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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF PRENYLQUINONES, PRENYLVITAMINS AND PRENOLS

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SUMMARY

Prenylquinones of the naphthoquinone type (menaquinones, phylloquinones) and the benzoquinone type (ubiquinones, plastoquinones, α-tocoquinone), the vitamin E chromanols and other prenylvitamins (A, D) and also prenols can be separated by adsorption or reversed-phase high-performance liquid chromatography (HPLC). Several solvent systems are described for the different prenyllipids. Those prenyllipids which possess the same or similar retention times in adsorption chromatography are well resolved by reversed-phase HPLC, *e.g.*, solanesol from geranylgeraniol, the ubiquinone homologues, menaquinone homologues and phylloquinone homologues. The retention times of β-carotene (provitamin A) and of some basic quinone compounds, such as *p*-benzoquinone, 1,4-naphthoquinone and tocol, are given for comparison. The relationship between the chemical structure and the retention time of a prenyllipid is discussed.

INTRODUCTION

The group of prenyllipids that occur in nature consists of different fat-soluble isoprenoid lipids, the carbon skeleton of which is either fully or partly derived from the isoprenoid pathway[1–3]. Carotenoids with β-carotene (provitamin A) and vitamin A[4], prenols[5] and sterols including vitamin D are pure prenyllipids[6,7]. The prenylquinones found in plant, animal and bacterial cells contain an isoprenoid side-chain, which is bound to a non-isoprenoid benzoquinone or a naphthoquinone nucleus; they represent mixed prenyllipids. To this group also belong the prenylvitamins K_1 and K_2, which are prenylnaphthoquinones, and the E vitamins, which represent cyclic forms (chromanols) of reduced prenylbenzoquinones[1–8]. The photosynthetic pigments of green plants, the chlorophylls and carotenoids, represent prenyl pigments.

There have been some reports on the application of high-performance liquid chromatography (HPLC) to the separation of prenylquinones and prenylvitamins. In 1977 we reported that the prenylquinones found in plant extracts can be separated by adsorption HPLC[9]; the application of a column switching valve, instead of

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gradient elution, was found to be very useful for shortening the retention time of the more polar compounds and allowed the simultaneous separation of polar and non-polar prenylquinones by stable isocratic adsorption HPLC[9]. There is one report on the HPLC separation of non-isoprenoid substituted benzoquinones, naphthoquinones and anthraquinones[10]. More recently HPLC has been used for the resolution of a few menaquinone derivatives[11] and of some menaquinone homologues[12]. HPLC has also been applied to the determination of tocopherols in blood cells and plasma[13–16] and of vitamin A in foods[17], for the identification of vitamin D_3 and 7-dehydrocholesterol[18] and for the assay of fat-soluble vitamins in multivitamin tablets[19].

A great disadvantage of this previous work is the fact that the individual authors, concentrating mostly only on a special quinone or vitamin type, used different solvents, columns and packing materials that do not permit comparisons. In a preliminary report we showed that reversed-phase HPLC is very useful for the resolution of prenylquinones, carotenoids and chlorophylls[20]. We have now extended our studies to include more prenylquinone homologues and additional prenylvitamins and describe here several solvent systems for the separation of the various natural and synthetic prenyllipids by both adsorption and reversed-phase HPLC.

EXPERIMENTAL

Apparatus

A Siemens S100 chromatograph with a pneumatic syringe system (10- or 20-μl syringe) with a Zeiss spectrophotometer PM2D as the detector was used.

Columns

Stainless-steel columns (V4A), 125 and 250 mm long and 3 mm I.D. containing (a) LiChrosorb Si 60, 5 μm, and LiChrosorb-diol (Merck, Darmstadt, G.F.R.) and (b) LiChrosorb RP-8, 5 μm (Merck) were packed by the slurry method.

Solvents

Adsorption HPLC was carried out with hexane plus different amounts of dioxan. Reversed-phase HPLC was carried out with methanol plus different amounts of pure water. In HPLC the flow-rates for isocratic elution were 1–2 ml/min and the pressure was varied between 80 and 150 bar.

Chemicals

Most of the prenylquinones tested were gifts from Hoffmann-La Roche (Basle, Switzerland), which are gratefully acknowledged. Desmethylvitamin K_1 was a gift from Dr. D. R. Threlfall (Hull, Great Britain). Plastoquinone-9 was isolated from *Fagus* leaves[21]. Dehydrovitamin K_1 was isolated from etiolated barley seedlings[21]. Some prenylvitamins and ubiquinones were purchased from Merck, Serva (Heidelberg, G.F.R.) and Roth (Karlsruhe, G.F.R.). Before application all prenyl compounds were purified by partition and adsorption thin-layer chromatography.

