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## **Triterpenoids as chemotaxonomic tools to identify and differentiate genuine, adulterated and archaeological balsams**

Courel Blandine<sup>1\*</sup>, Adam Pierre, Schaeffer Philippe\*

*Université de Strasbourg, CNRS, Institut de Chimie de Strasbourg UMR 7177, F-67000*

*Strasbourg, France*

### **ABSTRACT**

Plant exudates have been extensively used in the past for different applications related to their olfactory, physical or medical properties. Their identification in archaeological samples relies, notably, on the characterisation of chemotaxonomic molecular markers but is often hampered by the severe alteration of their typical genuine molecular signature due to ageing. Among these exudates, those recovered from *Styrax*, *Liquidambar* and *Myroxylon* tree species -defined as balsams- have been exploited in the past for their pleasant scent and medical properties. They are characterized by a large variety of aromatic compounds, especially of the cinnamate and benzoate series, which can be used to assess their botanical source. These compound series may, however, be subject to alteration over time during exposure to environmental conditions in the case of archaeological material. As a result, their reliability as chemotaxonomic markers to characterise and discriminate archaeological balsams is problematic and questionable. We have, therefore, carried out a comparative analysis of various balsams (*Styrax officinalis*, *S. paralleloneurum*, *S. tonkinensis*, *Liquidambar styraciflua*, *L. orientalis* and *M. balsamum var. pereirae*) using gas chromatography-mass spectrometry to search for more reliable solid lipid markers. The results revealed that, even in the case of extended alteration/removal of cinnamic and benzoic acid derivatives, a distinction between styrax, liquidambar, Peru and Tolu balsams remains possible based on the distribution of pentacyclic triterpenes. The predominance of

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<sup>1</sup> Present address: The British Museum, Great Russell Street, London WC1B 3DG, United Kingdom

\* Corresponding authors.

E-mail addresses: BCourel@britishmuseum.org (B. Courel), p.schaeff@unistra.fr (P. Schaeffer).

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uncommon 6-oxygenated derivatives of oleanolic acid among triterpenoids could be specifically related to styrax resins. Liquidambar gums were characterised by the predominance of oleanonic acid together with uncommon 3-*epi* oleanolic and ursolic acids, whereas Peru balsam was rich in cinnamic and benzoic acids, but devoid of triterpenoids. Thanks to the high specificity of some of these triterpenes, their low volatility and robustness towards alteration processes, they can be considered as reliable molecular tools for the unambiguous identification of archaeological balsams. In addition to archaeological applications, these triterpenic markers might also find an application for the detection of counterfeit balsams.

### **Keywords:**

Styrax, Liquidambar, Myroxylon, GC/MS, sumaresinolic acid, 3-*epi* oleanolic acid

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## 1. Introduction

Balsams are defined as plant exudates containing a large variety of aromatic compounds, especially cinnamic and benzoic acids along with their related alcohol and ester derivatives [1,2]. According to this definition, only the sticky resinous material collected from plant species belonging to the genera *Styrax* (Styracaceae), *Liquidambar* (Altingiaceae, formerly included within Hamamelidaceae) and *Myroxylon* (Fabaceae) can be considered as “true” balsams [1,2]. The antibacterial, antifungal and expectorant properties of these substances were already known by Akkadian, Egyptian, Roman, Greek, Carthaginian, Inca and Amerindian populations. Balsams were used to treat, among others, chronic diseases of the respiratory tract (*e.g.*, cold and asthma) and the women pains [3-9], as well as by embalmers to avoid body putrefaction [10-12]. Moreover, due to the presence of volatile aromatic compounds, balsams were used for fumigation during religious rituals or for formulation of perfumes, ointments and cosmetics [8,13,14]. Nevertheless, the relatively similar healing and olfactory properties of the various resins known as balsams led to a major mix-up at the botanical and linguistic level. *Stricto sensu*, the resins collected from *Styrax* species (Styracaceae) should be named “styrax” whereas those obtained from *Liquidambar* species (Altingiaceae) should be referred to as “liquidambar” or “storax”. However, this semantic distinction is not systematically made and, in the literature, the term “styrax” has been assigned interchangeably to both resins. By contrast, the denomination of Tolu balsam and Peru balsam is well defined and refers to exudates from *Myroxylon balsamum* and *Myroxylon pereirae*, respectively - or *Myroxylon balsamum* var. *balsamum* and *Myroxylon balsamum* var. *pereirae*, some botanists considering that only a single species divided into two varieties exists [1]. Additionally, some of these resins are extremely rare because of a low yield of exudation. Such is the case for the resin from *S. officinalis* since the plant produces very little resinous material by notching the bark. This rarity has even led some botanists to raise serious doubts about the real existence of this balsam [1]. Climatic and environmental conditions have a major impact on the production of the resin of *S.*

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*officinalis*. Thus, resin production seems to be exclusively restricted to warm climate regions. This environmental dependence for resin production was known since the Antiquity, compelling therefore Greeks to import the coveted resin from Phoenicia [3,8].

As suggested by the abundance of texts reporting on the use of balsams in the past, it is likely that organic substances containing balsams might be encountered in archaeological contexts. In this respect, balsams have indeed been identified as constituents of the organic residue found in an Egyptian censer [15] or as an ingredient of an organic coating on a decorated skull unearthed at Nahal Hemar cave (Israel) and dated to the Pre-Pottery Neolithic B period (ca. 8200 –7300 cal. BC) [16]. It thus appears that reliable chemotaxonomic criteria based, in particular, on lipid markers allowing unambiguous identification of archaeological balsams are desirable and have to be established.

Previous molecular studies of balsams, reviewed by Pauletti *et al.* [7] and Custódio and Veiga-Junior [2], were however mainly dedicated to fresh material and focused on the characterisation of volatile and fragrant compounds, comprising monoterpenoids and ester derivatives of cinnamic, benzoic, *p*-coumaric and ferulic acids in *S. benzoin* [15,17-20], *S. paralleloneurum* [9,19], *S. tonkinensis* [9,20], *L. styraciflua* [21], *L. orientalis* [9,21-23] and *Myroxylon pereirae* [24] and some specificity of the distributions of these compounds with respect to their source could be evidenced. Nevertheless, in the case of archaeological balsams, these compounds cannot be easily used to recognise the nature of the plant species since they are prone to alteration, as illustrated by the case of the balsam identified on the skull of the Nahal Hemar cave [16]. In the latter case, the distributions of the cinnamic and benzoic acid derivatives, although useful to relate the substance on the skull to a balsam-type resin, were too altered to allow a distinction to be made between a resin from *S. officinalis* and *L. orientalis* - the two possible botanical sources in the eastern Mediterranean region -. Alteration processes which had apparently affected cinnamic and benzoic acid derivatives comprised partial loss by evaporation due to high volatility and, possibly, cross-reactions (transesterification) with other

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constituents of the organic substance on the skull [16]. In addition, the predominant cinnamic and benzoic esters in balsams are potentially prone to hydrolysis and, similarly to unsaturated fatty acids [e.g. 25], the double bond of cinnamates might be cleaved by oxidative processes. Therefore, other species-specific molecular tools are required for the reliable identification of balsams (e.g. *Styrax* sp., *Liquidambar* sp. or *Myroxylon* sp.) notably when altered material is met (e.g., during archaeological excavations).

Plant triterpenoids are potentially good candidates for this purpose since their structures present a higher resistance and lower volatility compared to the compounds mentioned above and a number of studies on resinic lipids have demonstrated the high species-specificity of some triterpenoids (e.g., [26-29]). In addition to the archaeological interest mentioned above, the identification of resins using triterpenes might also be useful for the detection of counterfeit balsams. As a matter of fact, triterpenes might allow not only the unambiguous correlation with the precise botanical source of the balsam to be made, but also identification of resins from other sources (e.g. dammar resin, notably) that may have been used for adulteration. Indeed, because of limited supply and high cost of some balsams, substitution resins have often been used. For instance, the gum of *L. orientalis* has been an alternative of the Mediterranean styrax and the resin of *Altingia excelsa* (Altingiaceae) was used as a substitute of styrax and liquidambar resins [1]. The trade of imitations and adulterated products is even more problematical and appears to be a long-standing practice, Pliny the Elder reporting in the 1<sup>st</sup> century AD some fraud cases [5]. Even nowadays, the material sold on the market often turns out to be adulterated most generally with dammar resin or colophony, as reported by several authors [1,30], and observed by ourselves during chemical analyses of commercially-available resins.

However, despite representing potential chemotaxonomic markers, there are only few studies dedicated to the triterpene composition of balsams (*S. tonkinensis*: [9,31]; *L. orientalis*: [32]). We have therefore carried out a systematic and detailed molecular study of the triterpenoid

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distributions from styrax and liquidambar resins using GC/MS in order to explore their potential as chemotaxonomic indicators. For completeness, resins from different parts of the world have been investigated when they were available and unequivocally assigned to a botanical origin. Thus, in the case of Styracaceae, we have investigated two resin samples of *S. officinalis* native from the Middle East and the Mediterranean area [8], as well as resin samples of *S. tonkinensis* (South Eastern Asia) and *S. paralleloneurum* (Malaysia and Indonesia; [33]). Most significantly, the lipid distribution of the resin of *S. officinalis* is presented here for the first time. As a representative of the Altingiaceae family, we have examined the lipid content of the gum of *L. orientalis*, which is endemic from Anatolia in Turkey, and that of the resin of *L. styraciflua* native from North and Central America [1]. Finally, the occurrence of triterpenes was investigated within Peru balsam (*M. balsamum* var. *pereirae*) which find its origin in the Americas [1,2]. Several plant materials (aerial parts from *S. officinalis*, *M. balsamum* var. *balsamum* and *M. balsamum* var. *pereirae*) were also analysed, but the outcome of the lipid analysis of these materials will only be discussed in supplementary materials as it is not the primary focus of the present paper.

## 2. Experimental

### 2.1. Plant and resin material

Preliminary GC/MS analysis of the lipids from a large set of commercial balsams has revealed that most of them were adulterated. Therefore, we looked insofar as possible to obtain certified exudates or plant elements (Table 1). Bark, leaves and seed samples of *S. officinalis* were collected in October 2014 on a shrub from the Botanical Garden of Strasbourg (France). Two fresh resin samples of *S. officinalis* were collected in 2015 as part of the study, one in Yodfat (Israel) kindly provided by Dr. A. Nissenbaum (Weizmann Institute of Science, Israel), Prof. Z. Ammar (Bar Ilan University, Israel), Prof. M. Kislev (Bar Ilan University, Israel) and Prof. A. Dafni (Haifa University, Israel), the other one in the Botanical Garden of Montpellier (France) - authentication done by V. Girard (ISEM, University of Montpellier, France) -.

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Additionally, resins from *S. tonkinensis* (“Siam benjoin”) and *S. paralleloneurum* (“Sumatra benjoin”) were investigated (gift from E. Dodinet, Laboratoire Archéologie TRACES, University of Toulouse, France). Liquidambar resins (*L. orientalis* and *L. styraciflua*) have been purchased from the company of fragrance and aroma ROBERTET. Peru balsam was gathered in December 2016 by the company Rivera Balsam (J. Paul Rivera S.A. de C.V., El Salvador) and kindly provided for the study. Specimens of leaves and branches of *M. balsamum* (L.) Harms var. *balsamum* from Belgium (from Jardin colonial Laeken) and of *M. balsamum* (L.) Harms var. *pereirae* (Royle) Harms (from Democratic Republic of Congo, I.N.E.A.C.) were given by Dr. Marc Reynders and Mr. Franck van Caekenberghe (Botanic Garden of Meise).

