Original article

Effect of species and ecological conditions on ellagitannin content in oak wood from an even-aged and mixed stand of *Quercus robur* L. and *Quercus petraea* Liebl.

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Abstract – Species effects and ecological conditions on ten heartwood ellagitannins (vescalin, castalin, roburins A-E, grandinin, vescalagin and castalagin) and ellagic acid were investigated in a 100 years old stand of 5 ha located in western France (La Petite Charnie State Forest, Sarthe). The sample included a total of 286 trees (118 sessile oaks, 158 pedunculate oaks and 10 individuals with an intermediate morphology) located in three ecological zones (plateau, slope, small valley). The main factor influencing oak extractives level was botanical species. The ecological zone effect appears negligible. Pedunculate oak is generally richer in ellagitannins (48.4 mg/g against 34.4 for sessile oak), although a clear boundary between the two species cannot be established. Ellagitannin content was found to be correlated with ring width for pedunculate oak and not for sessile oak. The vescalagin/castalagin ratios differed between the two species (0.69 for *Quercus robur* against 0.53 for *Quercus petraea*). The distribution of ellagitannin contents is not strongly structured spatially.

ellagitannin / oak wood / *Quercus robur* L. / *Quercus petraea* Liebl. / variability / ecological conditions

Résumé – Effet de l'espèce et des conditions écologiques sur le contenu du bois en ellagitanins dans un peuplement équien de chêne (*Quercus robur* L., *Quercus petraea* Liebl.). Les effets de l'espèce et des conditions écologiques sur le contenu du duramen externe de dix ellagitanins (vescaline, castaline, roburines A à E, grandinine, vescalagine, castalagine) et de l'acide ellagique ont été étudiés dans un peuplement équien (100 ans) de chêne d'une surface de 5 ha située dans l'ouest de la France (forêt domaniale de La Petite Charnie, Sarthe). L'échantillon total se composait de 286 arbres (118 chênes sessiles, 158 chênes pédonculés et 10 chênes intermédiaires) répartis en mélange dans trois zones écologiques du peuplement (plateau, pente et fond de vallon). Le facteur principal qui influence la teneur en ellagitanin est l'espèce botanique, alors que le facteur « zone » est négligeable dans les conditions expérimentales considérées. Le bois de chêne pédonculé est plus riche en ellagitanins que celui du chêne sessile (48,4 mg/g pour le chêne pédonculé ; 34,4 mg/g pour le chêne sessile), mais une distinction claire entre les deux espèces ne peut être établie. Une corrélation entre la teneur en ellagitanin et la largeur de cerne est observée pour le chêne pédonculé à la différence du cas du chêne sessile. Le rapport vescalagine/castalagine est plus élevé pour le chêne pédonculé que pour le chêne sessile (0,69 et 0,53). La structuration spatiale est faible.

ellagitanin / bois de chêne / *Quercus robur* L. / *Quercus petraea* Liebl. / variabilité intrapeuplement / conditions écologiques

1. INTRODUCTION

Ellagitannin content in oak wood (*Quercus robur* L. and *Quercus petraea* Liebl.) is an important choice criterion in cooperage since these highly reactive chemicals interact with wine phenolics during the maturation of wine in oak barrels [11, 18, 22, 33, 35, 38, 43]. Several research groups have already investigated the influence of the botanical species (*Quercus robur* L. and *Quercus petraea* Liebl.) in relation to ecological factors (soil, climate), topography, rhythmic growth [4, 12, 26, 36] as well as the geographic location of the trees on ellagitannin content in oak wood. Total ellagitannin content was studied in wood of one or both species originating from several forests of Central and Northern France (Cîteaux,

Tronçais, Lavault, Grosbois, etc.) [28, 32, 39]. A large set of wood samples from South-West of France has also been studied [13, 14]. The major conclusion of these studies is that oak species effect on ellagitannin content largely predominates over geographic effect. Oak species differ substantially whatever their provenance, although the difference in total phenolics between species is lower among trees from mixed stand than from the trees originating from different locations [30]. Furthermore, a large proportion of the total variation among progeny was attributed to forest origins, but genetic or environmental causes could not be clearly separated [31].

However, the main problem in the previous cited reports is that the sampled stands were more or less monospecific. Thus, the effects of species and ecological conditions are often difficult to discriminate. Differentiation for ellagitannin amounts between trees within a stand was also to be taken into account

