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Elm bark components and their potential influence on bark beetle feeding

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Abstract

In order to better understand the chemical factors involved in the host feeding selectivity by *Scolytus*, components were extracted from elm bark and studied. Sampled trees were located in an elm stand that survived the Dutch twig elm disease pandemia, and from elm clones from throughout the Iberian Peninsula. The main samples studied were from *Ulmus minor*, and the other samples from *U. pumila*, *U. laevis* and several hybrids. The main compounds in the extracts obtained from current-year twigs were aliphatic hydrocarbons (particularly heptacosane and nonacosane) and from 2- to 4-year-old stems were triterpenes. Three of the triterpenes were identified for the first time in *Ulmus* (lupenol, alnulin, and ilexol) and two in *Ulmaceae* (moretenol and betulin). Differences occurred in the compounds isolated from *U. minor* from the two locations, and between species.

Key words: *Ulmus*, bark extract, triterpenes, aliphatic hydrocarbons, gas chromatography-mass spectrometry, *Scolytus*.

Resumen

Componentes de la corteza del olmo y su posible influencia en la alimentación de los escolítidos

Se analizaron los componentes de la corteza de ramillas de olmos, a fin de determinar los factores químicos involucrados en la selección, por parte de los escolítidos del olmo (*Scolytus*), de árboles para su alimentación. Las muestras fueron recogidas en árboles de una olmeda que ha sobrevivido a la pandemia de grafiosis y de ejemplares de distintas procedencias de la península Ibérica en un banco clonal. La principal especie estudiada fue *Ulmus minor*. Se tomaron además muestras de *U. pumila*, *U. laevis* y diversos híbridos. Los compuestos más abundantes en los extractos obtenidos de ramillas del año en curso fueron hidrocarburos alifáticos (principalmente heptacosano y nonacosano) y en las ramillas de 2º a 4º año triterpenos pentacíclicos. Tres de los triterpenos (lupenol, alnulina e ilexol) fueron identificados por primera vez en *Ulmus* y dos (moretenol y betulina) en *Ulmaceae*. Se observaron diferencias en los compuestos detectados en *U. minor* de las dos localizaciones así como entre especies.

Palabras clave: *Ulmus*, extracto de corteza, triterpenos, hidrocarburos alifáticos, cromatografía de gases-espectrometría de masas, *Scolytus*.

Introduction

The fungal pathogen of Dutch elm disease, *Ophiostoma novo-ulmi* Brasier, can spread from infected to healthy elms either by root contact between infected and healthy trees or by spore transmission by bark beetles. New infections by root grafts only occurs between close trees and is far less frequent and damaging than by pathogen transmission via bark beetles of the genus *Scolytus*. During the dispersion phase, bark beetles that emerge from fungus infected elm wood, land on the crowns of healthy trees and feed on twig-crotches. Many of these beetles carry spores of the pathogen, which are then introduced into the current-year xylem through the feeding wounds. Following spore germination, the fungus spreads throughout the whole tree resulting in the dysfunction of the xylem and,

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therefore, in death of the tree. These dead trees are then colonized by adult bark beetles to reproduce, thus closing the cycle (e.g. Webber and Brasier, 1984).

Although feeding on twigs is the pivotal stage in disease transmission, many of the steps and factors involved in the process are still imperfectly known. It is assumed that bark beetles can fly directly to appropriate trees in response to host volatile compounds (primary attraction) or can haphazardly find host elms and accept them according to favourable olfactory or gustatory signals emitted over a short range (Byers, 1995). Both behaviours appear to occur in different species of *Scolytus* spp., e.g. primary attraction in the case of *S. ventralis* to *Abies grandis* (Macias-Samano *et al*., 1998) and random finding of *Carya ovata* by *S. quadrispinosus* (Goeden and Norris, 1965). In both cases, chemical compounds are involved. For the «primary attraction» strategy, volatile compounds which are effective for over a long range (some hundred meters) can be attractants or repellents, and the balance between both kinds of compounds determines if the host will be accepted or rejected. In the second strategy, bark beetle-plant contact becomes necessary, and compounds can be stimulants or deterrents. The balance between positive and negative stimuli leads the insects to accept or reject the plant as a feeding substrate (Miller and Strikler, 1984).