## 2.2. Lipid extraction and sample preparation

The samples were extracted under sonication using a mixture of DCM/MeOH (1:1, v/v). An aliquot of the total lipid extract (TLE) recovered after filtration through celite and elimination of the solvents under reduced pressure was acetylated using acetic anhydride (1 mL) and *N*-methylimidazole (20 µL, catalyst) for 20 min at ambient temperature. After removal of the solvents and excess reagents, the acetylated TLE was methylated with diazomethane in diethylether. Following removal of the excess of diazomethane and solvents under a gentle stream of N<sub>2</sub>, the derivatized organic extract was fractionated on a silica gel column yielding an apolar fraction eluted with a mixture of DCM/EtOAc (8:2, v/v) and a more polar fraction eluted with DCM/MeOH (1:1, v/v). The latter was not further investigated.

## 2.3. GC/MS

GC/MS analyses were performed on a Thermo GC Trace gas chromatograph equipped with a HP5 MS column (30 m x 0.25 mm x 0.1 µm) and a temperature programmable injector linked to a Thermo TSQ Quantum mass spectrometer. Helium was used as carrier gas and the oven was programmed as follows: 70 °C (1 min), 70 °C - 200 °C (10 °C/min), 200 °C - 320 °C (4 °C/min), isothermal at 320 °C for 40 min. The mass spectrometer was operating in electron



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impact mode (70 eV) with a scan range of  $m/z$  50-700. In some cases, chemical ionisation (CI) analyses were performed using isobutane.

## 2.4. GC/TOF

GC-TOF analyses were carried out on a Agilent Technologies 7890A gas chromatograph equipped with a split/splitless injector (used in the splitless mode), a DB5-UI column (20 m x 0.18 mm; 0.18  $\mu$ m film thickness). The GC was coupled to a time-of-flight (TOF) JEOL Accu TOF GCV JMS-T100GCV mass spectrometer operating in the EI mode (70 eV) or field ionization (FI) mode with a scan range of  $m/z$  35-800. Helium was used as carrier gas and the temperature program was identical to that described in section 2.3.

## 3. Results

### 3.1. Phenylpropanoids in *Styrax*, *Liquidambar* and *Myroxylon* balsams

The balsams of *Styrax*, *Liquidambar* and *Myroxylon* species are characterised by the presence of ester derivatives of cinnamic and benzoic acids (Fig. 1, 2 and 3 respectively), this molecular assemblage being responsible for the specific pleasant flavour of the resins. In MS, detection of the ester derivatives of cinnamic and benzoic acids mainly relies on the presence of typical fragments respectively at  $m/z$  131 and 105 (Fig. S1). However, in some cases, the mass spectra of these compounds are relatively “poor” in terms of structural information (i.e., absence of molecular ions and of diagnostic fragmentation ions) preventing unambiguous structural assignments of the alcohol moieties of benzoic acid and cinnamic esters.

#### 3.1.1. *Styrax*

Overall, GC/MS investigation of the cinnamate and benzoate derivatives from the four *styrax* resins investigated in this study revealed significant differences in their molecular distributions (MS data in Supplementary Information, Fig. S1 and Table S1). Indeed, the predominance of ester derivatives of cinnamic acid vs. benzoic acid was observed in the resins of *S. officinalis*

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(Fig. 1a and 1b) and *S. paralleloneurum* (Fig. 1c), whereas the opposite occurred in the exudate of *S. tonkinensis* (Fig. 1d).

More specifically, the two resin samples of *S. officinalis* are characterised by the predominance of cinnamyl cinnamate **1** (Fig. 1a and 1b), followed by methoxyeugenol **2**, cinnamic acid **3** and benzyl cinnamate **4** as other quantitatively important constituents. Cinnamaldehyde **5**, vanillin **6**, eugenol **7**, vanillic acid **8**, benzyl benzoate **9**, cinnamyl benzoate **10**, ferulic acid **11**, cinnamyl alcohol **12**, propyl cinnamate **13**, coniferyl alcohol **14** and 3-phenylpropanyl cinnamate **15** were detected as minor constituents, compounds **12-15** being exclusively observed in the sample from Israel. The presence of 3-phenylpropanyl cinnamate **15** (trace amounts) in a Styracaceae resin may be seen as a contradiction with the statement of Pastorova et al. [23] who have suggested that this compound could be specific to the Hamamelidaceae gums. Nevertheless, when present in high relative amounts, 3-phenylpropanyl cinnamate **15** can still be considered as a chemotaxonomic marker for Hamamelidaceae gums since it is a predominant constituent of Liquidambar (see § 3.1.2 and Fig. 2). The structure of compound **16** - the latter being particularly abundant in the styrax resin from Israel and to a lesser extent in that from France - could not be fully established although a mass fragment at  $m/z$  167 suggests the presence of a syringyl moiety [33]. Compound **16** is absent from the bark and from the others aerial parts of the sample of *S. officinalis* investigated (see Supplementary material) and seems to be specifically associated to the resinous material.

Cinnamic acid **3** largely predominates the lipid distribution of the balsam exuded from *S. paralleloneurum* (Fig. 1c), in accordance with previous studies [1,9]. It is associated with *p*-coumaryl cinnamate **17**, cinnamyl cinnamate **1**, benzyl cinnamate **4** and coniferyl cinnamate **18** (in decreasing order of relative abundance). In addition to minor amounts of compounds **5-11**, **14** and **15**, also present in the resin of *S. officinalis*, small amounts of 4-hydroxybenzaldehyde **19**, chavicol **20**, 4-hydroxybenzoic acid **21**, *p*-coumaryl alcohol **22**, *p*-coumaric acid **23**, coumaryl benzoate **24**, coniferyl benzoate **25** and 3-(4-hydroxyphenyl)-propyl cinnamate **26**

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were detected. The rather low relative amounts (compared to free cinnamic acid) of esters of cinnamic acid (*e.g.*, compounds **1**, **4**, **17** and **18**) could be associated to a low-grade resin [19] or could be inherent to the resin of *S. paralleloneurum*.

In contrast to the resins of Styracaceae described above, the lipid extract of *S. tonkinensis* is characterised by abundant ester derivatives of benzoic acid (*m/z* 105; Fig. 1d). Indeed, coniferyl benzoate **25** represents by far the major compound in the exudate of *S. tonkinensis* along with vanillin **6**, benzyl benzoate **9**, *p*-coumaric acid **23** and *p*-coumaryl benzoate **24** occurring as minor constituents [9,18]. The importance of **25** was already noticed by Hovaneissian *et al.* [9] who proposed to use this compound as a marker for Siam benzoin. GC/MS analysis revealed small quantities of compounds **2-4**, **7**, **8**, **10**, **11**, **14**, **19** and **20**, also found in *S. officinalis* and *S. paralleloneurum* (with the exception of **19** and **20** for the latter resin), together with the benzoate derivatives **27** to **29**.

### 3.1.2. Liquidambar

The chromatographic profiles corresponding to the apolar lipids of the gums from *L. orientalis* and *L. styraciflua* (Fig. 2) unveiled substantial similarities and clearly differ from those of the resins of Styracaceae by the strong predominance of cinnamyl cinnamate **1** and 3-phenylpropanyl cinnamate **15**. Although trace amounts of **15** were detected in some styrax resins (see above), its high abundance together with that of **1** is a typical feature of freshly collected resins from liquidambar as shown by Pastorova *et al.* [23]. Among the minor compounds, the presence of cinnamic acid **3**, benzyl cinnamate **4**, cinnamyl alcohol **12**, *p*-coumaryl cinnamate **17** and 3-phenylpropanol **30** in both liquidambar was observed. Other compounds were identified exclusively in the American liquidambar (*L. styraciflua*), namely cinnamyl benzoate **10**, *p*-coumaryl alcohol **22** and 3-(4-hydroxyphenyl)-propyl cinnamate **26**. Apart from a few exceptions such as that of benzyl benzoate **9** not detected in our liquidambar gums, the molecular distributions of cinnamates and benzoates are close to those described in the literature for liquidambar exudates [9,22,23,35].

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### 3.1.3. Peru balsam (*Myroxylon balsamum* (L.) Harms var. *Pereirae*)

Ester derivatives of cinnamic and benzoic acids were also identified in Peru balsam (Fig. 3). Benzyl benzoate **9** prevails in the lipid extract - as already observed in a 18<sup>th</sup> century Peru balsam from the Vigani Cabinet [36] - and cinnamic acid **3** with benzyl cinnamate **4** figure among the main constituents. Compounds **31** to **33** were also detected in a relatively large amount in the lipid extract of Peru balsam, but their structures were difficult to establish based solely on GC/MS data (MS shown in Fig. S2). Compounds **31** and **33** are likely to be phenylpropanoid derivatives as they show characteristic MS base peaks of benzoate and cinnamate derivatives ( $m/z$  105 and  $m/z$  131, respectively). Similarly, **32** is characterised by a main fragment at  $m/z$  91 as commonly observed for aromatic compounds. However, being out of scope of our study which primarily aims at looking for triterpenoid markers, no further structural investigation was carried out. Finally, in accordance with the work of Seo et al. [24], E-nerolidol was also identified in significant amount.

### 3.2. Lignan and neolignan derivatives in aromatic balsams

Investigation of the two resins of *S. officinalis* revealed the occurrence of the neolignans egonol **34** and homoegonol **35** belonging to the benzofuran group (EI mass spectra shown in Supplementary, Fig. S3). The relative abundance of **34** and **35** in the sample from Israel was particularly marked (Fig. 1b) and has likely been induced by unintentional addition of small bark fragments during resin collection. This was further confirmed by the occurrence of **34** and **35** as predominant neolignans in the bark from *S. officinalis* (Fig. S4a). The same compounds were also found in the fruits from the plant (Fig. S4c), together with related derivatives **36-40**, the structure of which being proposed based on the interpretation of their mass spectra. It is noteworthy that **34** and **35** were also identified in trace amount in the resin of *S. paralleloneurum* and *S. tonkinensis* (Fig. 1). According to the literature, it appears more broadly that egonol and homoegonol are found in aerial parts (leaves, stems, seeds, bark) of trees from the genus *Styrax* ([7] and references herein, [37-39]).

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Lignans are constituted of two monolignol units (*e.g.*, coniferyl alcohol) linked at the carbon position C8-C8' (examples of EI mass spectra are shown in Supplementary, Fig. S5). Besides pinoresinol **41** ubiquitous in the Plant Kingdom [40,41], the two resins from *S. officinalis* contain styraxin **42** (also occurring in the bark as shown in Fig. S4a), sesamin **43** and piperitol **44** (Fig. 1a and 1b). As far as we know, since its characterisation by Ulubelen *et al.* [42] in the aerial parts of *S. officinalis*, styraxin **42** has not been reported to occur in other *Styrax* species and in any other plants. Hence, styraxin **42** could represent a specific marker of *S. officinalis* and could be used as an indicator for the use of *S. officinalis* resin in an archaeological context, as attested by its identification in an 8000 years-old decorated skull [16].

Aside from pinoresinol **41** which was detected in the twigs from *M. balsamum var. balsamum* (Fig. S6), none of the lignans and neolignans described above was identified in the liquidambar gums, in the Peru balsam and the other aerial parts of the Myroxylon species investigated in this study.

### 3.3. Triterpenoids in styrax resins

There are only a few reports in the literature on the triterpenoid content of Styracaceae. These include the triterpenoids from the stem-bark of *S. japonica* [43], from the leaves of *S. camporum* [44], and, surprisingly, there is only one triterpenoid study on the resin of a member of the genus *Styrax* (*S. tonkinensis*; [31]). The triterpenoid content of the resins from *S. officinalis* and *S. paralleloneurum* have not been investigated to date, with the exception of the article by Yayla *et al.* [45] reporting triterpene saponins from *S. officinalis*. This limited number of studies might be partly explained by their very low abundance relative to cinnamate and benzoate derivatives in the resins.