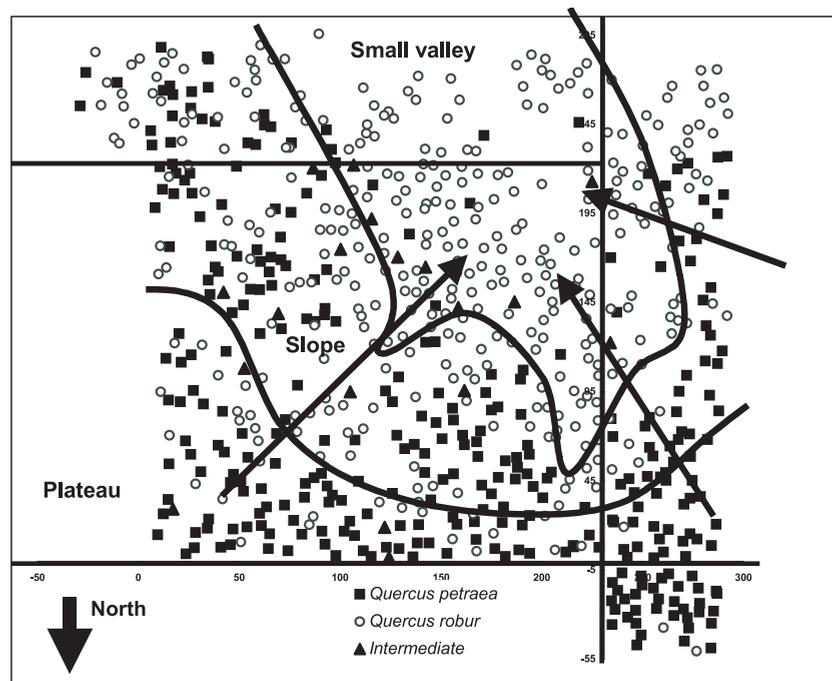* Corresponding author: puechjl@ensam.inra.fr

Figure 1. Map of La Petite Charnie stand. Arrows indicate the slope towards the valley.

by using numerous experimental sets of trees. Besides, tree age effects and heterogeneities in the distribution of ellagitannin in wood tissues could influence conclusions and render interpretations difficult [27, 28].

The aim of the current study is to contribute to clarify the respective influence of botanical species and site conditions on chemical composition of ellagitannins in oak heartwood. The studied stand was an average aged stand of 100 years from seed in which we had the opportunity to sample all the 286 constitutive trees (118 sessile oaks, 158 pedunculate oaks, 10 individuals with an intermediate morphology) which grew under the same silvicultural conditions. Given that those trees were distributed according to three ecological zones (plateau, slope, small valley) the effects of these ecological conditions and species were investigated globally and individually. Different statistical methods could be performed in this study. The current research focused on chemical composition of oak extractives to obtain a reliable database for the investigated site. The aim of this paper is to analyze the ellagitannin variations within a stand. The species, ecological factors and spatial distribution effects on ellagitannin concentrations are investigated.

2. MATERIALS AND METHODS

2.1. Wood sampling

The sampled stand (compartment 26, La Petite Charnie State Forest, latitude: 48.08 °N, longitude: 0.17 °W) is located in the western part of France. This stand was described by Bacilieri et al. [2]. The climate is typically Atlantic, temperate and wet: mean rainfall

is 880 mm per year and mean temperature is 11 °C. The geological substratum is composed of Ordovician red sandstone. The mean elevation of the stand is 140 m. The stand is included in a continuous forest of 700 ha, consisting mostly of naturally regenerated stands of sessile and pedunculate oaks. The sampled stand covers 5 ha and contains 287 adult trees (one beech tree and 286 oak trees therefore the density is 57 trees per ha. The stand consists of three ecological zones: small valley, plateau, intermediate (and regular) slope. On the northwestern part of the stand (plateau) the soil is well drained and composed of sand and slit. The south-eastern part (small valley) is characterized by humid clayish soil.

Both oak species (*Quercus robur* L. and *Quercus petraea* Liebl.) cover the stand (Fig. 1). However, we can observe a significant correlation between oak species distribution, with *Quercus robur* being dominant in the small valley and *Quercus petraea* on the plateau. The natural regeneration from seeds of the stand occurred in 1899–1900. During autumn 1998, 2000 and 2001 all the trees were cut down. Thus all the trees under investigation were approximately of the same age (100 years). The species were identified using Factorial Discriminant Analysis on 34 leaf markers [2].

A total of 286 trees (118 sessile oaks, 158 pedunculate oaks and 10 individuals with an intermediate morphology) were studied. The three studied ecological zones are represented in Figure 1. The species distribution between zones is as follows: pedunculate oaks (plateau: 17, slope: 57, small valley: 84 trees), sessile oaks (plateau: 52, slope: 62, small valley: 4 trees), intermediate oaks (plateau: 2, slope: 2, small valley: 6).

For each oak tree a 10 cm thick disk was cut at 1.30 m. From this disk a diametrical strip 10 cm wide and oriented North-South was extracted through sawing. After sapwood exclusion, sampling was carried out by shaving 10 cm-long zones (approximately 35–40 rings) located at both extremities of each diametric strip (corresponding to outer heartwood). The wood material from the two extremities

of the diametric strip were mixed so that a single powder sample is available for each of the 286 trees. This sampling should minimize the influence of heterogeneities in ellagitannin content within the trunk [28]. The shavings were ground down to obtain a powder with linear dimensions equal to or less than 0.5 mm. Newly felled trees were used and all the procedures were performed identically for all trees.

2.2. Analysis

2.2.1. HPLC

Ellagitannins were extracted with an acetone-water mixture (7:3). The acetone was evaporated and the samples filtered on Millipore filtration membranes. The quantification of ellagitannins was performed using a HPLC method. The HPLC line consisted of equipment from Millipore-Waters: a 490 E multiwaved detector, two Model 510 pumps, a Model 717 automatic injector, System Interface Module (SIM) and Maxima 820 software (Millipore-Waters) were used.

An RP 18 LiChrospher$^\circledR$ column (250 × 4 mm, 5 μm) (Merck, Darmstadt, Germany) and a precolumn from the same supplier (4 × 4 mm, 5 μm) were used to separate and determine the ellagitannins.

