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Chemotaxonomical investigations of fossil and extant beeches. I. Leaf lipids from the extant *Fagus sylvatica* L.

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Abstract

The chemistry of fossil plants can help in precising their taxonomic affinities as well as their taphonomic history. The lipids from mature leaves of *Fagus sylvatica* L. were thus characterised to identify the components potentially informative that might be preserved in fossil beeches. Gas chromatography–mass spectrometry analyses of the lipids revealed a complex mixture comprising more than 60 identified constituents, belonging to diverse chemical families (e.g., sterols, fatty lipids, acyclic isoprenoids, triterpenes, glycerols, esters). Although most of the identified components are reported here for the first time in the European beech, they predominantly correspond to rather common compounds in Angiosperms. Nevertheless, their co-occurrence may constitute a useful fingerprint in further chemotaxonomic investigations of beeches. The newly reported compounds also include degradation products of plant lipids and fungal markers, showing that these degradation markers are to be considered as molecules related to the leaves in further taphonomic studies. ***To cite this article: T.T. Nguyen-Tu et al., C. R. Palevol 6 (2007).***

Résumé

Étude chimiotaxonomique de hêtres fossiles et actuels. I. Lipides foliaires du *Fagus sylvatica* L. actuel. La chimie des plantes fossiles peut aider à préciser leurs affinités taxinomiques et leur histoire taphonomique. Les lipides de feuilles matures de *Fagus sylvatica* L. ont donc été caractérisés, afin d'identifier les composés potentiellement informatifs qui pourraient être préservés dans les hêtres fossiles. L'analyse de ces lipides par spectrométrie de masse a révélé une mixture complexe de plus de 60 molécules identifiées, appartenant à des familles chimiques variées. La plupart des composés identifiés sont décrits ici pour la première fois chez *F. sylvatica*. Ils correspondent majoritairement à des constituants communs chez les Angiospermes, mais leur présence concomitante pourrait constituer une empreinte chimique pertinente pour les futures études chimiotaxonomiques sur le hêtre. Certains des composés nouvellement identifiés correspondent à des produits de dégradation de lipides végétaux et à des marqueurs fongiques, montrant que de tels marqueurs de

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dégradation peuvent contribuer significativement aux lipides des fossiles. ***Pour citer cet article : T.T. Nguyen-Tu et al., C. R. Palevol 6 (2007).***

Keywords: *Fagus sylvatica* L.; Fagaceae; Lipids; Leaf; Gas chromatography; Mass spectrometry; France

Mots clés : *Fagus sylvatica* L. ; Fagaceae ; Lipides ; Feuille ; Chromatographie en phase gazeuse ; Spectrométrie de masse ; France

1. Introduction

Chemotaxonomy has been widely used to help in precising the taxonomical affinities of plants (e.g. [6,59]). It is mainly based on the comparative characterisation of the chemistry of the studied taxa and the identification of biomarkers, characteristic compounds typical of a given taxonomic rank. Chemotaxonomy can also be successfully applied to fossil plants, provided that organic matter is preserved, which is often the case (e.g., [13,24,40]). Among plant chemicals, lipids are the most commonly studied in palaeobotany. Indeed, due to their hydrophobic nature, lipids are expected to remain in close association with a buried macrofossil rather than migrating into the embedding sediment. Consequently, several studies have established the chemical composition of lipids extracted from fossil plants and demonstrated their chemotaxonomic value, based on comparison with extant counterparts (e.g., [32,38]).

The genus *Fagus* L. (Fagaceae) is well represented in the fossil record since the Early Cenozoic (e.g., [10]). It includes 10 species in two sub-genera distributed in North America and Eurasia. *Fagus* exhibits a high intraspecific morphological and genetic variability that may lead to an overestimation of its past and present specific variability (e.g., [9,10]). Moreover, as stressed by Denk and Meller [11], the preservation of a number of the most-diagnostic characteristics of beeches is not favoured during the taphonomy and fossilisation processes. Therefore, chemotaxonomic studies of fossil and extant specimens may provide further insights into the taxonomy of the genus *Fagus*.

This initiating study focuses on the chemical characterisation of leaf lipids of the European beech, *Fagus sylvatica* L. While the chemistry of *F. sylvatica* leaves was abundantly studied in the past, no authors detailed the chemical composition of its total lipids, in spite of the quantitative importance of this lipid pool in leaves (e.g., [37,66]). Indeed, previous studies focused on the analysis of selected classes of leaf constituents: volatiles [27,56,61], cutin [48], tannins [2], epicuticular waxes [19,20,21,25,35], vitamins [28], and the main components of a Bligh–Dyer-type extract [49,50]. Moreover, previous studies mainly dealt with the chemistry of summer leaves. However, total leaf lipids probably reach their full development at the beginning of autumn, as do epicuticular waxes [45,47]. Furthermore, for deciduous trees, autumn leaves have a much higher probability to be fossilised than summer ones. Therefore, this study aims at characterising the chemistry of total lipids extracted from autumn leaves of *F. sylvatica*, in order to further help in chemotaxonomic and taphonomic investigations of fossil and extant beeches.

