A RAPID METHOD FOR THE IDENTIFICATION OF SMALL QUANTITIES OF LIPID-SOLUBLE VITAMINS AND QUINONES IN BIOLOGICAL MATERIAL*

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Increased knowledge of the function of fat-soluble vitamins and quinones in the living organism has made their rapid separation and identification of considerable interest. Various methods for their separation by paper chromatography are reported in the literature[1,3]. These methods, however, are not directly applicable to the original extracts of plant or animal tissues, since vitamins and quinones are usually present in very small concentrations and are masked by large amounts of accompanying lipids. These interfering substances cause poor separation of the vitamins and quinones. A preliminary purification of the extract, usually by saponification and column chromatography, is necessary before paper chromatographic methods can be applied. The preliminary treatments, however, very often lead to a partial destruction of the quinones and vitamins and thus result in considerable losses. Transformation products that were not originally detectable in the extracts may then become prominent. Furthermore, most of the fat-soluble quinones and vitamins are extremely sensitive to light and oxygen, and may be partially destroyed during the usual chromatography on paper (10 to 20 h). For the study of the function and biosynthesis of these compounds it was desirable to develop a rapid paper chromatographic method for their separation and identification, which could demonstrate the presence of these substances in biological systems before the application of the further purification processes.

By testing various papers and solvents we have been able to develop a simple and rapid method for the separation of vitamins and naturally occurring quinones. In comparison with existing chromatographic methods the present technique has several advantages, resulting from the rapidity of the chromatogram development (1 to 2 h) and the high adsorption capacity of the paper used (Schleicher and Schüll, No. 288). The quantity of the whole cell extract which can be applied to the paper is about 10 times greater than can be used with silicone- or paraffin-impregnated papers. This makes it possible to locate compounds present in extracts in such extremely low concentrations that they could not previously be detected. The substantially higher absorption capacity is also important for the separation of the components for further analytical purposes.

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EXPERIMENTAL

In the text and the figures the following abbreviations are used for the separated compounds:

A	= Vitamin A	PQ_9	= Plastoquinone A (9 isoprene units in side chain)
C	= β-Carotene	PQB	= Plastoquinone B (side chain unknown)
Chl	= Chlorophyll a + b	Q_{10}	= Coenzyme Q_{10}
D_2	= Vitamin D_2	T	= α-Tocopherol
K_1	= Vitamin K_1	TQ	= α-Tocopherylquinone
Ph	= Phaeophytin	X	= Xanthophylls

The tested compounds were either bought commercially when available or isolated from spinach chloroplasts. A sample of synthetic plastoquinone 9 was kindly supplied by Hoffmann-LaRoche & Co.

Solvents

The choice of the solvent depended on the particular separation to be undertaken. Cyclohexane was found to be convenient for the separation of K_1 and Q_{10} from β-carotene. For the separation of the other quinones and vitamins from one another a mixture of cyclohexane–benzene (3:7), pure benzene, chloroform–benzene (1:1), and chloroform were used successfully.

Application of samples to paper

In general, samples were applied to the paper from ethanol, cyclohexane or isooctane solution. Samples in acetone were also satisfactory if care was taken to keep the spot size small.

Development of chromatogram

Ascending chromatography was used routinely; for small quantities chromatostrips were used, but when larger amounts of lipid were to be separated, large sheets of paper were used as separation media. Circular chromatography worked equally well with the above solvents, and was found especially convenient when test substances were employed, as well as for the quantitative analysis of natural extracts, when distinct separation of compounds in single, not overlapping, bands was required. All chromatograms were run at room temperature and in the dark to avoid destruction of the lipids.

Detection methods

1. Examination in daylight and in ultra-violet light.

2. Spraying with saturated solution of $SbCl_3$ in chloroform (A, β-carotene, carotenoids). D_2 gave a distinct brownish yellow color.

3. Spraying with a mixture of equal volumes of α,α'-dipyridyl (0.5 %) and $FeCl_3$ (0.2 %) in 95 % ethanol (tocopherol).

4. Use of the neotetrazolium spray according to LESTER AND RAMASARMA[4]: quinones were first reduced by immersing the paper in 0.1% solution of sodium borohydride for 30 sec. Excess borohydride was destroyed by dipping the paper in 0.1 N HCl for 2 sec. The chromatogram was then immersed in a solution containing

0.25% neotetrazolium chloride in 0.25 *M* potassium phosphate, pH 7.0 (hydroquinones). Naphthoquinones gave immediately an intense red color (K_1, menadione, 1,4-naphthoquinone and phthiocol). Benzoquinones (TQ, Q_{10}, PQ_9, PQB) showed red colored spots only after heating at 100° for 60 sec. Thus the neotetrazolium reaction allows a distinction between derivatives of naphtho- and benzoquinones. TQ gave an intense spot of deep violet, while PQ's showed only a pale red. Therefore care has to be taken not to miss the PQ bands when the method is applied to natural extracts.

