Analysis of the Seed Lipids of *Aleurites montana*

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The seed lipids of the Chinese tung-oil tree *Aleurites montana* are composed of 97.2% triglycerides, 2% phospholipids and of 0.8% glycolipids. In the triglycerides 67% of all fatty acids are α-eleostearic acid (configuration $C_{18:3}$, Δ^9 *cis,* Δ^{11} *trans,* Δ^{13} *trans*). This acid is bound in the triglycerides in the 1,3 position. Via mercury II-acetate adducts and subsequent chromatography on silica gel a separation of this fatty acid from the seed fatty acids is possible. By this procedure an α-eleostearic acid-free tung-oil mixture is obtained, which consists by one half of $C_{18:2}$, 25% of $C_{18:1}$ and by 25% of the saturated fatty acids $C_{16:0}$, $C_{18:0}$ and $C_{14:0}$.

Introduction

The seed-oil of *Aleurites* trees is an indispensable primary product of the Chinese lacquer industry (Fang and Que, 1981; Fang *et al.*, 1985). The culture of the Chinese tung-oil tree is more and more endangered by air pollution in particular by the actual SO_2 concentrations reached in China.

In earlier studies we had analyzed the leaf lipids and the fatty acid composition of *Aleurites montana* grown under the increased content of 700 ppm CO_2 in the atmosphere (He *et al.*, 1996a + b) as well as of plants grown under 0.3 ppm SO_2 in the atmosphere (He *et al.*, 1997). These experiments have shown that not only the thylakoid membrane but also mitochondrial and cell membranes, such as the tonoplast, plasmalemma and the endoplasmatic reticulum, suffer changes in dependence on these environmental factors.

In addition to these analyses on plant physiological changes due to environmental factors, an analysis of the oil of seeds of *Aleurites montana*, as it is produced under now-a-days Chinese environmental conditions, is given in the present paper. The oil of *Aleurites montana* and *Aleurites fordii* seeds is known as tung-oil and is according to work by Hopkins and Chisholm (1962) as well as by Fang and Que (1981) (compare also Gunstone *et al.* (1965, 1994)) supposed to be composed of up to 80% of α-eleostearic acid, which is an octadecatrienoic fatty acid with conjugated double bounds having Δ^9 *cis,* Δ^{11} *trans,* Δ^{13} *trans* configuration. In this publication we report on the composition of tung-oil, which not only was obtained by cold pressing of the seeds but also by extraction under boiling temperatures with a mixture of methanol/chloroform (v/v, 1:1). In order to obtain all lipids from the seeds we determine the position of the α-eleostearic acid in the triglyceride and describe the isolation of this $C_{18:3}$ fatty acid with conjugated double bounds from the oil via mercury II-acetate adducts.

Material and Methods

Aleurites montana seeds

Seeds for the lipid extraction came form the Central South Forestry University in Zhuzhou/Hunan, People's Republic of China. The seeds were harvested in October/November 1995. The analysis of the seeds was carried out in April 1996. In the meantime the seeds had been stored at −20 °C in order to exclude aging of the lipids.

Lipid preparation

21 g of dry *Aleurites* seeds were broken in a mortar and the obtained 19.9 g of seed cores were extracted with 150 ml methanol/chloroform (1:1,

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v/v) at 85 °C for 2 hours under back-flow conditions. After cooling down of the seed brei a further extraction with 100 ml acetone and 50 ml diethylether was carried out on a glass sinter funnel. From this lipid extract the seed lipids were taken up with diethylether in order to obtain a defined lipid fraction.

Lipid analysis

By chromatography on a silica column (Silica gel, mesh size 0.05–0.2, Merck, Darmstadt) the lipids were separated into a polar and a non-polar fraction. Elution of the triglycerides was done with chloroform and the glycolipids were thereafter extracted with acetone and phospholipids with methanol. After thin layer chromatography on silica gel, phospholipids and glycolipids were quantitatively determined according to earlier described methods (He *et al.*, 1996b) (Table I). For the gas chromatographic analysis the triglycerides were first subjected to a mild alcaline hydrolysis with simultaneous transesterification of the fatty acids. 50 mg triglyceride were diluted in 1 ml diethylether and were shaken for 10 minutes at 20 °C after addition of 1 ml 0.4 N KOH-methanol. After the subsequent addition of 4 ml water and 2 drops of ethanol the methyl esters were taken up in petrolether (40°-60 °C boiling point). The gas chromatographic analysis was carried out with a Hewlett Packard gas chromatograph (Type 5890, Series II) on a 10 m long capillary column with the polar stationary phase being ethylene glycole succinate. The carrier gas was nitrogen. The column temperature was 190 °C and that of the detector and of the injection block 300 °C. For the identification of the fatty acid methylester authentic carbonic acids were used. Hydration of the fatty acids was carried out with platin oxide as catalysator. During the transesterification of the triglycerides by 1 hour cooking with 5% water-free methanolic HCl the α-eleostearic acid produced approx. 5–7 isomerization products.