2. Materials and methods

2.1. Plant materials

Samples of *F. sylvatica* were collected in a rural area (experimental forest of Breuil-Chenue, 'Parc naturel régional du Morvan', France; [46]) where urban pollution is minimal. Several leaves were collected on several branches at the same height (approximately 1.5 m) of several trees, in order to minimize biases linked to intraspecific variability and obtain an average chemical composition representative of the leaves. The leaves were collected in October 2004, examined under microscope the day after collection and then oven dried for 48 h at 45 °C. The dried leaves were subsequently stored in the dark at 5 °C until analysis (less than two weeks). A voucher specimen has been deposited at the herbarium of the 'Laboratoire de paléobotanique, université Paris-6', France.

2.2. Lipid extraction and fractionation

The extraction was performed combining 20 leaves that corresponded approximately to 1 g of dry biomass. Leaves were crushed in a mortar and powdered to <500 μm. The leaf powder was ultrasonically extracted for 20 min with 30 ml dichloromethane/methanol (2:1; v/v). The mixture was centrifuged (10 min at 4000 rpm) and the extracted lipids were recovered with the supernatant. The procedure was repeated six times on the obtained residue. The combined extracts were concentrated by rotary evaporation and then dried under nitrogen gas. The extract yield was determined by weighting the dried combined extracts.

The total extract was then fractionated using column chromatography on alumina (13 g, Sigma-Aldrich 507C ~ 150 mesh) deactivated to Brockmann grade IV by adding 0.1 weight % of distilled water. The non-polar fraction (1) was recovered eluting with 52 ml of heptane. The slightly polar fraction (2) was recovered eluting with 39 ml of toluene. The medium polar fraction (3) was recovered eluting successively with 13 ml of dichloromethane and 52 ml of dichloromethane/methanol (9:1; v/v). The obtained fractions were concentrated by rotary evaporation and further dissolved in approximately 3 ml of heptane, toluene, and dichloromethane, respectively.

2.3. Quantification

The main components of the three studied fractions were quantified using (1) an internal standard (i.e. tetratriacontane for fraction 1 and 1-tricosanol for fractions 2 and 3) introduced in the fractions in known amount, (2) the GC peak areas of the component and the standard, and (3) the response factor in gas chromatography (GC) of the measured component. The latter was obtained from calibration curves of the standard and the measured component, or compound with similar chemical structure, polarity, and molecular weight if no reference compound was available.

2.4. Derivatization

The hydrocarbon fraction was analysed as such, while the other two fractions were converted into trimethylsilyl derivatives prior to analyses. Aliquots (30 μl) of the fractions were dried in a stream of nitrogen and then trimethylsilylated by reaction with 30 μl of *N,O*-bis-(trimethylsilyl)trifluoroacetamide (BSTFA) for 1 h at 80 °C. After cooling, the aliquot was dried under a gentle stream of nitrogen and redissolved in 30 μl of appropriate solvent (toluene for fraction 2 and dichloromethane for fraction 3).

2.5. Gas chromatography–mass spectrometry analysis

Gas chromatography (GC) analyses were performed on a Varian GC 3900 gas chromatograph fitted with a fused silica capillary column coated with VF5-MS (50 m × 0.32 mm i.d., 0.25 μm film thickness). The GC operating conditions were as follows: increase from 80 °C to 100 °C at 10 °C min^{-1}, then increase to 325 °C at 4 °C min^{-1}, followed by an isothermal period of 30 min at 325 °C. Helium was used as carrier gas at a constant flow of 2 ml min^{-1}. The splitless injector and FID detector temperatures were held at 350 °C.

Gas chromatography–mass spectrometry (GC–MS) analyses were carried out using an Agilent 6890N gas chromatograph coupled with an Agilent 5973N mass spectrometer, scanning the range 35–800 Da, electron impact voltage 70 eV, interface temperature 250 °C, source temperature 220 °C. The chromatographic conditions were the same as above. The compounds were identified by comparison of their retention time and mass spectrum with those of reference compounds or literature data (see Table 1 for references), and interpretation of mass spectrometry fragmentation patterns.

3. Results and discussion

The total lipids recovered after dichloromethane/methanol extraction of *F. sylvatica* leaves accounted for 11.3 wt % of the dried leaves. These lipids comprise 0.5% (0.6 mg) of non-polar fraction, 7.8% (9.4 mg) of slightly polar fraction and 17,1% of (20.7 mg) of medium polar fraction (i.e. eluted with heptane, toluene and a mixture of dichloromethane and methanol, respectively, see experimental section). The GC–MS analyses of these fractions revealed that beech lipids correspond to a complex mixture of more than 60 identified constituents (Table 1), and the chromatogram of the most abundant fraction is shown in Fig. 1. When all the fractions are considered, sterols constitute the main compound class of the extract, with β-sitosterol being by far the dominant molecule, with 1276 μg g^{-1} of dry biomass (Table 2, Fig. 1). Several sterols identified here in the beech lipids correspond to widespread phytosterols (i.e. brassicasterol, stigmasterol, campesterol, cycloartenol). Except brassicasterol, all have been previously described in beech leaves or, at least, in other parts of *F. sylvatica*, such as wood or seeds [20,33,43,50]. In addition, classical oxidation and biodegradation products of these sterols (i.e. hydroxy-, oxo- and unsaturated derivatives; [26,41]) are detected in significant amounts (Table 1, Fig. 1). Fungal sterols, such as ergosterol and obtusifoliol [14,23], and derivatives, are also identified in beech leaves (Table 1). Their occurrence can be explained by the presence of epiphytic fungi on several leaves, as revealed by microscopic examination (Fig. 2). Indeed, as most deciduous leaves, beech leaves host a number of endophytic and epiphytic fungi (e.g., [39]). The presence of degradation products of phytosterols and of fungal sterols were not previously reported in the common beech, which is likely due to the lower maturity of previously investigated leaves.

