The occurrence of polyprenols in seeds and leaves of woody plants

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The contents of the heterogenous group of polyisoprenoids was found about two orders of magnitude lower in seeds than the amount of polyprenols and/or their carboxylic esters accumulated during vegetation season in leaves. In contrast to leaves, no seeds were found containing more than 0.5 mg of these lipids per gram of dry tissue. Almost 50% had less than 0.01 mg/g — the amount which is the limit of detection by the procedure used in this work. In gymnosperms (10 representatives of *Cupressaceae, Pinaceae* and *Taxaceae*) the polyprenol spectra in seeds and in needles were similar. In angiosperms (25 representatives of 13 botanical families) the polyisoprenoid mixture in seeds resembled the minor, additional subfamily found in leaves.

Terpenoids (syn., isoprenoids), biosynthesised from isopentenyl pyrophosphate are the largest group of natural compounds. The number of known structures, doubling every decade, could soon reach 10^5. Among them, polyisoprenoids i.e. uncyclized = linear polymers of the C_5 unit have been very often found in the examined tissues.

The occurrence of long chain poly-*cis*-prenols accumulating in leaves in the majority of the hundreds plant species analysed has been well documented (Swieżewska *et al.*, 1994; Jankowski *et al.*, 1994). Numerous species of *Spermatophyta* belonging to: *Asetraceae, Euphorbiaceae, Laureaceae, Magnoliaceae, Moraceae, Pinaceae, Rosaceae, Sapindaceae* and *Tiliaceae* accumulate in leaves family(ies) of polyprenols, of chain length C_{35-500}, in amounts approaching approx.i 5% of dry weight. However, in other plant organs the amounts of polyprenols were found to be rather low, with exception of wood of *Betula*

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Abbreviation: TLC, thin-layer chromatography.

verrucosa (Lindgren, 1965), and it seems that only in photosynthetic tissues distinct accumulation of polyprenols and perhaps their biosynthesis takes place. Recent isolation of new polyisoprenoid structures differing from the previously known ones in the number and configuration of the C=C double bonds indicates considerable heterogeneity of these lipids, obvious in extracts from leaves of plants containing them in moderate amounts (Suga *et al.*, 1989). As shown in the case of the all-*trans*-rubber-producing *Eucommia ulmoides* various plant organs (leaves, roots, seed coat) exhibit structural diversity of polyprenoids (Bamba *et al.*, 2001), i.e. both poly-*cis*- and poly-*trans*-prenols of different chain length were observed.

Polyprenols
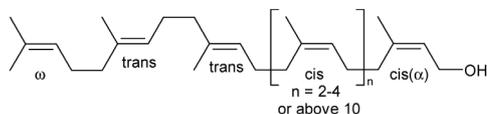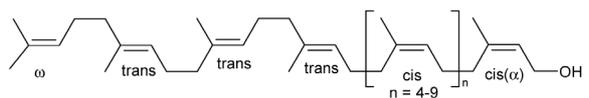
Dolichols
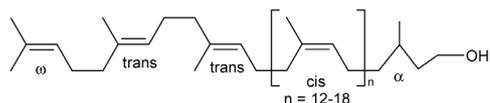
Scheme 1. Structures of polyprenols and dolichols.

The role of phoshopolyisoprenoids as universal coenzymes and carriers of sugar residues for glycosylation in a variety of biosynthetic reactions is well documented (Hemming, 1992). However, only recently the role of the dominating free polyisoprenols and their carboxylic esters as components of the antioxidant machinery located in the cell membranes has been uncovered (Bergamini *et al.*, 2004). The protective role of polyisoprenoids against reactive oxygen and nitrogen species has induced interest of pharmaceutical industry with these lipids. There-

fore, finding a source of easily obtainable polyisoprenoids of high purity should be of both scientific and economic interest. Ravi *et al.* (1984) have shown for a limited number of dicotyledonous species that seeds contained low amounts of dihydropolyprenols (dolichols), and mixtures of equal amounts of polyprenols and dolichols were found in seeds of monocotyledons. To extend those data we compared the content and the type of polyisoprenoids in seeds of other easily available species of large evolutionary distance, known to accumulate polyprenols in leaves. The spectra of polyprenols from seeds and needles of gymnosperms were found to be similar whereas a complex mixture of polyisoprenoids was found in the seeds of angiosperms. The mixture consisted of a family of polyprenols resembling the minor, additional subfamily of polyprenols found in leaves and of a family of dolichols. The range of chain lengths of those polyprenols and dolichols was similar.