A binary gradient was used with the following elution conditions: solvent A, 0.1% phosphoric acid in water; solvent B, water-methanol solution (50:50); flow rate 0.8 mL/min; gradient, 0 to 16% B in 45 min, 16 to 90% B in 5 min, 90% B (constant gradient) for 5 min, 90 to 100% B in 15 min, 100% B (constant gradient) for 10 min, 100% to 0% B in 5 min.

The ellagitannins were detected by their UV-sorption at 240 nm using a diode-array detector (Waters$^\circledR$ 990). Identification was achieved by co-chromatography with purified references and by spectres comparison. Quantification was achieved using calibration with purified ellagitannins provided by INRA (Montpellier). Ten ellagitannins (vescalin, castalin, roburin A-E, grandinin, vescalagin and castalagin) and ellagic acid were quantified in each oak wood sample.

2.3. Statistical treatment

Statistical treatments were carried out using SAS software [37] and SGS (http://kourou.cirad.fr/genetique/software.html).

2.3.1. Principal Component Analysis (PCA)

PCA was carried out for all the ellagitannins traits (individual ellagitannin content, ellagic acid content, total ellagitannin content, percentage of individual ellagitannin, vescalagin/castalagin ratio). The 2-D variables graphs were plotted for 22 variables. Variance explained by each axis was calculated.

2.3.2. Correlation analysis

Correlation analysis was performed to assess possible relationships between total ellagitannin content and ring width, as well as between total ellagitannin content and vescalagin/castalagin ratio. Breavis – Pearson correlation coefficients and probabilities were calculated.

2.3.3. Spatial analysis

We have used the SGS software [8] available at the following address: http://kourou.cirad.fr/genetique/software.html. The spatial structure of continuous quantitative traits can be analysed by applying a distance measure. The mean distance between all pairs of individuals belonging to a given distance class serves as the measure of spatial structure. The mean over all pairs provides the reference value indicating absence of spatial structure. Values below the reference show positive autocorrelation and those higher indicate negative spatial autocorrelation. The SGS program computes transformed values of each trait using the z-transformation. This transformation is necessary to avoid problems with changing scales among different traits ([9] in [8]).

2.3.4. Variance analysis

Variance analysis was performed for the following traits: individual ellagitannin content, ellagic acid content, total ellagitannin content, percentage of individual ellagitannin, and vescalagin/castalagin ratio. First, the following effects were analyzed by one-way ANOVA: species effect for the global set (all the trees, regardless of the ecological zone), species effect in one ecological zone in which the two species were intimately mixed with large number of trees for both species (slope). Second, two-way ANOVAs were performed: the first one assuming significant interaction between ecological zone and species effects, the second one under the hypothesis of not significant interaction between them. For this analysis, intermediate individuals were excluded due to the small sample size (10 trees).

The general linear models procedure was applied for this purpose. A Student-Newman-Keuls test was carried out for each variable.

2.3.5. Total ellagitannin content in oak wood

The total ellagitannin content in oak wood was measured for both species. The measures were made in the range from 0 to maximum ellagitannin level (120 mg/g of dry weight of wood) with intervals of 5 mg/g.

2.3.6. Differentiation functional analysis (DFA)

DFA was carried out using all ellagitannin traits (individual ellagitannin content, ellagic acid content, total ellagitannin content, percentage of individual ellagitannin, vescalagin/castalagin ratio). Two canonical functions were calculated and oak samples were projected on a 2-D plan.

3. RESULTS AND DISCUSSION

Ellagitannin contents for each species are shown in Tables I and II. The values of ellagitannin content and their percentages were comparable to those reported by other authors for European oak wood [5, 16, 28, 30]. As in these previous studies, a high natural variability of wood extractives was observed. However, the large sample size led to important conclusions.

Table I. Means and distribution parameters of sessile oak ellagitanin.

Descriptive	Vescalin	Castalin	Roburin A	Roburin B	Roburin C	Grandinin	Roburin D	Vescalagin	Roburin E	Castalagin	Ellagic acid	Total ellagitanin
Mean	0.73	0.48	1.67	2.45	2.12	2.74	3.23	6.41	3.00	11.82	2.00	34.68
95% CIM*	0.62–0.84	0.42–0.55	1.51–1.83	2.18–2.72	1.81–2.43	2.44–3.03	2.81–3.64	5.79–7.03	2.71–3.30	10.93–12.71	1.79–2.21	31.81–37.56
Minimum–maximum	0.05–3.96	0.07–2.26	0.27–4.78	0.39–8.66	0.28–10.54	0.31–9.55	0.17–11.64	1.27–15.88	0.46–9.34	3.75–30.26	0.00–8.38	8.62–94.28
Std.	0.61	0.34	0.88	1.48	1.71	1.61	2.27	3.41	1.61	4.89	1.15	15.85

* CIM - Confidence Interval for Mean.

Table II. Means and distribution parameters of pedunculate oak ellagitanin.