Therefore, it is essential to understand the chemical factors involved in the host-selection process. Since *Scolytus scolytus* and *S. multistriatus* are the main vectors of *O. novo-ulmi* (Pajares, 1987; Webber, 1990), the chemicals susceptible to play a role in this process have been mainly searched for in these species. In several studies, the stimulant activity of several compounds was shown by means of laboratory bioassays. One such stimulatory compound, first isolated by Baker and Norris (1967), is a pentacyclic triterpene from *U. americana*. Later, Meyer (1975) isolated two pentacyclic triterpenes with formulas $C_{30}H_{52}O$ and $C_{30}H_{50}O$, having similar properties. Other compounds reported to have phagostimulatory activity are: p-hydroxybenzaldehyde (Baker *et al*., 1968), (+)-catequin-7-β-D-xylopiranoside and (-) lupeyl cerate (Doskotch and Chatterji, 1970; Doskotch et al., 1973), and two dihydroxybenzenes (resorcin and hydroquinone) (Meyer and Norris, 1974). The opposite effect, that is, feeding deterrence, was shown in juglone (5-hydroxy-1,4 naphtoquinone) from *Carya ovata* (Gilbert *et al*., 1967).

On the long range «primary attraction» hypothesis, two benzaldehydes (vanillin and syringaldehyde) were found to be attractants for *S. multistriatus* (Meyer and Norris, 1967). The sesquiterpene α-cubebene, considered to be part of the pheromone complex, may be responsible for higher attraction of *S. scolytus* and *S. multistriatus* to pruned branches (Byers *et al.*, 1980). A monoterpene from *U. procera* (= *U. minor*), - (-) limonene, worked as a synergist of 4-methyl-3 heptanol, an aggregation pheromone of *S. scolytus* (Blight *et al.*, 1980).

The age of the twigs seems to have some effect on the feeding preference. For example, *S. multistriatus* preferred to feed on elderberry pith discs containing bark extracts from second to fourth year twigs of *U. americana*, when compared to bark extracts of current-year twigs collected at the beginning of the growing season (Norris and Baker, 1967). Evidence exist that, among second to fourth year twigs, *S. multistriatus* often feeds on younger twigs than *S. scolytus* (Webber, 2000).

Despite the limited knowledge on host selection and colonization processes of elm bark beetles, some evidence of differential interspecific preferences of bark beetles to elms have been found. In experiments with *U. procera* (= *U. minor*) and *U. glabra* saplings, Webber and Kirby (1983) showed that most *S. scolytus* and *S. multistriatus* preferred to feed on *U. minor*. When feeding preferences of *S. multistriatus* for either *U. minor* or *U. laevis* were compared, *U. minor* was again most preferred (Sachetti *et al.*, 1990; Piou, 2002),as was also *U. pumila* by both bark beetle species over *U. glabra* and *U. laevis* (Webber, 2000). Volatile and non-volatile chemical compounds may be responsible for such preferences.

Thus, we have compared the chemical composition of the phloem of different groups of elm trees. A first objective was to determine if different chemical profiles exist between 2-to-4 year and current year *U. minor* twigs. A second one was to find out if bark extracts from *U. minor* trees from a stand that survived the Dutch Elm Disease pandemia differed from those of trees throughout the Iberian Peninsula, where the disease is present. Thirdly, we have investigated differences in bark composition among *U. minor*, *U. pumila*, and *U. laevis*.