METHODS

Materials. Leaves of angiosperm plants were collected in September 1996 in the Arboretum of the Institute of Dendrology, Polish Academy of Sciences in Kórnik, and in the Botanical Garden of the Polish Academy of Sciences in Powsin. Seeds were from the Arboretum of the Institute of Dendrology in Kórnik; most of them were collected in 1996. In a few cases they were from earlier collections. Chromatographic materials were from Merck, Darmstadt (Germany); all solvents and other chemicals were of analytical grade from POCh Gliwice (Poland).

Preparation of samples for analysis. Leaves harvested to paper envelopes were immediately chilled, then put unfrozen into lyophilization jar within a few hours after harvesting and dehydrated in vacuum for 36 h. Seeds were dehydrated without the external parts. From the lyophilized material 1 g samples were taken for homogenization in 10 ml

of hexane using a Janke & Kunkel Ultra turrax T25 homogenizer at 24 000 rev/min. for 2 min. The homogenates were transferred into 0.8 × 6 cm Pasteur pipettes clogged with a piece of glass wool. After filtering the solvent the residue was leached with 10 ml of acetone and finally with 10 ml of chloroform/methanol, 2:1 (v/v) mixture.

Study of lipid substances. The three extracts and the lipids fractions were analysed by thin-layer chromatography (TLC) on Kiesel gel plates in solvent A (hexane/toluene, 2:1, v/v), solvent B (ethyl acetate/toluene, 1:19, v/v) and solvent C (chloroform/methanol/water, 65:25:4, by vol.). For checking the type of polyprenols RP-18 plates and acetone/methanol, 9:1, v/v (solvent D) were used.

The presence of spots of polyisoprenoids was revealed with the aid of anisaldehyde spray reagent (5% anisaldehyde and 5% H_2SO_4 in ethanol) and subsequent heating at 100°C. The characteristic greenish-gray colour of a spot is a useful indicator of the presence of a polyisoprenoid on the plate. For detecting polyprenols on RP-18 plates the spots were visualized with iodine vapours and, after evaporation of iodine, with the anisaldehyde spray.

The quantities of polyprenols and their esters were measured by three methods:

◆ **1**. Comparison with the area of two spots similar in size of increasing amounts of cochromatographed standards — the neighbour points on both sides within the calibration curve.

◆ **2**. Measuring the weight (with the accuracy 0.1 mg or 0.001 mg) of purified polyisoprenoid preparations kept previously overnight under vacuum in an exicator over granulated paraffin wax applied as a trap for residual hydrophobic solvents.

◆ **3**. Comparison of the area (calculated with the use of software Maxima 820–Waters, U.S.A.) of chromatographic peaks of isoprenologues separated on reversed phase HPLC column with the area of the peak of an internal standard — isoprenologue of different chain length therefore different elution time. The discrepancies between method 2 and 3 were below 25%.

Saponification of lipids was performed according to Stone *et al.* (1967) by treating the lipid sample isolated from 1 g of material dissolved in 1 ml of benzene with 1 ml of 1 M KOH in 85% aqueous ethanol for 1 h at 90°C. The unsaponifiable lipids were isolated by three subsequent extraction of the hydrolysate diluted with 1 ml of water with 3 ml of ethyl ether, 3 times. The combined ether extract was washed with 5 ml of water. After evaporating the solvent with stream of N_2 the unsaponifiable lipids were dissolved in 0.3 ml of hexane and separated on a Lichroprep Si60 column packed into 0.8 × 6 cm Pasteur pipettes clogged with a piece of glass wool.

The lipid sample was applied in hexane and the column eluted with two 5 ml portions of 5% ethyl ether in hexane (fractions 1 and 2) and with two 5 ml portions of 12% ethyl ether in hexane (fractions 3 and 4). The presence of polyprenols in the four fractions was checked by TLC (solvents A and B) and the fractions containing free polyisoprenoid alcohols were taken for further analysis.