Table 1
Composition of the lipids extracted from *Fagus sylvatica* L. mature leaves (dichloromethane/methanol extract)
Tableau 1
Composition des lipides extraits de feuilles matures de *Fagus sylvatica* L. (extrait dichlorométhane/méthanol)

Compound	*Series distribution*[a]	Rt[b]	Fraction[c]	formula	M_w[d]	Characteristic fragments[e]	*reference for MS*[f]
steroids							
brassicasterol		43.8	3	$C_{28}H_{46}O$	398	TMS: **125**, 255, 380, 455, 470	[29]
stigmasta-3,5-diene		47.3	1	$C_{29}H_{48}$	396	**81**, 147, 381, 396	[15]
epiergosterol		49.1	3	$C_{28}H_{44}O$	396	TMS: 253, 337, **363**, 468	[14]
ergosterol		49.4	3	$C_{28}H_{44}O$	396	TMS: 201, 337, **363**, 468	[14]
campesterol		49.8	3	$C_{28}H_{48}O$	400	TMS: **129**, 343, 382, 472	
stigmasterol		50.1	3	$C_{29}H_{48}O$	412	TMS: **83**, 129, 255, 394, 484	[57]
7α-OH-sitosterol		50.3	3	$C_{29}H_{50}O_2$	430	TMS: 129, 253, **484**, 574	[26]
β-sitosterol		51.2	3	$C_{29}H_{50}O$	414	TMS: **129**, 357, 381, 396, 486	
stigmastanol (= dehydrositosterol)		51.6	3	$C_{29}H_{52}O$	416	TMS: **215**, 283, 298, 473, 488	
obtusifoliol (= 4,14-dimethylergosta-8,24(28)-dien-3-ol)		51.7	3	$C_{30}H_{50}O$	426	TMS: 227, **393**, 483, 498	[14]
cycloartenol		52.0	2	$C_{30}H_{50}O$	426	TMS: 339, 365, **393**, 408, 498	
7β-OH-sitosterol		52.3	3	$C_{29}H_{50}O_2$	430	TMS: 129, **484**	[26]
24 methylenecycloartan-3-one		52.6	2	$C_{31}H_{50}O$	438	95, 313, 423, **438**	
4-stigmasten-3-one (= β-sistostenone)		52.7	2	$C_{29}H_{48}O_2$	412	**124**, 229, 289, 412	
24-methylenecycloartanol		52.9	2	$C_{31}H_{52}O$	440	TMS: 379, 407, **422**, 512	[67]
7-ketositosterol		55.3	3	$C_{29}H_{48}O_2$	428	TMS: 129, 161, 390, **500**	[26]
phytyl derived compounds							
neophytadiene		22.7	1,2,3	$C_{20}H_{38}$	278	**68**, 82, 95, 123, 278	[16]
6,10,14-trimethyl pentadecan-2-one (phytone)		22.8	2	$C_{18}H_{36}O$	268	**43**, 58, 71, 250	
(*Z*)-1,3-phytadiene		23.3	1,2,3	$C_{20}H_{38}$	278	**81**, 82, 95, 123, 278	[16]
(*E*)-1,3-phytadiene		23.7	1,2,3	$C_{20}H_{38}$	278	**82**, 95, 123, 278	[16]
6,10,14-trimethyl-2-methylenepentadecanal		24.7	2	$C_{19}H_{36}O$	280	**84**, 97, 125, 280	[51]
2,6,10,14-tetramethylpentadec-2(E)-enal		26.5	2	$C_{19}H_{36}O$	280	**84**, 97, 126, 280	[51]
Z-phytenal		29.1	2	$C_{20}H_{38}O$	294	**84**, 97, 139, 149	[53]
E-phytenal		29.9	2	$C_{20}H_{38}O$	294	**84**, 97, 140, 149	[53]
phytol		30.6	2,3	$C_{20}H_{40}O$	296	TMS: 75, 123, **143**	
3-methylidene-7,11,15-trimethylhexadecan-1,2-diol (phytyldiol)		32.8	3	$C_{20}H_{40}O_2$	312	TMS: **73**, 143, 147, 353, 456	[54]
phytyl esters	*alkyl chain: C_6-C_{20} (C_{16})*	(**54.6**)	1,2,3	$C_nH_{2n-2}O_2$	(**534**)	95, **123**, . . ., 278, 296, . . ., $M^{+\bullet}$	[42]
other compounds comprising an acyclic isoprenoid chain							
geranyl acetone		12.4	2	$C_{13}H_{22}O$	194	**43**, 69, 136, 151, 194	
farnesyl acetone		24.5	2	$C_{18}H_{30}O$	262	**69**, 81, 136, 262	
4,9,13,17-tetramethyl-4,8,12,16-octadecatetraenal		32.2	2	$C_{22}H_{36}O$	316	**69**, 81, 93, 137, 149, 316	
4,8,12,16-tetramethylheptadecan-4-olide		34.3	2	$C_{21}H_{40}O_2$	324	**99**, 114, 126, 324	[55]
squalene		43.0	1,2	$C_{30}H_{50}$	410	**69**, 81, 95, 410	
α-tocopherol		48.1	3	$C_{29}H_{50}O_2$	430	TMS: 73, **237**, 502	
α-tocopherol ester	*alkyl chain: C_{14}, C_{16} (C_{16})*	(**76.0**)	2	$C_{30+n}H_{52+2n}O_2$	(**668**)	165, **430**, $M^{+\bullet}$	[42]
β-tocopherol ester	*alkyl chain: C_{14}, C_{16} (C_{14})*	(**66.5**)	2	$C_{29+n}H_{50+2n}O_2$	(**626**)	151, **416**, $M^{+\bullet}$	[42]
phylloquinone (vitamin K1)		51.4	2	$C_{31}H_{46}O_2$	450	186, 198, 225, **450**	
lipids with *n*-alkyl chain							
isopropyl esters	*alkyl chain: C_{12}, C_{14} (C_{12})*	(**17.2**)	2	$C_2H_{2n}O_2$	(**242**)	**43**, 60, 102, M-42$^{+\bullet}$	