GC/MS investigation of the triterpenoids from *S. officinalis*, *S. paralleloneurum* and *S. tonkinensis* resin showed that they belong to the oleanane, and, to a lesser extent, to the ursane family (Fig. 4a), and can be divided into three group series:

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- (a) triterpenoids relatively common in the Plant Kingdom, namely oleanonic (**45**), oleanolic (**46**) and ursolic (**47**) acids (EI mass spectra shown in Supplementary; Fig. S7a-c)
- (b) rather rare triterpenoids having the same main fragments in MS as **45-47**, but with molecular ions shifted upwards by 14 or 16 mass units (**48-50**; Fig. S7d-f).
- (c) uncommon triterpenoids displaying a base fragment at  $m/z$  201 and a minor fragment at  $m/z$  278 in their mass spectra (**51** and **52**; Fig. S7g-h).

For the compounds from the second series, the absence or very low intensity of molecular ions under the conditions used (triple quadrupole, EI, 70 eV) initially prevented us to propose a generic structure. However, GC-HRMS analysis of the same compounds using a TOF mass analyser under either EI or FI allowed the molecular mass of **48** ( $M^+$  484), **49** ( $M^+$  526) and **50** ( $M^+$  528) to be unambiguously determined. These data, in combination with the fragmentation pattern observed, are in good agreement with the mass spectra of triterpenoids belonging to the sumaresinolic acid generic series reported in a commercial Tolu balsam (*Myroxylon balsamum*) by Wahlberg and Enzell [30]. It should however be mentioned that the exact nature of this commercial Tolu balsam has to be questioned, as noted by the authors themselves, and it is likely that this “Tolu” balsam was in fact a counterfeit made with a mixture of resins of different botanical origins comprising Dipterocarpaceae resin (see discussion below in paragraph 4). The EI mass spectra of compounds **48** (3-oxo-sumaresinolic acid), **49** and **50** (sumaresinolic acid) are shown in Supplementary Fig. S7d-f. In agreement with the data from Wahlberg and Enzell [30], the lack of reactivity of the hydroxyl group at C-6 towards acetylation confirmed that it is located at a sterically-hindered position (i.e., at the axial position).

The uncommon triterpenoids of the third series (**51** and **52**) belong to the oleanane series as well and were exclusively detected in the resin of *S. tonkinensis*. The presence of a fragment at  $m/z$  278 in their mass spectra might be ascribed to a retro-Diels-Alder type fragmentation of the

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ring C of a triterpenoid having a  $\Delta^{12}$  double bond and a carbomethoxy group at C-17 ( $m/z$  262; [46]) plus a hydroxyl group on the C/D/E ring moiety, as further confirmed by the loss of H<sub>2</sub>O ( $m/z$  260) from the  $m/z$  278 fragment (EI mass spectra and fragmentation pattern shown in Supplementary; Fig. S7g-h). The fact that this hydroxyl group could not be acetylated suggests that it is located at C-19 at a sterically-hindered position (i.e.,  $\alpha$  stereochemistry) [47]. The mass spectrum of **51** (Fig. S7g; Supplementary Information) is in agreement with published data [48]. For compound **52**, the molecular ion could not be observed under EI (Fig. S7h, Supplementary Information), but was unambiguously present using CI with isobutane as reactant gas ( $[M+H]^+$  at  $m/z$  529). It can thus be proposed that compound **52** represents the C-3 acetylated derivative of 3 $\beta$ ,19 $\alpha$ -dihydroxy-olean-12-en-28-oic acid (siaresinolic acid).

### 3.4. Triterpenoids in liquidambar gums

The triterpenoid distributions of the two liquidambar gums are closely related (Fig. 4b) and are characterised by the presence of derivatives of oleanolic and ursolic acids (presence of typical fragments at  $m/z$  203 and  $m/z$  262; [46]). They comprise oleanonic acid **45** and its 3-*epi* isomer **53**, as described by Huneck [32], along with oleanolic acid **46**, maslinic acid **54**, ursonic acid **55** and 3-*epi*-ursolic acid **56**. Additionally, 6 $\beta$ -hydroxy-3-oxo-olean-12-en-28-oic acid **48** (3-oxo-sumaresinolic acid) was detected, this compound being reported to occur in the cones of *L. styraciflua* [49]. Furthermore, oleanolic and oleanonic aldehydes **57** and **58** bearing the aldehyde group at C-28, as shown in MS by the occurrence of a retro-Diels-Alder type fragment at  $m/z$  232, were detected. Finally, constituents from the lupane series comprising lupeol **59** and 6 $\beta$ -hydroxy-3-oxo-lup-20(29)-en-28-oic acid **60** were identified. The latter has also been reported to occur in the cones from *L. styraciflua* [49,50].

## 4. Cinnamate and benzoate derivatives *versus* triterpenoids as lipid markers to differentiate between balsams

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Fresh styrax resins, liquidambar and Peru balsam can be differentiated by their content in phenylpropanoids as summarized in Table 2. Cinnamyl cinnamate **1**, cinnamic acid **3** and coniferyl benzoate **25** represent the main constituents of the resin from *S. officinalis*, *S. paralleloneurum* and *S. tonkinensis* respectively whereas cinnamyl cinnamate **1** with 3-phenylpropanyl cinnamate **15** prevail in both *L. orientalis* and *L. styraciflua* gums. By consequence, in the case of fresh, undegraded material, each of those compounds described above can be regarded as specific biomarkers allowing a distinction to be made between styrax and liquidambar resins as well as within the *Styrax* species. Furthermore, the distribution of ester derivatives of cinnamic acid and benzoic acid in the Peru balsam, dominated by benzyl cinnamate **4** and benzyl benzoate **9**, also clearly differs from those observed in the styrax and liquidambar resins. However, given their structures, these aromatic derivatives are sensitive towards alteration processes and their genuine distributions will likely be lost and/or strongly modified in the case of severely altered material, as is generally the case with archaeological findings. Furthermore, the lower molecular weight homologues (*e.g.*, free cinnamic and benzoic acids) will be lost preferentially over time because of their high volatility and water solubility (leaching). Additionally, the relative distribution of the aromatic ester derivatives can depend upon the quality of the resins [19], which hinders quantitative analysis from being used as a discriminating tool. Intermolecular esterification reactions may also occur during ageing processes, leading to the neoformation of cinnamate and benzoate derivatives absent from fresh balsams. For instance, such a possibility may explain the presence of a number of high molecular weight ester derivatives of cinnamic and benzoic acids detected in a 8000-years old organic coating from a decorated human skull [16]. Following these considerations, ester derivatives of cinnamic and benzoic acids on their own are not reliable markers to allow a clear discrimination to be made between balsams, notably in the case of ancient material unearthed at archaeological sites.



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For this reason, triterpenoids were investigated in order to evaluate their potential as species-specific biomarkers. The styrax resins studied are all characterised by the occurrence of sumaresinolic acid **50** and its derivatives (**48** and **49**; Fig. 4a), in accordance with the molecular description of a *S. tonkinensis* resin reported by Wang et al. [31]. In addition, the composition of the resin of *S. tonkinensis* contrasts with other styrax resins by the presence of siaresinolic acid **51** and its C3-ketone analogue **52**. Despite a thorough search for the presence of compounds **50** and **52**, they could not be detected in the other balsams investigated, which led us to explore their potential as taxonomic markers for styrax resins.

According to the literature, it appears that sumaresinolic and siaresinolic acids are not restricted to the *Styrax* genus but also occur in the aerial parts of plant species from other genera (e.g., [51-56]). Nonetheless, none of these plants produce resinous exudates containing ester derivatives of cinnamic and benzoic acids. Certainly, 3-oxo-sumaresinolic acid **48** was identified in the cones of *L. styraciflua* [49,57] and minute amount of **48** was also found within the two liquidambar balsams investigated (Fig. 4b), but not in association with **49** and **50**. Compounds **48** and **50** were reported by Wahlberg and Enzell in a commercial Tolu balsam as well [30,58] and in collection material [36]. However, the identification of dammarane skeletons including dammaradienone, 20*R*-hydroxy-dammarenone and 20*S*-dammarenediol [36,58] within the “Tolu balsam” triterpenoids suggests that these Tolu balsams investigated were adulterated by resinous material from Dipterocarpaceae (dammar resin; [59,60]) - as envisaged by the authors themselves [30,36] - which consequently brings into question the origin of sumaresinolic acid in the balsams. Unfortunately, we did not manage to obtain certified Tolu balsam from *Myroxylon balsamum* var. *balsamum* to carry out our own investigation but GC-MS analysis of certified Peru balsam (*M. balsamum* var. *peirerae*) from El Salvador attested to the complete absence of triterpenoids within this exudate. It is also worth noting that sumaresinolic derivatives **48-50** could not be detected in the aerial part of *S. officinalis* (Fig. S4), suggesting that these compounds are inherent to the resinous material.

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Hence, we suggest that the co-occurrence of the triterpenoids **48-50** together with ester derivatives of cinnamic and benzoic acids could be considered as characteristic of resins from *Styrax* species. Furthermore, among the resins investigated, only *Styrax* species did contain the neolignans **34** (egonol) and **35** (homoegonol), in agreement with the literature ([7] and references herein, [37-39]). Because of that, they can be considered as additional specific molecules which may be used to recognise styrax resins. It is however important to note that **34** and **35** are typical constituents not only of styrax resin, but also of the aerial parts of the plants (bark and seeds; Fig. S4a, c). Therefore, their presence within the resin lipids may be attributed to the contribution of bark, for example, that could have been collected while harvesting the resin.

Liquidambar resins are characterised by a high abundance of oleanonic acid **45** along with a low amount of oleanolic and ursolic acids having the uncommon 3 $\alpha$ -OH stereochemistry (**53** and **56**). This triterpenoid assemblage can be defined as specific to liquidambar since it has not been observed with the balsams of other botanical origin.

The present study shows that triterpenoids represent powerful taxonomic tools for the characterisation of higher plant resins, allowing a clear distinction between styrax resins and liquidambar gums to be made using molecular criteria. The Peru balsam investigated was characterised by a complete lack of triterpenoids which questions the findings from previous studies of the literature on the triterpenic content of Peru and Tolu balsam [30,58]. Finally, the lipid content of balsams from Myroxylon would need to be carefully investigated given the presence of numerous (and sometimes predominant) compounds with uncharacterised structures.

### Appendix

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## Conflict of interest

The authors declare that they have no conflict of interest.

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## Figure caption

**Figure 1.** Gas chromatograms (TIC) of the apolar fraction from the lipid extract obtained from a) the resin of *S. officinalis* originating from France (Botanical Garden of Montpellier); b) the resin of *S. officinalis* collected in Israel (Yodfat); c) the resin of *S. parallelon*; d) the resin of *S. tonkinensis*. Alcohols and carboxylic acids are analysed as acetates and methyl esters, respectively.

**Figure 2.** Gas chromatograms (TIC) of an apolar fraction isolated from the lipid extract obtained from a) the gum of *L. orientalis*; b) the gum of *L. styraciflua*, both purchased from the fragrance company ROBERTET. For an enlarged zone of the triterpenes, see Fig. 4b. Alcohols and carboxylic acids are analysed as acetates and methyl esters, respectively.

**Figure 3.** Gas chromatograms (TIC) of an apolar fraction isolated from the lipid extract of Peru balsam (Rivera Balsam, El Salvador). Alcohols and carboxylic acids are analysed as acetates and methyl esters, respectively.

**Figure 4.**  $m/z$  189+201+203 mass fragmentograms showing the triterpenoids content a) in the styrax resins; b) in the liquidambar gums. Alcohols and carboxylic acids are analysed as acetates and methyl esters, respectively.