HPLC of polyisoprenoid alcohols was performed on a Nucleosil 120-C18, 5 μm column 4.6 × 250 mm using a Waters apparatus and a linear gradient from 20% to 50% of a mixture of hexane/isopropanol — 7:3 in a mixture of methanol/isopropanol/water (12:8:1, by vol.).

Polyisoprenoid standards serving as reference substance came from the local „Collection of Polyprenols" (Institute of Biochemistry and Biophysics, PAS, Warszawa, Poland). The other test lipid substances used (fatty acids, triglyceride and phospholipid mixtures) were prepared from sunflower oil or were from Larodan Fine Chemicals (AB, Malmo, Sweden).
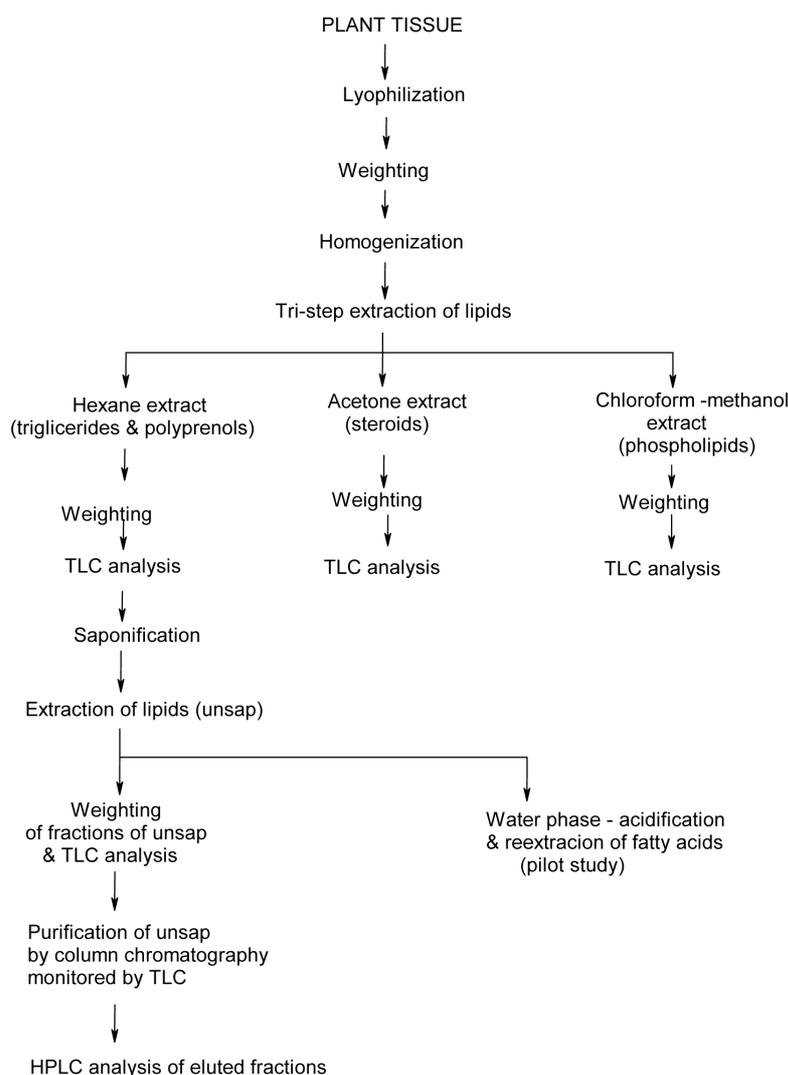
RESULTS

The lipid substances in seeds of gymnosperms

Free polyisoprenoid alcohols and their carboxylic esters were present exclusively in the first-hexane extract, but they could be observed by TLC only after chromatography on silica (the penultimate step on Scheme 2). The contents of substances present in the acetone and chloroform-methanol fractions were estimated gravimetrically and the lipids were investigated only summarily by TLC. In the acetone extracts from oily seeds e.g. *P. cembra* some triglycerides could be observed whereas the largest amounts of steroids are present in the extract from *T. orientalis*. The main components of the chloroform-methanol extract were phospholipids: phosphatidylcholine approx. 50%, phosphatidylserine approx. 10%, phosphatidylethanolamine approx. 10% and cardiolipine approx. 5%, (TLC on silica gel, solvent C).