Materials and Methods

Sampling sites

Healthy trees were sampled from two different locations: the Rivas-Vaciamadrid elm stand and the DGCN-

ETSIM elm clone collection. The first area, located in the south east of the Autonomous Community of Madrid, comprises nearly 300 surviving *U. minor* trees that are approximately 60year-old (Cabrera, 2000). The second site contains a collection of 5- to 12-year-old seedlings of elm clones from throughout the Iberian Peninsula, as well as several exotic elm species.

Plant material

Sampling of twig bark with phloem was carried out in 2001 and 2002 between the end of May and the beginning of June, within the spring flight period of elm bark beetles. All trees sampled in Rivas-Vaciamadrid were *U. minor*, while samples from the elm clone collection were from *U. minor*, *U. laevis* and *U. pumila* and from several hybrids provided by the Dutch Breeding and Conservation program obtained from *U. glabra* and *U. minor*, and different Asian species (*U. wallichiana* and *U. pumila*). Two-to-4-year old twigs were sampled from three different orientations on the selected trees to homogenize the sample and reduce positional effect (i.e. light, wind). In some of the Rivas-Vaciamadrid trees, current year twigs were also sampled. The total number of samples collected was 79 from the 2-to-4 year-old twigs and 7 from current-year twigs (Table 1).

Preparation of extracts

Samples were brought into the laboratory and the bark with phloem was stripped from them the same day, and then ground into small pieces (3-5 mm). Approximately 30 g from each sample was extracted for 48 h in the dark, at room temperature, with 200 ml of petroleum ether/diethyl ether (1:1). The extract was then decanted, treated with anhydrous sodium sulphate and filtered. The solvent was removed from the extract in a nitrogen stream. For the analyses, 10 mg of the dried extract were dissolved into 2 ml of petroleum ether-diethyl ether.

Table 1. Number of samples per location and species

	U. minor	*U. laevis*	*U. pumila*	Dutch hybrids
Rivas-Vaciamarid	42 (7*)	—	—	—
Elm clone collection	27	3	2	5

* Current-year twigs.

Chromatographic analysis

Chromatographic separation and identification of the extracted compounds were achieved by gas chromatography/mass spectrometry (GC-MS) using an Agilent 6890N gas chromatograph connected to an Agilent 5973N mass detector (EI, 70 eV) and equipped with a 30 m × 0.25 mm i.d PTE-5 capillary column (0.25μm film thickness). The working conditions were: split (1:20), injector temperature, 250 ºC; temperature of the transfer line to the mass spectrometer, 300 ºC; column temperature, 60 ºC during the split period (2 min), followed by increases in temperature at 5ºC/min to 200 ºC, then at 10 ºC/min to 300 ºC, and then at 300º C for 15 min. Electron ionization (EI) mass spectra and retention times were used to assess the identity of the compounds, by comparing them with those of standards when available and in a database (Wiley 275 Mass Spectra Database, 2001). Percent concentration values were directly calculated from total ion chromatogram (TIC) peak areas.

Results

The major components in petroleum ether/diethyl ether extracts of elm bark corresponded to two main groups: aliphatic hydrocarbons (11 compounds, with 21 to 31 C atoms), and pentacyclic triterpenes (8 compounds). β-Sitosterol was also identified. It is important to note that each bark sample did not necessarily contain all of these triterpenes, a fact which is useful to discriminate among groups of samples, as will be discussed later. Additional constituents of twig bark extracts were also identified in lower concentrations, such as scopoletin and isofraxidin.

Components from current-year and 2-to-4 year-old twigs in Rivas-Vaciamadrid

Aliphatic hydrocarbons were the most abundant components in extracts of current year twig bark, with an average of 69.0% of total peak area (range 58.8%-73.0%), whereas bark extracts from older twigs averaged only 6.7% of total area, with a wider range of concentrations, between 2.1% and 23.3%. Heptacosane and nonacosane reached the highest percentage among compounds of this group in all current-year samples (Fig. 1 and 2).

