# Seasonal Variation in the Composition of Epicuticular Waxes of *Quercus robur* Leaves

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*Quercus robur*, Fagaceae, Leaves, Epicuticular Wax Composition, Seasonal Variation

The epicuticular leaf waxes of *Quercus robur* were analyzed continuously over a two years vegetation period with preparation every week from April to November.

The folded leaflets in buds have waxes quite different in yield and composition from those of mature leaves. They contain homologous series of hydrocarbons, wax esters, primary alcohols, fatty acids and triterpenoids from the beginning, but not aldehydes. After leaf unfolding a dynamic biosynthesis of alcohols, aldehydes and fatty acids is observed in May and June. Wax content is doubled per dry weight or in $cm^2$ leaf surface area and 80-fold per one leaf in that time. During leaf development tetracosanol becomes the dominant epicuticular wax component comprising *ca.* 40% of the wax.

In both years of the study a reactivation of wax ester biosynthesis is observed in October and November. Esters with chain length $C_{36}$ and $C_{38}$ increased particularly. From July to November the wax composition remained nearly constant within mean values and their standard deviations. Within the two years studied most values concerning wax composition are reproducible and are therefore genetically determined. In spring the growing processes are influenced by climatic factors.

## Introduction

The epidermal cells of mature leaves of *Quercus robur* are covered with a continuous wax layer superimposed with numerous wax crystalloids in the shape of fringed edged platelets [1]. Similar surface structures have already been shown in other *Quercus* species [2]. The epicuticular wax of mature leaves consists of homologous series of *n*-alkanes (6.4%), wax esters (1.1%), aldehydes (38.8%), primary alcohols (36.0%), fatty acids (6.1%) and in addition triterpenols (3.6%) and their esters with fatty acids (0.5%) [3]. Leaves of *Quercus petrea* have nearly the same wax composition [2]. Recent studies on the deciduous trees *Tilia tomentosa* [4, 5] and *Fagus sylvatica* [6–8] showed a dynamic biosynthesis of distinct wax lipids after leaf emerging from buds and therefore seasonal and developmental variations within the wax composition and their surface wax ultrastructures. In this paper the changing chemical composition of epicuticular waxes during leaf development and all over one vegetation period are studied continuously of *Q. robur*.

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## Materials and Methods

Leaves of the oak *Quercus robur* L. were harvested from an isolated tree, aged about 12 years, which was grown in the garden of the Botanical Institute, at the University of Cologne. Leaf samples were collected from 24th April to 19th November 1990 and from 6th May to 14th November in 1991. Three branches of about 25 cm in length (30–40 g leaves) were harvested twice a week in April, May and June and then once a week for the rest of the season. Areas of representative leaves were measured using a planimeter. Dry weights of leaves were determined after heating them at 105 °C for 3 h. Surface waxes were extracted from leaves by immersion in chloroform (twice for 1 min each). The waxes were separated on a silica gel column in three fractions using the solvents *n*-pentane, 2-chloropropane and methanol. Subsequently the constituents were analyzed by TLC with toluene as the solvent. FID-GC was carried out on a OV-1 (10 m) fused silica capillary column. The column temperature was programmed from 140 to 320 °C as required [9, 10]. Monthly mean and standard deviation values plotted in the figures were, in most cases, derived from 4 replicates.

## Results

In the present investigation we studied developmental and seasonal factors affecting the composition of leaf surface waxes in *Quercus robur*. The leaf waxes were analyzed every week throughout the vegetation period in the years 1990 and 1991.

The first emergence of leaves from their buds was observed on 24th April in 1990. A spell of cold weather in 1991, delayed leaf emergence until the 6th May. Even these very young leaflets, newly unfolded from their buds were found to contain a continuous wax layer, consisting of homologous series of hydrocarbons, wax esters, primary alcohols, fatty acids and triterpenols as well as triterpenol fatty acid esters. In this early developmental stage, no aldehydes were detected in 1990. In both years epicuticular waxes were found in amounts of about 0.60% per unit dry weight, giving about 20 µg wax per $cm^2$ leaf surface area or *ca.* 50 µg wax per leaf in the early developmental stage. In May and June the waxes were analyzed twice a week and then once a week for the rest of the season up to 19th November in 1990 and up to 14th November in 1991. The number of samples analyzed in 1990 and 1991 was 37 and 32 respectively.