In Table 1 are shown the amounts of lipids from seeds of ten species of gymnosperms extracted according to Scheme 2. Most of the seed lipids were extracted with hexane and after removal of the dominating triglycerides (after alkaline hydrolysis) gave the fraction of unsaponifiable lipids. The last column of Table 1 shows the amount of polyprenols present in fraction 3 (eluted with 12% ethyl eter) from the silica gel column, estimated by com-

PLANT TISSUE
↓
Lyophilization
↓
Weighting
↓
Homogenization
↓
Tri-step extraction of lipids

Hexane extract
(triglicerides & polyprenols)

Acetone extract
(steroids)

Chloroform -methanol
extract
(phospholipids)

Weighting

Weighting

Weighting

TLC analysis

TLC analysis

TLC analysis

Saponification
↓
Extraction of lipids (unsap)

Weighting
of fractions of unsap
& TLC analysis

Water phase - acidification
& reextracion of fatty acids
(pilot study)

Purification of unsap
by column chromatography
monitored by TLC
↓
HPLC analysis of eluted fractions

Scheme 2. Analytical procedure applied to the seeds.

unsap., unsaponifiable lipids.

paring the intensity and size of the TLC spot with that obtained for a known amount of pure pentadecaprenol cochromatographed on the same silica gel TLC plate, solvent system B, detection with anisaldhyde reagent (Stone *et al.*, 1967).

dles are up to 100 times higher. Although there is some similarity in the spectra of *T. nucifera* to that of *T. baccata* (Fig. 1g), both *Taxaceae*, apparently the most complex spectrum of polyisoprenoids was observed in extracts from *T. baccata* where the first family

Table 1. The amounts (% of dry weight) of lipid substances extracted from seeds of gymnosperms

Species	Hexane[a]	Acetone[a]	Chl–Met[a]	Total[a]	UNSAP[a]	Isoprenoids[b]
Abies alba	24.66	6.61	2.02	33.29	2.251	Not detect
Cedrus atlantica	35.19	3.86	1.75	40.80	5.110	Not detect
Larix decidua	7.00	0.69	1.92	9.61	0.368	0.001
Picea abies	28.04	1.38	1.50	30.92	0.364	0.010
Pinus cembra	53.85	1.61	3.27	58.73	0.337	0.012
Pinus nigra	26.11	2.73	1.56	30.40	0.437	0.014
Pinus silvestris	27.33	1.28	1.94	30.55	0.252	0.015
Taxus baccata	25.81	4.63	0.78	31.22	0.117	0.003
Thuja orientalis	11.23	1.34	1.61	14.18	0.301	0.005
Torreya nucifera	45.72	1.49	2.05	49.26	0.331	0.055

[a]The content of lipids in: hexane, acetone, chloroform/methanol (2:1, v/v), extracts and unsaponifiable lipid (UNSAP) fractions were estimated gravimetrically. [b]Contents of polyisoprenoids calculated from the area of chromatographic spot.

Seeds of *C. atlantica* and *A. alba* contain up to 20 times more unsaponifiable lipids than seeds of other gymnosperm plants studied. Most of these substances migrate on silica gel TLC plates in solvent B as two distinctive spots (R_F = 0.42 and 0.31) slightly slower than polyisoprenoids (R_F = 0.48–0.54, solvent B) but the colour of their derivatives with anisaldehyde differs from that of polyisoprenoids or cholesterol and β-sitosterol (R_F = 0.27). These interesting lipids dominate the unsaponifiable lipids and they interfere with their isolation and identification (Wellburn & Hemming, 1966).