Descriptive	Vescalin	Castalin	Roburin A	Roburin B	Roburin C	Grandinin	Roburin D	Vescalagin	Roburin E	Castalagin	Ellagic acid	Total ellagitanin
Mean	1.08	0.65	3.25	3.10	2.54	3.20	4.28	11.04	3.39	15.78	1.84	48.35
95% CIM*	0.97–1.20	0.59–0.71	2.92–3.58	2.83–3.37	2.26–2.81	2.86–3.54	3.84–4.72	10.15–11.93	3.09–3.69	14.82–16.73	1.70–1.98	45.06–51.65
Minimum–maximum	0.12–3.89	0.10–1.99	0.57–12.83	0.57–8.63	0.36–9.65	0.47–11.51	0.62–13.07	2.95–35.47	0.29–10.86	5.39–41.29	0.50–5.37	14.10–134.67
Std.	0.72	0.39	2.09	1.70	1.72	2.17	2.82	5.66	1.91	6.07	0.88	20.94

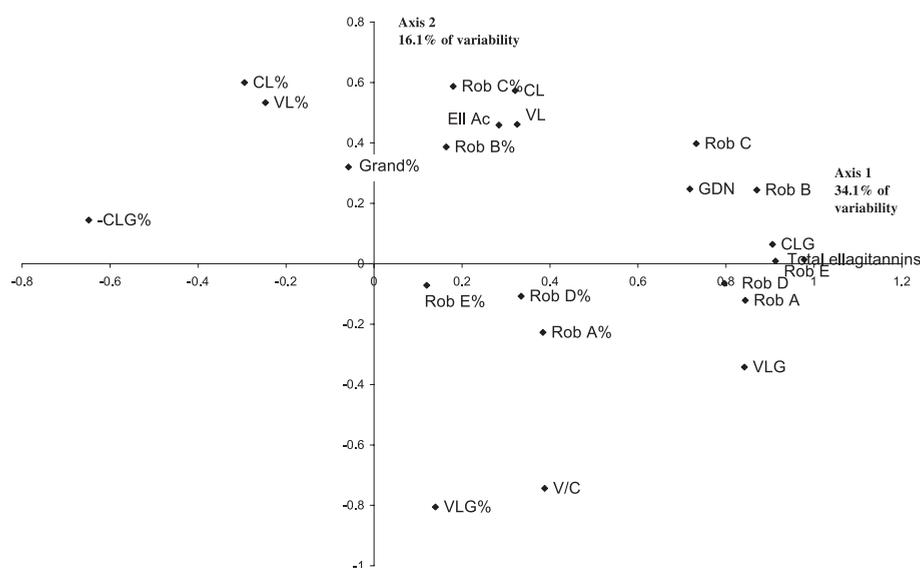* CIM - Confidence Interval for Mean.

Figure 2. Principal Component Analysis. Variables projection.

3.1. Correlation between studied parameters

The 2-D PCA projection of variables is presented on Figure 2. The first principal component axis explains 34.1% of the total variation and is closely correlated with some parameters such as total ellagitannin content, vescalagin, castalagin, grandinin and roburin A-E content. The second axis explains 16.1% of the total variation and is related to castalin, vescalin, percentage of vescalin, castalin, roburin A and C. The PCA plot indicates the relation between variables. The measured traits can be roughly grouped as follows: total ellagitannin content, vescalagin, castalagin, grandinin and roburin A-E content, which are negatively correlated with the percentage of castalagin and are not correlated with castalin, vescalin and ellagic acid content as well as with the percentage of vescalin, castalin, vescalagin, roburin B, C and E. Moreover one can observe a negative correlation between castalin, vescalin and ellagic acid content and the percentage of vescalin, castalin, roburin A and C on the one hand and the percentage of vescalagin and vescalagin/castalagin ratio on the other hand.

The significance of these correlations is that the increase of the total ellagitannin content, corresponding to an increase in all individual ellagitannins content except for vescalin and castalin, is mostly the result of an increase in roburin A-E, grandinin and vescalagin, whereas the castalagin content increases more slowly. Roburin A-E, grandinin and vescalagin contents rose gradually (their percentages are poorly correlated or not correlated with total growth), while the castalagin increase declined (its percentage is negatively correlated with total growth). Vescalin, castalin and ellagic acid are the structural units of other ellagitannins under investigation and related with them by formation – hydrolysis pathways [24,42]. However the absence of correlation between these two groups emphasizes the complex character of these transformations.

The total and individual ellagitannin content, except ellagic acid, were found to be correlated with ring width for peduncu-late oak. The best correlation is for castalagin and total ellagitannin content: $r^2 = 0.41$ (0.1%) and 0.39 (0.2%) respectively ($n = 158$). The correlation for total ellagitannin content is presented in Figure 3. No significant correlation was observed for sessile oak, in contradiction with the findings of Snakkers et al. [39], who found a positive correlation for sessile oak. This result was observed in each ecological zone, although the mean values and deviation of ring width were approximately the same for both species (2.81 ± 0.48 mm for sessile oak and 2.52 ± 0.38 mm for pedunculate oak).

An anatomical interpretation of this result can be proposed. As large "grained" wood (i.e. wide annual rings) of both sessile and pedunculate oaks is known to have a higher proportion of latewood [1, 7, 10, 15, 17, 19, 20, 25] it can be inferred that the ellagitannin content in latewood tissues is higher than in earlywood (at least for pedunculate oak). This inference corresponds to the results described by Masson et al. [27] who observed a higher ellagitannin content in latewood tissues (in that case, for both sessile and pedunculate oaks). However, we cannot completely exclude the possibility of the following artefact: as all the examined heartwood samples have the same length (10 cm), large ring samples exhibit lower age from the sapwood/heartwood limit and, thus, higher ellagitannin content as it is well known that the lower-aged wood possess a higher ellagitannin level [23, 28].

