The Use of Argentation Chromatography for the Analysis of Fatty Acid Esters of Polyenes: The Structure of Carotenoid Esters of Aglais urticae (Lepidoptera, Insecta)

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Argentation Chromatography, Fatty Acids, Carotenoid Esters, Insect Lipids

Introduction

The importance of lipid metabolism to insects is shown by the dietary requirement for some fatty acids, steroids, and other terpenes, which are vital factors for embryogenesis, metamorphosis and metabolism. Unsaturated fatty acids play a major role in insects, and especially the Lepidoptera show a special need for polyunsaturated acids. During the study of insect carotenoids the widespread occurrence of carotenoid esters has been established. Until now, no work has been done in this field of insect biochemistry. If only small amounts of material are available, work on this subject mostly suffers from the difficulty to isolate pure substances, which are free of other lipid esters. Neutral glycerides are often predominant and therefore prevent an unequivocal identification of the fatty acids by convenient methods like GC. Therefore the native esters must be studied using e.g. the reversed-phase partition TLC developed by Egger. But the plant material, studied so far, mostly contains a homologous series of saturated fatty acids. In the case of unsaturated acids direct methods of analysis have been used. As shown in this paper argentation chromatography, which has been widely used in the separation of lipids, can be successfully applied to whole carotenoids esters. The results could be confirmed by mass spectrometry of the esters.

Materials and Methods

Animals

Egg batches of Aglais urticae were collected in the field. The larvae were reared in the laboratory at room temperature under natural light conditions during the summer season. They were fed with Urtica dioica. Pupation occurred in the rearing cages. Few days after the ecysis the pupae were collected and frozen till further processing.

Isolation of carotenoids

Extraction of Aglais pupae

The pupae were lyophilized and homogenized in cold acetone with an “Ultra-Turrax”. Further ex-
tractions were done with acetone and methanol until the solutions remained free of carotenoids. From the combined extracts the carotenoids were transferred to petroleum ether (boiling range 50–70 °C) by addition of concentrated solution of \((\text{NH}_4)_2\text{SO}_4\). After repeatedly washing with distilled water the pigment solution was dried with anhydrous \(\text{Na}_2\text{SO}_4\) and concentrated under reduced pressure.

**Extraction of Tagetes and Aesculus**

Known lutein di- and monoesters were isolated from the flowers of *Tagetes* spec. \(^{15}\) and from autumn leaves of *Aesculus hippocastanum* \(^{12}\) for comparison with those esters, which were extracted from *Aglais* pupae. In *Tagetes* lutein is combined with the saturated fatty acids stearic acid (18:0), palmitic acid (16:0), and myristic acid (14:0), to form the diesters distearate, stearate-palmitate, dipalmitate, palmitate-myristate, and dimyristate. The corresponding monoesters were prepared by partial saponification (ca. 15 min) of the diester mixture. As the two hydroxy groups of lutein are chemically different two positionally isomeric monoesters with each fatty acid are obtained, which were separated by chromatography on silica gel-G. The 3'-isomers, esterified at their allylic hydroxyls, predominate over the 3-isomers as they are more resistant to alkaline treatment.

In *Aesculus* the lutein esters only contain the saturated palmitic acid (16:0) and the highly unsaturated linolenic acid (18:3). The diesters are dipalmitate-palmitate-linolenate, and dilinolenate. Additionally, the corresponding monoesters occur as a mixture of the isomers. Autumn leaves contain large amounts of lipids, which interfere with the chromatographic separation of the pigment esters. Part of lipid can be precipitated by storing the pigment solution in petroleum ether at \(-30 \, ^\circ\text{C}\).

The isolation of these lutein esters of plant origin was performed in the same way as described for the *Aglais* carotenoids, but the material has not been lyophilized, and for extraction only acetone was used. Chromatography was done by partition on silica gel-G.

**Chromatography (TLC)**

**Silica gel-G system**

The carotenoids were fractionated on 0.5 mm thick layers of silica gel-G, which were not activated prior to use. Development was performed with a mixture of petroleum benzene (boiling range 100–140 °C) and propanol-2 (1:30:8; v/v) \(^{16}\). This system provides excellent partition chromatography with high separation accuracy and capacity. The single fractions obtained were further purified by rechromatography in the same system once or twice, mainly to remove lipids.