Table 1 (*Continued*)

Compound	*Series distribution*[a]	Rt[b]	Fraction[c]	formula	M_w[d]	Characteristic fragments[e]	*reference for MS*[f]
methyl esters	*alkyl chain: C_{14}–C_{28}, $C_{18:1}$, $C_{18:2}$ (**C_{16}**, C_{28})*	(**24.9**)	2	$C_nH_{2n}O_2$	(**270**)	**74**, 87, . . ., $M^{+\bullet}$	
n-acids	*C_9–C_{28}, $C_{18:1}$, $C_{18:2}$, $C_{18:3}$ (**C_{16}**, C_{28})*	(**27.7**)	2,3	$C_nH_{2n}O_2$	(**256**)	TMS: 73, 117, 132, 145, . . ., **M-15**$^+$, $M^{+\bullet}$	
n-alkenes	*C_{14}–C_{27}, (**C_{26}**)*	(**39.1**)	1	C_nH_{2n}	(**378**)	**55**, 69, 83, . . ., $M^{+\bullet}$	
n-alkanes	*C_{12}–C_{33} (**C_{27}**)*	(**41.1**)	1	C_nH_{2n+2}	(**380**)	**57**, 71, 85, . . ., $M^{+\bullet}$	
n-alkan-2-ols	*C_{23}–C_{29} (**C_{27}**)*	(**45.5**)	2,3	$C_nH_{2n+2}O$	(**396**)	TMS: 73, 75, 117, . . ., **M-15**$^+$, $M^{+\bullet}$	
n-alkan-2-ones	*C_{25}, C_{27}, C_{29} (**C_{27}**)*	(**45.5**)	2	$C_nH_{2n}O$	(**394**)	58, **59**, . . ., $M^{+\bullet}$	
n-aldehydes	*C_{20}–C_{32} (**C_{28}**)*	(**47.0**)	2	$C_nH_{2n}O$	(**408**)	**82**, 96, . . ., $M^{+\bullet}$	
nonacosan-10-ol		47.1	3	$C_{29}H_{60}O$	424	TMS: 73, 229, 369	
n-alkan-1-ols	*C_{14}–C_{30} (**C_{28}**)*	(**48.4**)	2,3	$C_nH_{2n+2}O$	(**410**)	TMS: 75, 103, . . ., **M-15**$^+$, $M^{+\bullet}$	
p-coumaric esters	*alkyl chain: C_{18}–C_{22} (**C_{20}**)*	(**56,8**)	3	$C_nH_{2n-10}O_3$	(**516**)	TMS: 179, 192, **219**, **236**, . . ., $M^{+\bullet}$	[58]
benzyl esters	*alkyl chain: C_{24}, C_{26}, C_{28} (**C_{28}**)*	(**57.6**)	2	$C_nH_{2n-8}O_2$	(**514**)	91, **108**, . . ., M-109$^+$, M-91$^+$	[18]
wax esters	*C_{36}–C_{46}, $C_{38:1}$, $C_{40:1}$ (**C_{44}**)*	(**72.4**)	1,2	$C_nH_{2n}O_2$	(**648**)	57**, 257**, 313, 341, . . ., $M^{+\bullet}$	
glycerol esters							
1-palmitoleylglycerol (1-monopalmitolein)		38.7	3	$C_{19}H_{36}O_4$	328	TMS: 147, 237, **369**, 457	
1-hexadecanoylglycerol (1-monopalmitin)		38.9	3	$C_{19}H_{38}O_4$	330	TMS: 147, 239, **371**, 459	
1-heptadecanoylglycerol (margarin)		40.7	3	$C_{20}H_{40}O_4$	344	TMS: 147, 253, **385**, 473	
1-linolenoylgycerol (1-monolinolenin)		42.0	3	$C_{21}H_{36}O_4$	352	TMS: 147, 261, **393**, 481	
1-monostearoylglycerol (1-monostearin)		42.4	3	$C_{21}H_{42}O_4$	358	TMS: 147, 267, **399**, 487	
pentacyclic triterpenoids							
α-amyrenone		51.0	3	$C_{30}H_{48}O$	424	189, 203, **218**, 424	[34]
α-amyrin		51.7	3	$C_{30}H_{50}O$	426	TMS: 189, 203, **218**, 498	[34]
dodecandral		53.3	3	$C_{30}H_{46}O_4$	470	203, **216**, 496, 614	
oleanonic aldehyde		54.2	3,2	$C_{30}H_{46}O_2$	438	189, **203**, 232, 409, 438	[64]
oleanolic acid		54.3	3	$C_{30}H_{48}O_3$	456	TMS: 73, **203**, 320, 482, 585	[5]
oleanolic aldehyde		54.6	3,2	$C_{30}H_{48}O_2$	440	TMS: 190, **203**, 232, 512	
ursolic acid		54.9	3	$C_{30}H_{48}O_3$	456	TMS: **73**, 203, 320, 482, 585	[5]
hederagenin		58.0	3	$C_{30}H_{48}O_4$	472	TMS: 73, **203**, 320, 570, 673	[5]
miscellaneous							
ionene		9.9	1	$C_{13}H_{18}$	174	**159**, 174	
α-ionone		11.8	2	$C_{13}H_{20}O$	192	93, **121**, 136, 192	
β-ionone		13.3	2	$C_{13}H_{20}O$	192	43, 123, **177**, 192	
loliolide		21.2	3	$C_{11}H_{16}O_3$	196	**75**, 178, 212, 253, 268	