RESULTS AND DISCUSSION

Chromatography of pure compounds

In the procedure described here, all known fat-soluble vitamins and various naturally occurring quinones have been separated successfully. The efficiency of the Schleicher and Schüll filter paper No. 288 for the separation of lipid-soluble quinones and vitamins is due to its content of alumina. On ordinary filter paper, quinones, vitamins and β-carotene are separated only poorly or not at all. Reverse phase chromatographic techniques using vaseline, paraffin, or silicone as the primary phase are limited by the small amount of material which can be applied to the paper and by the impossibility of eluting the separated compounds for further analysis without simultaneous extraction of the impregnated oil. Alumina paper, however, has an obvious advantage in its higher absorption capacity. It has the additional advantage of absorbing quinones and vitamins more strongly than β-carotene. Moreover, the separated compounds can be eluted uncontaminated. Quinones isolated from natural extracts by this new technique showed absorption spectra in the ultraviolet region which were in good agreement with the data reported in the literature.

Cyclohexane and a mixture of cyclohexane and benzene (7:3) were found to be convenient for the separation of vitamin K_1 from β-carotene and from other quinones. This procedure also produced the purest β-carotene. For the isolation of PQ_9, Q_{10} and T, a mixture of cyclohexane and benzene (3:7) or pure benzene was used. TQ was best separated from accompanying lipids with the benzene–chloroform mixture (1:1), and for vitamin D_2 and A pure chloroform provided good separation. Typical chromatostrips are represented in Fig. 1.

The adsorption sequence on the alumina paper follows the lipid character of the compounds being chromatographed. Molecules with a high lipid solubility (*e.g.*, hydrocarbons) are adsorbed to a much smaller extent than others with more hydrophilic groups. In the case of the quinones, one might assume that their lipid character, and consequently their order of migration, are determined by the length of the side chain in position 3. However, it will be seen that this is only partially true. Thus, PQ_9, a 5,6-dimethyl-3-solanesyl-benzoquinone with 9 isoprene units (C 45) in the side chain has a higher R_F than Q_{10}, a 5,6-dimethoxy-2-methylbenzoquinone with 10 isoprene units (C 50) in position 3. Thus, methoxy-groups in place of methyl-groups lower the R_F value considerably. The structure of the above mentioned compounds is shown in Fig. 2. TQ, a 5,6-dimethylbenzoquinone with a 3'-hydroxy-phytyl side chain (C 20) compared with PQ_9 and Q_{10} has, as may be expected, a significantly lower mobility on the chromatogram. For vitamin K_1 (2-methyl-3-phytyl-naphthoquinone) which has a phytyl group (as does TQ) but no hydroxyl group, one could expect a chromatographic mobility somewhat greater than that of TQ.

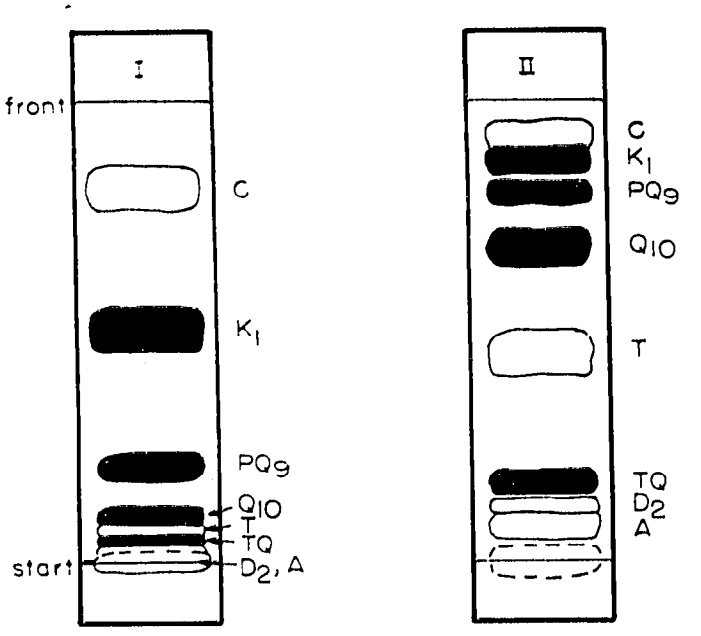

Fig. 1. Paper chromatogram of lipid-soluble vitamins and quinones. Solvent for I, cyclohexane–benzene (7:3); for II, benzene. Dark spots indicate quinones.