Unfolding of the leaves from their buds is followed by a dynamic biosynthesis of wax lipids, documented in Tables I and II and in Fig. 1–6. Table I summarizes the epicuticular wax composition at the beginning of leaf development, 10 days after leaf unfolding and the mean values of mature leaves from July to November in both years.

### *Developmental and seasonal factors*

The amount of wax during a vegetation period was correlated of both with dry weight and surface area of the leaves. The change in these two growth parameters and in the amount of wax produced are shown in Fig. 1 for both years of the study.

In 1990 the very young leaflets which had just emerged from their buds have a dry weight of about 26.1% on 24th April, the first sample taken in that year. In the next two weeks this value remains nearly constant but thereafter increases rapidly until the end of June when dry weights remain nearly constant and at a mean value of $\bar{x}_{24}$ = 44.8 ± 2.6% from July to November (Fig. 1 A). Newly emerged leaflets have a leaf area of approximately 1.3 $cm^2$. The leaf areas increase slowly in April, but then rapidly in May and reach an area of $\bar{x}_{24}$ = 30.9 ± 3.1 $cm^2$, which remains unchanged in the following months (Fig. 1 B).

In 1991 there was a spell of cold weather in April. As a result, the first emerging of leaflets from their buds occurred 12 days later, on 6th May, in that year (Fig. 1). The dry weight of the first sample is much greater (34.3%) than that for 1990. However this value decreased during May to 24.7% the level of 1990, and then in June increased up to a mean value of $\bar{x}_{16}$ = 42.4 ± 2.3% the period from July to November (Fig. 1 A).

Emerging leaflets in 1991 contained more organic substances than those of leaflets in 1990. Biosynthesis may already be active during the retarded unfolding of these leaves. In both years the

Table I. Yield and composition of epicuticular leaf waxes of *Q. robur* in 1990 and 1991; wax lipid classes in % dry weight.

| | 1990 | | | 1991 | | |
|---|---|---|---|---|---|---|
| | 24. April | 4. May | $\bar{x}_{25} \pm s$ (July–November) | 6. May | 16. May | $\bar{x}_{16} \pm s$ (July–November) |
| Dry weight [%] | 26.1 | 26.0 | 44.8 ± 2.6 | 34.3 | 27.0 | 42.4 ± 2.3 |
| Leaf area [$cm^2$] | 1.3 | 6.6 | 30.9 ± 3.1 | 1.3 | 3.9 | 47.5 ± 5.6 |
| % Wax/dry weight | 0.61 | 1.0 | 1.18 ± 0.13 | 0.6 | 0.33 | 1.10 ± 0.13 |
| µg Wax/$cm^2$ leaf surface | 17.5 | 19.7 | 53.5 ± 8.7 | 24.0 | 8.69 | 48.1 ± 4.8 |
| µg Wax/one leaf | 44 | 261 | 3245 ± 702 | 64 | 67 | 4574 ± 756 |
| Hydrocarbons | 0.05 | 0.04 | 0.04 ± 0.01 | 0.04 | 0.04 | 0.04 ± 0.01 |
| Wax esters | 0.19 | 0.05 | 0.04 ± 0.02 | 0.13 | 0.07 | 0.05 ± 0.02 |
| Aldehydes | – | 0.05 | 0.27 ± 0.09 | 0.02 | 0.03 | 0.22 ± 0.07 |
| Alcohols | 0.11 | 0.53 | 0.44 ± 0.06 | 0.16 | 0.29 | 0.42 ± 0.05 |
| Fatty acids | 0.31 | 0.46 | 0.24 ± 0.08 | 0.17 | 0.07 | 0.23 ± 0.06 |
| Triterpenols | + | + | 0.01 ± 0.01 | 0.01 | 0.01 | 0.09 ± 0.01 |
| Triterpenol esters | + | + | 0.01 ± 0.01 | + | + | 0.01 ± 0.01 |