RESULTS AND DISCUSSION

Adsorption HPLC

The separation of quinones and prenylquinones and vitamin K and E compounds (for chemical structures, see Fig. 1) can be achieved with considerable success using adsorption HPLC and increasing mixtures of dioxan in hexane as the solvent system. A mixture of 0.3% dioxan in hexane resolves β-carotene, phylloquinone K_1, plastoquinone-9 and α-tocopherol from a plant lipid extract. At 0.5% and higher dioxan levels the ubiquinones are resolved; the Q-homologues, however, are not resolved by adsorption HPLC (Table I). With 1% and 1.5% dioxan in hexane all plant prenylquinones are resolved; α-tocoquinone appears rather late, however (Fig. 2). This system works with test mixtures and natural plant extracts. For a more rapid resolution of α-tocoquinone in addition to the other prenylquinones a column switching valve can be applied, as described previously[9].

Tocopherols are completely separated by adsorption HPLC (Fig. 3). The retention time increases with decreasing number of methyl groups in the chromanol ring from α-tocopherol (three methyl groups), β- and γ-tocopherol (two methyl groups), δ-tocopherol (one methyl group) to tocol (no methyl groups). γ-Tocopherol with the two methyl groups in the *meta*-position (7,8-dimethyltocol) is resolved later than β-tocopherol, which contains two methyl groups in the *para*-position (5,8-dimeth-

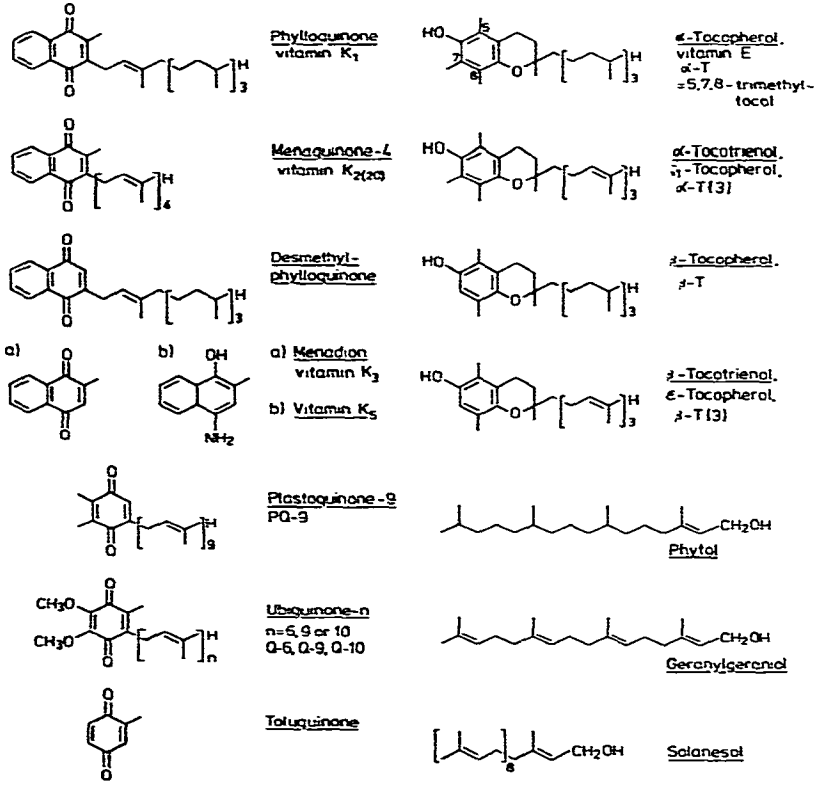

Fig. 1. Structures of some prenylquinones, prenylvitamins and prenols.

TABLE I

SEPARATION OF PRENYLQUINONES, VITAMIN K_1 AND K_2 COMPOUNDS (PHYLLOQUINONES AND MENAQUINONES) AND PRENOLS BY ADSORPTION HPLC

Column packings: P1 = LiChrosorb Si, 60.5 μm; P2 = LiChrosorb-diol, 10 μm. Solvents, dioxan in hexane: S1 = 0.5%; S2 = 0.8%; S3 = 1.0%; S4 = 1.25%; S5 = 0.3%; S6 = 0.1%. Detection with a Zeiss PM2D spectrophotometer at the wavelengths indicated (λ, nm). Temperature: ambient.