In Table 2 and Fig. 1 are shown polyisoprenoid spectra in seeds and needles of gymnosperms. The spectra of polyisoprenoids in seeds of all three *Pinus* species are the same and in the seeds and needles of *Pinaceae* are very similar and simple (C_{80}-prenoid dominates, Fig. 1f) although their contents in needles are up to 100 times higher.

with C_{75} dominant overlaps the second where C_{80} and C_{110} polyisoprenoids dominate. Seeds of *T. nucifera* (Fig. 1i, Fig. 2 lower line) contain several times more polyisoprenoids than other examined seeds, however, it is only approx. 5% of the amounts present in needles (Fig. 2 upper line). The mixture of polyisoprenoids from seeds of *T. orientalis* (*Cupressaceae)* is jointly dominated by both α-un, and α-saturated types of polyisoprenoids with chain length C_{85} and C_{90} (Fig. 1h). Apart from the presented taxonomic similarities the occurrence of both types of polyisoprenoids in the seed extracts is indicated by unsymmetrical (*P. nigra* and *T. nucifera,* Figs. 1f and 1i) or split (*T. baccata* and *T. orientalis,* Figs. 1g and 1h) peaks on the chromatograms. The ratio of lipids migrating like dolichols to those which behave like polyprenols is different: *P. abies* and *P. nigra* = 0.2, *T. nucifera* <0.1, *T. orientalis* = 2.2.

Table 2. Comparison of polyisoprenoids in seeds and needles of gymnosperms

Species	Prenologues (number of isopren units)[a, b]	Contents (% of dry weight)[c]
Larix decidua		
Seeds	15, **16**, 17, 18	0.001
Needles	14, 15, **16**, 17, 18, 19	0.11
Picea abies		
Seeds	14, 15, **16**, 17, 18	0.010
Needles	14, **15**, **16**, 17, 18	0.55
Pinus nigra		
Seeds	12, 13, 14, 15, **16**, 17, 18, 19	0.010
Needles	13, 14, 15, **16**, 17, 18,	0.22
Taxus baccata		
Seeds	12, 13, 14, **15**, 16, 17, **18**, 19, 20, 21, **22**, 23, 24, 25	0.003
Needles	16, 17, **18**, 19, 20, 21, **22**, 23, 24	0.37
Thuja orientalis		
Seeds	15, 16, **17**, **18**, 19, 20	0.005
Needles	14, 15, **16**, **17**, 18, 19, 20, 21, 22, 23	0.21
Torreya nucifera		
Seeds	14, 15, 16, 17, **18**, 19, 20, 21, **22**, 23, 24, 25	0.055
Needles	14, 15, 16, **17**, **18**, 19, 20, 21, **22**, 23, 24	1.10

[a]The numbers refer to the isoprene units of polyisoprenoid alcohols. [b]Dominating isoprenologues are indicated in bold. [c]Contents calcutated from HPLC peak areas.

## The lipid substances in seeds of angiosperms

Table 3, similary to Table 1 shows the weights of the lipid extracts obtained according to Scheme 2 from seeds of angiosperms. Almost half of the analysed seeds contained, in 1 g sample, polyisoprenoids in amounts to small to be identified and estimated during the applied procedure.

Representative HPLC spectra of polyisoprenoids isolated from seeds and leaves of five angiosperms according to Scheme 2, are presented in Table 4 and Fig. 1. Due to the low amount of polyisoprenoids only the main dominating component of the mixture could be recognised as a polyprenol or dolichol. The seeds contain at least 2 times more dolichols than polyprenols. The ratio of lipids migrating like dolichols to those which behave like polyprenols is for *A. rubrum* = 1.8 (Fig. 1c), *H. rhamnoides* = 2.7 (Fig. 1e), *F. silvatica* = 2.9 (Fig. 1d), *T. cordata* = 4.3 (Fig. 1b), *Q. rubra* = 5.1 (Fig. 1a).