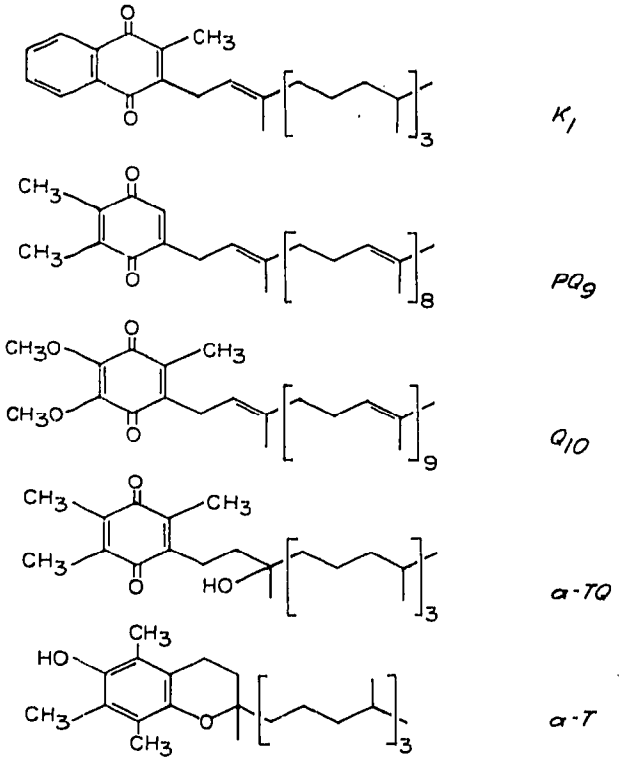

Fig. 2. Comparison of the structures of quinones and vitamins from spinach leaves.

Its R_F value, however, is even higher than those of the long side chain quinones PQ_9 and Q_{10}. This fact must be ascribed to the possession by the napthoquinone ring of a lipid character apparently greater than that of benzoquinone. In spite of its phenolic hydroxyl and its short side chain of only 15 C atoms, T, the chromanol from of TQ,

shows a higher R_F than TQ. This behavior, and that of vitamin K_1, prove that double ring structures possess a higher lipid solubility than single ring systems. Thus, comparison of structure and R_F values makes it clear that apart from the length of the polyisoprenoid side chain, the arrangement of substituents in the quinone molecule is of essential significance for the position on the chromatogram.

Various other solvents such as isooctane, petroleum ether, carbon tetrachloride, and toluene, or mixtures thereof, were tested for separation of lipid soluble quinones and vitamins. They also provided good separation, but had no advantages over the solvents described. More polar solvents such as pyridine, methanol and acetone were of no value, since all fat-soluble substances tested had R_F values higher than 0.9 in these solvents.

In Table I the R_F values of quinones and vitamins, as well as some plant pigments, are listed for the different solvents. The values of the tested substances varied within narrow limits, especially when natural extracts were applied. There was, however, no change in the order of migration.

Normally, the walls of the chromatographic tanks were lined with filter paper moistened with solvent. If this was not done, higher R_F values were obtained. In Table I, in case (*a*), where the glass tank was saturated with the solvent, the solvent travelled much farther from the starting point per time unit than in case (*b*), where the solvent partially evaporated from the chromatogram. Thus, when calculating R_F's, higher values were obtained. This fact has to be taken into account when highly volatile solvents are used, and particularly when, as in the present instance, short development times are employed (1–2 h). Therefore, in all further experiments the walls were covered with filter paper in order to ensure saturation of the tank with the solvent and to obtain reproducible R_F values.

Chromatography of plant extracts

The method described here has been used successfully for examining various plant extracts. It might also be employed for animal tissue extract analysis, a possibility now under investigation. β-Carotene, the principal compound accompanying and often masking quinones and vitamins in natural extracts, runs almost with the solvent front, while the chlorophylls and xanthophylls remain practically on the starting line. Thus, these normally interfering substances do not affect the separation of the other lipids. Fig. 3 shows chromatograms of a freshly prepared acetone extract from spinach leaves.

Large amounts of extraneous fatty material in the extract, however, have a considerable influence on the R_F values of the quinones and may cause poor separation. This is particularly true for extracts from whole leaves. Therefore, purification by column chromatography should precede the separation on paper. Repeated paper chromatography is also advisable. The application of the new technique to plant extracts therefore quickly provides information as to whether quinones or tocopherols are present, before further purification and final isolation are carried out. When preliminary column chromatography is employed, the method is very useful in detecting and identifying the quinones in the various fractions eluted. Its application as a test of purity of the isolated compounds is also of great significance.