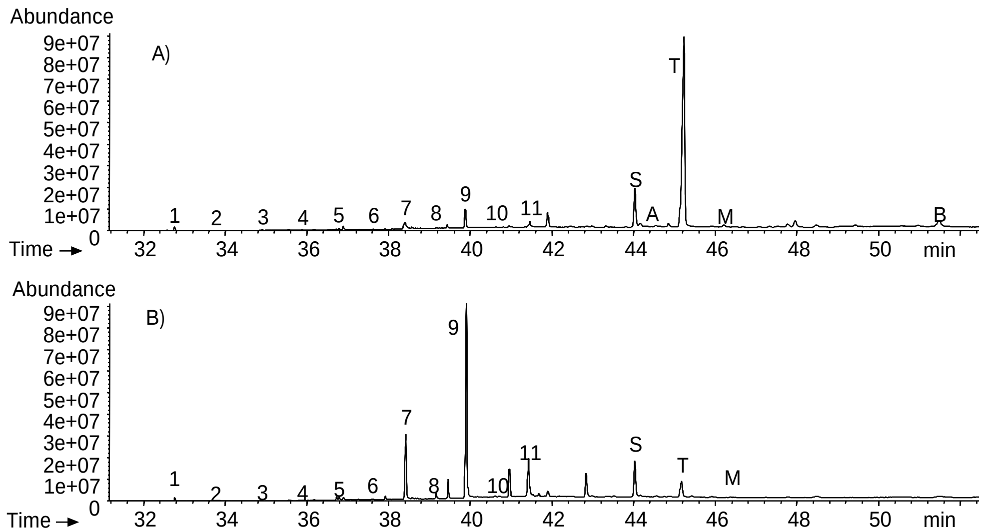

Figure 1. Total ion chromatogram of *Ulmus minor* bark extracts A) from 2-to-4 year twigs, and B) from current year twigs. Twigs were taken from the same tree in the Rivas-Vaciamadrid (M-RV122). 1, heneicosane; 2, docosane; 3, tricosane; 4, tetracosane; 5, pentacosane; 6, hexacosane; 7, heptacosane; 8, octacosane; 9, nonacosane; 10, triacontane; 11,hentriacontane; S, β-sitosterol; A, β-amirina; T, lupenol; M, moretenol; B, betulin.

Four pentacyclic triterpenes plus β-sitosterol were characteristic of twig bark extracts of *U. minor*, the only elm species present in Rivas-Vaciamadrid stand. The overall mean for these five compounds was 69.9% of total area in 2-to-4-year-old bark samples. Although differences between trees were high (22.2%-85.5%), for 90% of the samples in this age group, triterpenes averaged more than 60% of total area. Lupenol was the major compound (average 53.8%), but also showed the highest standard deviation (± 15.2%). Thus, its occurrence significantly varied between trees (3.7%-71.2%), but only in three of the 42 trees studied was the presence of lupenol lower than 45% of total area.

All triterpenes and β-sitosterol appeared in much lower percentages in the current year twig extracts: average of 8.4% of total area and a range of concentrations between 4.6% and 14.1%. In this group of samples, β-sitosterol had the highest percentage (5.8%), followed by lupenol (2.2%) (Figures 1 and 2).