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	Sample number	Plant element	Provenance
<b>Styracaceae, <i>Styrax</i> sp.</b>			
<i>S. officinalis</i>	2982	resin (+ bark)	Yodfat, Israel, collected in 2015
	3020	resin	Botanic Garden of Montpellier, France, collected in 2015
	-	fruits	Botanic Garden Strasbourg, France, collected in October 2014
	-	leaves	
	-	bark	
<i>S. parallelon</i>	2984	resin	Sumatra (Indonesia)
<i>S. tonkinensis</i>	2986	resin	Bought in Syria
<b>Altingiaceae (formerly included within Hamamelidaceae), <i>Liquidambar</i> sp.</b>			
<i>L. orientalis</i>	-	gum	ROBERTET
<i>L. styraciflua</i>	-	gum	ROBERTET
<b>Fabaceae, <i>Myroxylon</i> sp.</b>			
<i>M. balsamum</i> <i>var. balsamum</i>	19373049-F	leaves	Botanic Garden of Meise (Belgium) – <u>source</u> : Jardin colonial Laeken, Bruxelles (Belgium)
	19373049-B	branches	
<i>M. balsamum</i> <i>var. pereirae</i>	-	Peru balsam	J. Paul Rivera S.A. de C.V., El Salvador, date of production: December 2016
	19546864-F	leaves	Botanic Garden of Meise (Belgique) –
	19546864-B	branches	<u>source</u> : Institut National pour l'Etude Agronomique du Congo Belge, Democratic Republic of Congo

**Table 1** Sample list of resins and aerial parts from *Styrax*, *Liquidambar* and *Myroxylon* species.

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	predominant ester derivatives of cinnamic or benzoic acid	triterpenoids	Other markers
<b>Styrax resins</b>			
<i>S. officinalis</i>	cinnamyl cinnamate <b>1</b>	sumaresinolic acid <b>50</b> and its derivatives <b>48</b> and <b>49</b>	egonol <b>34</b> and homoegonol <b>35</b> (in bark)
<i>S. paralleloneurum</i>	cinnamic acid <b>3</b>		
<i>S. tonkinensis</i>	coniferyl benzoate <b>25</b>	sumaresinolic and siaresinolic acids ( <b>50</b> and <b>52</b> ) along with their derivatives ( <b>48</b> , <b>49</b> and <b>51</b> )	+ styraxin <b>42</b>
<b>Liquidambar gums</b>			
<i>L. orientalis</i>	cinnamyl cinnamate <b>1</b> and	oleanonic acid <b>45</b> (major) with 3-epi-oleanolic acid <b>53</b> (minor)	-
<i>L. styraciflua</i>	3-phenylpropanyl cinnamate <b>15</b>		
<b>Myroxylon balsams</b>			
Peru balsam	Benzyl benzoate <b>9</b>	-	-

**Table 2** Molecular criteria for discriminating between styrax resins, liquidambar resins and Peru balsam.

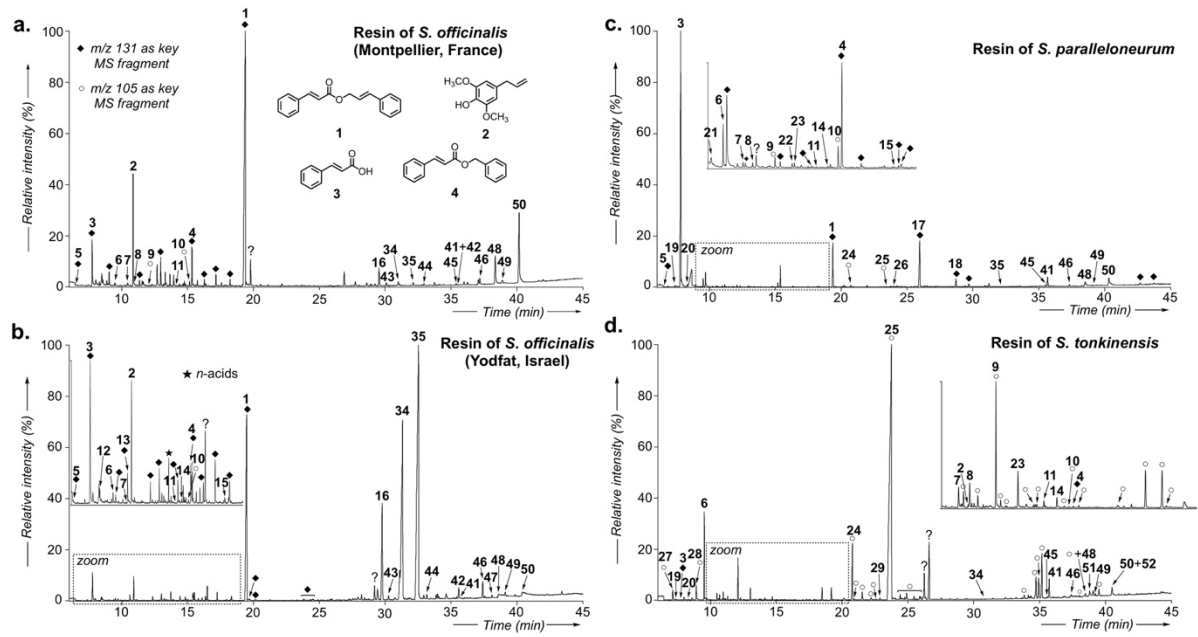
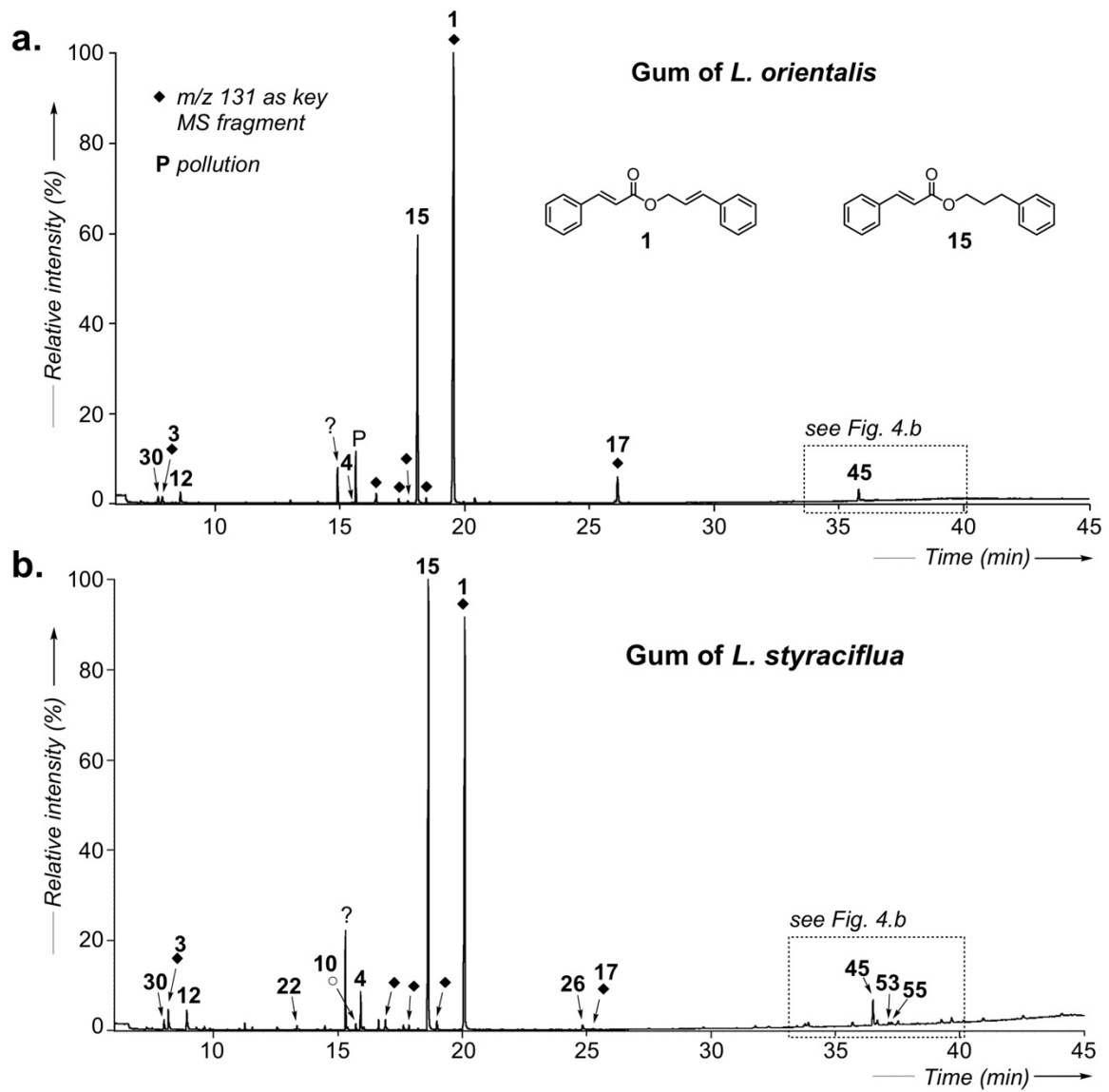