Packing		P1	P1	P1	P1	P1	P1	P1	P1	P1	P2	P2
Column length (cm)		25	25	25	25	25	12.5 + 25	12.5 + 25	10 + 20	12.5 + 25	20	20
Column diameter (mm)		3	3	3	3	3	3	3	6	3	3	3
Solvent		S5	S1	S2	S3	S4	S5	S1	S1	S3	S5	S6
Pressure (bar)		105	105	105	105	105	120	120	80	20	90	90
Flow-rate (ml/min)		1.9	1.9	1.9	1.9	1.9	1.3	1.3	4.5	1.3	1.8	1.8
Compounds	*λ (nm)*	*t_R (min)*										
Quinones:												
p-Benzoquinone	250	–	–	–	–	–	16.4	12.7	–	8.9	–	–
1,4-Naphthoquinone	250	–	–	–	–	–	14.0	10.8	–	7.7	–	–
Anthraquinone	250	–	–	–	–	–	–	8.2	–	6.1	–	–
Prenylnaphthoquinones:												
Dehydrovitamin K_1	260	–	–	–	–	–	–	4.0	9.9	–	–	–
Desmethylvitamin K_1	260	–	–	–	–	–	5.2	4.4	10.5	3.3	–	–
Vitamin K_1 = K_1-4	260	3.2	2.4	2.1	1.9	1.7	4.2	3.6	9.0	3.0	0.6	0.7
Menaquinone-4 = K_2 (20)	260	–	–	–	2.3	–	–	–	–	3.6	–	–
Menaquinone-9 and -10 = K_2 (45) and K_2 (50)	260	–	–	–	–	–	–	–	–	3.7	–	–
Menadione = vitamin K_3	260	–	–	–	–	–	9.6	8.2	–	5.8	–	–
Vitamin K_3	260	–	–	–	–	–	9.6	8.2	–	5.8	–	–
Prenylbenzoquinones:												
Plastoquinone-9	260	4.5	3.1	2.6	2.1	1.9	5.3	–	11.5	–	0.6	0.7
Ubiquinone-6	260	–	22	13.3	10.9	9.3	–	–	–	–	2.1	2.9
Ubiquinone-9	260	–	22	13.3	10.9	9.3	–	–	–	–	2.1	2.9
Ubiquinone-10	260	–	22	13.3	10.9	9.3	–	–	–	–	2.1	2.9
α-Tocopherol	290	13.1	10.4	7.8	7.0	6.4	–	–	–	–	2.7	4.0
α-Tocoquinone	265	–	–	–	67.0	50.0	–	–	–	–	12.3	18.7
Prenols:												
Phytol	205	–	–	–	13.0	–	–	–	–	–	–	–
cis-Geranylgeraniol	205	–	–	–	14.8	–	–	–	–	–	–	–
trans-Geranylgeraniol	205	–	–	–	18.6	–	–	–	–	–	–	–
Solanesol	205	–	–	–	18.6	–	–	–	–	–	–	–