[a] Carbon number range (maximum, submaximum).

[a] Gamme de variation de la longueur de chaîne (maximum, sous-maximum).

[b] Retention time (minutes), values in parenthesis correspond to the series maximum.

[b] Temps de rétention (minutes), les valeurs entre parenthèses correspondent au maximum de la série.

[c] Elution fraction: heptane (1), toluene (2) and dichloromethane/methanol (3).

[c] Fraction d'élution: heptane (1), toluène (2) et dichlorométhane/méthanol (3).

[d] Molecular weight; weights in parenthesis correspond to the series maximum.

[d] Masse moléculaire, les masses entre parenthèses correspondent au maximum de la série.

[e] Fragments in bold correspond to the base peak of the mass spectrum, TMS indicate that the compound was identified as trimethylsilyl derivative.

[e] Les fragments en gras correspondent au pic de base du spectre de masse, TMS indique qu'un composé a été identifié après triméthylsilylation.

[f] When no reference is indicated, the compound was identified by interpretation of mass spectrometry fragmentation pattern and/or comparison with NIST database.

[f] Quand aucune référence n'est indiquée, le composé a été par interprétation de ses caractéristiques de fragmentation en spectrométrie de masse et/ou par comparaison avec la base de données NIST.

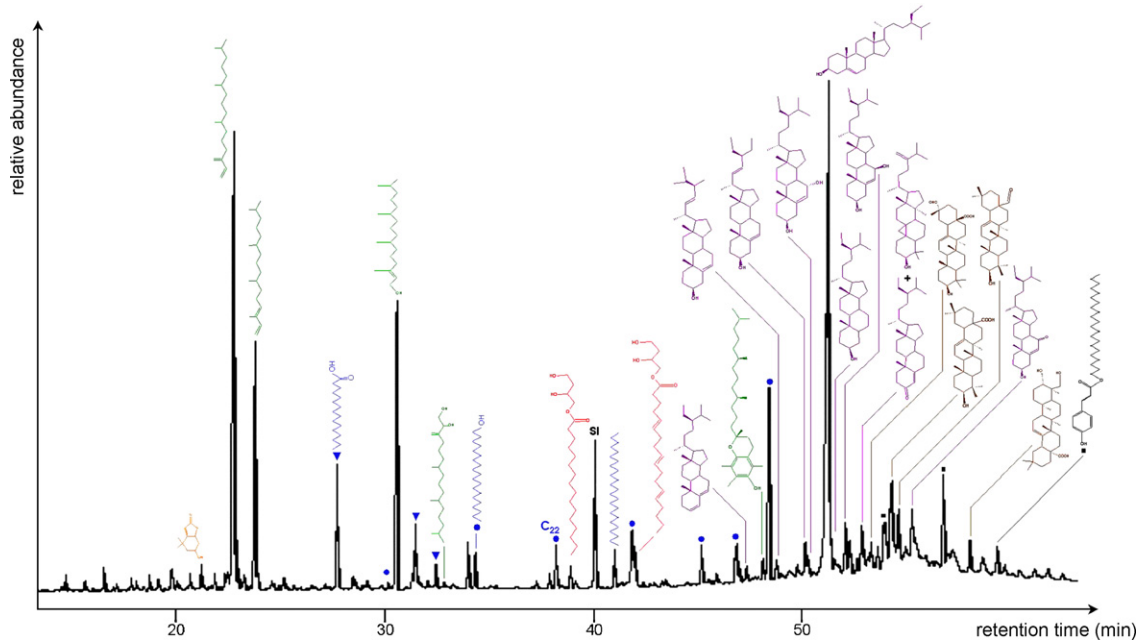

Fig. 1. Chromatogram of the main fraction of the lipids extracted from *Fagus sylvatica* L. leaves. This fraction was eluted with dichloromethane and methanol and compounds were analysed as trimethylsilyl-derivatives. IS: internal standard.
Fig. 1. Chromatogramme de la principale fraction des lipides extraits de feuilles de *Fagus sylvatica* L. Cette fraction a été éluée au dichlorométhane et au méthanol et ses composés identifiés en tant que dérivés triméthylsilylés. IS: standard interne.