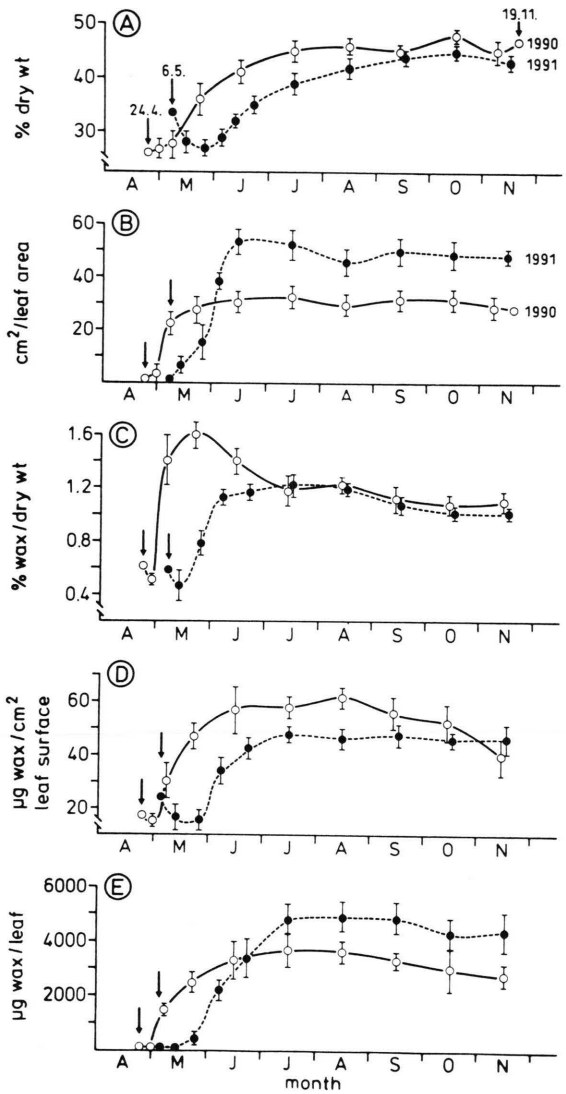


Fig. 1. Seasonal variations and developmental factors in epicuticular waxes from *Quercus robur* leaves in 1990 and 1991. A, dry weight of leaves in %; B, leaf size in $cm^2$; C, % surface wax per dry weight; D, µg wax per $cm^2$ leaf surface; E, µg wax per one leaf.

leaflets grew very rapidly by May and reach a constant size in the middle of June, $\bar{x}_{16} = 47.3 \pm 5.6\ cm^2$ (Fig. 1 B). The leaf area reached in 1991 was much higher (47.5 $cm^2$) than those of 1990 (30.9 $cm^2$). The amount of epicuticular waxes present in leaflets emerging from the buds is nearly equal in the two years of the study at 0.61% wax per dry weight in 1990 and 0.57% in 1991. In both years, these values decrease during the following 10 days, but then increase rapidly. Similar, nearly constant values of wax contents are reached at the end of June in both years with mean values $\bar{x}_{24} = 1.18 \pm 0.13\%$ for 1990 and $\bar{x}_{16} = 1.10 \pm 0.13\%$ for 1991. As may be seen in Fig. 1 C, the two curves of increasing wax content for May and June in the two years of the study differ markedly.