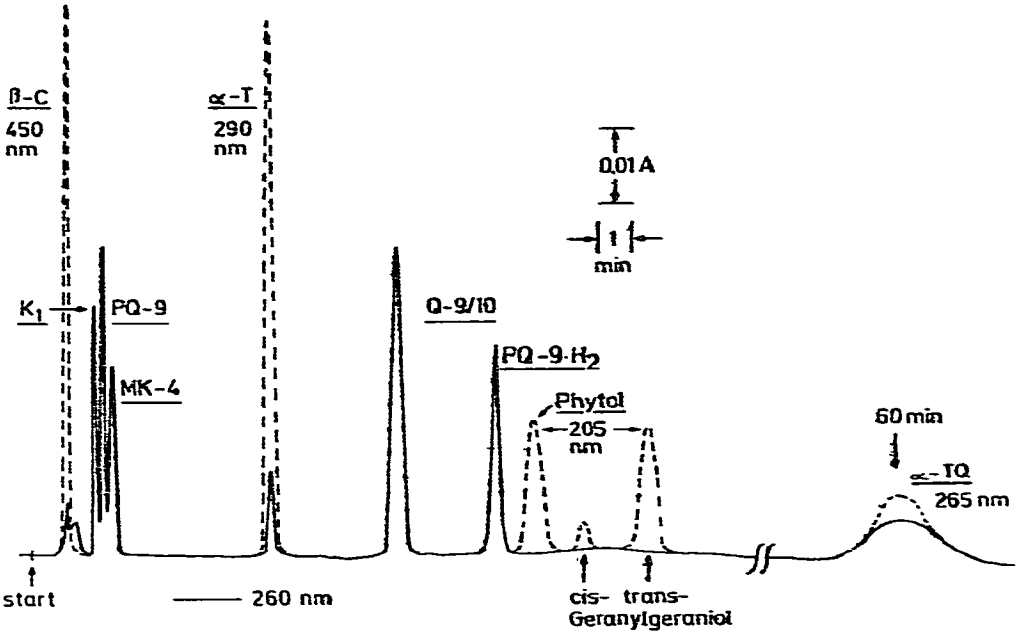

Fig. 2. Adsorption HPLC of prenylquinones and prenols. Column, 250 × 3 mm I.D., LiChrosorb Si 60, 5 μm; solvent, 1% dioxan in hexane; flow-rate, 1.9 ml/min; pressure, 105 bar; standard detection wavelength, 260 nm. By using other detection wavelengths prenols show up (205 nm) and the peaks of β-carotene (450 nm) and tocopherols (290 nm) are increased (dashed lines). β-C = β-carotene; K_1 = phylloquinone; MK-4 = menaquinone-4; PQ-9 = plastoquinone-9; PQ-9·H_2 = plastohydroquinone-9; Q-9/10 = ubiquinone-9 and -10; α-TQ = α-tocoquinone; α-T = α-tocopherol. A = absorption units.

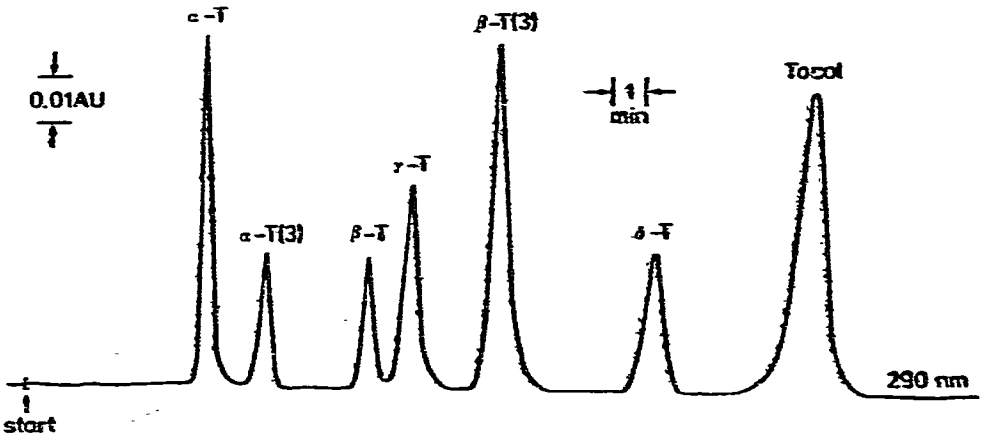

Fig. 3. Adsorption HPLC of tocopherols and tocotrienols. Column, 250 × 3 mm I.D., LiChrosorb Si 60, 5 μm; solvent, 1.5% dioxan in hexane; flow-rate, 1.9 ml/min; pressure, 105 bar; standard detection wavelength, 290 nm. α-T = α-tocopherol; α-T(3) = α-tocotrienol; β-T = β-tocopherol; β-T(3) = β-tocotrienol; γ-T = γ-tocopherol; δ-T = δ-tocopherol. AU = absorption units.

yltocol), α- and β-tocotrienol, with three additional double bonds in the prenyl chain, appear after α- and β-tocopherol, respectively. The elution sequence given here has repeatedly been verified with purified substances from different sources; it is in full agreement with the theoretical prediction of the relationship between chemical structure and retention time. The sequence originally given in a preliminary report[20] (Fig. 4) proved to be wrong.