**Reversed-phase partition chromatography**

Precoated cellulose layers were partially impregnated \(^8\) with a 10 per cent solution (w/w) of liquid paraffine in petroleum benzene (boiling range 100–140 °C). For this the cellulose sheets were put into a tank containing the paraffine solution. The run was stopped, when the solvent front was 3–4 cm below the upper edge of the plate. Then the plates were dried in an air stream for complete evaporation of the petroleum benzene. The pigment solutions were spotted on the not impregnated part of the cellulose layer. Separation of substances occurs only in the paraffine part. As developing solvent acetone-methanol 2:1 (v/v) + 3 per cent water was used for monoesters with 1 free hydroxyl group, a ratio of 4:1 + 1.5 per cent water was suitable for diesters. The mixtures were saturated with paraffine.

**Argentation chromatography**

\(\text{AgNO}_3\) in the stationary phase: For the preparation of plates silica gel-G was mixed with an aqueous solution of \(\text{AgNO}_3\) under red light (silica gel-G/water/\(\text{AgNO}_3 = 4:8:1\); by weight). The layers were dried at 110 °C for 1–1.5 hours, and then allowed to cool in the drying oven. Prior to use the application zone was inactivated by moistening with petroleum ether in order to avoid formation of artifacts. The most fitted developing solvent was found to be a mixture of \(n\)-hexane, diethyl ether, and propanol-2 (12:3:1; vol.). The runs were done in absolute darkness.

\(\text{AgNO}_3\) in the mobile phase: For this the paraffine impregnated cellulose layers were used in a reversed-phase partition system. Development was performed with methanol-water (50:1; v/v) saturated with \(\text{AgNO}_3\). This kind of chromatography was usually performed in a two-dimensional way: normal reversed-phase chromatography in the first direction, and in the second with the \(\text{Ag}^+\) containing developing solvent.

In this system a demixing of the solvent is demonstrated. If the solvent reaches the upper edge of the plate (\(\alpha\)-front), the silver ions migrate about half the distance only (\(\beta\)-front), what can be demonstrated by photoreduction of the silver (cf. Fig. 3). Consequently, separation by argentation partition chromatography refers to the \(\beta\)-front.

The solubility of silver nitrate in acetonitrile provides no useful basis for argentation partition...
chromatography as proposed by Morris. Even at high silver concentrations no complexing with the applied substances was observed, probably owing to the binding of Ag⁺ to the solvent itself. The use of silver fluoroborate (AgBF₄) instead of nitrate resulted in no superior procedure despite of its good solubility in aqueous methanol.

**Sample purification for mass spectrometry**

The ester fractions were eluted from the paraffine impregnated cellulose with acetone. Part of the paraffine could be removed by storage at −30 °C, most of it was separated by two runs on silica gel-G plates. The substances were purified further by adsorption on layers of CaCO₃/MgO, followed by chromatography on silica gel 60 HR after blank runs in methanol and n-hexane. As developing solvent a mixture of n-hexane and methanol (50:1; v/v) was used. The zones were eluted with a minimum of acetone.

**Quantitative estimation of carotenoids and fatty acids**

The calculation of pigment fractions eluted from the silica gel chromatogramm was based on the specific extinction coefficients as described previously. The total ester fractions were determined in the same way. The single esters, which are obtained by reversed-phase chromatography of each ester fraction from the silica gel plate, were calculated by densitometry of the chromatograms (carotenoid absorption). From the relative portion of each ester and the absolute amount of the total fraction the absolute quantity of the single ester carotenoid can be computed. As the fatty acids of these esters could be analysed the amounts of these acids bound to carotenoid could be determined, too. This indirect method of calculation was the only reasonable way, since the pigment ester fractions were not free of lipid contamination, which would have caused marked errors in the determination of the liberated fatty acids by e.g. gas chromatography.