The second most abundant compound class after the sterols corresponds to acyclic isoprenoid molecules; among them, phytadienes are predominant (Table 1, Table 2, Fig. 1). Phytadienes were shown to be artificially produced from phytol by numerous analytical procedures such as acid-hydrolysis, saponification, and high-temperature GC injection [17]. However, phytadienes probably constitute original components of *F. sylvatica* since (1) they are detected in the trimethylsilylated extracts, and (2) Grossi et al. [17] suggested that analysis of trimethylsilylated extracts does not produce any phytadiene as artefacts. Along with phytadienes, phytol, phytone, phytenals, and other oxygenated phytyl compounds are detected in substantial amounts in the leaf lipids extracted from *F. sylvatica* (Table 1). These compounds mainly correspond to various intermediates in the degradation of the phytyl chain of chlorophylls. To a lesser extent, they also may be derived from other compounds comprising a phytyl chain (e.g., tocopherols, phylloquinone, phytylalkanoates; [3,8,51,52,54,55,63]). These phytyl degradation compounds are reported here for the first time in beech lipids. Phytol is also present as a series of wax esters of phytol with acid moieties comprising 6 to 20 carbons and maximizing at C_{16} (Table 1); such a series has been described previously in leaf lipids of *F. sylvatica*, with a similar distribution pattern [50].

Table 2
Quantification of the main constituents of *Fagus sylvatica* leaf lipids
Tableau 2
Quantification des principaux constituants des lipides foliaires de *Fagus sylvatica*

Constituent	Abundance*
β-sitosterol	1276.0
neophytadiene	1096.0
oleanolic acid	631.2
(*E*)-1,3-phytadiene	491.1
phytyl hexadecanoate	456.0
phytol	370.0
n-octacosan-1-ol	314.8
n-octacosanal	290.0
n-hexadecanoic acid	275.2
n-heptadecane	268.3
squalene	109.6
eicosanyl-*p*-coumarate	97.0
4-stigmasten-3-one	38.9
α-tocopherol	7,0

* mg g^{-1} of dry biomass.
* mg g^{-1} de biomasse sèche.

Other acyclic isoprenoid compounds are identified, the most abundant being the ubiquitous squalene, which has been described previously in beech seeds and wood (Tables 1 and 2; [20,43]). Polyunsaturated acyclic isoprenoid ketones and aldehydes are also identified here for the first time in *F. sylvatica* (Table 1). These compounds are rather