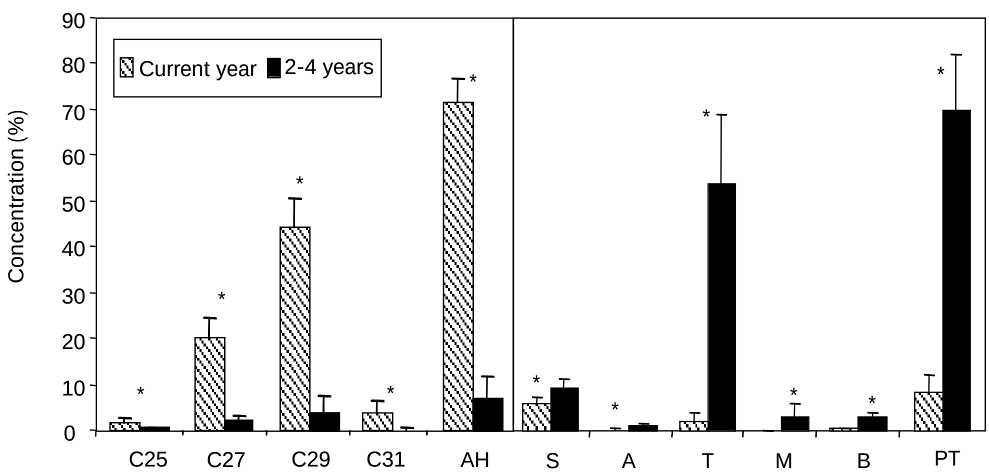

Figure 2. Relative concentrations (%) of major compounds in *U. minor* current -year twigs and 2-to 4-year-old twigs. Bars represents mean ± sd. C25, pentacosane; C27, heptacosane; C29, nonacosane; C31, hentriacontane; AH, aliphatic hydrocarbons; S, β-sitosterol; A, β-amyrin; T, lupenol; M, moretenol; B, betulin; PT, pentacyclic triterpenes. Asterisks denote significant differences ($P < 0.01$).

Components from trees in different locations: Rivas-Vaciamadrid and elm clone collection

In this case, bark extracts from 2-to-4-year old twigs of *U. minor* from different locations were studied. Twenty seven trees were sampled from the DGCN-ESTIM elm clone collection (EC) and 42 from the Rivas-Vaciamadrid elm stand (RV).

Extracts from EC trees contained aliphatic hydrocarbon concentrations which in average were almost three times higher (18.09 ± 13.56%) than in RV trees (6.7 ± 4.6%). For both groups of samples, the most abundant hydrocarbons were nonacosane (12.2 ± 10.6%) in EC and 3.7 ± 3.3% in RV) and heptacosane (3.2 ± 2.2% in EC and 2.1 ± 1.1% in RV), their percentages showing significant variations. On the contrary, concentrations of tripertenes in RV samples were higher (69.9 ± 12.4%) than in the EC samples (48.3 ± 14.6%), particularly of lupenol which reached average concentrations of 53.8%. However, β-sitosterol concentrations were higher in EC extracts (18.7 ± 5.9%) than in those from RV (9.4 ± 2.0%) (Fig. 3 and 4).

Components from different elm species

The DGCN-ETSIM elm clone collection allowed to study the chemical compositions of three elm species and some European x Asiatic hybrids. 2-to-4 year old twigs extracts were used in this comparative analysis.

Whereas *U. minor* contained high concentrations of lupenol, four new pentacyclic triterpenes were obtained from the other elm species and hybrids. Alnulin was only found in *U. laevis* (0.28-8.53%) and the Dutch hybrids (0.47-5.91%), and ilexol was present in all *U. pumila* (2.59-3.27 %) and some of the Dutch hybrids (0.00-1.76%). High amounts of epifriedelinol were detected in *U. pumila* (average 47.4%) and in some of the hybrids (average 13.6%), while concentrations of friedelin were higher in the hybrids (average 26.3%, with a wide range of concentrations from 1.48% to 61.43%) than in *U. pumila* (average 7.0%). Differences in the presence of other triterpenes (β-amyrin, moretenol or betulin) were minor and non-significant. Therefore, Dutch Hybrids showed the most complex chemical composition of the species under study, with the eight identified triterpenes ocurring in their bark extracts. *U. pumila* and *U. laevis* were each missing one of the triterpenes and *U. minor* had the most simple composition, with only five of the eight triterpenes (Figure 5 and Table 2).