DISCUSSION

The presented results are in support of the universal occurrence of small amounts of a complex mixture of polyisoprenoids in seeds. In spite of the accumulation of considerable amounts of polyprenols being common in plants it was found that dolichyl- and not polyprenyl-phosphates function as sugar lipid carriers in glycoprotein biosynthesis in plants (Elbein, 1979). At the same time it was noticed that occasionally, preparations of C_{85-105} dolichols extracted from mammalian organs contain also up to 20% of C_{55} dolichol or C_{50} polyprenol, i.e. unusually short chain length polyisoprenoids for animal tissues (Mańkowski *et al.*, 1976; Radomińska-Pyrek *et al.*, 1979).
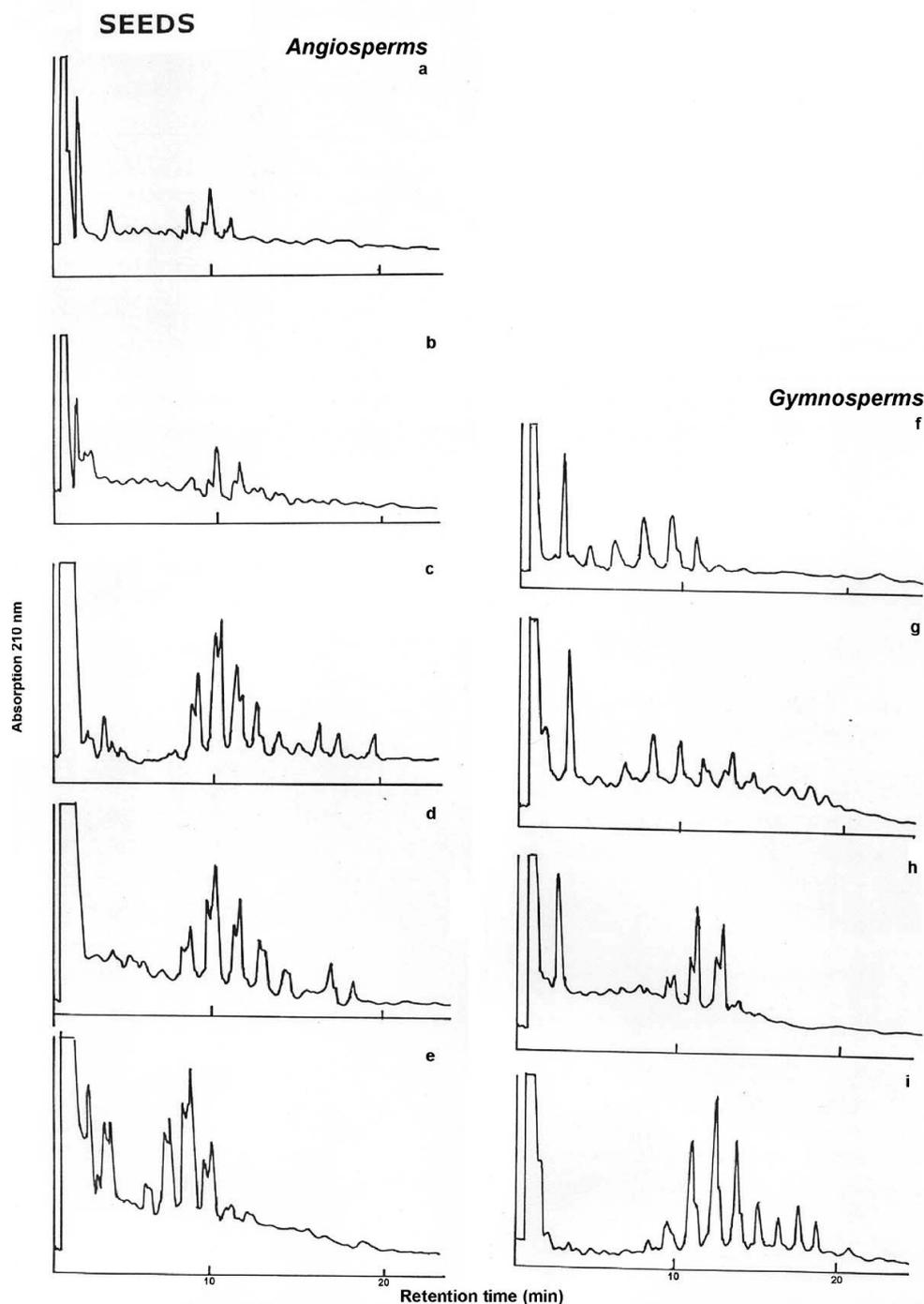
Figure 1. Polyisoprenoid spectra from seeds.

a, *Q. rubra*; b, *T. cordata*; c, *A. rubrum*; e, *H. rhamnoides*; f, *P. nigra*; g, *T. baccata*; h, *T. orientalis*; i, *T. nucifera*.

