**Analysis of plant polyisoprenoids**

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**Summary**

Polyisoprenoid alcohols are representatives of high-molecular terpenoids. Their hydrocarbon chains are built of 5-100 and more isoprene units giving rise to polymer molecules that differ in chain-length and/or geometrical configuration. Several structural variants of polyisoprenoids have been found in nature. Following the hydrogenation status of the α-terminal isoprene residue, polyisoprenoid alcohols are subdivided into polyprenols (α-unsaturated) and dolichols (α-dihydro-polyprenols, i.e. α-saturated counterparts). Polyprenols are common constituents of plant photosynthetic tissues, while dolichols are found in yeast and animal cells and in root tissue.

Polyisoprenoid alcohols are synthesized in all living cells and their accumulation is significantly increased with the age of the tissue. The biological function of polyisoprenoid phosphates is well established (e.g. cofactors of protein glycosylation and biosynthesis of GPI-anchor) while polyisoprenoid alcohols are postulated to act as modulators of biomembrane properties. They have also been found to participate in cellular response to environmental stress.

In this chapter methods of analysis of polyisoprenoid alcohols in various plant materials are described.

keywords: polyprenol; dolichol; TLC: HPLC

**1. Introduction**

Polyisoprenoid alcohols are highly hydrophobic isoprenoid products synthesized in all living cells. Despite their regular structure comprised of numerous (5 > n > 100 and more) isoprene units, several structural variants have been described *(1)*. Firstly, hydrogenation status of the α-terminal double bond in the molecule decides whether a polyisoprenoid alcohol belongs to the subgroup of polyprenols (α-unsaturated) or dolichols (α-saturated) type. Polyprenols are considered as typical components of plant photosynthetic tissues and bacterial cells while dolichols are found in plant roots and all animal and yeast cells *(1)*. Although considered as unique in nature, ω-saturated polyprenols have also been noted in the leaves of some plant species *(2)*.

Secondly, the diversity of the chain-length resulting from the number of isoprene units polymerized in the molecule of natural polyisoprenoid alcohols which might be a few and reach more than one hundred. Interestingly, this variation is much more highly pronounced in the case of plant polyprenols than mammalian dolichols *(1)*. In all eukaryotic cells polyisoprenoids are always accumulated as mixture of homologues commonly called a ‘family’ with one component being most abundant and a Gaussian-like distribution of homologues. Interestingly, in some plant tissues a two- or even three-family pattern of polyisoprenoid alcohols has been noted *(3)*.

Thirdly, the occurrence of the double bond in the isoprene units gives rise to geometrical isomers *trans* (*E*) or *cis* (*Z*). The most typical polyisoprenoid alcohols are either of the di-*trans* (ω-t2-cn-3) or the tri-*trans* (ω-t3-cn-3) type, however all-*trans* (ω-tn-1) and α-*trans* (ω-t3-cn-4-t) variants have also been reported *(4)*.

Thus, due to the natural heterogeneity, polyisoprenoids with their modest molecules composed of the repetitive, head-to-tail condensed, isoprene units require attention during the procedure of isolation and quantitative analysis.

Biosynthesis of the polyisoprenoid hydrocarbon skeleton is achieved due to the combined activity of a short-chain *trans*-prenyltransferase (usually farnesyl diphosphate synthase, in some plant species geranylgeranyl diphosphate synthase) providing a precursor for a *cis*-prenyl transferase (CPT) which elongates the isoprenoid chain until the polyprenyl diphosphate of a desired chain length is produced - summarized in *(5)*. Subsequent dephosphorylation *(6)* of the polyprenyl diphosphate is, when a dolichol is to be formed, followed by hydrogenation of the α-terminal isoprene unit. This very last step is catalyzed by polyprenol reductase which has been recently discovered in humans *(7)*. It is also worth mentioning that plant dolichols are ‘mosaic’ isoprenoid compounds built from the isopentenyl diphosphate molecules derived from both the methylerythritol phosphate (MEP) and mevalonate (MVA) pathways *(8)*.