Fractionation of fatty acids

Mercury II-acetate adducts of the fatty acid methyl ester mixture of the triglycerides were obtained according to the procedures described by Jantzen and Andreas (1959, 1960). For this purpose 500 mg fatty acids were incubated for 6 days under anaeobic conditions with 2.7 g mercury II-acetate dissolved in 50 ml methanol at room temperature. Then methanol was removed by distillation and for the removal of residues of methanol taken up twice with benzene with both components removed by distillation. The adducts were dissolved in benzene and for the removal of insoluble components filtered over a paper filter with a cotton layer. For the separation of the adducts the filtrate dissolved in benzene was given on a silica gel column (Silica Gel, Merck, Darmstadt, Germany, 0,05–0.2 mesh; the column dimensions were 2.4 cm Ø / 18 cm h which gives a column volume of 81 ml). Saturated fatty acids were eluted with 240 ml benzene, the monoenoic fatty acids (monoadducts) with 240 ml diethylether, the dienoic fatty acids (diadducts) with 160 ml of a mixture of propanol/acetic acid (100:1, v/v) according to Klenk and Tschöpe (1963) and the tri- and polyenoic fatty acids (tri- and polyenoic adducts) with 160 ml of a mixture of methanol and acetic acid (1:1, v/v). After the subsequent regeneration of the fatty acids by 1 hour shaking at 20 °C with a mixture of 15% HCl, methanol and petrol ether (1:3:2, v/v) the gas chromatographic analysis of the obtained fractions was carried out.

Enzymatic splitting of the triglyerides

For position analysis of fatty acids 50 mg triglyceride in 40 ml 0.06 M phosphate buffer 0.15 M NaCl, 0.01 M $CaCl_2$ and 0.01% sodium deoxycholate were emulgated by ultrasonic treatment and after the addition of 0.7 ml lipase from *Rhizopus arrhizus* (Boehringer, Mannheim) or lipase from pig pancreas (Serva, Heidelberg) incubated under continuous shaking, for 90 minutes at 37 °C. Thereafter the fatty acids were taken up by extracting 3 times with 20 ml petrol ether/diethylether (1:1, v/v). Diglycerides were extracted from the incubation solution by extracting 3 times with 30 ml chloroform and for the splitting of the fatty acid subjected to a mild alcaline hydrolysis.

Results and Discussion

From 21 g *Aleurites* seeds, after the removal of the seed shells, 19.9 g "pure seeds" were obtained. The seed/nut represents 95% of the fruit. An extraction of the seed lipids with a mixture of boiling methanol/chloroform (1:1, v/v) and a subsequent

dissolution of the lipids in diethylether showed, that *Aleurites* seeds are composed of 37–41% of their dry weight of lipids. These lipids represent a yellow oil. Differing lipid contents are due to the stage of maturity of the seeds. The column chromatographic fractionation of this yellowish oil on a silica gel column leads to the result that this oil is composed of 97.2–98.3% of the typical storage lipid triglyceride. The remainders are glycolipids and phospholipids which make up for 0.83 respectively 1.95%. A thin layer chromatographic quantitative analysis of these polar lipids yielded the composition as summarized in Table I. The main components of the phospholipids are phosphatidylcholine, phosphatidylglycerol and phosphatidylinositol, and the main component of the glycolipids are monogalactosyldiglyceride and sulfoquinovosyldiglyceride. These glycolipids and phospholipids represent the components of the oleosome membranes and cell membranes of the germ structure.