Recently, multiple polyisoprenoids of different structure are observed in individual plant tissues (Rezanka & Votruba, 2001; Bamba *et al.*, 2001; Skorupińska-Tudek *et al.*, 2003; Bamba *et. al.*, 2004) thanks to the application of rapidly developing research techniques es-

pecially high performance liquid and super-critical fluid chromatography combined with mass spectrometry. Their use allows for more exact physical separation of polyisoprenoids or for estimation of the content of different polyisoprenoids present in the same chro-

Table 3. The amounts (% of dry weight) of lipid substances extracted from seeds of angiosperms

Species	Hexane [a]	Acetone [a]	Chl-Met [a]	Total [a]	UNSAP [a]	Isoprenoids [b]
Acer circinatum	10.14	2.43	2.30	14.87	0.36	0.003
Acer ginnala	22.09	12.14	2.39	36.62	0.52	0.025
Acer pseudoplatanum	9.79	1.21	1.76	12.76	0.43	0.003
Acer rubrum	15.00	11.84	1.96	28.80	0.38	0.002
Acer saccharinum	2.51	1.80	1.80	6.11	0.15	Not detect
Carpinus betulus	4.74	0.69	1.13	6.56	0.24	Not detect
Corylus colurna	55.42	0.78	1.88	58.08	0.37	Not detect
Cotonoaster horizontalis	3.22	1.31	0.90	5.43	0.22	Not detect
Cotonoaster multiflora	4.11	0.93	1.12	6.16	0.18	Not detect
Crategus submollis	2.34	0.60	1.04	3.98	0.16	Not detect
Fagus silvatica	26.54	1.11	1.28	28.93	0.21	0.003
Hippophe rhamnoides	9.73	4.07	1.59	15.39	0.34	0.003
Laburnum anagyroides	7.23	1.16	3.16	11.55	0.18	0.013
Prunus avium	36.28	1.10	2.74	40.12	0.31	0.007
Quercus rubra	14.12	9.24	3.16	26.52	0.29	0.006
Rosa canina	9.20	0.89	1.72	11.81	0.27	Not detect
Sambucus nigra	30.16	0.64	1.21	32.01	0.34	Not detect
Shepherdia argentea	9.83	1.65	2.23	13.71	0.38	Not detect
Staphylea trifoliata	21.99	0.54	1.54	24.07	0.28	0.003
Symphoricarpos albus	20.20	0.54	1.28	22.02	0.33	Not detect
Tilia cordata	25.47	0.60	1.84	27.91	0.27	0.002
Ulmus glabra	36.36	0.84	2.50	39.70	0.40	0.005
Ulmus laevis	33.53	0.56	2.86	36.95	0.35	0.005
Viburnum lantana	12.93	3.32	5.04	21.29	0.56	Not detect
Viburnum opulus	10.93	5.53	6.00	22.46	0.55	Not detect

[a]The content of lipids in: hexane, acetone, chloroform/methanol (2 :1, v/v) extracts and unsaponifiable lipid (UNSAP) fractions were estimated gravimetrically. [b] Contents of polyisoprenoids calculated from the area of chromatographic spot.

matographic peak. The results obtained by the application of these methods generally confirm the universal occurrence of heterogeneity of these lipids encountered repeatedly in the past (Mankowski *et al.,* 1976; Sasak *et al.,* 1977) and strongly support a demand for the elucidation of the physiological meaning of this phenomenon. One may speculate that, the presence of different polyisoprenoids should be a result of their specific cellular functions. However, except dolichyl phosphate necessary for functioning in complex biosynthesis of oligosaccharide moiety or glycosylphosphatidylinositol anchors of glycoproteins, common in all eukariots, no function has been attributed and confirmed to other polyisoprenoids.

To answer the question of specificity of metabolic processes utilizing polyisoprenoids several approaches have been used. A wide range

Table 4. Comparison of polyisoprenoids in seeds and leaves of angiosperms

Species	Prenologues (no of isopren units)[a, b]	Content (% of dry weight)[c]
Acer rubrum		
Seeds	16,**17**,18,19,20	0.003
Leaves	10,11,**12**,13	0.2
Hippophae rhamnoides		
Seeds	15,16, **17,**18,19	0.003
Leaves	10,**11**,12 15,16, 17,18,**19**,20,21,22,	0.1
Fagus silvatica		
Seeds	15,16, **17**,18,19	0.003
Leaves	**10**,11 14,15,**16**,17,18,19,20,21,22,23	0.2
Quercus rubra		
Seeds	16,**17**,18,19	0.006
Leaves	10,**11**,15,16,**17**,18, 19, 20	0.2
Tilia cordata		
Seeds	16,**17**,18,19,20	0.003
Leaves	**10,11**,12 16,17,18,19,**20**,21,22,23...30	0.1