**Densitometry**

Thin-layer scanning of the reversed-phase partition chromatograms was done with a double-beam densitometer ("Cromscan"; Joyce, Loebl & Co., Ltd.). The standard instrument with the normal sample holder was used. From a chromatogramm of the esters, which had been applied as a 30-40 mm long band, a ca. 25 mm broad strip was cut, mounted in the sample holder, and covered with the plastic frame spacer. The optical parameters were: blue filter (465 nm); slit 0503 (5.0 × 0.3 mm); grey wedge 2.0 OD. The specimen/record ratio was 1:3. For record range expansion those cams were chosen, which produced maximal peak heights. Measurements were performed in reflectance and transmission operation. The peak areas were automatically integrated and corrected for the baseline. For comparison the areas were also determined by the gravimetrical method. In preceding studies it was shown, that in both, reflectance and transmission measurements, a linear relationship exists between pigment quantity and integrator counts.

**Spectroscopy**

Visible-light absorption spectra were recorded with a Zeiss spectral photometer type DMR 21 using glass cuvettes of 1 cm pathway. Mass spectrometry was performed with a Varian Mat CH 5 mass spectrometer equipped with the direct inlet system. The probes were heated to 230 °C. Electron energy was 70 eV. Acceleration potential was 3 kV. Perfluorokerosene was used as mass marker.

**Chemical reactions**

Saponification was done with 3 per cent Na-methylat in methanol (w/w). Acetylations were performed with acetic anhydride in dry pyridine (1:5; v/v). Allylic hydroxyl groups were etherified with 0.1 M BF₃ in ethanol.

**Chemicals and chromatographic materials**

All chemicals were purchased from Merck (Darmstadt). Most of them were of analytical grade ("p.A."); Na-methylat and BF₃-ether complex were for laboratory use ("LAB"). Chemicals were used without further purification. Pyridine was dried over KOH platelets. Liquid paraffine, and those solvents, which were used to purify samples for mass spectrometry, were of ultra pure grade ("Uvasol"). Silica gel-G and silica gel 60 HR were obtained from Merck, the precoated cellulose layers (F 1440, plastic sheets) were a product of Schleicher and Schüll (Dassel, West-Germany).

**Results**

**Identification of the carotenoid fractions**

The carotenoid extract of *Aglais* pupae separates into five fractions on the silica gel chromatogramm.

Fraction 1; hRF = 82: β-Carotene (β, β-carotene)

The pigment exhibited the visible-light absorption maxima at 450 and 477 nm in hexane. It changes
not during saponification and is identical in its chromatographic and spectroscopic properties with synthetic \( \beta,\beta \)-carotene.

Fraction 2; \( hR_F = 66 \): Diester of lutein

The electronic spectrum of this fraction showed maxima at 423, 446, and 475 nm in ethanol, demonstrating a chromophoric system identical with that of \( \beta,\varepsilon \)-carotene. Saponification yields a more polar end product, which is identical with lutein (fraction 5). Hereby two intermediates occur, which run parallel with the fractions 3 and 4 on the chromatogram. Both products form monoacetates, which are not separable by chromatography. Therefore, the two intermediates are positionally isomeric monoesters, derived from a lutein diester.

Fraction 3; \( hR_F = 37 \): 3'-monoester of lutein

This fraction, also showing the visible light absorption maxima of the \( \beta,\varepsilon \)-chromophore, yields lutein on saponification without the occurrence of intermediate products. The formation of a monoacetate and of a monoether was shown, the latter of which can be transferred to lutein-3'-ether by alkaline treatment. Therefore, the allylic hydroxyl group of the lutein molecule must be free.

Fraction 4; \( hR_F = 29 \): 3'-monoester of lutein

The light absorption properties coincide with those of the fractions 2 and 3. Saponification results directly in the formation of lutein. During acetylation a monoacetate is produced, which is chromatographically identical with that one obtained from fraction 3. But in contrast fraction 4 yields no ether. This means, that the position of the acyl group must be allylic. (Treatment of the allylic ester with acid alcohol results in a slow replacement of the ester group by an ether group, thus forming lutein-3'-ether, which runs slightly below fraction 4 on the chromatogram.)