No correlation was found between individual ellagitannin percentage and ring width for both species.

The correlation between total ellagitannin content and vescalagin/castalagin ratio was studied for the whole set (tree zones, two species, $n = 276$), for sessile oaks (for each of three zones), for pedunculate oaks (for each of three zones), for small valley (both species), slope (both species) and plateau (both species). Although one can observe significant correlation for each species (more pronounced for pedunculate oak: $r^2 = 0.29$ (0.2%), than for sessile one : $r^2 = 0.24$ (0.99%)), the correlation substantially increased in the whole set, where both
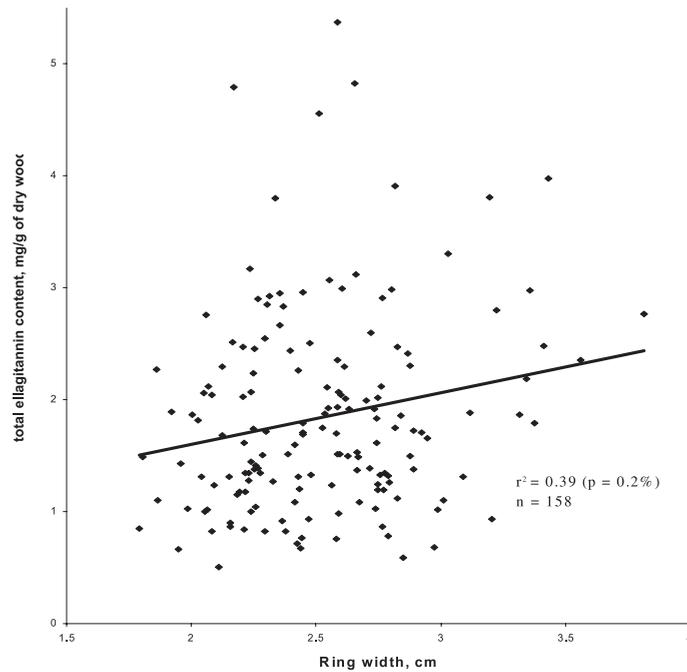
Figure 3. Correlation between ring width and total ellagitannin content for pedunculate oak.
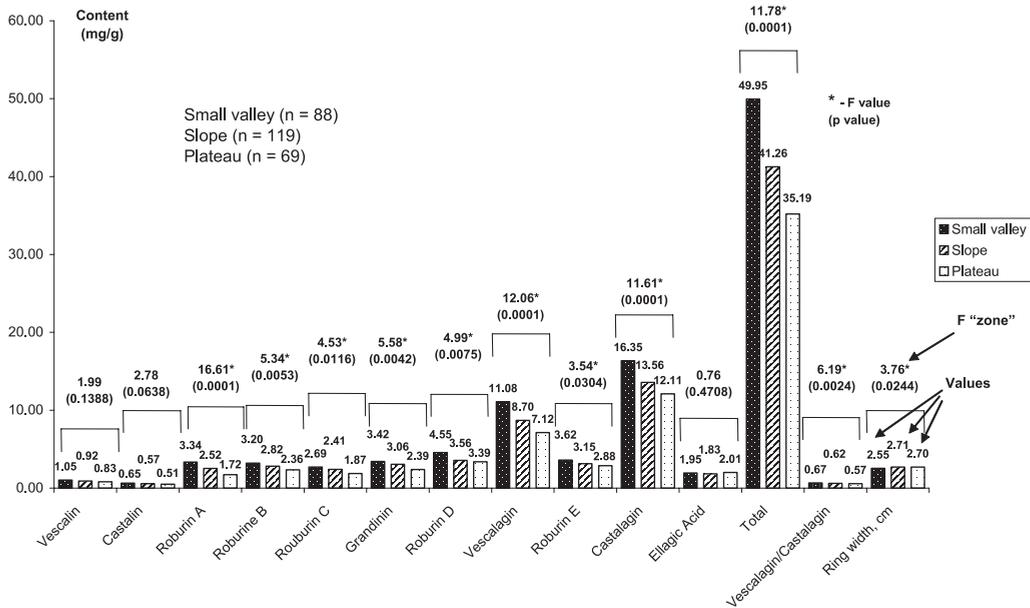

Figure 4. One-way ANOVA results for the zone effect as tested on the whole set of sessile and pedunculate oaks.

species are represented: 0.37 (0.1%). This result was expected because as it will be shown below, pedunculate oak contained a higher level of both ellagitannin content and vescalagin/castalagin ratio than sessile oak.

3.2. Species differentiation

No difference was observed for the content of major ellagitannins of the same species in the three ecological zones: three zones for pedunculate, but only two zones (slope and plateau) for sessile were used in the analyses because of the scarcity of the last species in the valley. The detailed results for zone differentiation in Figure 4 correspond to the whole set of sessile and pedunculate oaks.