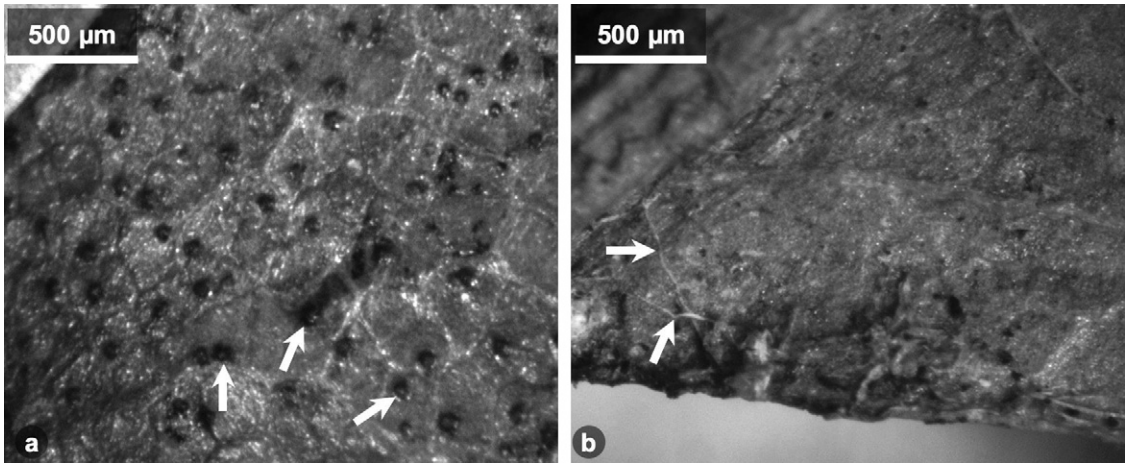

Fig. 2. Typical zones of *Fagus sylvatica* L. leaves invaded by fungi. (**a**) Fructifications of epiphytic fungi breaking through the epidermis (e.g., arrows). (**b**) Mycelium growing between the main nerve and the margins (e.g., arrows).
Fig. 2. Zones des feuilles de *Fagus sylvatica* L. typiquement envahies par des champignons. (**a**) Fructifications de champignons épiphytes perçant l'épiderme (par exemple, flèches). (**b**) Mycélium se développant entre la nervure principale et les marges (par exemple, flèches).

common in plant oils and can correspond either to degradation products or to biosynthetic intermediates of squalene or other terpenes (e.g., [12,60,62]). Vitamins are also detected in the lipids extracted from beech leaves (Tables 1 and 2): α-tocopherol (i.e. vitamin E), which has been previously reported in beech leaves [28], and phylloquinone (i.e. vitamin K1), which is described here for the first time. Tocopherols are also present as wax esters (C_{14}, C_{16}) of α- and β-tocopherols (Table 1). These compounds that are described here for the first time in *F. sylvatica* have not been frequently reported in the literature, probably because their high molecular weight made them difficult to detect at the temperatures generally used for GC analyses. Nevertheless, they may correspond to rather common plant compounds since (1) their moieties are widespread among plants and (2) they were detected in various Angiosperms (e.g., [42]).

Several series of aliphatic homologues are also detected in substantial amounts in the leaf lipids extracted from *F. sylvatica*; most of them have been previously described in its leaf lipids, with similar distribution patterns [20,31,35,49,50]. These series exhibit a marked carbon number predominance. The most abundant series show an even over odd predominance; they correspond to *n*-alkan-1-ols, *n*-aldehydes, *n*-acids, and, to a lesser extent to methyl esters, isopropyl esters and *n*-alkenes (Tables 1 and 2, Fig. 3). The absolute abundance determined for octacosanal should be considered cautiously, since Naß et al.[35] showed that quantification of aldehydes is often non-reproducible. However, these authors pointed out that the quantification biases were remarkably low in the case of *F. sylvatica* extracts. Otherwise, the natural occurrence of methyl esters in lipids is uncommon. Esterification of carboxylic acids upon extraction with solvent mixture containing methanol was previously attributed to the presence of clays in the experimental mixture [1,36]. To test the methylation of acids without clays, a standard acid (C_{17}) was added to a piece of leaf and submitted to the extraction procedure. The corresponding ester was formed in substantial amounts and it was concluded that the methyl esters detected corresponded to a part of the original fatty acids that had been methylated upon extraction. Hence, the distribution showed in Fig. 3 is a combination of fatty acid and methyl ester distribution. Series with an odd over even predominance are also present: *n*-alkanes, *n*-alkan-2-ols and *n*-alkan-2-ones (Table 1 and 2, Fig. 3). *n*-Alkan-2-ones are generally considered as oxidation products of *n*-alkanes, *n*-alkan-2-ols corresponding to intermediates in this conversion [7,49,50,65]. The similarity in the distribution of these three series of compounds supports this hypothesis (Fig. 3). The occurrence of such degradation products in beech lipids may be due to the maturity of the studied leaves. As previously noticed by Lockheart [30], the *n*-alkane distribution of *F. sylvatica* leaf lipids is markedly dominated by the C_{27} homologue, which seems to be typical of the *Fagus* species living in the European cool temperate forest (e.g., *F. sylvatica, F. crenata*) when compared with species living in the warmer areas of Asia and Eurasia (e.g., *F. grandifolia, F. japonica, F. orientalis*), which exhibit significant amounts of the C_{25} and C_{29} homologues along with the C_{27}. Nonacosan-10-ol is also identified