Discussion

The nature of twig bark extracts from several elm species and twigs of different age was analyzed. Ma-

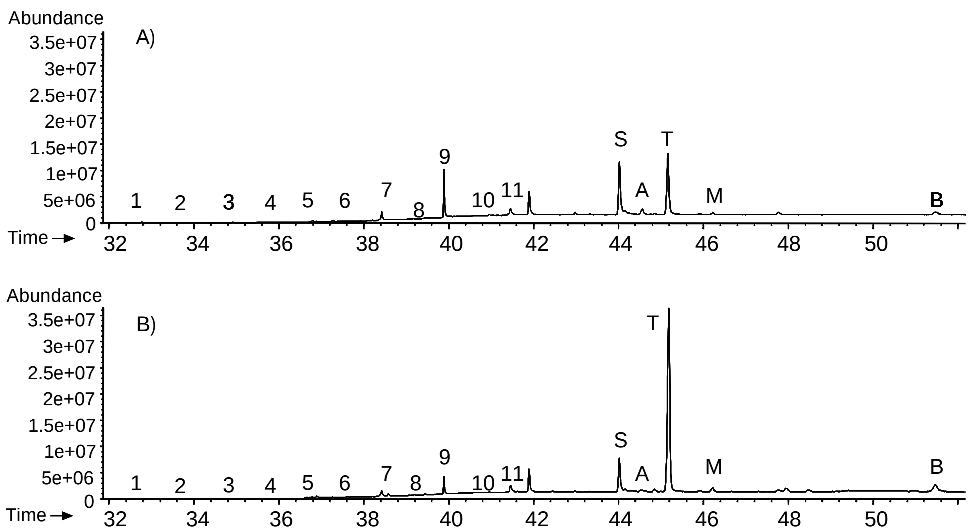

Figure 3. Total ion chromatogram of *Ulmus minor* bark extracts, from A) Elm clone collection twig, and from B) Rivas-Vaciamadrid twigs. 1, heneicosane; 2, docosane; 3, tricosane; 4, tetracosane; 5, pentacosane; 6, hexacosane; 7, heptacosane; 8, octacosane; 9, nonacosane; 10, triacontane; 11, hentriacontane; S, β-sitosterol; A, β-amyrin; T, lupenol; M, moretenol; B, betulin.