The species status strongly influences oak wood extractives. Most ellagitannins differed by their content as a function of the species. In Figures 5 and 6, major results are presented for the complete stand and for slope, where sessile and pedunculate
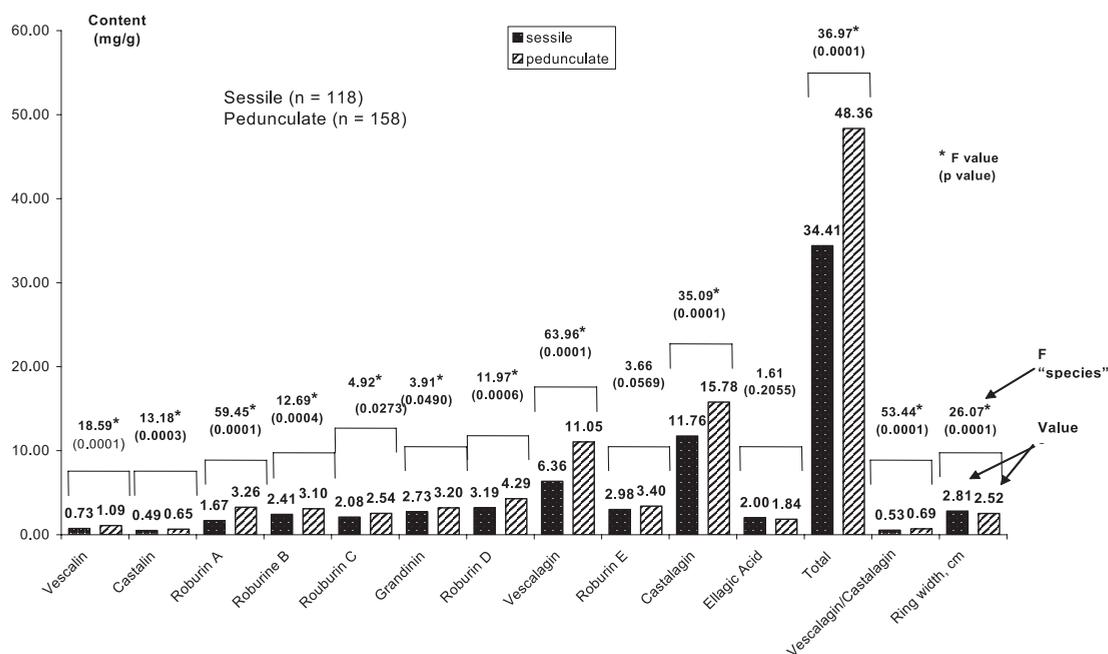
Figure 5. One-way ANOVA results for the species effect as tested on the whole set of sessile and pedunculate oaks.
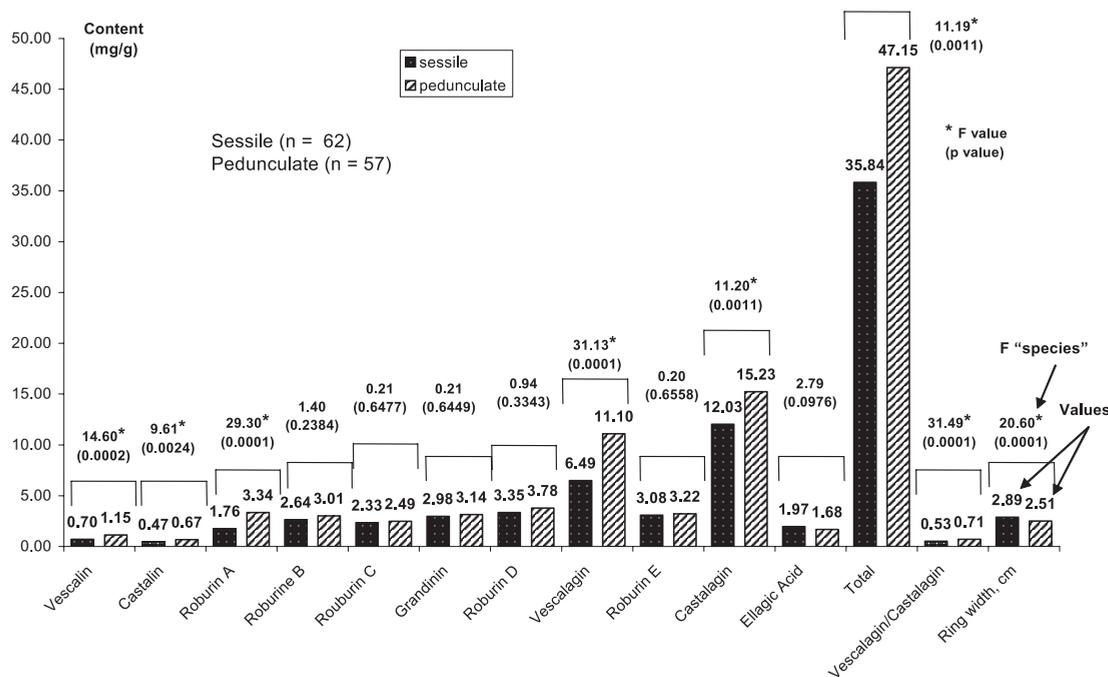

Figure 6. One-way ANOVA results for the specie effect as tested on the same ecological zone that is the slope.

trees are in equal proportions (62 sessile and 57 pedunculate oaks). Mean ellagitannin content and the concentrations of some major ellagitannins (vescalin, roburin A, vescalagin and castalagin) in pedunculate oak (48.36 mg/g for total content, 15.78 mg/g for castalagin and 11.05 mg/g for vescalagin) are substantially higher than in sessile oak (34.41 mg/g, 11.76 mg/g and 6.36 mg/g respectively), in agreement with previous studies [5, 14, 16, 28–30].