The vital biological function of dolichyl phosphate found in a minute amount in eukaryotic cells comes from its involvement as a cofactor in the biosynthesis of *N*-, *O*- and *C*-glycosylated and GPI-anchored proteins. In bacterial cells polyprenyl phosphate fulfills this function and also serves as cofactor for the biosynthesis of peptidoglycan. Several excellent reviews summarize these data *(9-12)*.

The function of the cellular predominant chemical forms of polyisoprenoids, polyprenols and dolichols accumulated as free alcohols and carboxylic esters remains elusive. Since their accumulation is considerably increased in senescing eukaryotic cells and upon pathological and adverse environmental conditions *(13-15)* their role in cell defense against stress has been suggested. Moreover, polyisoprenoids being the structural components of the biological membranes affect their chemico-physical properties. Extrapolating the results of the biophysical experiments (phospho)polyisoprenoids have been suggested as membrane fluidizers, permeabilizers and vesicle fusion inducers *(16-18)*.

Additionally, the observed species-specific composition of the polyprenol mixture in plant photosynthetic tissues gives rise to the possibility of their exploitation as chemotaxonomic markers *(19,20)*.

As briefly summarized above, analyses of the structure and content on polyisoprenoids have been performed in various biological matrixes. In this chapter the reader will find a detailed description of the analytical method currently used in our laboratory to analyze plant polyisoprenoids. The procedure of polyisoprenoid analysis described below might be used for all types of tissues and the protocol has been developed for 3-week old Arabidopsis seedlings.

**2. Materials**

Use analytical or, when indicated, HPLC grade reagents and ultrapure water (MiliQ, prepared by purifying deionized water to attain a resistivity of 18 MΩ cm at 25°C)). Work inside the ventilation hood while using organic solvents. Use only glass laboratory equipment (tubes, pipettes, flasks, bottles, etc.), plastic materials must not be used. Tubes for storage of lipids and organic solvent solutions should be equipped with teflon seals. Carefully follow all waste disposal regulations when disposing of waste materials.

**Hydrolyzing mixture:**

Weigh 1.2 g NaOH and transfer to a screw-capped tube. Add 0.6 mL water (see **Note 1**) and vortex. Add 3.3 mL ethanol, 3.95 mL toluene. and 0.005 g pyrogallol (see **Note 2**).This amount is sufficient for 10 samples each of 3g fresh weight.

**HPLC mobile phases (see Note 3) :**

Solvent A: add 900 mL methanol to a 1 L screw-capped bottle. Add 100 mL water and mix carefully. Use HPLC grade methanol and MiliQ water.

Solvent B: add 500 mL methanol, 250 mL 2-propanol and 250 mL hexane to a 1 L screw-capped bottle and mix carefully. Use HPLC grade solvents.

**TLC solvents (see Note 4):**

S-TLC: mix 90 mL toluene with 10 mL ethyl acetate in a TLC tank.

RP-TLC: add a desired volume of acetone to a TLC tank.

**Silica gel for column chromatography:**

Load 40 g silica gel powder (Silica gel 60, 0.040-0.063 mm) into a 250 mL screw-capped bottle. Add 100 mL hexane and mix (see **Note 5**).

**Solvents for column chromatography:**

2% E/H: Add 98 mL hexane to a 100 mL screw-capped bottle. Add 2 mL diethyl ether and mix (see **Note 8**).

15% E/H: Add 170 mL hexane to a 250 mL screw-capped bottle. Add 30 mL diethyl ether and mix (see **Note 8**).

**Internal standard:**

Load 5 mg of Prenol-14 into a screw-capped tube. Add 5 mL hexane and mix carefully, store the solution at -20º C (see **Note 6**).

**Extraction solvent:**

C/M: add 100 mL methanol and 100 mL chloroform to a 250 mL bottle, mix. This amount is sufficient for 10 samples (3 g fresh weight each). Use methanol and chloroform of p.a. grade (see **Note 7**).