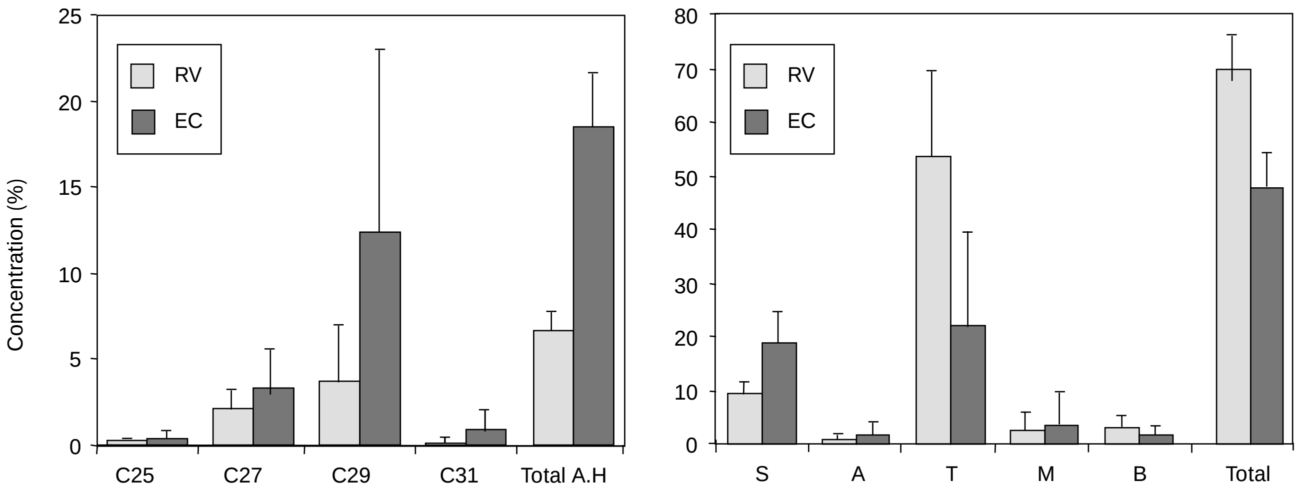

Figure 4. Relative concentrations of major compounds in extracts from Rivas-Vaciamadrid (RV) elm stand twigs and elm clone collection (EC) twigs. Each bar represents mean ± sd. C25, pentacosane; C27, heptacosane; C29, nonacosane; C31, hentriacontane; AH, aliphatic hydrocarbons; S, β-sitosterol; A, β-amyrin; T, lupenol; M, moretenol; B, betulin; PT, pentacyclic triterpenes. Asterisks denote significant differences ($P < 0.01$).

jor constituents belonged to two groups of compounds: aliphatic hydrocarbons (AH) and pentacyclic triterpenes (PT). Both groups are characteristic constituents of plant epicuticular waxes with important water repellent and protection functions (Baker, 1982). These compounds are not very volatile and for insects to detect them would require direct contact the bark.

Eight different PT were identified. Three of them are new to the Ulmaceae family: lupenol, alnulin, and ilexol. Two more are identified for the first time in *Ulmus*: moretenol and betulin. β-Sitosterol was previously reported as occurring in the bark of *U. americana* (Baker and Norris, 1968), and β-amyrin, friedelin and epifriedelinol in *U. minor* leaf extracts (Wegener, 2002). Neither friedelin nor epifriedelinol were found in *U. minor* bark extracts in this study.

AH are common compounds found in plants, particularly those with odd C atom numbers (Baker, 1982).

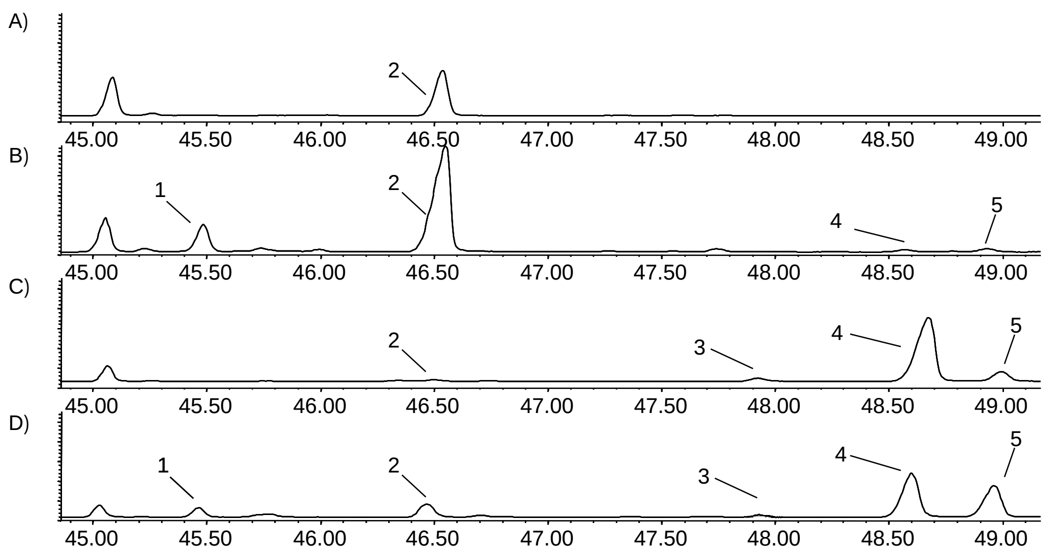

Figure 5. Total ion chromatogram of twig bark extracts of three elm species, A) *Ulmus minor;* B) *U. laevis;* C) *U. pumila,* and, D) Dutch hybrids.1, alnulin; 2, lupenol; 3, ilexol; 4, epifriedelinol; 5, friedelin.