Fig. 1



**Fig. 2**

# Version avant révision

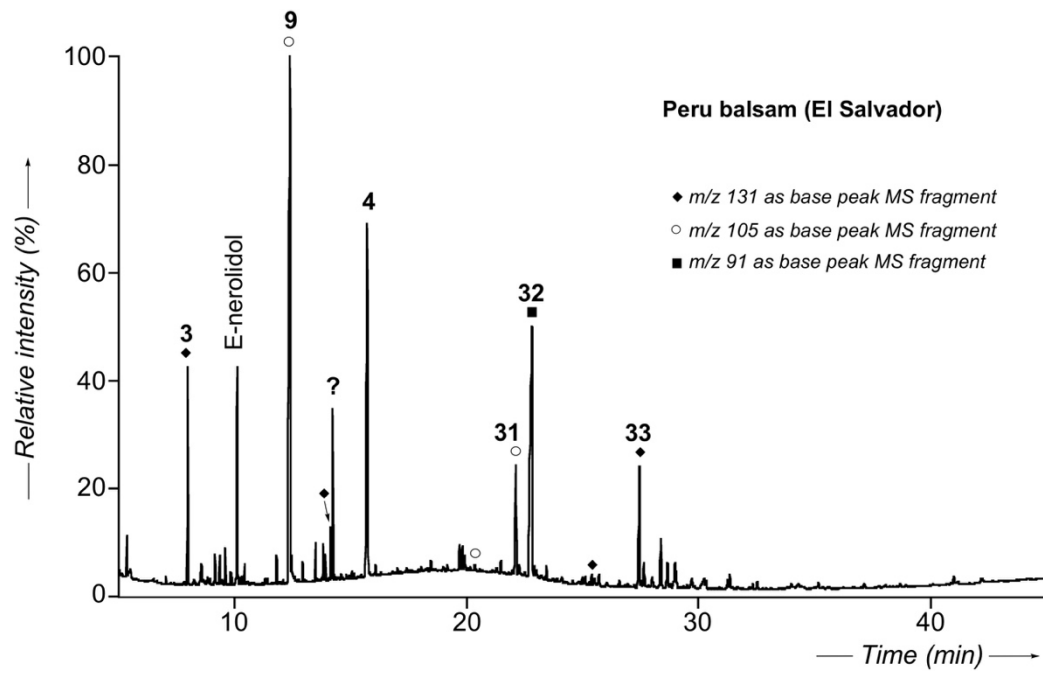


Fig. 3

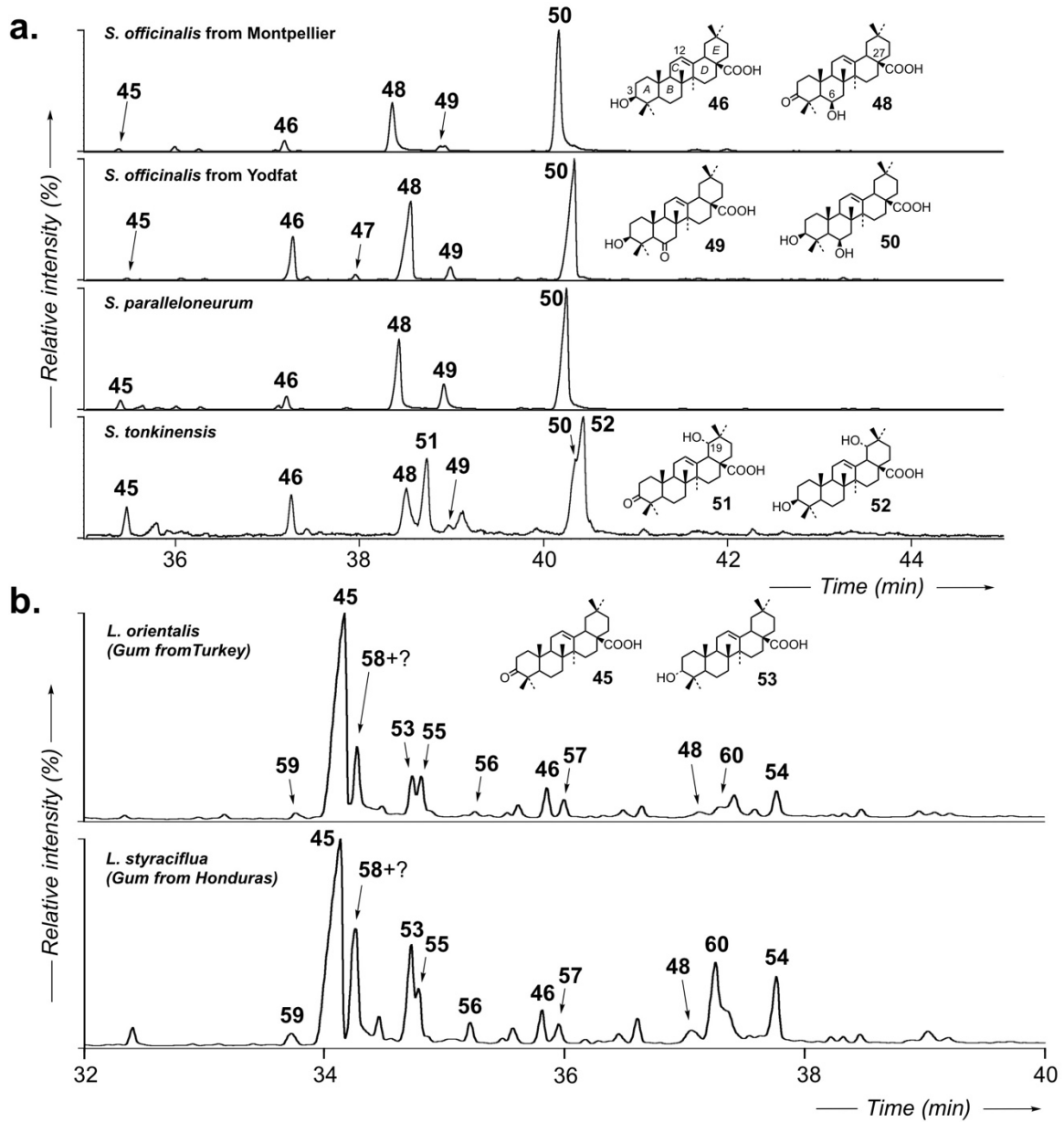
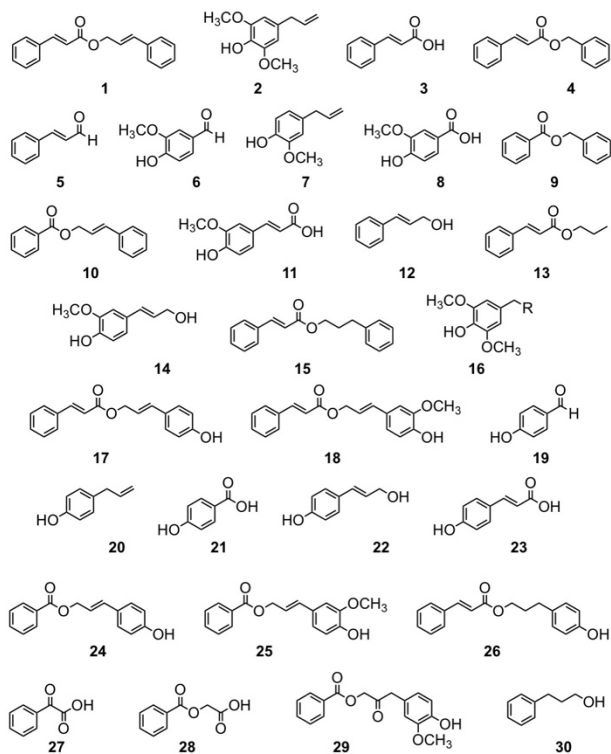
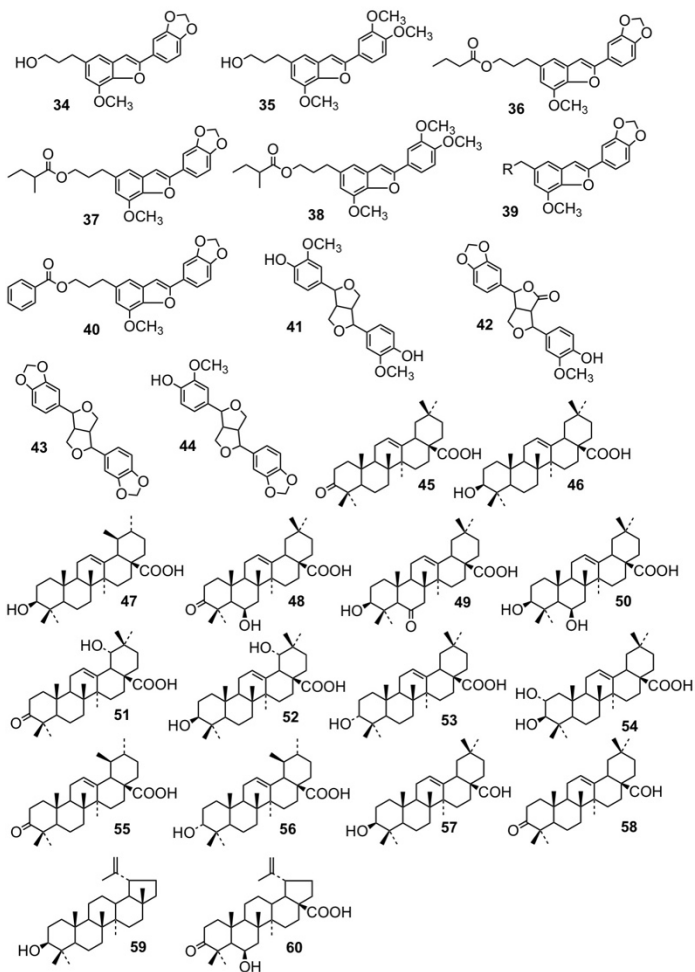


Fig. 4

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31-33: unknown structures



Appendix



# Version avant révision

1 **Supplementary Information**

2

3 **Triterpenoids as chemotaxonomic tools to identify and differentiate genuine, adulterated**  
4 **and archaeological balsams**

5 Courel Blandine<sup>1\*</sup>, Adam Pierre, Schaeffer Philippe\*

6 *Université de Strasbourg, CNRS, Institut de Chimie de Strasbourg UMR 7177, F-67000*

7 *Strasbourg, France*

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<sup>1</sup> *Present address: The British Museum, Great Russell Street, London WC1B 3DG, United Kingdom*

\* Corresponding authors.

*E-mail addresses* : BCourel@britishmuseum.org (B. Courel), p.schaef@unistra.fr (P. Schaeffer).

## Investigation of the triterpenoid content of plant materials

### 1. Triterpenoids in the aerial parts from *S. officinalis*

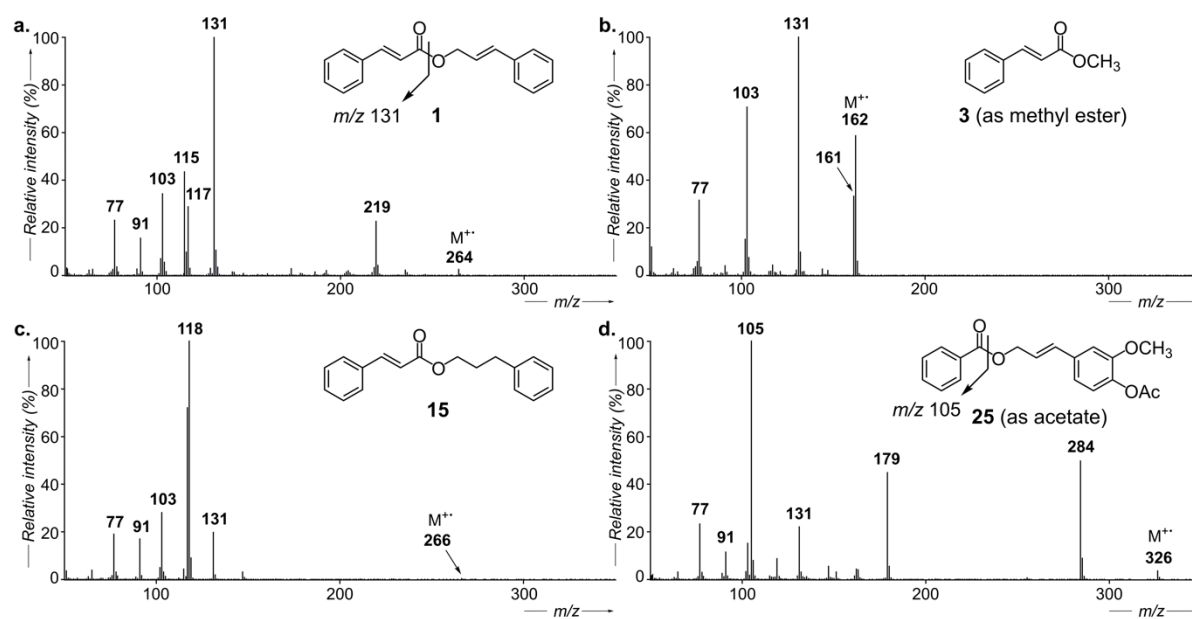
The triterpenoid distributions from the aerial parts (bark and leaves) of *S. officinalis* are clearly distinct from those observed in the resins, except for the co-occurrence of the non-specific oleanolic and ursolic acids **46** - numbers refer to the structures shown in Fig. S8 - and **47**. The triterpenoids detected comprise betulin **61**, erythrodiol **62**, oleanolic aldehyde **57** and betulinic acid **63** (Fig. S4). It is worth noting that the seeds were devoid of triterpenoids and comprised predominantly lignin/neolignan derivatives (see above; Fig. S4c).