**3. Methods**

Carry out all the procedures at room temperature unless specified otherwise, all the manipulations with solvents should be performed inside the ventilation hood.

**Polyisoprenoid isolation:**

Cut fresh plant tissue into pieces with scissors, weigh approx. 3g and place in a homogenization screw-capped tube (see **Note 9**). Add 10 μL of the internal standard (see above) and 20 mL CM extraction solvent and homogenize the sample with a mechanical homogenizer (we use Ultra-Turrax homogenizer for approx. 5 min) (see **Note 10**). Put the sample into a dark place for 24h at ambient temperature, shake the tube occasionally (see **Note 11**). After that time add 5 mL chloroform and 2 mL water (see **Note 12**). Wait 15 min until phases are separated. Transfer the lower (chloroform) phase to the next screw-capped tube using a Pasteur pipette (see **Note 13**). Re-extract the tissue by adding 12 mL chloroform to a homogenization tube containing the upper methanol-water phase and vortex. Wait 15 min. until phases are separated. Collect the lower (chloroform) phase and pool both lower phases. and evaporate this crude extract to dryness (oily residue) under a stream of nitrogen or by using an evaporator (see **Note 14**).

**Hydrolysis:**

Add 0.8 mL of the hydrolyzing mixture to the sample (see **Note 15**). Close the tube tightly and incubate in a pre-warmed water bath at 97°C for 1h. Let the tube cool (10 - 15 min) and add 2 mL water and 2 mL hexane to the cooled hydrolyzed mixture and vortex carefully. Wait 10 min until the two phases are separated (see **Note 16**). Collect the top organic (hexane) phase and put into a new previously weighed tube. Re-extract the hydrolyzed mixture three times by adding 2 mL hexane each time and pool all four hexane phases. (see **Note 17**). Evaporate hexane under a stream of nitrogen. Dissolve the remaining (unsaponifiable) lipids in 0.4 mL hexane.

**Polyisoprenoid purification:**

Place three tubes in a rack (see **Note 18**). Into the first tube insert a standard glass Pasteur pipette plugged with a small piece of cotton wool (see **Note 19**). Fill this column with a prepared silica gel suspension up to the final column volume of 2.5 mL (see **Note 20**). Check whether the column is correctly packed (see **Note 21**). Wash the column with 3 mL hexane. Load unsaponifiable lipids on the top of the column (see **Note 22**). Rinse the sample tube three times with 0.4 mL hexane, load these solutions on the top of the column too. When the entire volume of hexane is down in the tube, rinse the column with an addional 1 mL of pure hexane. When almost all hexane is down in the tube transfer the column to a second tube. Elute the column with 6-8 mL of 2% E/H (see **Note 23**) and transfer the column to a third tube. Continue the elution using 14-15 mL of 15% E/H. Evaporate all three fractions under a stream of nitrogen and dissolve each remaining residual material in 0.2 mL hexane.

**Polyisoprenoid analysis:**

**TLC analysis:**

Use both the silica gel and RP HPTLC plates. Spot 5 μL of the sample from each tube on both TLC plates (see **Note 24**). Spot standards of polyprenols on a separate lane(s) beside the sample. Insert the silica gel and RP HPTLC plates into a tank with S-TLC and RP-TLC solvent, respectovely. After approx. 15 – 20 min. when the solvent front arrives close to the plate edge (approx. 0.5 cm below) take the plate out, mark the solvent front and dry inside the ventilation chamber (warm but not hot fan might be used). Insert dry TLC plates into the tank (TLC tank or other container with a lid) containing iodine crystals to stain the chromatograms (Fig.1).

**HPLC analysis:**

Transfer the three obtained fractions to a pre-weighed vial or screw-cap tube for HPLC analysis. Evaporate solvents under a stream of nitrogen, estimate the amount of lipids gravimetrically and dissolve in a calculated volume of 2-propanol (see **Note 25**) to obtain the final lipid concentration 3 mg per mL. Vortex the samples carefully and store in the refrigerator. Inject 50 μL of each sample to the HPLC, use a manual injector or autosampler.