[a]The numbers refer to the isoprene units of polyisoprenoid alcohols. [b]Dominating isoprenologues are indicated in bold. [c] Contents calcutated from HPLC peak areas.

of specificity was found, from absolute — only one substrate (with acceptance of the same polyisoprenoid structures with different chain length) to preference of the "wrong" lipid when the enzymes were assayed *in vitro* towards different polyisoprenoid substrates, Jankowski *et al.* (1989), Krag (1998). However, recent identification (Gao *et al.*, 2004) of multienzymatic complexes utilizing dolichyl phosphate, located in the intact yeast endoplasmatic reticulum, suggest their increased catalytic specificity. Although cells grown *in vitro* efficiently take up and utilise radiolabelled polyisoprenoids or their precursors supplied into the medium, even investigation with the use of grown *in vitro* mutant cells de-
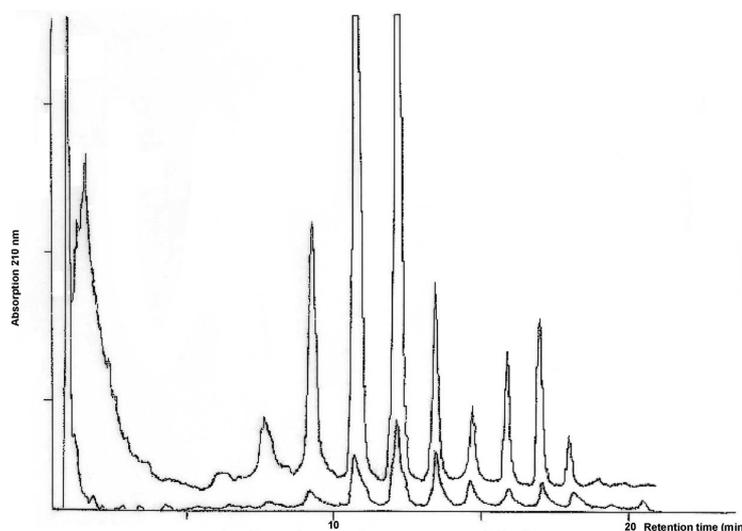
Figure 2. Polyisoprenoid spectra of leaves (upper) and seeds (lower) from *T. nucifera*.

fective in the biosynthesis of dolichol from polyprenol does not allow an unequivocal confirmation of utilization of polyprenyl phosphate in the cellular glycoprotein synthesis (Jankowski, 1987; Krag, 1998). Also, the postulated role of dolichol as a cellular substrate for the biosynthesis of dolichyl phosphate in germinating soybean in the presence of high activity of dolichyl phosphate phosphatase is not easy to rationalize (Ravi *et al.*, 1986).

In yeast, the content and polyisoprenoid spectrum depend on the phase of growth and/or starving condition. Induction by stress of a synthesis of the second family of longer dolichols could be a necessary cellular mechanism for the adaptation to new conditions of cultivation (Sato *et al.*, 2001; Szkopinska *et al.*, 2002).

In higher plants, biosynthesis of polyisoprenoids is a very complex system of connections between different cellular compartments and metabolic pathways (Rohmer, 1999). Therefore, the multiple families of polyisoprenoids encountered in plant tissues could be a product of different biosynthetic pathways simultaneously or sequentially active in different conditions of vegetation.

When both types of isoprenoids are present in the extract in appreciable amounts it is possible to separate them by the careful use of simple chromatographic separation methods (Jankowski & Chojnacki, 1991). However, if a complex mixture of microgram quantities of polyisoprenoids is extracted from the tissue together with large amounts of lipids with similar properties it is necessary to upscale the procedure and to use disposable analytical columns in the multistep purification before the final analysis allowing their separation and estimation of the content of individual lipid (Bamba *et al.*, 2001). Due to the low content of polyisoprenoids in seeds their use as a taksonomic marker is of limited value especially that unequivocal identification of particular member of polyisoprenoid spectrum requires very precise work, disposable vessels and costly instruments.

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