marked transformation trends of the molecular assemblages of biogenic lipids, depending on whether the transformation occurred in the presence of the mineral fraction of the soil studied or not. In both species (pine and juniper), the lipid evolution from leaf to litter was typically characterized by the oxidation of terpenoidal compounds or, in more advanced phases, by their defunctionalization with accumulation of the corresponding cyclic hydrocarbons. However, in the presence of the soil mineral fraction, most of the oxygen-containing compounds were depleted and a considerable concentration of alkanes remained as a major fraction of the lipid fraction. These results suggest polymerization or condensation of lipid compounds on the organo-mineral matrix of the soil, in particular humic substances, which would result due to the fact that the less reactive compounds would be those remaining in free, solvent-extractable forms.

Polar compounds

Although differences were observed between pine and juniper samples when the polar compounds were grouped together in the van Krevelen diagrams (Fig. 6), their evolution during the transformations from leaf to soil showed common characteristics (Fig. 6S A–C), similar to those observed in the case of lipid compounds.

In the case of pine, it was observed that the greatest increase in molecular diversity occurred during litter formation, where alkanolic acids, presumably from microbial metabolism, and linear polyalcohols appeared as additional groups of major compounds (Fig. 6S A). However, as the evolution of the polar fraction in the soil progressed, a simplification of the polar compound fraction occurred, in which acids and disaccharides hardly remained as main compounds (Fig. 6S B and C).

Regarding juniper samples (Fig. 6), the evolution was similar to pine, although there was a higher diversity of compounds, both in the needles and the litter. This latter was the sample that presented the greatest complexity as cyclitols and monosaccharides. These compounds were preserved from the needles, where an enrichment in linear polyols was also observed (Fig. 7S A). The final evolution in the soil led to a simplified composition, in which alkanolic acids, disaccharides and, to a lesser extent, polyols, produced in earlier stages, survived (Fig. 7S B and C).

Conclusion

To the best of our knowledge, this study represents the first comparative assessment based on the absolute quantitation of up to 148 compounds (free lipids, LMWC and acids) present in pine and/or in juniper extracts during the course of their transformation in the soil. In this preliminary study, different characteristic compounds which may be associated with the different stages of transformation were suggested. Differences were also observed in the two investigated forest species.

In general, the evolution of the lipid and LMWC compound assemblages of both juniper and pine samples monitored by van Krevelen diagrams showed a simplification, i.e. a decreasing molecular diversity: in the case of lipids, terpenoids were the most abundant

in needles and litter of both species, whereas alkanes were the predominant compound family in the case of soil lipids; the lipid fraction found in the intermediate evolution stage (i.e. litter) of both species presents a great molecular complexity attributed to the mixture of biogenic compounds with their transformation products. Regarding polar compounds, alkanolic acids and disaccharides tend to accumulated in the soil.

This work is a preliminary investigation demonstrating that the methodology used allows changes that occur during soil transformations to be monitored. Using experimental designs similar to the one used in this research, with a higher number of samples and other environmental scenarios, with different soil types and forest species, could be useful to establish objective quantitative criteria to assess factors involved in the impact of vegetation on the composition of organic matter in soils.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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