### 2. Triterpenoids in plant material from *Myroxylon* species

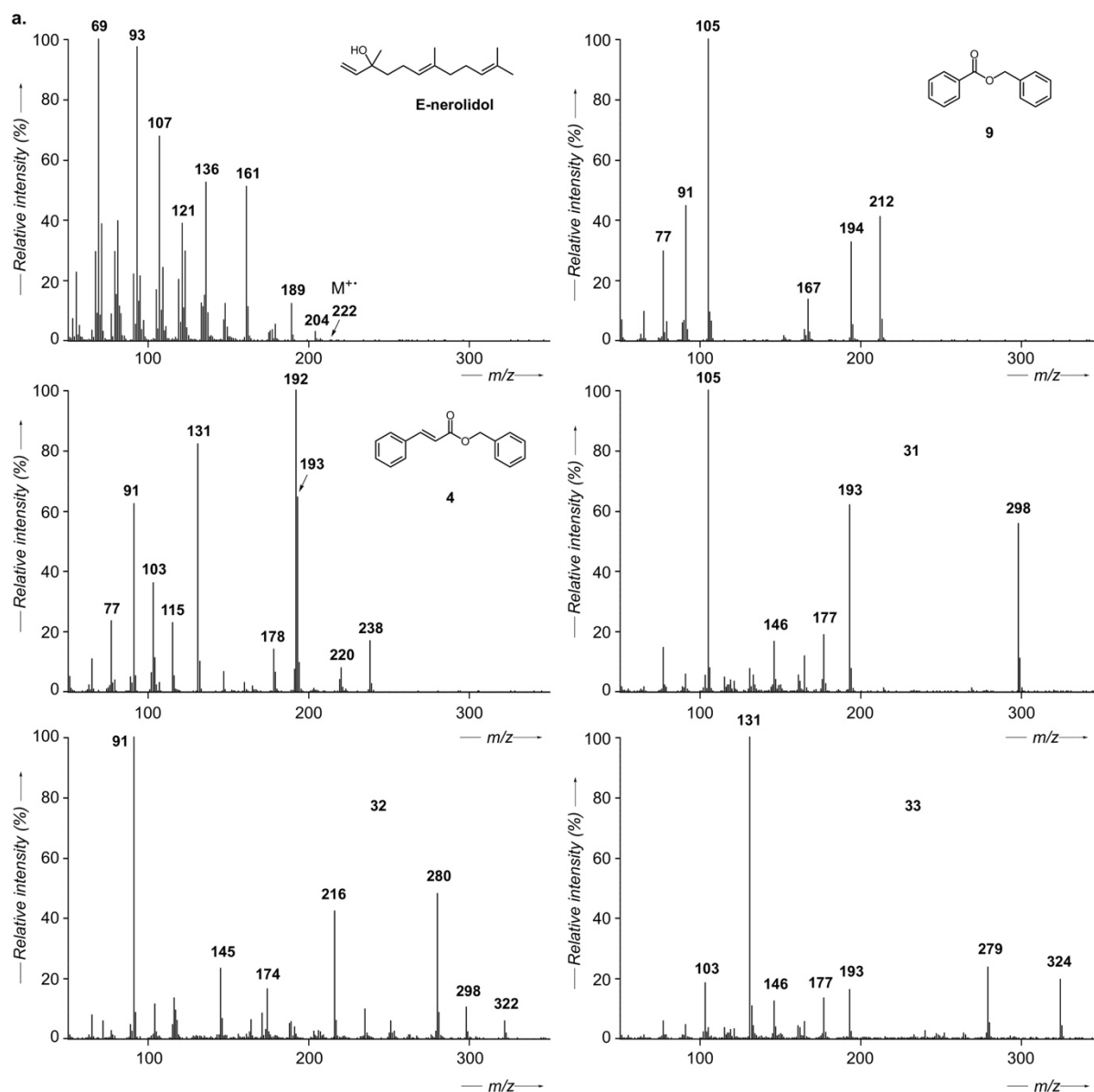
No triterpenes were detected by GC/MS in the Peru balsam (*Myroxylon balsamum* var. *Pereirae*) whereas the apolar lipid fractions obtained from the aerial parts of *M. balsamum* var. *balsamum* and *M. balsamum* var. *pereirae* (Fig. S6) have revealed the presence of various triterpenoids in leaves and branches along with other lipids comprising sesquiterpenoids, sugars, phenolic compounds, fatty acids, isoflavonoids and phytosteroids. The branches and leaves are notably characterised by the presence of lupeol **59**,  $\beta$ -amyrin **64** and 11-oxo- $\beta$ -amyrin **65** (Fig. S6a and S6b). In addition, an unusual oleanane derivative bearing a methoxy function at C-11 (**66**) and a diene (**67**) which could be a degradation product of **66** was exclusively observed in the leaves. Based on a previous study carried out on triterpenoids from the leaves of *M. balsamum* [1], an  $\alpha$ -stereochemistry for the methoxy group at C-11 could be suggested for **67**. Furthermore, the C-2,3 hydroxylated triterpenoids **68-71** were also reported to occur in the leaves [1].

[1] L. Mathias, I.J.C. Vieira, R. Braz-Filho, E. Rodrigues Filho, A new pentacyclic triterpene isolated from *Myroxylon balsamum* (syn. *Myroxylon peruiferum*), J. Brazil. Chem. Soc. 11 (2000) 195-198.

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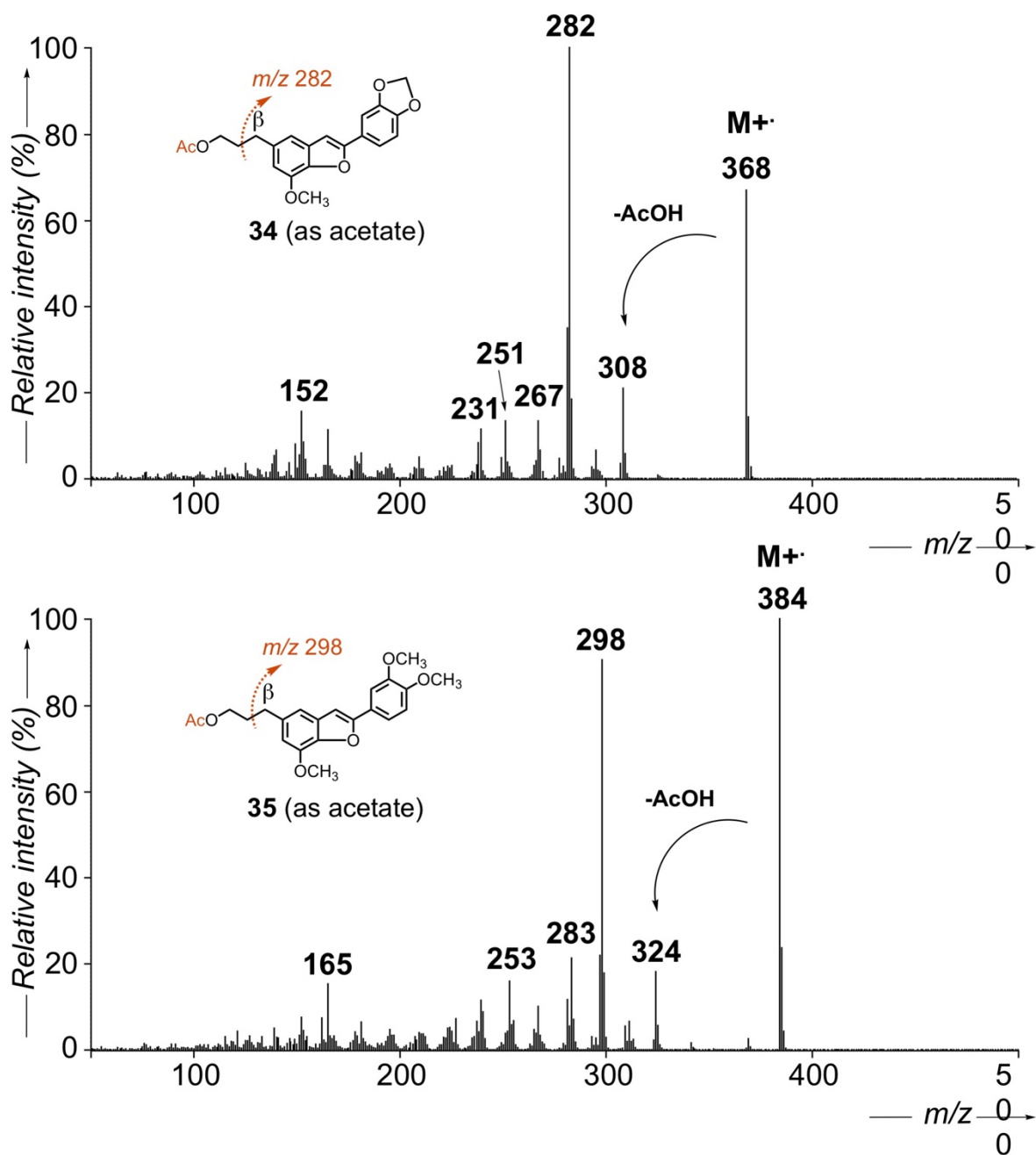


**Figure S1.** Mass spectra (EI, 70 eV) of a) cinnamyl cinnamate **1**; b) cinnamic acid **3** (as methyl ester derivative); c) 3-phenylpropanyl cinnamate **15**; d) coniferyl benzoate **25** (as acetate derivative).



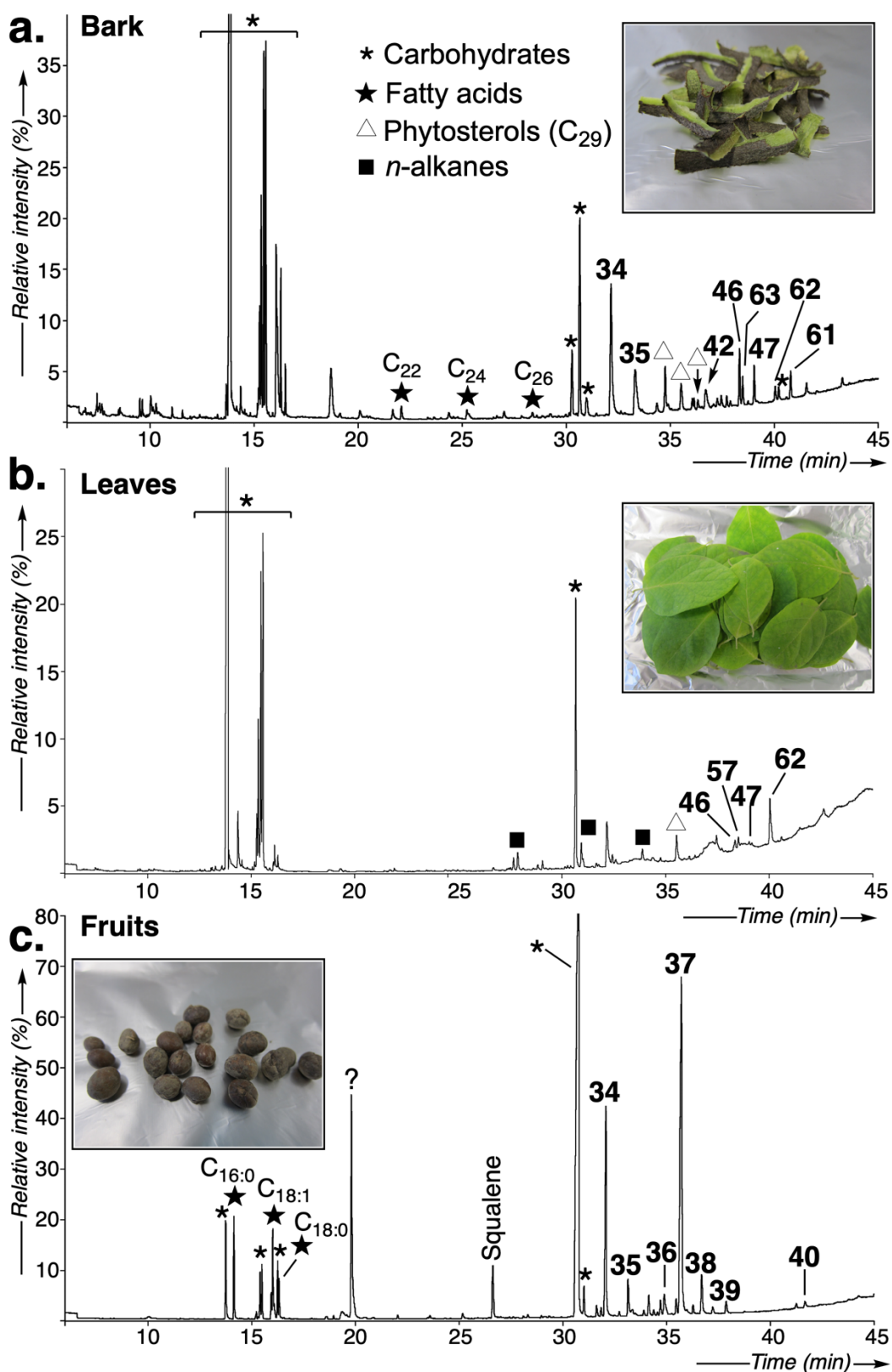
**Fig S2.** Mass spectra (EI, 70 eV) of major compounds detected in Peru balsam. Compounds 31-33: unknown structures.