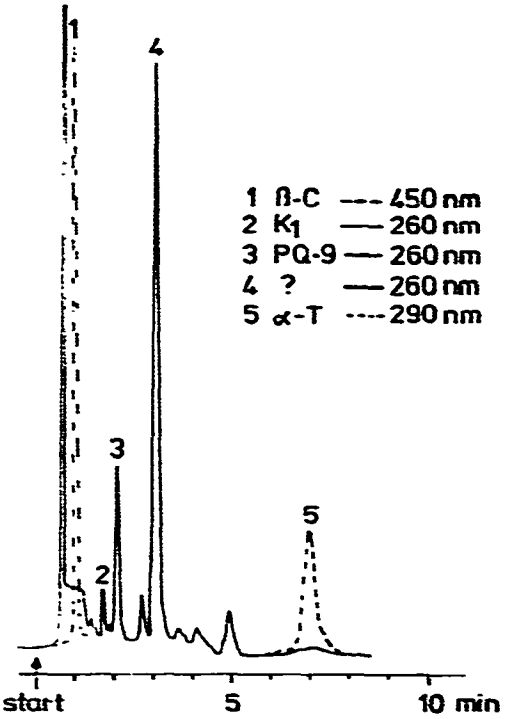

Fig. 4. Separation of the prenylquinones from a lipid extract from the envelope of spinach chloroplasts by adsorption HPLC. Column, 250 × 3 mm I.D., LiChrosorb Si 60, 5 μm; solvent, 0.5% dioxan in hexane; flow-rate, 1.5 ml/min; pressure, 145 bar. β-C = β-carotene; K_1 = phylloquinone; PQ-9 = plastoquinone-9; α-T = α-tocopherol.

The two C_{20} prenols phytol (one double bond) and geranylgeraniol (four double bonds) are resolved as separate peaks as well as *cis*- and *trans*-geranylgeraniol. The latter is, however, not separated from the C_{45} prenol solanesol (Table I). Also, other prenyllipids that differ only in three double bonds are separated by adsorption HPLC. Menaquinone-4 is resolved after phylloquinone K_1, β-tocotrienol after β-tocopherol and α-tocotrienol after α-tocopherol. Adsorption HPLC also resolves the non-isoprenoid quinones. The retention time decreases in the order benzoquinone > naphthoquinone > anthraquinone. The presence of an additional methyl group reduces the retention time; thus menadione appears before 1,4-naphthoquinone and vitamin K_1 before desmethylvitamin K_1. Menaquinone homologues are not resolved by adsorption HPLC. The retention time of mono-dehydrovitamin K_1, with two double bonds in the prenyl side-chain, is between that of vitamin K_1 and men-

aquinone-4, which contain one and four double bonds, respectively, in the prenyl side-chain.

As in adsorption TLC it is not possible to separate fully desmethylvitamin K_1 from mono-dehydrovitamin K_1. More polar substituents in the benzoquinone ring increase the retention time. Ubiquinone-9, with two methoxy groups in the ring, is resolved much later than plastoquinone-9, which also has the C_{45} solanesyl chain.

The elution sequence of prenylquinones is not changed with increasing dioxan content in hexane using the same adsorbent, LiChrosorb Si 60. With LiChrosorb-diol, however, the sequence is changed: ubiquinone-9 and -10 appear before α-tocopherol (P2 in Table I), whereas with LiChrosorb Si 60 they are resolved after α-tocopherol. A different mobility of ubiquinone and α-tocopherol was also found in

TABLE II

SEPARATION OF PRENYLQUINONES AND VITAMIN K COMPOUNDS (K_1 HOMOLOGOUS AND MENAQUINONE HOMOLOGUES) BY REVERSED-PHASE HPLC

Column packing: LiChrosorb RP-8, 5 μm. Solvents, water in methanol: S1 = 0%; S2 = 3%; S3 = 4%; S4 = 4.75%; S5 = 8%; S6 = 10%; S7 = 15%; S8 = 20%; S9 = 50%. Detection in UV light at wavelengths indicated (λ, nm). Detector: Zeiss PM2 DLC spectrophotometer. Column: 25 cm × 3 mm I.D. Temperature: 30°C.