With the new method, several quinones and α-tocopherols were found in chloroplasts and quantasomes of spinach. Some of these quinones were identified as K_1,

TABLE I

R_F VALUES FOR NATURALLY OCCURRING QUINONES, FAT-SOLUBLE VITAMINS AND PLANT PIGMENTS ON SCHLEICHER AND SCHUELL NO. 288 PAPER

Compounds	*Cyclohexane*	*Cyclohexane–benzene (7:3)*		*Cyclohexane–benzene (3:7)*	*Benzene*	*Benzene–$CHCl_3$ (1:1)*		*$CHCl_3$*	*Isooctane*	*Pet. ether (b.p. 30–60°)*	*CCl_4*	*Toluene*	
		(a)	*(b)*	*(a)*		*(a)*	*(b)*					*(a)*	*(b)*
β-Carotene	0.51	0.81	0.98	0.84	0.92	0.87	0.95	0.94	0.13	0.32	0.79	0.86	0.90
K_1	0.20	0.50	0.90	0.78	0.87	0.85	0.94	0.91	0.08	0.10	0.53	0.79	0.87
PQ_9	0.08	0.18	0.23	0.77	0.82	0.85	0.94	0.91	—	0.05	0.18	0.78	0.87
Q_{10}	0.05	0.09	0.16	0.47	0.70	0.83	0.90	0.91	—	—	0.13	0.55	0.85
T	—	0.05	0.13	0.26	0.46	0.48	0.64	0.82	—	—	0.10	0.32	0.78
TQ	—	—	0.05	0.05	0.14	0.41	0.50	0.73	—	—	0.05	0.09	0.65
D_2	—	—	0.05	0.08	0.13	0.40	0.41	0.59	—	—	—	0.09	0.52
A	—	—	—	0.04	0.11	0.29	0.31	0.51	—	—	—	0.09	0.38
Chl a + b	—	—	—	—	—	0.04	0.04	0.08	—	—	—	—	—
X	—	—	—	—	—	—	—	0.08	—	—	—	—	—
Ph	—	—	—	—	0.17	0.47	0.58	0.77	—	—	—	0.09	0.74

(a) Walls of tank lined with solvent-wetted filter paper.
(b) Walls of tank not lined as in (a).

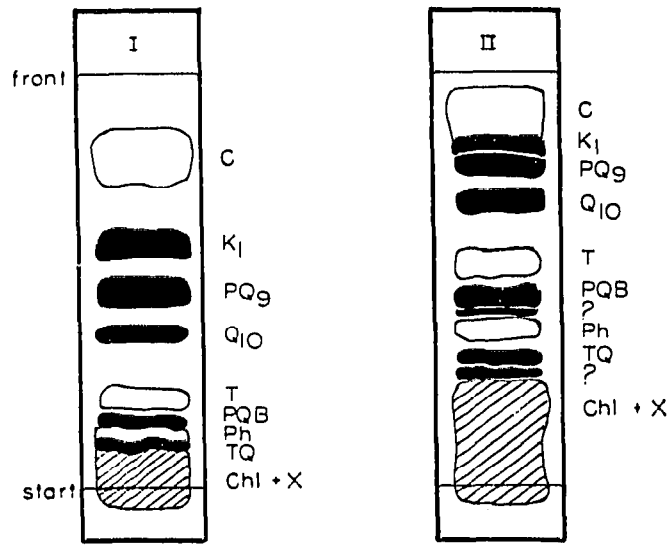

Fig. 3. Paper chromatogram of an acetone extract from spinach leaves. Solvent for I, cyclohexane–benzene (7:3); for II, benzene. Dark spots indicate quinones.

PQ_9, PQB and TQ. Q_{10}, present in the leaves of spinach, was not detected in chloroplasts and quantasomes. Further study is required for the identification of other quinones present in lower concentrations, as indicated on the chromatograms. These might correspond to plastoquinone C and other tocopherylquinones which recently have been shown to occur in chloroplasts[5].

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SUMMARY

A rapid method for separation and identification of naturally occurring quinones and fat-soluble vitamins on commercially available filter paper with alumina filler is described. The R_F values for different solvents are tabulated. The application of the method to natural extracts is discussed.

NOTE ADDED IN PROOF

When the chromatograms are immersed in the neotetrazolium chloride solution before they are taken into the sodium borohydride solution, the sensitivity of revealing the quinones as red spots is increased considerably. Heating is then to be omitted.

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