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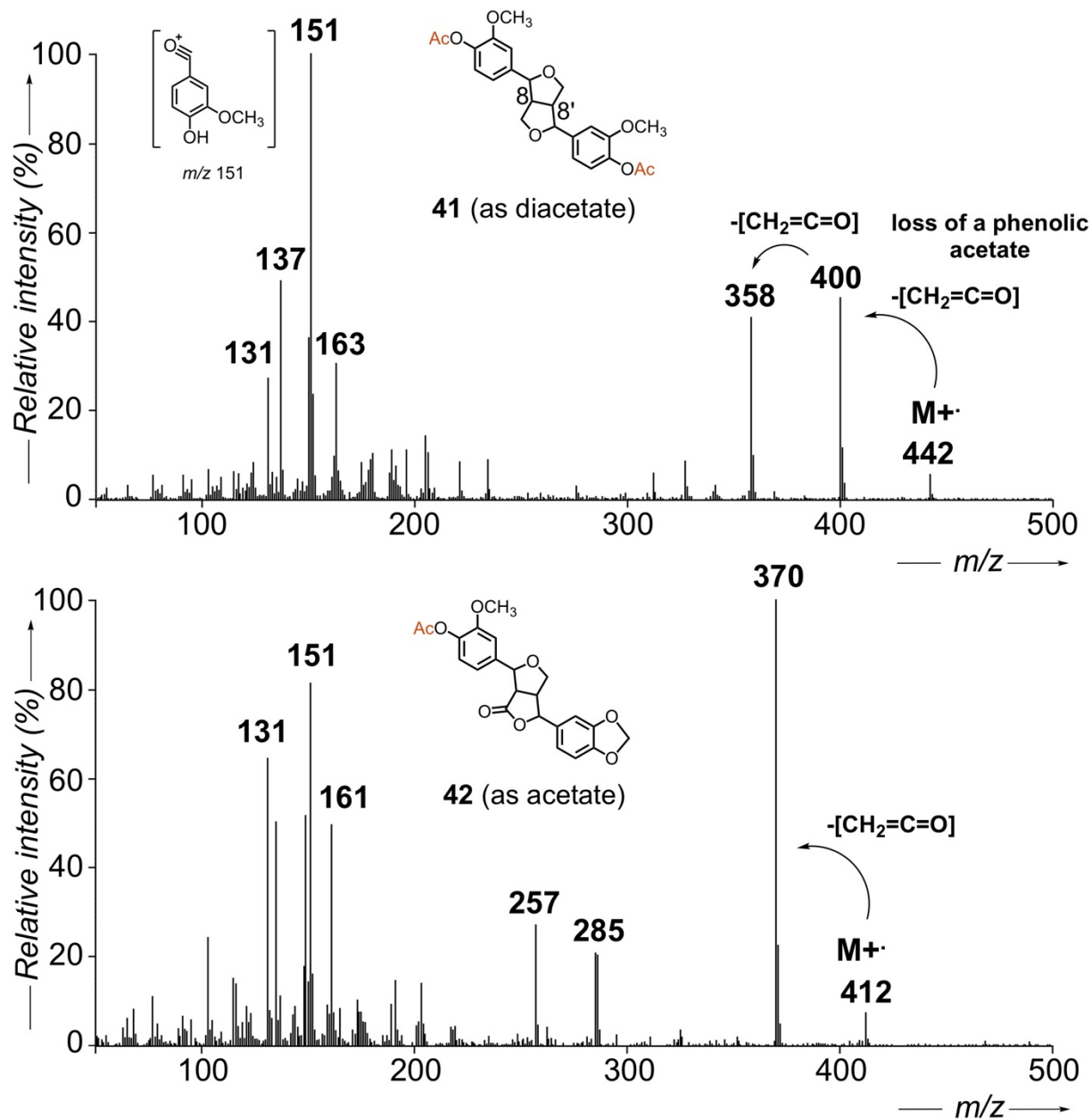
**Figure S3.** Mass spectra (EI, 70 eV) of egonol **34** and homoegonol **35** as acetate derivatives.

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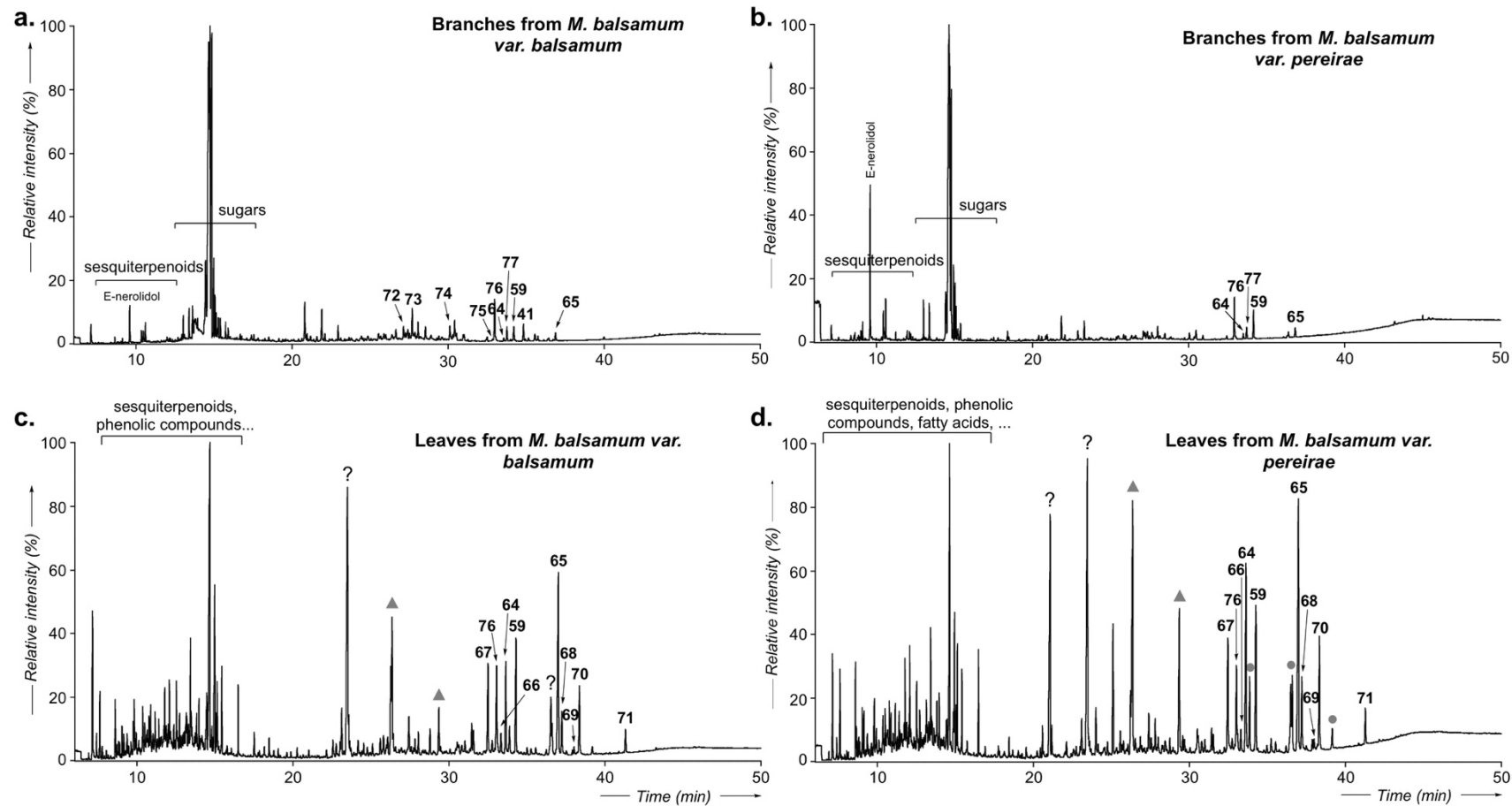
**Figure S4.** Gas chromatograms of an apolar fraction isolated from the lipid extract obtained from aerial parts of *S. officinalis*: a) bark; b) leaves; c) fruits (Botanical Garden of Strasbourg, France). Alcohols and carboxylic acids are analyzed as acetates and methyl esters, respectively.

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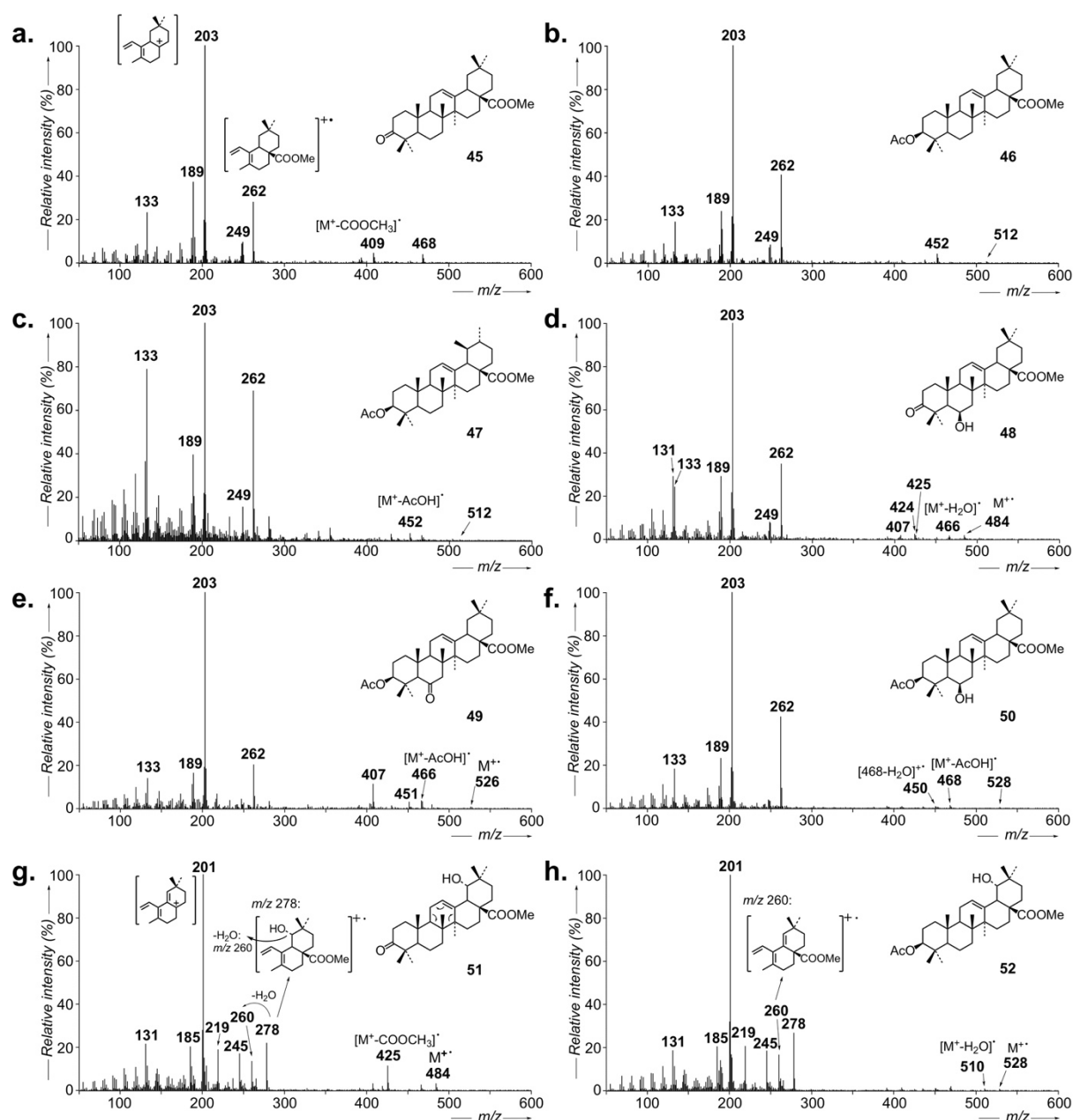
**Figure S5.** Mass spectra (EI, 70 eV) of pinoresinol **41** and styraxin **42** as acetate derivatives.