Fraction 5; \( hR_F = 14 \): Lutein (\( \beta,\varepsilon \)-carotene-3,3'-diol)

The shape of the electronic spectrum and the positions of the peaks revealed the chromophoric system of \( \beta,\varepsilon \)-carotene. The pigment is resistant towards alkali treatment. Acetylation yields two isomeric monoacetates, which are separable on silica gel, and a diacetate. With BF\(_3\)/ethanol a monoether is formed. All properties of this pigment correspond with those of lutein isolated from Pieris pupae.

The results show, that Aglais pupae only contain two carotenoids, \( \beta \)-carotene and lutein, the latter of which occurs both, as free pigment and as mono- and diesters.

**Fatty-acid analysis of the lutein esters**

The ester fractions eluted from the silica gel chromatogram were further studied by reversed-phase chromatography. Such a system provides pure partition chromatography, and substances of a homologous series are separated both according to the lengths of the aliphatic chains of the same degree of saturation and according to the number of double bonds in chains of equal lengths. The introduction of a double bond into a molecule gives a change in its partition coefficient roughly equivalent to that produced by a shortening of the chain length of two methylene groups. Thus "critical pairs" are possible, which are separated by other methods.

**Di esters**

The lutein diester fraction of Aglais separates into three zones (\( P_{I-III} \)) on paraffine impregnated cellulose (Fig. 1). Two other, very weak zones were not investigated further. Co-chromatography with the known diesters of Tagetes and Aglais showed, that the lowest (i.e. the less polar; \( P_{III} \)) diester of Aglais run together with the dimyristate (14:0 + 14:0) of Tagetes and with the palmitate-linolenate (16:0 + 18:3) of Aesculus; the upper (most polar;
Aglais diester showed the same $R_F$-value as the dilinolenate (18:3+18:3) of Aesculus (Fig. 1).

According to the Martin-relation in homologous molecules a linear function exists between their $R_m$-values [$R_m = \log(1/R_F - 1)$] in a partition system and their molecular differences, for example the lengths of the carbon chains. As seen in Fig. 2 the $R_m$-values of the known diesters (C$_{28}$ - C$_{36}$) and monoesters (as acetates C$_{16}$ - C$_{29}$) of Tagetes are linearly arranged. In the case of the Aglais diesters the effective chain lengths of their fatty acids are 24, 26 and 28 C-atoms as revealed by the $R_m$-values. These values are a little reduced, i.e. these esters are more polar than expected for saturated fatty acids of the calculated chain lengths. So unsaturated fatty acids are to be supposed.

Fig. 2. Linear relationship between $R_m$-values of lutein esters and the effective number of carbon atoms of their fatty acids. ○ — Tagetes monoester acetates C$_{14}$—C$_{29}$, diesters C$_{28}$—C$_{36}$ (all saturated fatty acids). ● — Aglais monoester-acetates C$_{14}$ and C$_{16}$, diesters C$_{24}$—C$_{28}$ (all unsaturated fatty acid) (cf. Fig. 1).

Evidence for unsaturated acids was produced by argentation chromatography, which is based on the ability of olefines to form labile complexes with Ag$^+$ ions$^{14,18}$. This method has been widely used in the separation of lipids, and it was a question, whether it was applicable to the analysis of fatty acids esterified with polyenes. As shown in Fig. 3 a clear differentiation between carotenoid esters of saturated and unsaturated fatty acids is possible by two-dimensional reversed-phase chromatography using a Ag$^+$ containing solvent for the second run. In the case of saturated fatty acids the esters show no or only little (shorter acids) mobility, whereas unsaturated acids change their partition coefficient due to complexing with Ag$^+$ ions, resulting in a strong increase in the relative mobility of the esters. But, as in reversed-phase chromatography both number of double bonds and number of C-atoms are involved, an unequivocal result on the degree of unsaturation can not be obtained by this method. Therefore the Aglais esters were chromatographed by adsorption on AgNO$_3$-impregnated silica gel, which permits separation according to number, position and geometry of the double bonds, ir-

Fig. 3. Two-dimensional separation of lutein esters with saturated and unsaturated fatty acids on paraffine impregnated cellulose. 1. run: solvent without silver ions (the strip on the left represents the positions of the esters after the first run). 2. run: argentation partition chromatography. The dashed line shows the silver front (β-front). Punctuated zones are Tagetes monoester-acetates (upper) and diesters (lower) with saturated fatty acids. Black zones are Aglais monoester-acetates (upper) and diesters (lower) with unsaturated fatty acids (cf. Fig. 1).