Wax amounts are also correlated with $cm^2$ leaf surface area and a single leaf. In 1990, an amount of 15.5 µg wax was found per $cm^2$ of leaf surface area. This value decreased within the next 10 days but then increased very rapidly in May. In June amounts of wax reached a constant value of $\bar{x}_{24} = 53.5 \pm 8.7$ µg wax/$cm^2$ for the period July to November. With an initial value of 24 µg wax/$cm^2$ leaf surface area, the amount found in 1991 is greater than in 1990. The decreasing phase in May 1991 is more prolonged than in 1990, but beginning in June a rapid increasing phase is found resulting in a mean value of $\bar{x}_{16} = 48.1 \pm 4.8$ µg wax/$cm^2$ leaf surface area by the end of July, only a little lower than in 1990 (Fig. 1 D).

At the initial stage of leaf unfolding, leaflets have nearly the same wax content (44 µg in 1990 and 64 µg in 1991). After 10 days amounts of wax increase very rapidly up to a constant value in June 1990 of $\bar{x}_{24} = 3245 \pm 702$ µg wax/leaf. In 1991, the increasing phase is slightly retarded. In that year, the amounts of wax per leaf are attributable to the greater leaf size $\bar{x}_{16} = 4574 \pm 756$ µg wax/leaf (Fig. 1 E).

Changes in the class of wax lipids during a vegetation period have been studied in detail. In the figures these are represented as: total (a) and individual (b) components. All values in the figures are described per unit dry weight (Fig. 2–6). The lipid classes with respect to % of surface wax are summarized in Table II. Fig. 2–6 and Table II show the results of analyses of the individual wax components in 1991.

*Hydrocarbons*

Hydrocarbons are found in an homologous series ranging from $C_{21}$ to $C_{35}$ over the whole season. In the first days of leaf development they are represent about 7% of the wax content. In May and June this value decreases and in the following months increases again to about 5% wax in November 1991 (Table II). *n*-Alkanes are present in similar concentrations during the whole vegetation

Table II. Composition of epicuticular wax lipids of *Q. robur* during a vegetation period in 1991 (in % wax, $\bar{x} \pm s$).

| | Hydrocarbons | Wax esters | Aldehydes | Alcohols | Fatty acids | Triterpenols Triterpenol esters |
|---|---|---|---|---|---|---|
| May 6 | 6.9 ± 0.7 | 19.1 ± 5.2 | | 44.0 ± 15.0 | 24.3 ± 8.5 | 1.4 ± 0.3 |
| May | 2.4 ± 0.3 | 7.2 ± 1.4 | 8.8 ± 3.0 | 70.5 ± 1.7 | 9.5 ± 2.5 | 0.4 ± 0.3 |
| June | 2.0 ± 0.5 | 3.2 ± 0.9 | 27.8 ± 7.2 | 50.6 ± 8.3 | 13.2 ± 2.6 | 3.3 ± 2.5 |
| July | 2.9 ± 0.6 | 2.5 ± 0.2 | 23.0 ± 1.6 | 36.3 ± 2.6 | 24.5 ± 3.2 | 7.4 ± 0.8 |
| August | 3.3 ± 1.0 | 3.2 ± 1.2 | 22.7 ± 0.6 | 38.2 ± 0.5 | 23.2 ± 2.4 | 8.5 ± 0.9 |
| September | 4.5 ± 0.3 | 3.2 ± 0.6 | 21.8 ± 0.5 | 37.5 ± 2.8 | 18.7 ± 1.8 | 8.1 ± 1.0 |
| October | 4.4 ± 0.3 | 7.7 ± 2.1 | 13.6 ± 1.9 | 41.3 ± 4.0 | 18.3 ± 1.8 | 9.6 ± 0.8 |
| November | 5.2 ± 0.2 | 7.1 ± 0.3 | 12.9 ± 1.4 | 44.9 ± 1.9 | 16.0 ± 1.8 | 8.4 ± 1.2 |