However, a better discrimination of species is found in the complete set (Fig. 5) than in the slope (Fig. 6). Hence, a "zone" effect is superimposed onto a "species" effect in the overall sample.

The percentage of individual ellagitannin is a less relevant characteristic for species discrimination. It is significant only for the following ellagitannins: roburin A (41.52 prob. 0.0001), grandinin (19.21 prob. 0.0001), vescalagin
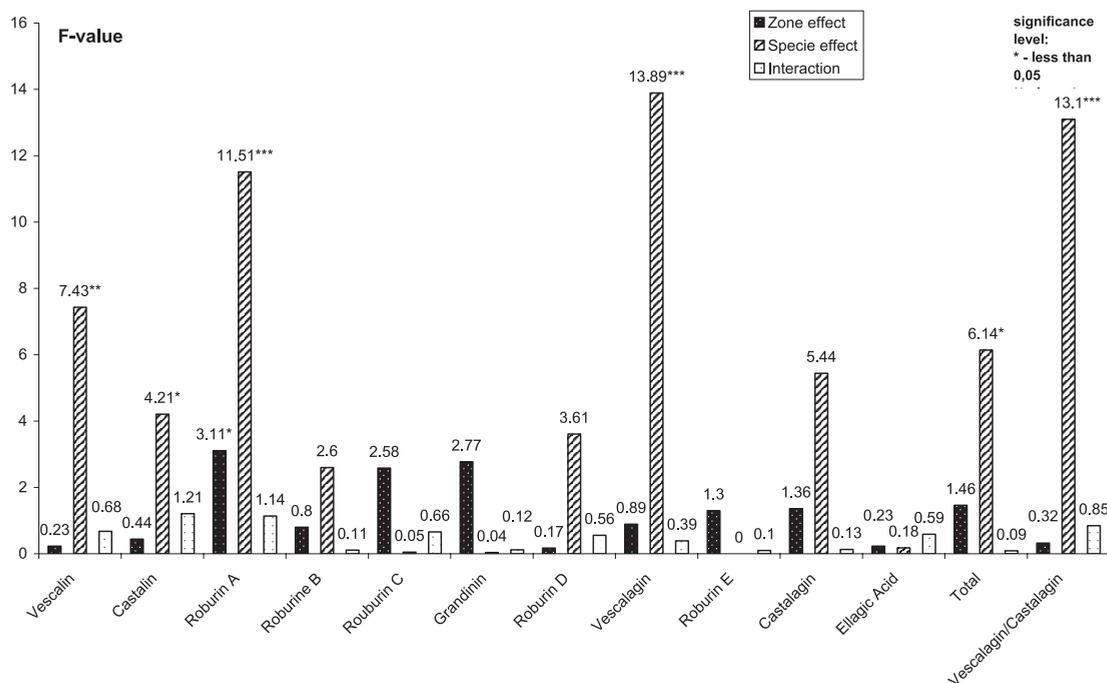
Figure 7. Two-way ANOVA results for ecological zone, species effects and interaction.

(49.13 prob. 0.0001), roburin E (58.59 prob. 0.0001) and also for the vescalagin/castalagin ratio (53.44 prob. 0.0001). This last index is higher for *Quercus robur* (0.69 against 0.53 for *Quercus petraea*). It was reported earlier that this index could be used to distinguish different oak (*Quercus*) and chestnut species (*Castanea sativa*) [34, 44]. The present data confirm this hypothesis.

The results of the two-way ANOVA(s) are presented in Figure 7. A two-step approach was performed to evaluate the influence of each factor: species, ecological zone as well as the interaction between them. In the first step, species and ecological zone effects were calculated by assuming that they are independent. This step represents a somewhat rough approach, because of the pronounced correlation between species distribution and ecological zone (Fig. 1). Therefore, in the second step, the model took into account the interaction between these factors. Model estimation values allowed the assessment of model reliability.

The analysis of the interaction between species effect and ecological zone effect demonstrates the predominance of the species effect. Neither zone effect, nor species – ecological zone interactions are significant. This result shows that the "zone" effect found in the mixed lot is conditioned by species effect and not by ecological zone effect.

As a consequence, the distribution of total ellagitannin content was studied with the objective to better characterise species differences. The DFA analysis (two canonical functions) was used for this purpose.