Fig. 4. Argentation adsorption chromatogramm of lutein diesters on silica gel-G. A — Tagetes: Saturated fatty acids. B = Aesculus (from top to bottom): Dipalmitate, palmitate-linolenate, dilinolenate. C — Aglais: Dilinoleate, linoleate-linolenate, dilinolenate (fractions A$_1$, A$_{11}$, A$_{111}$).
respective of the lengths of the carbon chains. The applicability of argentation adsorption chromatography on fatty-acid esters of polyenes is demonstrated in Fig. 4. The five *Tagetes* diesters, all with saturated fatty acids (28:0 – 36:0), run in a single fraction, which is less adsorbed. The same behaviour is shown by the dipalmitate (32:0) of *Aesculus*; the dilinolenate (36:6) is strongly adsorbed due to the polarity of its silver complexes; the palmitate-linolenate (34:3) runs between the two others, as expected. Concerning the *Aglais* diesters the polarity of the main fraction (AIII) is identical to that of the *Aesculus* dilinolenate, thus demonstrating the existence of six double bonds in its fatty acids. For the two other *Aglais* fractions five resp. four double bonds are to be expected according to their minor adsorption (fractions AII and AIV).

In order to associate these adsorption zones with those zones obtained by reversed-phase chromatography, the esters separated as silver complexes were rechromatographed in the normal reversed-phase system. Thereby the identity of AIII and P1, AII and PII, A1 and PIII was shown. That means that the sequence of the diesters in the partition system completely matches their degree of unsaturation. From the equivalence of one double bond to two methylene groups and the effective chain length the number of C-atoms of the fatty acids of the *Aglais* diesters can be calculated:

- Fraction P1 = AIII: 24 + (2 × 6) = 36 C-atoms;
- Fraction PII = AII: 26 + (2 × 5) = 36 C-atoms;
- Fraction PIII = A1: 28 + (2 × 4) = 36 C-atoms.

Accordingly, the total length of the fatty acids is the same for each diester. Fraction P1, possessing five double bonds in the fatty acids, must be a mixed diester with one dienoic and one trienoic acid probably. In this case fraction P1 may be the symmetrical diester of the trienoic acid, and fraction PIII the corresponding one of the dienoic acid. Thereby the *Aglais* diesters P1, PII and PIII would be identical to the dilinolenate (18:3 + 18:3), the linolenate-linoleate (18:3 + 18:2), and the dilinoleate (18:2 + 18:2). As the argentation method separates not only according to the number of double bonds but also according to their position and configuration, the identical chromatographic properties of P1 = AIII to the dilinolenate of *Aesculus* strongly support this interpretation. Evidence was obtained both by partial saponification of the diesters to monoesters (see below), and by mass spectrometry of the diesters.

The fractions P1 and PII were isolated in a preparative scale by chromatography on paraffine impregnated cellulose layers; PIII yielded no sufficient amount. The mass spectrum of P1 (Fig. 5) showed the molecular ion at $m/e = 1088$ (weak) as expected for the dilinolenate of lutein. Prominent peaks were at $m/e = 810$ (M-278) (base peak) and at $m/e = 532$ (M-278-278). Ions at $m/e = 996$ (M-92) and $m/e = 982$ (M-106) result from loss of toluene resp. xylene from the polyene chain of the carotenoid. Other intense peaks originate by multiple fragmentations: $m/e = 718$ (M-278-92), $m/e = 704$ (M-278-106), $m/e = 440$ (M-278-278-92), $m/e = 426$ (M-278-278-106), $m/e = 397$ (M-278-278-135), $m/e = 374$ (M-278-278-158), $m/e = 357$ (M-278-278-175). The fragment of 158 mass units origi-
nates in the polyene chain, whereas elimination of 135 and 175 mass units are caused by cleavage of the 7,8 and 9,10 double bonds. The mass differences of 278 units are caused by the loss of one and two fatty acids from the molecular ion; they correspond to a 18:3 fatty acid like linolenic acid. The mass spectrum of P_II showed the molecular ion at m/e = 1090 as calculated for the linolenate-linoleate of lutein. Different to P_I two monoester ions were observed at m/e = 810 and m/e = 812 respectively. Like in the case of P_I the peak at m/e = 532 refers to the deacylated lutein diester. The known losses of toluene and xylene were observed, too. The differences of 278 and 280 mass units correspond to a 18:3 and a 18:2 fatty acid, thus supporting the mixed nature of the P_II diester.