period. Main components have carbon atom chain lengths $C_{27}$, $C_{25}$ and $C_{29}$ (Fig. 2). This fraction also contains alkenes about 5%. Alkenes with even carbon numbers are predominant in this wax, especially $C_{24}$, $C_{26}$, $C_{28}$, $C_{30}$ alkenes. In GC their RT values are just below those of the corresponding alkanes. In GC-MS spectra they show the characteristic fragmentation of alkenes, all fragments are two units lower than the alkanes. Fragments like those resulting from a dehydratization splitting of primary alcohols are found with the characteristic fragments *m/z* 83 and 97. The same fragmentation patterns are observed for Δ1-heptadecen [12, 13]. Therefore it is concluded, that the double bond in these alkenes is at position Δ1 [14]. Unsaturated hydrocarbons are found throughout the season. The amounts and the pattern of development of hydrocarbons is virtually identical in the vegetation period 1990 and 1991.

A remarkably large proportion, nearly 20%, of waxes from the first preparation is composed of an homologous series of wax esters (Table II). This value decreases very rapidly in the following 2 months to a minimum of about 3% of wax, but increases again in October and November (Fig. 3).

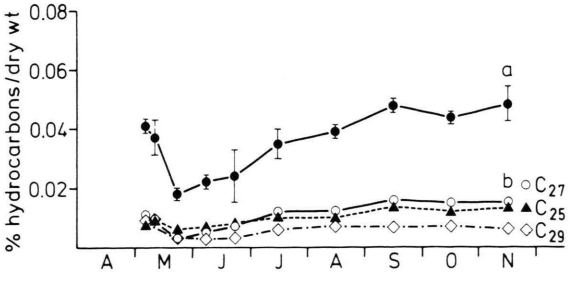


Fig. 2. Seasonal variation in hydrocarbons of *Quercus robur* leaf waxes in 1991. a, total hydrocarbons; b, individual components.

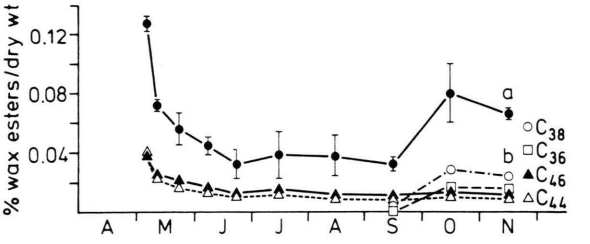


Fig. 3. Seasonal variation in wax esters of *Quercus robur* leaf waxes in 1991. a, total wax esters; b, individual components.

Wax esters have chain lengths from $C_{36}$ to $C_{52}$ with main components $C_{44}$ and $C_{46}$ in concentrations of 20% to 30%, each. At the beginning of October a distinct accumulation of wax esters was observed up to *ca.* 8% of the wax. This increase in the total of esters is caused by the synthesis of only two esters, with chain length $C_{36}$ and $C_{38}$. $C_{36}$ wax esters are mainly composed of octadecanoid acid and octadecanol and $C_{38}$ wax esters of eicosanoic acid and octadecanol. GC-MS spectra of these wax esters show characteristic fragments for protonic acids *m/z* 285 for $C_{36}$ and *m/z* 313 vor $C_{38}$ esters [15]. This increasing in the amount of the $C_{36}$ and $C_{38}$ wax esters was reproducible in the two years studied. Increasing in these two esters occur at the same time and in similar concentrations and are characteristic for oak leaves (Fig. 3).

### *Aldehydes*

Aldehydes were absent from the leaf wax of the initial developmental stage of the unfolding leaf on 24th April 1990 and were detected for the first time 10 days later on 4th May 1990 in rapidly increasing amounts of about 28% of the wax content. Due to a spell of cold weather in April 1991, with

temperatures under 0 °C on April 17 [11], leaves emerged 12 days later on 6th May. The wax of these leaflets which had just unfolded from their buds already contained traces of aldehydes in the first samples. Throughout May the trend was one of increasing amounts of aldehyde. The aldehyde curve reach a maximum at the end of June in 1991, in contrast to 1990 with a maximum at the end of May. In both years similar mean values for aldehydes from July to November were found (Table I). In Fig. 4 aldehyde curves for 1990 and 1991 are compared with each other. In both years aldehydes are present in chain length from $C_{20}$ to $C_{32}$ with $C_{26}$ and $C_{28}$ as the main components in concentrations of *ca.* 20–40% each (Fig. 4). In 1992 there was again a spell of cold weather in April. The leaves emergence was retarded until 5th May in that year and wax of these leaflets already contained traces of aldehydes.