Table 2. Relative abundance of pentacyclic triterpenes in twig bark extracts of three elm species and hybrids

	Alnulin	Lupenol	Ilexol	Epifriedelinol	Friedelin
U. minor	0[a]	+[b]/++[c]	0	0	0
U. laevis	+	++	0	–	–
U. pumila	0	– –	+	++	+
Dutch hybrids	++	–	–	++	++

[a] Absent (0); present in very low concentration (– –); present in low concentration (–); present in high concentration (+); present in very high concentration (++). [b] Trees from the elm clone collection. [c] Trees from Rivas Vaciamadrid.

In the elm extracts analyzed, the most abundant hydrocarbons were pentacosane, heptacosane, nonacosane, and hentriacontane, with 25, 27, 29, and 31 C atoms, respectively. Their concentrations were characteristically higher in *U. minor* extracts of current year twigs than in 2-to-4-year twigs. The fact that these compounds were detected in twigs from both ages suggests that the lower feeding preference of elm bark beetles (EBB) on current year twigs extracts found in one tree (see Pajares *et al.* in this volume), would be more related to a low level of stimulants than to the presence of deterrents. EBB might distinguish between current year and older twigs by the absence of certain stimulating compounds, choosing as a feeding substrate the thicker phloem of older twigs. In addition, plant compounds of common occurrence, such as AH found in elm bark extracts, would not be very efficient as discriminating stimuli in the host finding and accepting processes for a monophagous insect.

More complex chemical profiles were found in 2-to-4-year old twigs, in which the most abundant compounds were PT. Differences in these constituents were significant between elms from the two locations studied, as well as from the species analyzed. Regarding location, elm clone collection (EC) bark extracts had higher AH concentrations, whereas PTs were more abundant in the Rivas-Vaciamadrid elm stand (RV) samples. Although twigs were of the same age in this experiment, trees were not. RV trees are older (ca. 60 years) and higher (Cabrera, 2001) than younger EC specimens, which were planted less than 12 years ago. Thus, the observed chemical differences might be related to the different ages of the trees. Bark extracts of 2-to-4-year-old twigs from EC trees have shown chemical profiles, aliphatic hydrocarbons > pentacyclic triterpenes, which were intermediate between 2-to-4-year-old twigs and current-year twigs from old RV trees.

For the group of 2-to-4-year-old twigs samples, PTs were the most interesting compounds obtained, since they presented differences that allowed to discriminate between species. European elms, such as *U. minor* and *U. laevis*, were richer in lupenol than Asian species (*U. pumila*), the latter being characterized by high concentrations of epifriedelinol and friedelin. Hybrids had epifriedelinol and friedelin in comparable amounts. Ilexol was also isolated from *U. pumila* and elm hybrids.

The great variability in PT composition and concentration between elm species and twig ages, and the fact that they represent an important part of the epicuticular waxes of twigs, make them very interesting in relation to EBB feeding behavior. The presently identified PTs have the same formulas $C_{30}H_{52}O$ and $C_{30}H_{50}O$, as those isolated by Baker and Norris (1967) and Meyer (1975) from *U. americana*, and found to be stimulant for EBB.

Another group of compounds isolated from the elm extracts, different from those mentioned above, were hydroxycoumarins, particularly scopoletin and isofraxidin. Scopoletin was present in almost all extracts in small amounts. No significant differences were observed between groups of samples from the 2-to-4-year-old twigs. However, its concentrations in these were tenfold higher (0.1%) than in current-year twigs (0.01%). This hydroxycoumarin, previously reported to occur in *U. minor* and *U. pumila,* was shown to inhibit mycelial growth of *Ophiostoma ulmi* in culture (Valle *et al.*, 1997). Isofraxidin was only obtained from *U. pumila*. Even though some hydroxycoumarins, such as aesculetin and fraxetin, from non-host tree species, were shown to produce an antifeeding effect on *S. multistriatus* (Norris, 1977), it seems unlikely that both compounds here would play such a role in EBB feeding.

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