Solvent		S1	S2	S3	S4	S5	S6	S7	S8	S9
Pressure (bar)		140	150	150	155	160	180	180	200	250
Flow-rate (ml/min)		1.4	1.3	1.3	1.3	1.2	1.2	1.2	1.0	0.8
Compound	*λ (nm)*	*t_R (min)*								
Quinones:										
p-Benzoquinone	245	–	–	–	1.1	1.2	1.4	–	1.7	2.2
Toluquinone	245	–	–	–	1.2	1.3	1.4	–	1.8	3.1
1,4-Naphthoquinone	245	–	–	–	1.2	1.4	1.5	–	2.1	6.7
Anthraquinone	245	–	–	–	1.4	1.7	1.9	–	3.3	41.2
Prenylnaphthoquinones:										
Dehydrovitamin K_1	260	–	2.6	–	–	7.2	–	–	–	–
Desmethylvitamin K_1	260	–	2.6	3.3	3.8	7.4	–	–	–	–
Vitamin $K_1$1 = K2 (5)	260	1.2	1.4	1.4	1.5	1.8	2.2	2.7	4.9	–
Vitamin K_1-2	260	1.4	1.7	1.7	1.9	2.7	3.6	5.8	15.2	–
Vitamin K_1-3	260	1.7	2.2	2.3	2.7	4.5	7.2	15.1	–	–
Vitamin K_1-4 (phylloquinone)	260	2.1	2.8	3.4	4.2	8.7	–	–	–	–
Vitamin K_1-5	260	2.9	4.9	5.6	7.3	–	–	–	–	–
Vitamin K_1 cyclohexanone	260		1.5		1.7	2.5	2.9	3.9	8.3	–
Menaquinone-4 = K_2 (20)	260	1.5	2.1	2.4	2.9	5.1	8.5	–	–	–
Menaquinone-9 = K_2 (45)	260	6.5		–	–	–	–	–	–	–
Menaquinone-10 = K_2 (50)	260	9.1		–	–	–	–	–	–	–
Menadion = vitamin K_3	260	1.1	–	1.2	1.3	1.5	1.6	1.8	2.3	12.2
Vitamin K_5	260	–	–	1.2	1.3	1.5	1.7	1.8	2.3	12.2
Prenylbenzoquinone derivatives:										
Plastoquinone-1	260	–	–	1.3	1.4	1.6	1.8	2.2	3.5	–
Plastoquinone-9	260	4.4	8.5	12.3	18.7	–	–	–	–	–
Ubiquinone-6	260	1.9	2.6	3.0	3.8	–	–	–	–	–
Ubiquinone-9	260	3.6	6.5	9.0	13.1	–	–	–	–	–
Ubiquinone-10	260	4.8	9.6	14.1	21.7	–	–	–	–	–
α-Tocoquinone	265	–	1.8	2.1	2.5	5.0	–	12.0	–	–

silica gel TLC on changing the solvent system: ubiquinone exhibits a lower R_F value than α-tocopherol in solvent 1 (light petroleum b.p. 50–70°C–diethyl ether, 9:1), whereas in solvent 2 (chloroform–carbon tetrachloride, 3:2) the ubiquinone moves above α-tocopherol[8].

Reversed-phase HPLC

For the resolution of the non-isoprenoid quinones by reversed-phase HPLC one has to use methanol with a high water content (20–50%) as the solvent; the retention time increases from benzoquinone to naphthoquinone and anthraquinone (S9 in Table II). Methyl groups as substituents in the quinone nucleus result in higher resolution times (*e.g.*, for toluquinone > benzoquinone and for menadione > naphthoquinone, S8 and S9 in Table II). With prenylquinones one obtains a good resolution with a lower water content (0–10%) in methanol as the solvent. The retention times increase with increasing number of isoprene units in the side-chain (S1–S4, Table II), as can be seen for the homologues of vitamin K_1 (from K_1-1 to K_1-5), of menaquinones (MK-1, MK-4, MK-9, MK-10), of ubiquinones (UQ-6, UQ-9, UQ-10) and two plastoquinones (PQ-1 and PQ-9).

Vitamin K_1 with a phytyl side-chain (one double bond) appears later than the corresponding menaquinone-4, which has a geranylgeranyl side-chain (four double bonds). Dehydrovitamin K_1 (dehydrophylloquinone), with two double bonds in the side-chain, is resolved between vitamin K_1 and menaquinone-4. The absence of a methyl group (desmethylvitamin K_1) results in a shorter retention time than that of the corresponding methyl derivative (vitamin K_1). More polar substituents in the benzoquinone ring (*e.g.*, two methoxy groups in ubiquinone-9) decrease the retention time considerably compared with plastoquinone-9, which also possesses the solanesyl (C_{45}) chain.

Reversed-phase HPLC is also very suitable for the separation of other prenylvitamins and for prenols (Table III and Fig. 5). Those compounds with fewer double bonds (α-tocopherol, β-tocopherol, vitamin D_3 and phytol) are resolved earlier than those with more double bonds (α-tocotrienol, β-tocotrienol, vitamin D_2 and geranylgeraniol, respectively). Vitamin D_2 and D_3, which differ only in one double bond, give peaks that are very close together. The retention times are also very similar for vitamin A alcohol and aldehyde (Table III). The possession of more methyl groups in the chromanol ring increases the retention time, as can be seen from the sequence tocol (no methyl group), δ-tocopherol (one methyl group), β-tocopherol (two methyl groups), α-tocopherol (three methyl groups) (S6, Table III). There is also a very good separation of the prenol homologues geranylgeraniol and solanesol (S3 and S5, Table III). Neither adsorption nor reversed-phase HPLC separated menadione (a 2-methyl-1,4-naphthoquinone) from vitamin K_5 (a 4-amino-2-methylnaphthol).