*Alcohols*

From the beginning primary alcohols are the main constituent of the wax lipids in oak leaf wax. After leaf unfolding a rapid biosynthesis of alcohols especially tetracosanol is observed. The alcohols account for about 70% of the wax at their maximum in the seasonal curve and average about

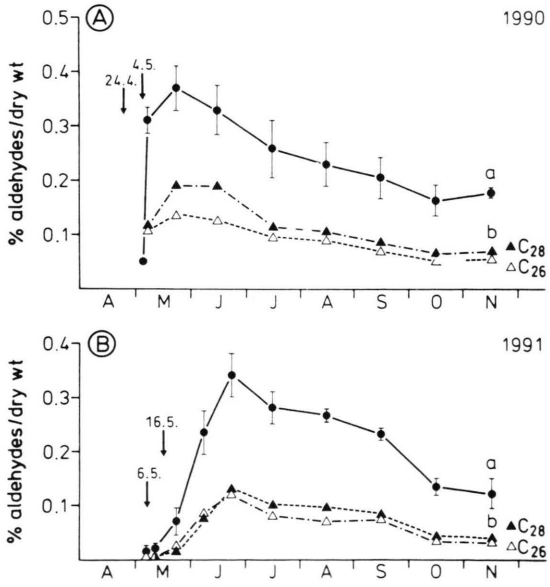


Fig. 4. Seasonal variations in aldehydes of *Quercus robur* leaf waxes in 1990 (A) and 1991 (B). a, total aldehydes; b, individual components.

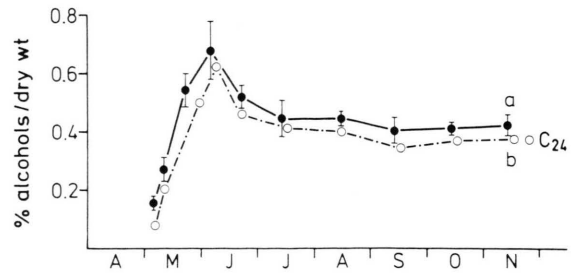


Fig. 5. Seasonal variations in alcohols of *Quercus robur* leaf waxes in 1991. a, total alcohols; b, individual components.

40% throughout the season. The homologous series of alcohols shows chain lengths ranging from $C_{16}$ to $C_{34}$ with one main component, tetracosanol, dominating at more than 90% of the total. In both years the amounts and composition of the alcohols and the course of their seasonal figures are similar (Table I, Fig. 5).

*Fatty acids*

In the initial stage of leaf unfolding fatty acids occur in relatively large amounts about 20% of wax. They decrease in amount during May and June and increase again in July, when the synthesis of the other wax lipids stops and maintain an average of about 20% of the wax for the remainder of the season (Fig. 6).

Fatty acids are found in an homologous series ranging from $C_{14}$ to $C_{32}$. Hexadecanoic and octadecanoic acid are the main components in this lipid class at the initial developmental stage, but later the synthesized of hexacosanoic and octacosanoic predominates. In both years, the amount of fatty acid, their composition and the change in amount in the course of the growing reason are very similar (Table I, Fig. 6).