The distribution total ellagitannin content in sessile and pedunculate oaks are shown in Figure 8. The continued smoothing curves were plotted to better visualise the distributions. The sessile and pedunculate samples present one-
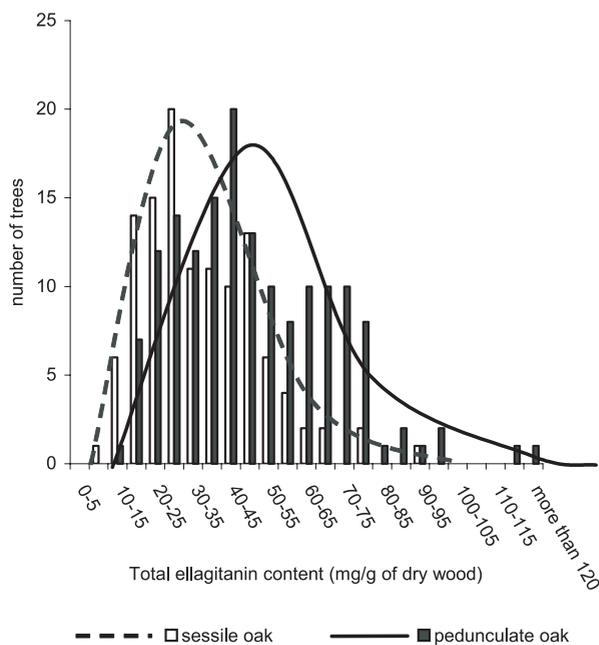

Figure 8. Distribution for total ellagitannin content in sessile and pedunculate oak trees (118 and 158 trees respectively).

peak distributions. Maximum distribution density was found to be located in a 25–30 mg/g interval for sessile oak and 45–50 mg/g for pedunculate oak. However, the natural variability of ellagitannin content in each species is very high and many oak samples cannot be identified on the basis of this single character, as shown by the DFA results.
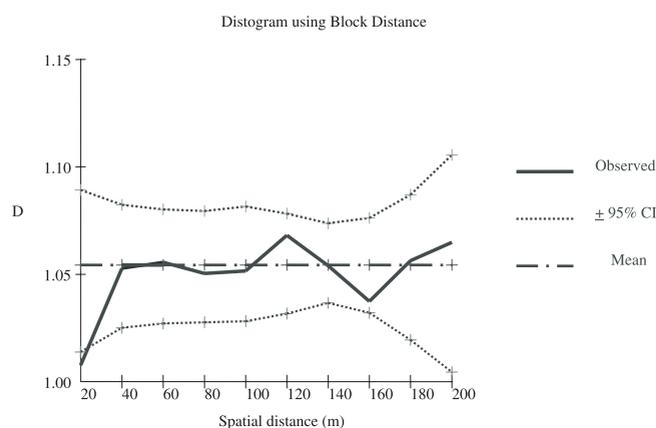
Distogram using Block Distance

Figure 9. Distogram using block distance with sessile and pedunculate oaks for ten ellagitannins. Values below the reference show positive autocorrelation and those higher indicate negative spatial autocorrelation.

3.3. Spatial distribution of ellagitannin variability

The analysis of ten ellagitannins in the two species shows a very weak spatial distribution in this stand (Fig. 9 and Tab. III). A weak spatial distribution is found for six of the 10 ellagitanins and total ellagitannins for the two species combined, for three ellagitanins in sessile oak and for four in pedunculate oak. This low spatial structure is surprising given that the stand is spatially structured for the species distribution, ecological conditions and gene resources. Several spatial studies were conducted with phenological, morphological and molecular markers in this stand. Significant spatial structure up to 40 and 70 m were found by Bacilieri et al. [3] with isozymes, morphology and phenology and by Streiff et al. [41] with microsatellite markers. Plant phenols, such as ellagitannins, constitute an important group of molecules involved in plant defence [21]. Sork et al. [40] have observed local adaptation against insect predation within a stand of *Quercus rubra*. In *Quercus suber*, Conde et al. [6] have found provenances differentiation for ellagitanins and other polyphenols. This low spatial organization could be due to a combination of ecological heterogeneity at local scale and genetic structure.

4. CONCLUSIONS

A mixed and even-aged high forest of *Quercus robur* L. and *Quercus petraea* Liebl. was investigated in this study. A statistically large set of samples (286 trees) was analyzed and treated by different statistical methods. HPLC technique was applied to quantify ellagitannin content in oak wood samples. There was a large variation in concentration of ellagitannins in the 286 trees samplings investigated. Several analytical indexes, like total ellagitannin content and content of major ellagitannins (vescalagin, castalagin, grandinin and roburin A-E) are closely correlated with each other.

The main factor influencing oak extractives level is botanical species. The factor of ecological zones is negligible. Pedunculate oak is generally richer in ellagitannins

Table III. Geographic distribution of ellagitannins within the stand (meter).

Ellagitannins	All species	Pedunculate oak	Sessile oak
Species distribution	Strong: 110 and 130		
10 ellagitannins	25 (weak)	–	–
Vescalin	–	–	–
Castalin	–	–	–
Roburin A	20 (weak)	–	170 (weak)
Roburin B	30 (weak)	–	20 (weak)
Roburin C	60–80 and 180 (weak)	80, 180 (weak)	–
Grandinin	–	180 (weak)	–
Roburin D	–	–	–
Vescalagin	25 (weak)	–	–
Roburin E	–	40–60 and 180 (weak)	–
Castalagin	30 (weak)		60 (weak)
Total ellagitannins	25 (weak)	–	60 (weak)

(48.4 mg/g vs. 34.4 for sessile oak). Even if the mean contents are statistically different between species, it is not clear cut, since the pedunculate oak contents overlap with those of the sessile oak. Ellagitannin content was found to be correlated with ring width in pedunculate oak but not in sessile oak.

In the future, the chemical composition could be correlated with other characters such as morphology, architecture, growth, wood quality and molecular genetic data.

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