Evidence for P_III as a diester of a 18:2 fatty acid is produced by the analysis of the monoesters, which were obtained by partial saponification of the diester fraction.

Monoesters

The lutein monoesters, both the native isomers (fractions 3 and 4) and those ones obtained from the diesters (fraction 2), behaved in all chromatographic systems completely identical. In the following, therefore, they are described together.

The 3-isomers as well as the 3'-isomers are separated on paraffine impregnated cellulose into two zones, the upper of which predominates (Fig. 6).

Because of their free 3-hydroxyl, being more polar than the one in 3'-position, the 3'-esters run above the corresponding 3-isomers as demonstrated by comparison with the monoesters of Aesculus. As the two isomers of Aglais obviously contain the same fatty acids, they were combined and acetylated. In this state the isomeric pairs behave identically on cellulose. According to their R_m values (Fig. 2) the effective chain lengths of the acetates are 14 and 16 C-atoms, i.e. 12 and 14 C-atoms for the fatty acids alone (fractions P_II^* and P_III^*). As shown by multiple development, however, the lower zone is more polar than the myristate-acetate of Tagetes (Figs 3, 7). The upper zone runs together with the linolenate-acetate of Aesculus, similarly being more polar than in the case of a 12:0 fatty acid. The unsaturation of the fatty acids of the two monoesters was established by argentation chromatography in the reversed-phase system (Fig. 3). The number of double bonds was obtained by argentation adsorption chromatography and comparison with the acetates of the monoesters of Tagetes (14:0; 16:0; 18:0) and Aesculus (16:0; 18:3). Following this (Fig. 8) the main fraction (A_1^*) contains a trienoic fatty acid, the other fraction (A_2^*) a mono- or dienoic acid. Work on an other subject showed, that in this system esters of saturated and mono-unsaturated fatty acids are not to distinguish. Therefore, the other fatty acid of the Aglais monoesters must have two double bonds.
Rechromatography of the argentation zones in the partition system revealed the identity of $P_I^*$ and $A_{II}^*$, and of $P_{II}^*$ and $A_I^*$. Now, the chain lengths of the fatty acids can be calculated:

- Fraction $P_I^* = A_{II}^*$: $12 + (2 \times 3) = 18$ C-atoms;
- Fraction $P_{II}^* = A_I^*$: $14 + (2 \times 2) = 18$ C-atoms.

Thus the monoesters and the diesters show the same fatty acids: linoleic (18:2) and linolenic (18:3) acid. They are esterified with lutein in the following way:

- Monoesters: $3$-linoleate, $3'$-linoleate ($P_{II}^*$);
  $3$-linolenate, $3'$-linolenate ($P_I^*$).
- Diesters: $3,3'$-dilinoleate ($P_{III}$);
  $3$-linoleate-$3'$-linolenate
  $3$-linolenate-$3'$-linoleate
  $3,3'$-dilinolenate ($P_I$).

Quantitative measurements

Quantitative measurements of the carotenoid fractions

Table I shows mean values of several measurements of the carotenoid content of a few days old pupae. Besides free lutein the lutein diester is the most prominent fraction. The $\beta$-carotene content of the pupae is very low. From the total amount of lutein (13.1 $\mu$g = 94.7 per cent) 48.6 per cent occur as diester, 20.6 per cent as the two monoesters, and 30.8 per cent as free lutein. From the combined monoesters 59.8 per cent refer to the $3'$-isomers probably due to the greater stability of the allylic ester bond as compared with the 3-isomers.

As the carotenoid content of animals is based on the dietary supply, the low $\beta$-carotene content means that Aglais larvae most selectively absorb (free) lutein from the ingested plant material.