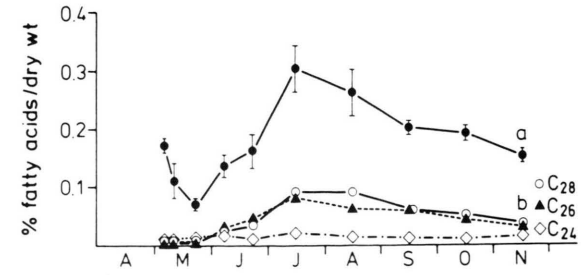


Fig. 6. Seasonal variation in fatty acids of *Quercus robur* leaf waxes in 1991. a, total fatty acids; b, individual components.

During leaf development a change in the composition of fatty acid is observed. Tetracosanoic acid averages about 10%, while hexacosanoic and octacosanoic acid each make up 20–30% of the acid fraction in the remainder of the season.

*Triterpenoids*

From the beginning of leaf unfolding oak leaf wax also contains triterpenoids. Taraxerol, β-amyrin, α-amyrin and lupeol were identified free as well as esterified with fatty acids [3]. Triterpenoids are found in concentration of about 1% of the wax in the initial stages and increase during June to about 8% and remained nearly constant over the remaining season (Table II).

## Discussion

In *Quercus robur* the folded oak leaflets in buds were found to contain a continuous epicuticular wax layer. The composition of these waxes is quite different from those of mature leaves. In the initial stages of leaf development the wax consist of homologous series of hydrocarbons, wax esters, alcohols, fatty acids with relative short chain lengths and triterpenoids. Aldehydes are absent from this wax. The consistency of this wax layer enables the unfolding movements of the leaflets and the growing process of the leaves in the growth stages which follow.

After the leaves have emerged from the buds, a very dynamic biosynthesis of organic cell substances and also of wax components was observed. Generally following a ten day delayed period, dry weight, leaf size and wax amounts increase very rapidly in May, and reach constant values at the end of June. Although the absolute values in the two years of the study differed, the curves for the above parameters followed a similar course. The large difference in the curves for May (Fig. 1) are attributable to climatic influence which had the effect of retarding leaf emergence in 1991 and also in 1992.

During leaf unfolding the biosynthesis of predominantly very long chained lipids is observed. Aldehydes are synthesized with chain length $C_{26}$ and $C_{28}$. Fatty acids are found in the same chain lengths $C_{26}$, $C_{28}$ and in addition $C_{24}$. Within the alcohols synthesis of only one chain length, $C_{24}$, predominated.

The seasonal curves of aldehydes and alcohols have a maximum in June while the seasonal curve for fatty acids reach a maximum in July, when the synthesis of the aldehydes and alcohols ceased. After leaf unfolding the biosynthesis of hydrocarbons remains low while the synthesis of wax esters is not continued further. At the beginning of October however the biosynthesis of wax esters is reactivated. In both years studied, the synthesis of wax esters with chain lengths $C_{36}$ and $C_{38}$ was detected at that time. Within the leaf waxes of oak trees tetracosanol is the dominant wax lipid accounting for about 40% of wax and is therefore responsible for the development of wax crystalloids in form of edged platelets on both surfaces of the oak leaf [1].

The wax composition of mature leaves from oak trees show a good agreement during the two years studied. The mean values of the individual lipid classes from July to November have nearly the same values and are always within the standard deviations. The wax composition is therefore very strongly genetically determined.

As the 1991 values show, the initial stage of leaf unfolding in spring can be influenced by climatic factors. While the emerging of buds is influenced by temperature and is therefore retarded in 1991 and 1992, the synthesis of organic substances did not stop at this time. The dry weight of the leaflets is much more higher (34.3%) in 1991 than that in 1990 (26.1%). The emerging of leaflets from these buds is first accompanied by a hydratization process. There then follows an activation of enzymes leading to a dynamic biosynthesis of distinct wax substances at the end of May. In the present study the biosynthesis of aldehydes ($C_{26}$, $C_{28}$), alcohols ($C_{24}$) and fatty acids ($C_{24}$, $C_{26}$, $C_{28}$) is found to be predominant.

An SEM study on the development of wax structures throughout the vegetation period and their correlation with the above chemical analysis will be reported in a further paper.

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