Because of its reproducibility, the HPLC of prenyllipids using one solvent system has advantages over gradient HPLC systems. Reproducibility is of importance for routine analyses. Plant lipid extracts contain prenylquinones and other prenyllipids which are very different in their lipid character, depending on the type and number of polar groups in their molecule. This often makes the separation of the different forms with one solvent system impossible, because the more polar prenylquinones are either not resolved from the column or only very late (more than 1 h), with a concomitant broadening of the peak. This problem can be avoided, however, by using two columns in combination with a column switching valve[9,20].

TABLE III

SEPARATION OF PRENYLVITAMINS AND PRENOLS BY REVERSED-PHASE HPLC

Column packing: LiChrosorb RP-8, 5 μm. Solvents, water in methanol: S1 = 0%; S2 = 3%; S3 = 4%; S4 = 4.75%; S5 = 8%; S6 = 10%; S7 = 15%; S8 = 20%; S9 = 50%. Detection: Zeiss PM2 DLC spectrophotometer at the wavelengths indicated (λ, nm). Column: 25 cm × 3 mm I.D. Temperature: 30°C.

Solvent		S1	S2	S3	S4	S5	S6	S7	S8
Pressure (bar)		140	150	150	155	160	180	180	200
Flow-rate (ml/min)		1.4	1.3	1.3	1.3	1.2	1.2	1.2	1
Compound	*λ (nm)*	*t_R (min)*							
Vitamins:									
Vitamin A alcohol	250	–	–	1.5	1.7	2.1	2.8	4.2	9.4
Vitamin A aldehyde	250	–	–	–	1.7	2.3	3.0	4.5	9.9
Vitamin A palmitate	250	2.3	–	–	6.3	–	–	–	–
β-Carotene (provitamin A)	450	–	4	5	7.2	–	–	–	–
Vitamin D_2	265	–	–	–	2.5	4.1	6.6	14.2	–
Vitamin D_3	265	–	–	–	2.5	4.2	6.8	14.6	–
α-Tocopherol (vitamin E)	290	–	2.1	2.4	3.0	6.0	9.0	–	–
α-Tocotrienol (ζ_1-tocopherol)	290	–	–	2.0	2.2	4.0	5.3	–	–
β-Tocopherol	290	–	–	–	2.8	4.8	8.1	–	–
β-Tocotrienol (ε-tocopherol)	290	–	–	1.9	2.1	3.1	4.7	–	–
δ-Tocopherol	290	–	–	2.2	2.5	4.2	6.9	–	–
Tocol	290	–	–	1.9	2.3	4.0	5.5	–	–
Prenols:									
Phytol	205	1.4	–	2.3	–	3.5	–	–	–
Geranylgeraniol	205	1.2	–	1.9	–	2.4	–	–	–
Solanesol	205	2.4	–	8.0	–	27.0	–	–	–

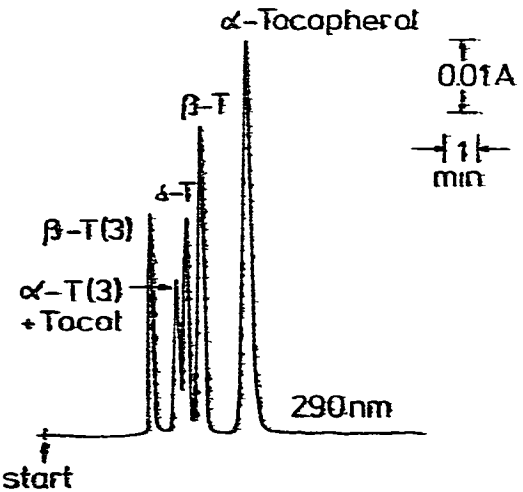

Fig. 5. Reversed-phase HPLC of tocopherols and tocotrienols. Column, 250 × 3 mm I.D. LiChrosorb RP-8, 5 μm; solvent, 8% water in methanol; flow-rate, 1.3 ml/min; pressure, 160 bar. A = absorption units. α-T, β-T, δ-T = α-, β- and δ-tocopherol; α-T(3), β-T(3) = α- and β-tocotrienol; Tocol = basic chromanol structure of tocopherols without a methyl group in the aromatic ring.