Table I. Absolute and relative quantities of carotenoid fractions in pupae of Aglais urticae. The fractions were obtained by TLC on silica gel-G.

<table>
<thead>
<tr>
<th>Silica gel fractions</th>
<th>$\mu$g per pupae</th>
<th>% of total carotenoids</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1) $\beta$-carotene</td>
<td>0.7</td>
<td>5.3</td>
</tr>
<tr>
<td>(2) lutein as diester</td>
<td>6.4</td>
<td>46.0</td>
</tr>
<tr>
<td>(3) lutein as 3-monoester</td>
<td>1.1</td>
<td>7.8</td>
</tr>
<tr>
<td>(4) lutein as 3'-monoester</td>
<td>1.6</td>
<td>11.7</td>
</tr>
<tr>
<td>(5) free lutein</td>
<td>4.0</td>
<td>29.2</td>
</tr>
<tr>
<td>total lutein</td>
<td>13.1</td>
<td>94.7</td>
</tr>
<tr>
<td>total carotenoids</td>
<td>13.8</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Quantitative measurements of single esters and fatty acids

The single esters of a silica gel fraction were scanned after their separation in the reversed-phase partition system by densitometry basing on the absorption of the carotenoid alcohol (Fig. 9). In order to compare different methods each chromatogram was scanned in remission and transmission operation and quantified both by the integrator of the densitometer and by gravimetry of the peak areas. The data on the diesters (Table II) and on the monoesters (Table III) show, that the results of the different methods are very consistent with each other. In the case of the diesters the dilinolenate is the most prominent fraction; the relative amount of dilinoleate is very low. Concerning the monoesters the trienoic acid predominates over the dienoic acid, too, reaching a $18:3/18:2$ ratio of about 4:1. Interestingly, the monoesters derived from the diesters correspond very well with the native ones in this respect. This fact indicates that a. the formation of the monoesters and the diesters is based on the same frequencies of the two fatty acids.
Remission:
Integration of reversed-phase partition chromatogramms on paraffine impregnated cellulose (CP).

Transmission:
Integration of reversed-phase partition chromatogramms on paraffine impregnated cellulose (CP).

Table II. Relative quantities of lutein diesters in *Aglais* pupae. Measurements were done by densitometric scanning (n = 7) of reversed-phase partition chromatograms on paraffine impregnated cellulose (CP).

<table>
<thead>
<tr>
<th>Measuring method</th>
<th>CP-fractions P_I (18:3+18:3)</th>
<th>CP-fractions P_II (18:3+18:2)</th>
<th>CP-fractions P_III (18:2+18:2)</th>
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<td>x ± S.E.M.</td>
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<td>Remission:</td>
<td>69.1 ± 1.0</td>
<td>25.9 ± 0.7</td>
<td>4.9 ± 0.6</td>
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<tr>
<td>Integration</td>
<td>68.7 ± 0.4</td>
<td>27.5 ± 0.1</td>
<td>4.0 ± 0.3</td>
</tr>
<tr>
<td>Gravimetry</td>
<td>70.2 ± 0.7</td>
<td>24.8 ± 0.6</td>
<td>5.0 ± 0.2</td>
</tr>
</tbody>
</table>

Transmission:
Integration of reversed-phase partition chromatogramms on paraffine impregnated cellulose (CP).

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<td>Gravimetry</td>
<td>70.2 ± 0.7</td>
<td>24.8 ± 0.6</td>
<td>5.0 ± 0.2</td>
</tr>
</tbody>
</table>

Table III. Relative quantities of lutein monoesters in *Aglais* pupae. The esters were native ones or obtained from the diester fraction by partial hydrolysis. Measurements were done by densitometric scanning of reversed-phase partition chromatogramms on paraffine impregnated cellulose (CP).