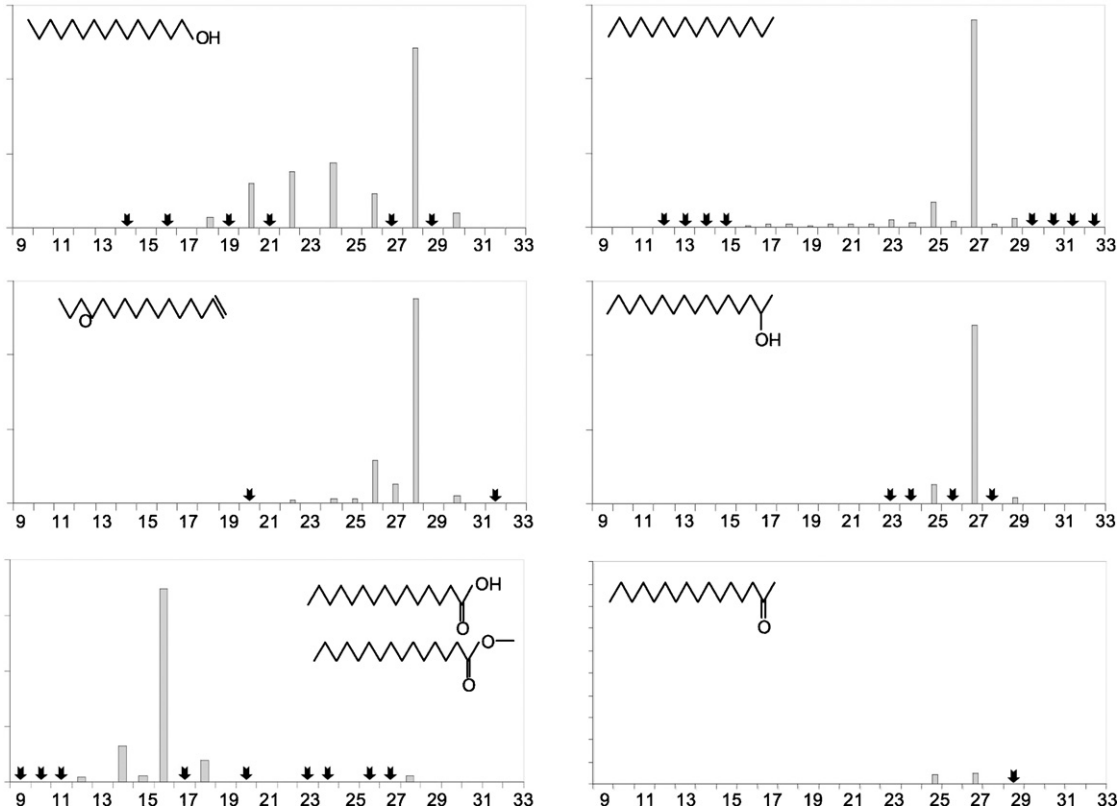

Fig. 3. Distribution of the main series of fatty lipids extracted from *Fagus sylvatica* L. Numbers in abscissa refer to chain length and arrows indicated when a homologue is detected but below the quantification level.
Fig. 3. Distribution des principales séries de lipides gras, extraites de *Fagus sylvatica* L. Les numéros en abscisse désignent la longueur de chaîne, et les flèches indiquent un homologue identifié, mais en dessous du seuil de quantification.

for the first time in the European beech (Table 1), although commonly observed in several Embryophytes [4,22]. Additionally, three series of long-chain esters are detected: wax esters, benzyl esters and *p*-coumaric esters (Table 1); their distribution is in agreement with previous characterisation of leaf lipids from *F. sylvatica* [20,35,50].

Several pentacyclic triterpenoids can be identified at the end of the chromatogram of fraction 3; they exhibit ursane or oleanane carbon skeleton (Tables 1 and 2, Fig. 1). Except α-amyrin that has been previously described in beech wood [43], all these triterpenoids are reported here for the first time in *F. sylvatica*. Most of them correspond to terpenoids rather common in plants, especially in Angiosperms, or to their degradation product. A number of monoacylglycerols is also detected in the leaf lipids of beech (Table 1, Fig. 1). These intermediates in the lipolysis and biosynthesis of triacylglycerols were previously described in the lipids extracted from the seeds of *F. sylvatica*, although with a narrower range of chain length [44]. Finally, four small terpenoids are detected in small amount (ionene, α-ionone, β-ionone, loliolide), they correspond to common compounds of Angiosperm essential oils, or their degradation products, and are reported here for the first time in *F. sylvatica*.

4. Conclusion and implications

This study thus allowed to precise the chemical composition of free lipids extractable from autumn leaves of the European beech (*Fagus sylvatica*). In addition to the constituents previously reported in specific lipid pools of *F. sylvatica*, a number of compounds are described here for the first time in this plant. These compounds are fairly abundant and generally correspond to molecules rather common in Angiosperms; they were not previously reported in beech lipids, probably because previous authors investigated more restricted lipid pools. Nevertheless, the co-occurrence and relative abundances of some of these compounds (e.g., phylloquinone, brassicasterol, nonacosan-10-ol, tocopheryl alkanoates, dodecandral, oleanonic triterpenoids, ursolic

acid, hederagenin) may constitute a chemical fingerprint to be used in further chemotaxonomic investigations of fossil and extant beeches. Otherwise, a number of the newly reported compounds correspond to degradation products of the main components of the leaves, emphasizing the quantitative importance of such products in mature leaf lipids. In the same way, the few fungal sterols detected in this study show that the epiphytic microflora might contribute significantly to the lipids extracted from leaves. The occurrence of such maturity markers in lipids extracted from leaves collected on trees in October are to be taken into account in further taphonomic studies; it may help to clarify the degradation/preservation stage of fossils. This investigation of total lipids from autumn leaves of *F. sylvatica* thus led to a description of beech lipids more complete than previously and should constitute a reference dataset in further chemotaxonomic and taphonomic investigations of fossil and extant beeches.

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