For the gas chromatographic analysis of the fatty acids the triglycerides were subjected to a mild alkaline hydrolysis with simultaneous transesterification of the fatty acids. The results obtained from the gas chromatographic analysis are summarized in Table II. For comparison purpose the fatty acids of the tung-oil obtained by cold pressing and those of the leaf lipids are also shown. Thus, two thirds of all fatty acids of the seed lipids are the octadecatrienoic fatty acid called α-eleostearic acid with conjugated double bounds and the Δ^9 *cis,* Δ^{11} *trans,* Δ^{13} *trans* configuration (Hopkins and Chisholm, 1962; Fang and Que, 1981; compare also Gunstone *et al.*, 19965, 1994). In addition to these unsaturated fatty acids common for storage fatty acids, such as linoleic acid with 19% and oleic acid with 9% occur. Saturated fatty acids such as palmitic acid, stearic acid and myristiic acid taken together make up for only 8%. As the gas chromatogram shows, also traces of a hydroxy-fatty acid occur (Davis *et al.*, 1949). According to Fang and Que (1981), the α-eleostearic acid is supposed to make up for up to 80% of total fatty acids. The different concentration given in the present paper is probably due to a different maturity stage of the seeds, since Fang and Que (1981) have shown that this acid increases in the seeds with increasing maturity, reaching a maximum in mature seeds. Finally, an extraction of seed lipids with different organic

Table I. Composition of seed lipids of *Aleurites montana* given as percent total lipids.

Total lipids	36.7–40.5% of seed cores*
Triglycerides	97.2
Phospholipids	
Cardiolipin	0.09
Phosphatidylethanolamine	0.13
Phosphatidylglycerol	0.68
Phosphatidylcholine	0.50
Phosphatidylinositol	0.56
Glycolipids	
Monogalactosyldiglyceride	0.41
Digalactosyldiglyceride	0.11
Sulfoquinovosyldiglyceride	0.28
Sterylglycoside	0.04

* Determination of total lipids of the seed cores yielded in three determinations a deviation width of 36.7–40.5% (an average of 38.7%) of dry matter. Phospholipids and glycolipids are averages of 8–10 individual determinations which deviate from the given mean by maximally ± 3%.

Table II. Fatty acid composition of seed lipids of *Aleurites montana,* the tung-oil and for comparison purpose the fatty acid composition of leaves of *Aleurites montana.*

Fatty acids	Leaves	Seeds of *Aleurites montana*	Tung-oil	Seeds of *Centranthus ruber*
$C_{12:0}$	0.2	-	-	-
$C_{14:0}$	1.0	0.1	0.1	0.1
$C_{16:0}$	11.9	6.3	7.8	6.3
$C_{16:1\ cis}$	0.5	-	-	0.3
$C_{16:1\ trans}$	1.0	-	-	-
$C_{16:2}$	1.1	-	-	-
$C_{16:3}$	2.3	-	0.7	-
$C_{18:0}$	2.0	1.7	3.4	3.0
$C_{18:1}$	2.2	9.0	9.0	6.0
$C_{18:2}$	8.2	18.7	24.7	50.8
$C_{18:3}$	68.7	-	0.5	1.6
$C_{18:3}$ (α-Eleostearic acid)	-	61.2	47.2	26.4
$C_{20:0}$	0.9	1.0	0.6	1.0
unknown	-	2.0	6.0	4.5
Saturated fatty acids	16.0	9.1	11.9	10.4
Unsaturated fatty acids	84.0	90.9	88.1	89.6
C_{16}-Fatty acids	16.8	6.3	8.5	6.6
C_{18}-Fatty acids	81.1	90.6	84.8	87.8

Fatty acids are given in percent total fatty acids. Seed lipids were extracted with boiling methanol and chloroform. Tung-oil comes from China and was obtained by cold pressing of seeds of *Aleurites montana.* Fatty acids of leaf lipids are described in earlier work (He *et al.*, 1996b). Fatty acid determinations are averages of 8–10 individual determinations. The given averages deviated by maximally ± 3%.

solvents also leads to a certain selection of triglycerides of differing fatty acid composition. In the analyzed tung-oil from China this acid makes up for only 47%, containing on the other side 25% linoleic and 5% of a yet unknown fatty acid. This unknown fatty acid exhibits a somewhat longer retention time than α-eleostearic acid and yields stearic acid after hydration.

α-Eleostearic acid is very labile and is only obtained by mild alcaline hydrolysis of the triglycerides with a 0.4 N KOH methanolic solution. Splitting of the triglycerides and transesterification of the fatty acids with 5% methanolic HCl yields up to 7 isomeres, which in the gas chromatographic analysis yield shorter or longer retention times in comparison to α-eleostearic acid. Hydration of these isomers with platin oxide as catalysator yields stearic acid.