Standards (quantitative - polyprenol and dolichol mixtures of known composition and qualitative - single polyprenol solution of known concentration) have to be injected at least once per each set of analyses.

The following HPLC method is routinely used in our laboratory:

column: RP-HPLC type, we use Zorbax Eclipse XDB-C18; 4.6 x 75 mm, 3.5 μm

flow rate: 1.5 mL/min

detection: UV set at 210 nm or diode array detector (DAD)

column temperature: 21ºC

gradient: a combination of linear gradients used is shown in Table 1.

After each run the HPLC system is equilibrated for 5 min. by elution with solvent A (see **Note 26**).

The obtained chromatogram is shown in Fig. 2.

**Result calculation:**

Compare the analyzed chromatograms (Fig.2) with that of qualitative standards, identify the signals corresponding to the retention time of the polyprenols of interest.

Integrate the signals in the chromatogram using HPLC software. Calculate the amount of polyisoprenoid according to the equation:

(g / g FW)

mx - amount of the analyzed compound (g)

ms - amount of the internal standard (g)

mt - amount of the tissue (g)

ls - number of double bonds in the internal standard molecule

lx - number of double bonds in the analyzed compound molecule

As - area of the integrated signal of the internal standard

Ax - area of the integrated signal of the analyzed compound

**4. Notes**

1. Add water to NaOH and vortex until a homogenous solution is obtained, then add ethanol, toluene and pyrogallol; upon addition of pyrogallol the solution changes color to brown. Use freshly prepared solution.
2. Prior to adding to the sample the hydrolyzing mixture has to be carefully vortexed until a well-mixed emulsion of solvents is formed, pyrogallol has to be well dissolved.
3. Solvents A and B should be carefully mixed until a homogenous solution is obtained. If necessary (if your HPLC system is not equipped with a degasser) remove air from the solvents e.g. bubble the solvents with helium for several minutes. If not used the HPLC solvent can be stored in a tightly capped bottles for 2-3 days preferably in the cold room. When used the solvents must be pre-warmed at room temperature.
4. Make sure the TLC tank is tightly covered with a lid and introduce S-TLC or RP-TLC solvent. Control the level of liquid – the sample origin on the TLC chromatogram should be above this level. TLC solvents may be eventually used for a few days, however, in order to preserve the composition of the mixture evaporation should be avoided.
5. Prepare a gel suspension a day before the chromatography is planned and store at room temperature. During this time the gel will swell and trapped air will be released.
6. Polyisoprenoid composition of the tissue of interest has to be estimated prior to the internal standard selection. For seedling and leaves of *Arabidopsis thaliana* plant we use Prenol -14.
7. Chloroform is an aggressive solvent - you should wear gloves. Make sure that the bottle for C/M mixture is tightly closed.
8. Ethyl ether is highly volatile and flammable - remember to work under the hood. Mix 2% E/H and 15%E/H until you get a clear solution.
9. When several samples are to be analyzed the homogenizer has to rinsed between the samples.
10. For 3g of fresh tissue 20 mL of C/M (1/1, by vol) should be used in order to obtain the ratio of 0.3/1/1 of water/methanol/chloroform. Using this ratio the tissue homogenate is suspended in a homogenous mixture of solvents.
11. We recommend to continue the extraction for 24h in darkness. Otherwise the extraction might be also performed for 2h at 37°C (water bath).
12. Add water and chloroform to obtain the ratio of the solvents water/ methanol/chloroform equal to 1/2/3. In these conditions two solvent phases should be separated while precipitated proteins together with tissue remnants should be recovered in the solvent inter-phase. For better and faster phase separation the sample might be centrifuged in a low-speed centrifuge (e.g. 2000 rpm). Alternatively samples might be cooled down on ice and warmed again in a warm water bath. Sometimes addition of a small aliquot of methanol or ethanol (50-100 μL) improves the separation.
13. Chosen tubes should be resistant to high temperatures, tightness of the cap should be checked. These tubes will be used for hydrolysis.
14. During evaporation you can add an aliquot (0.2 mL) of ethanol - it will facilitate getting rid of the remaining water.
15. Remember to dissolve the sediment completely. After adding the hydrolyzing mixture vortex the tube carefully. Make sure the cap is tightly closed.
16. If the phases do not separate easily add 2ml of saturated NaCl solution.
17. Remember to pool all the four hexane phases - the final volume is approx. 9 mL.
18. For column purification choose regular glass tubes, e.g. with a volume of 25 - 30 mL, mark them: 0%, 2%, 15%.
19. Too much cotton wool can drastically decrease solvent flow and consequently stop or delay the separation on silica gel. Before adding gel inside the pipette add some hexane and check the flow. If it is too low discard this pipette and make a new column.
20. Calculate gel volume using the equation:

V- volume

d - column diameter

h - gel height

1. The column is well-packed when the level of gel does not move upon touching, gently tap the column to speed up gel sedimentation.

To avoid drying of the column keep it standing on the bottom of the tube and fill it regularly with an eluting solvent.

1. Load the sample very carefully, to avoid damaging the gel surface touch the inner surface of the Pasteur pipette.
2. When purifying polyisoprenoids extracted from plant photosynthetic tissues continue the elution with 2%E/H until the first yellow band (carotenoids) is eluted out from the column.
3. We recommend 10 cm long pre-coated glass TLC plates, if required cut the plate prior to spotting the sample. The spots of analyzed lipids (‘origin’) should be located on the TLC plate above (approx. 0.3 – 0.5 cm) the solvent level in the TLC tank. A warm but not hot air fan (hair-drier) might be used to accelerate sample loading.

When the iodine-stained TLC plate is removed from the iodine tank it should be immediately ‘sandwiched’ between two clean glass plates or in a transparent plastic envelope. The chromatogram has to be immediately scanned since the iodine desorption is quite fast. Use gloves when working with iodine, avoid contact with cloth.

1. Some of compounds might not be easily soluble in 2-propanol. To get a clear solution warm the tube (use a water bath or a hair dryer).
2. In order to keep the HPLC column ready-to-use, flush the system with solvent A and subsequently solvent B for 10 min at the end of the analysis of a sample set. To avoid sample contamination, the HPLC injector should be rinsed (use a syringe) with 2-propanol (e.g. 100 μL) after each run.

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**Figure captions**

Fig. 1. TLC chromatograms of lipids extracted from 3 week-old seedlings of *Arabidopsis thaliana*. Three fractions (0%, 2% and 15%) obtained after separation of a unsaponifiable lipids on silica gel column and a qualitative polyprenol standard (polyprenol mixture composed of Pren-9, -11, up to -23 and Pren-25) were resolved on TLC (a) Silica gel and (b) RP-18 plates.

Please note that lipid(s) appearing as a strong band in fraction 2% do(es) not correspond to polyisoprenoid alcohol since Rf of this compound(s) on silica gel plate (a) precludes this possibility.

Fig. 2. HPLC/UV chromatogram of polyisoprenoid lipids isolated from *A. thaliana* 3-week-old seedlings. Analysis of a purified fraction containing polyisoprenoids (15%E/H) has revealed the presence of a family of polyprenols (Pren-9 to -13, Pren-10 dominating) and dolichols (Dol-14 to -18, Dol-15 dominating). Insert shows an enlarged fragment of the HPLC chromatogram with a family of dolichols. Corresponding signals as well as a signal of the internal standard - Pren-14 (IS) are indicated.

Table 1

HPLC gradient program used for separation of polyisoprenoid lipids isolated from Arabidopsis seedlings. A combination of linear gradients is used. For details see the paragraph HPLC analysis.

|  |  |  |
| --- | --- | --- |
| **Time (min)** | **% Solvent A** | **% Solvent B** |
| 0 | 100 | 0 |
| 20 | 25 | 75 |
| 28 | 10 | 90 |
| 30 | 0 | 100 |
| 39 | 0 | 100 |
| 40 | 100 | 0 |