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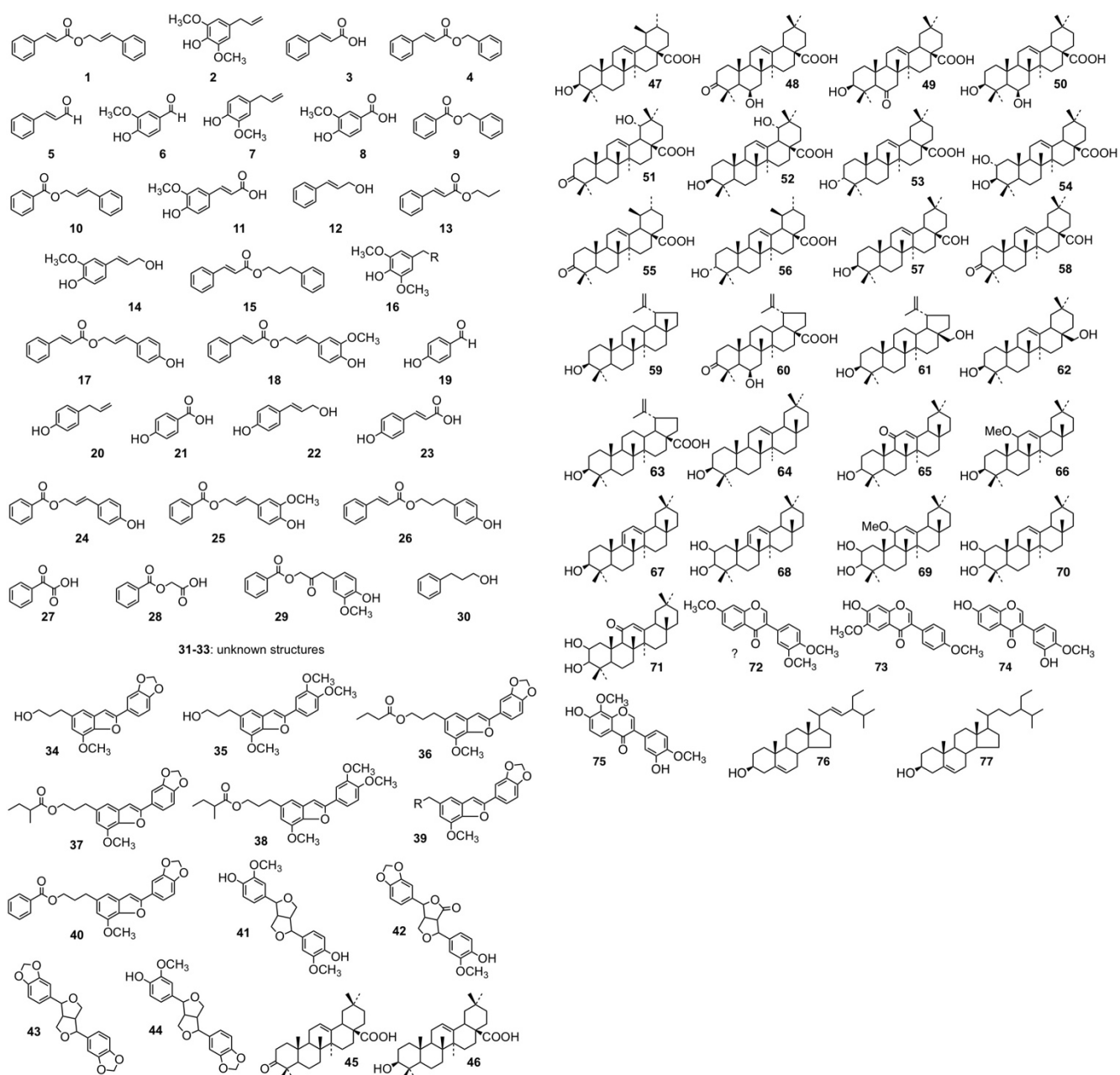
**Figure S6.** Gas chromatograms of the apolar fraction isolated from lipid extracts of a) branches of *M. balsamum* var. *balsamum*; b) branches of *M. balsamum* var. *pereirae*; c) leaves of *M. balsamum* var. *balsamum*; d) leaves of *M. balsamum* var. *pereirae*. Alcohols and carboxylic acids are analysed as acetates and methyl esters, respectively.





**Figure S7.** Mass spectra (EI, 70 eV) of acetate and/or methyl ester derivatives from triterpenic biomarkers of styrax resins: a) oleanonic acid **45**; b) oleanolic acid **46**; c) ursolic acid **47**; d) 3-oxo-sumaresinolic acid **48**; e) 3 $\beta$ -hydroxy-6-oxo-olean-12-en-28-oic acid **49**; f) sumaresinolic acid **50**; g) 3-oxo-siarsesinolic acid **51**; h) siarsesinolic acid **52**.

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**Figure S8.** Lipids identified in the resins investigated in the present study and in the aerial parts of *S. officinalis*, *M. balsamum* var. *balsamum* and *M. balsamum* var. *pereirae*.

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No.	Compound name	MW	Characteristic fragments (EI, m/z, %)
1	cinnamyl cinnamate	264	77 (23), 91 (16), 103 (34), 115 (44), 116 (10), 117 (29), <b>131</b> (100), 132 (11), 219 (23), 264 (3)
2	methoxyeugenol	236	91 (9), 103 (18), 119 (8), 131 (66), 147 (8), 162 (27), 163 (35), 167 (39), 193 (9), <b>194</b> (100), 195 (12), 236 (9)
3	cinnamic acid (Me)	162	51 (12), 77 (31), 102 (15), 103 (72), <b>131</b> (100), 132 (10), 161 (35), 162 (60)
4	benzyl cinnamate	238	51 (7), 65 (13), 77 (30), 89 (6), 91 (73), 92 (7), 102 (7), 103 (45), 104 (13), 115 (26), 116 (5), 131 (92), 132 (11), 147 (7), 178 (16), 179 (6), 191 (7), <b>192</b> (100), 193 (68), 194 (9), 220 (8), 238 (12)
5	cinnamaldehyde	132	77 (26), 78 (23), 103 (44), 104 (20), <b>131</b> (100), 132 (49)
6	vanillin (Ac)	194	81 (5), 109 (7), 123 (10), 151 (85), <b>152</b> (100), 194 (9)
7	eugenol (Ac)	206	55 (4), 77 (8), 91 (12), 104 (11), 131 (13), 133 (12), 149 (24), <b>164</b> (100), 165 (10), 206 (11)
8	vanillic acid (Me, Ac)	224	51 (2), 123 (11), 151 (89), <b>182</b> (100), 183 (12), 224 (6)
9	benzyl benzoate	212	51 (8), 65 (12), 77 (31), 91 (49), <b>105</b> (100), 106 (8), 107 (7), 167 (13), 194 (26), 212 (27)
10	cinnamyl benzoate	238	77 (20), <b>105</b> (100), 115 (33), 116 (9), 117 (10), 131 (1), 133 (12), 209 (6), 238 (7)
11	ferulic acid (Ac, Me)	250	51 (20), 53 (14), 79 (17), 89 (16), 91 (38), 97 (11), 105 (23), 124 (11), 135 (11), 136 (10), 138 (10), 145 (51), 147 (31), 150 (10), 151 (58), 152 (16), 153 (17), 162 (86), 163 (33), 175 (13), 177 (58), 179 (12), 193 (24), 194 (46), 195 (20), 207 (10), <b>208</b> (100), 209 (13), 222 (14), 236 (42)
12	cinnamyl alcohol (Ac)	176	51 (8), 77 (24), 91 (29), 92 (23), 103 (19), 105 (32), <b>115</b> (100), 116 (36), 117 (28), 133 (36), 134 (27), 147 (16), 176 (23)
13	<i>n</i> -propyl cinnamate	190	51 (11), 77 (35), 102 (15), 103 (65), <b>131</b> (100), 137 (20), 147 (13), 148 (21), 161 (4), 166 (8), 190 (7)
14	coniferyl alcohol (Ac, Me)	264	91(13), 103 (14), 119 (18), 124 (15), 131 (38), 147 (15), 151 (18), 162 (12), 163 (15), 179 (60), 180 (22), <b>222</b> (100), 223 (12), 264 (13)
15	3-phenylpropanyl cinnamate	266	51 (5), 77 (26), 91 (32), 102 (18), 103 (39), 115 (10), 117 (95), <b>118</b> (100), 119 (13), 131 (33), 147 (6)
16	Unknown structure	400 ?	103 (4), 131 (9), 151 (6), 152 (7), <b>167</b> (100), 209 (26), 323 (1), 340 (2), 400 (< 1)
17	<i>p</i> -coumaryl cinnamate (Ac)	322	77 (11), 103 (21), 105 (8), <b>131</b> (100), 132 (12), 133 (15), 149 (5), 235 (11), 280 (16)
18	coniferyl cinnamate (Ac)	352	77 (8), 103 (24), <b>131</b> (100), 132 (10), 179 (10), 265 (15), 310 (23), 352 (1)
19	4-hydroxybenzaldehyde	164	105 (4), <b>121</b> (100), 122 (82), 123 (6), 134 (9), 164 (24)
20	chavicol (Ac)	176	103 (7), 105 (18), 107 (10), 117 (9), 119 (7), 133 (79), <b>134</b> (100)135 (9), 176 (15)
21	4-hydroxybenzoic acid (Me, Ac)	194	77 (10), 91 (10), 93 (10), 105 (24), <b>121</b> (100), 152 (59), 162 (10), 194 (13)
22	<i>p</i> -coumaryl alcohol (Ac)	234	65 (11), 77 (20), 91 (20), 94 (28), 103 (25), 104 (12), 105 (40), 107 (34), 115 (24), 121 (47), 131 (50), 132 (28), 133 (64), 149 (92), 150 (72), <b>192</b> (100), 193 (16), 234 (19)
23	<i>p</i> -coumaric acid (Ac, Me)	220	89 (11), 91 (20), 118 (9), 119 (14), 120 (9), 131 (9), <b>147</b> (100), 178 (77), 189 (10), 220 (12)
24	<i>p</i> -coumaryl benzoate (Ac)	296	51 (3), 77 (19), <b>105</b> (100), 131 (13), 133 (15), 149 (31), 254 (22), 296 (5)
25	coniferyl benzoate (Ac)	326	77 (15), 91 (6), 103 (9), <b>105</b> (100), 119 (6), 131 (18), 162 (5), 179 (53), 284 (64), 285 (11), 326 (7)

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<b>26</b>	-	324 ?	77 (10), 103 (24), 107 (21), 131 (9), 133 (50), <b>134</b> (100), 135 (13), 176 (30)
<b>27</b>	benzoyl formic acid (Me)	164	51 (9), 77 (42), 92 (5), <b>105</b> (100), 106 (7), 122 (5), 135 (4), 136 (3), 164 (< 1)
<b>28</b>	(Me)	194	51 (10), 77 (39), 91 (3), <b>105</b> (100), 106 (8), 135 (6), 147 (4), 162 (5), 163 (3), 194 (17)
<b>29</b>	-	342	77 (24), 105 (98), 122 (11), <b>137</b> (100), 150 (66), 151 (5), 163 (30), 300 (82), 301 (17), 342 (8)
<b>30</b>	3-phenyl propanol (Ac)	178	77 (9), 91 (35), 105 (7), 115 (8), <b>117</b> (100), 118 (68), 119 (6), 178 (< 1)

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**Table S1:** MS data of the ester derivatives of cinnamic and benzoic acids. (Ac): Acetate derivative; (Me): Methyl ester derivative.