A position analysis by enzymic degradation with lipases from pig pancreas and lipases from *Rhizopus arrhizus* shows that α-eleostearic acid is bound in the 1,3 position in the triglyceride. In leaf lipids which do not contain triglycerides and which are essentially composed of glycolipids, phospholipids and waxes, this acid does not occur (He *et al.*, 1996b, 1997). In glycolipids and phospholipids from leaf lipids only the trienoic fatty acid linolenic acid occurs.

α-Eleostearic acid occurs in concentrations of 17–72% of total fatty acids in seeds of a number of higher plants of different taxonomic position (Table III). Thus, the acid occurs in *Euphorbiaceae* besides *Aleurites* also in *Garcia nutans* and in *Ricinodendron* and furthermore also in representatives of *Rosaceae*, of *Valerianaceae* and of the *Cucurbitaceae*. In seeds of *Centranthus ruber* (*Valerianaceae*) which contain only 32% lipids, α-eleostearic as shown by our analyses, makes up for 25% of total fatty acids (Hopkins and Chisholm, 1962).

Due to its excellent properties of polymerisation and oxidation, which are to be attributed to α-eleostearic acid and linoleic acid, the tung-oil is an important primary product of the Chinese laquer industry. On the other hand, due to its content of these acids, the oil is not consumable for human and animal nutrition. This view is not scientifically verified. It is rather assumed that the indigestibility of this oil is caused by a toxic protein (Mann, 1954). It is clear that multiply unsaturated fatty acids such as linoleic, linolenic, arachidonic and eicosatrienoic acid all with *cis*-configuration are essential fatty acids with vitamin character, whereas fatty acids with the same chain lengths but *trans*-configuration are not equally valuable. Deposits of *trans*-fatty acids, taken up with food, have been detected in storage- and organ fat of man and animals (summarized by Kaufmann and Mankel (1964)).

The isolation of α-eleostearic acid from tung-oil is not only possible by gas chromatography and high pressure liquid chromatography (HPLC) but

Table III. Distribution of α-eleostearic acid ($C_{18:3}$, Δ^9 *cis*, Δ^{11} *trans*, Δ^{13} *trans*) in seeds of different plants.

Plants	Concentration %	References
Aleurites montana (Euphorbiaceae)	47–61	Gunstone *et al.* (1994)
Aleurites fortidii	47–61	Gunstone *et al.* (1994)
Aleurites trisperma	39	Hopkins *et al.* (1969)
Garcia nutans (Euphorbiaceae)	72	Hopkins *et al.* (1969)
Ricinodendron rautanenii (Euphorbiaceae)	24	Chisholm and Hopkins, 1966)
Chrysobalanus icaco (Rosaceae)	22	Kaufmann und Sud (1960)
Licania rigida (Rosaceae)	17	Badami and Patil (1981)
Parinarium annamense (Rosaceae)	43	Badami and Patil (1981)
Parinarium insularium (Rosaceae)	64	Chisholm and Hopkins (1966)
Parinarium laurinum (Rosaceae)	30	Badami and Patil (1981)
Parinarium macrophyllum (Rosaceae)	23	Badami and Patil (1981)
Cotia chestnut (Rosaceae)	24	Hopkins and Chisholm (1962)
Prunus yeodensis (Rosaceae)	35	Chisholm and Hopkins (1966)
Prunus mahaleb (Rosaceae)		Chisholm and Hopkins (1966)
Centranthus ruber(Valerianaceae)	43	Hopkins and Chisholm (1962)
Centranthus macrosiphon (Valerianaceae)	50	Hopkins and Chisholm (1962)
Momordica charantia L. *(Cucurbizaceae)*	46–55	Verma and Aggarwal (1956)

also by mercury-II-acetate adduct formation according to the procedure of Jantzen and Andreas (1959, 1960) followed by chromatography of these adducts over silica gel. In a single separation step saturated fatty acids could be separated from monoenoic, dienoic and polyenoic fatty acids. The regeneration of these adducts to fatty acids with 15% HCl in methanol resulted in formation of 7 to 9 isomers of this labile α-eleostearic acid, exhibiting either shorter or longer retention times than α-eleostearic acid itself. Hydration of these isomers again led to stearic acid. By means of this method of adduct formation and chromatographic separation it is possible to separate from the tung-oil this acid indigestible for humans, thus obtaining 30% of the fatty acids, which are composed by one half of the essential linoleic, by 1/4 of oleic and by 1/4 of the saturated palmitic, stearic and myristiic acid.

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