<table>
<thead>
<tr>
<th>Origin of the fractions</th>
<th>Measuring method</th>
<th>CP-fractions P_I (18:3)</th>
<th>CP-fractions P_II (18:2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>x ± S.E.M.</td>
<td>x ± S.E.M.</td>
</tr>
<tr>
<td>monoesters native</td>
<td>Remission:</td>
<td>81.0 ± 1.5</td>
<td>19.0 ± 1.5</td>
</tr>
<tr>
<td></td>
<td>Integration</td>
<td>79.0 ± 1.9</td>
<td>21.1 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>Gravimetry</td>
<td>80.5 ± 1.6</td>
<td>17.9 ± 1.0</td>
</tr>
<tr>
<td>monoesters from the diesters</td>
<td>Remission:</td>
<td>81.3 ± 1.3</td>
<td>18.9 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>Integration</td>
<td>79.8 ± 0.6</td>
<td>20.3 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>Gravimetry</td>
<td>78.5 ± 0.8</td>
<td>21.5 ± 0.8</td>
</tr>
</tbody>
</table>

Table IV. Calculation of absolute amounts of the fatty acids bound in lutein di- and monoesters in *Aglais* pupae.

<table>
<thead>
<tr>
<th>CP-fractions of lutein esters</th>
<th>Bound lutein [%]</th>
<th>Ester [\mu g/pupa]</th>
<th>Bound fatty acids [\mu g/pupa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>(P_I) 18:3+18:3</td>
<td>69.1</td>
<td>4.4</td>
<td>8.5</td>
</tr>
<tr>
<td>(P_II) 18:3+18:2</td>
<td>25.9</td>
<td>1.7</td>
<td>3.1</td>
</tr>
<tr>
<td>(P_III) 18:2+18:2</td>
<td>4.9</td>
<td>0.3</td>
<td>0.6</td>
</tr>
<tr>
<td>total in diesters:</td>
<td>6.4</td>
<td>12.2</td>
<td>1.1</td>
</tr>
<tr>
<td>(P_I*) 18:3</td>
<td>81.0</td>
<td>2.2</td>
<td>3.2</td>
</tr>
<tr>
<td>(P_II*) 18:2</td>
<td>19.0</td>
<td>0.5</td>
<td>0.8</td>
</tr>
<tr>
<td>total in monoesters:</td>
<td>2.7</td>
<td>4.0</td>
<td>0.3</td>
</tr>
<tr>
<td>total per pupa:</td>
<td>9.1</td>
<td>16.2</td>
<td>1.4</td>
</tr>
</tbody>
</table>

* Values obtained by remission densitometry and integrator counts (cf. Tables II and III).

Discussion

Argentation chromatography has been shown to be a useful tool not only in the identification of free fatty acids or their triglycerides, but also for fatty-acid esters of polyenic alcohols like carotenoids. There is no loss of the known selectivity of this method by the eleven mostly conjugated double bonds of the carotenoid, which is also able to complex with silver ions. However, the quite different behaviour of carotenoid esters with saturated and mono-unsaturated fatty acids in the argentation partition system suggests that the ability of the carotenoid to bind with Ag⁺ is less pronounced, probably due to conjugation of the double bonds. The partition system described is mainly useful for the proof of unsaturation of the fatty acids; the number of the double bonds may be established by the adsorption method, which also may be conclusive in respect to position and geometry of these bonds.

Because of the high extinction values of carotenoids the analysis of native fatty-acid esters of these polyenes needs only minute amounts of material in a relative crude state. If there are no known esters of the same carotenoid available for comparison from a biological source, they can be synthesized from the carotenoid and the corresponding chloride of the fatty acid. Thus the combination of reversed-phase partition and argentation chromatography provides an excellent method for complete structure analysis of fatty-acid carotenoid esters.
The exclusive esterification of dietary lutein with poly-unsaturated fatty acids in *Aglais* pupae, as revealed by the method described, is highly significant for the physiology of the insect. Insects lack the ability for the synthesis of 18:2 and 18:3 acids and therefore depend on their dietary supply. The main fatty acid of green leaves is linolenic acid, indeed. As known so far polyenoic acids are incorporated at a high rate into structural lipids, especially into phospholipids. Their accumulation into eggs shows their significance for insect development. On the other hand dietary lack of polyenoic acids results in wing malformation at adult emergence. The binding of large amounts of these fatty acids to lutein in *Aglais* seems to serve as a stock for these essential factors comparable to the formation of glycerides. It is the question, whether the esterification of carotenoids is a necessary or a facultative way, which is omitted in the case of carotenoid lack.

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