HPLC can also be applied for the separation of the major chloroplast prenyl pigments of green plants, the chlorophylls and the carotenoids. The main compounds are resolved by adsorption and reversed-phase chromatography (Fig. 6). In reversed-phase HPLC the more polar carotenoids neoxanthine (with three hydroxy and one epoxy group), violaxanthine (with two hydroxy and two epoxy groups) and antheraxanthine appear before luteine (with two hydroxy groups), and the more polar chloro-

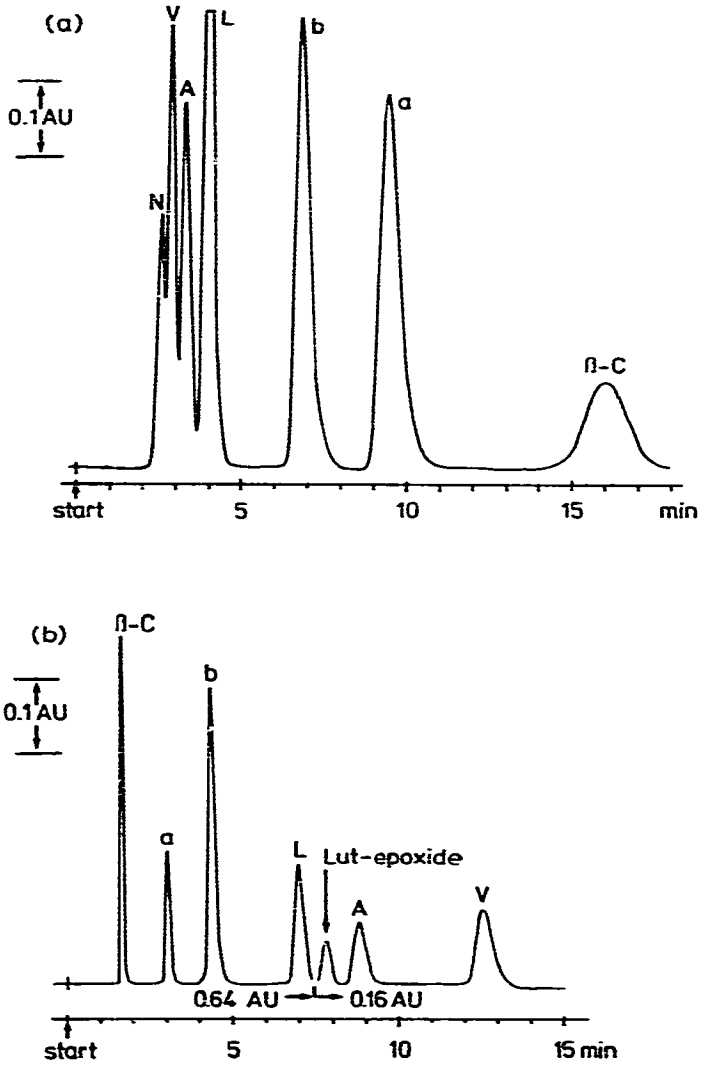

Fig. 6. (a) Reversed-phase and (b) adsorption HPLC (II) of plant prenyl pigments (chlorophylls and carotenoids) from radish seedlings. (a) Column, 250 × 3 mm I.D., LiChrosorb RP-8, 5 μm; solvent, 10% water in methanol; flow-rate, 1.2 ml/min; pressure, 160 bar. (b) Column, 250 × 3 mm I.D., LiChrosorb Si 60, 5 μm; solvent, 20% acetone and 5% dioxan in hexane; flow-rate, 1.5 ml/min; pressure, 150 bar. AU = absorption units. N = Neoxanthine; V = violaxanthine; A = antheraxanthine + luteine epoxide; L = luteine; b = chlorophyll b; a = chlorophyll a; β-C = β-carotene.

phyll b (with a CHO group) before chlorophyll a (which possesses a methyl group in the pyrrole ring). In adsorption HPLC β-carotene is resolved first, followed by the chlorophylls and the xanthophylls.

The results of this investigation show that the separation of prenylquinones, prenylvitamins and chloroplast pigments can be achieved by either adsorption or reversed-phase HPLC. Homologues that appear as one peak in adsorption HPLC are well resolved by reversed-phase HPLC. A combination of both methods can yield pure preparations for all of the natural prenyllipids investigated here. With lipid extracts from green plant material a pre-fractionation of the lipids by thin layer chromatography is recommended[21].

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