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SEPARATION AND ESTIMATION OF QUINONES AND α-TOCOPHEROL FROM *VICIA FABA* LEAVES

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SUMMARY

Six quinones (3 plastoquinones, vitamin K_1, ubiquinone and α-tocopheryl-quinone) and α-tocopherol have been separated by column chromatography and determined quantitatively in extracts of broad bean leaves. The column chromatography method is new and provides excellent separation of the above lipids from pigments and the glyceride lipids. The method may be adapted to a method of large scale isolation of these lipids. Determinations made suggest that plastoquinone A is the most abundant and that the other plastoquinones occur in much lower quantities, particularly in young leaves. Determinations also show that α-tocopherol occurs in leaves in similar concentration to plastoquinone A, but that tocopherylquinones occur in much lower concentrations.

INTRODUCTION

Since the discovery of plastoquinone by CRANE[1] in 1959, nine quinones (PQ A, B, C and D, α-, β- and γ-tocopherylquinone, vitamin K_1 and an unknown naphthoquinone[2]) have been reported in spinach chloroplasts[3]. As well as the chloroplast quinones, at least one quinone, coenzyme Q_{10} or ubiquinone, is present in leaves, probably located in the mitochondria. Vitamin E (α-tocopherol) has been found in a wide variety of plant tissue[4,5]. BOOTH suggested that at least part of the α-tocopherol is associated with the chloroplasts. Results with broad bean leaves[6] and extracted spinach chloroplasts[7] confirm this.

The importance of plastoquinone in electron transport has been shown[8-13], and stressed by CRANE[14]. The relative importance of the plastoquinones and tocopherylquinones in photosynthetic electron transport is under investigation in the author's laboratory and by others.

The methods employed in separation and purification of the quinones and α-tocopherol consist of paper[7,16-18], thin-layer[3,4,15,19,20] and column chromatography[1,2,17]. The adsorbents used in column chromatography often cause breakdown of the quinones[21,22], whereas thin-layer chromatography often results in streaking and poor resolution. A method was devised, and is reported here, using a short column for the separation of 3 plastoquinones, vitamin K_1, α-tocopherylquinone (α-TQ), α-

tocopherol (α-T) and coenzyme Q_{10} (Q_{10}) found in broad bean leaves. The method is suitable for rapid quantitative determination of the above quinones and α-tocopherol. The adsorbent used is almost identical to that used by many workers for thin-layer chromatography, but the "tailing" observed by the author on thin-layer plates, is eliminated using column elution.

MATERIALS AND METHODS

Vicia faba plants (Broad bean, Giant Windsor) were grown in a greenhouse for 3–4 weeks with supplementary light (approx. 700 ft. c. with 16 h day). The age of the plant in all experiments was calculated from the day of planting. For each experiment, approximately 30 g leaves were harvested, frozen in liquid air and ground with 40–50 g anhydrous disodium hydrogen phosphate. The finely ground leaves were lyophilized over-night at 4° (in later experiments at —6°) in the dark. The lyophilized leaves were reground, extracted with acetone (200 ml) and filtered. The residue was washed with acetone until almost colourless. The liquid extract was dried on a rotary evaporator and the green residue dissolved in chloroform. A known volume was removed for chlorophyll determination and the chloroform solution dried down once more. The residue was then redissolved in *n*-hexane (b.p. 67–70°) and a known volume applied to the top of a Kieselgel G–Celite (1:1, w/w) column. The extract was washed into the column with a small volume of *n*-hexane.

The column adsorbent was prepared by mixing equal quantities (by weight) of Celite and Kieselgel G (E. Merck). The mixture was thoroughly washed with ether, which removes a yellow contaminant, and dried at 100° for one hour. The column was prepared by making a slurry of the medium with *n*-hexane and carefully packing the column, 1.2 cm in diameter, to a height of 6 cm. The final packing was done under pressure from a nitrogen cylinder. The column was washed with a small volume (10 ml) of *n*-hexane before the application of the lipid extract.

Elution of the quinones was carried out with 60 ml of 0.5 %, 60 ml of 3.0 %, 60 ml of 10 % and finally 20 % diethyl ether in hexane, consecutively. Fractions of approximately 3.5 ml were collected, using a drop counter, beginning with the first eluate containing β-carotene. All fractions were dried down at room temperature and redissolved in 3 ml of 95 % ethanol. U.V. absorption spectra (200 mμ–300 mμ) were determined in 1 ml cuvettes before and after reduction with potassium borohydride in a Bausch and Lomb Spectronic 505 spectrophotometer. Determinations of α-tocopherol were made on 0.5 ml of the ethanol solutions.

Plastoquinones, α-tocopherylquinone, ubiquinone and vitamin K_1, were identified in the samples by their characteristic absorption spectra before and after reduction with potassium borohydride. The quinone in each fraction was identified and estimated using the following extinction coefficients:

PQ molecular extinction coefficient (oxidised minus reduced) = 15,000 at 225 mμ,
α-TQ $E^{1\%}_{1\,cm}$ (oxidised minus reduced) = 397 at 262 mμ,
Q_{10} $E^{1\%}_{1\,cm}$ (oxidised minus reduced) = 142 at 275 mμ,
vitamin K_1 $E^{1\%}_{1\,cm}$ (oxidised minus reduced) = 237 at 269 mμ.

Chlorophyll was determined by the method of ARNON[23] in a solution of 80 % acetone.

α-Tocopherol was determined using an adaptation of the method of BOOTH[18]. The α-tocopherol standards and test solutions in ethanol were made to 2 ml with ethanol. 0.5 ml α,α-dipyridyl solution (0.15 g/100 ml ethanol) and 0.5 ml $FeCl_3$ solution (0.15 g/100 ml ethanol) were added and the mixture allowed to stand in the dark for exactly 5 min. The optical density of the solution was then determined at 520 mμ. A standard curve was produced for each set of determinations using known concentrations of α-tocopherol.

RESULTS

During preliminary experiments, lipids were extracted from leaves in acetone and transferred into hexane. The hexane solutions were washed with distilled water to remove acetone and non-lipid water-soluble contaminants. The results of these experiments were extremely variable (more than was expected by simple variation in the leaf), and it was thought that the extraction and washing technique might be responsible. On a number of occasions, R263 was discovered. R263 has been shown to be a breakdown product of PQB[21] suggesting that this technique is, in fact, causing some inaccuracy, at least in the determination of PQB. It was decided to try a method of extraction which did not require transferring the extract from one solvent to another and washing. The method developed, involved the freezing and grinding of the frozen leaf material with anhydrous disodium hydrogen orthophosphate. This was chosen to aid in the neutralisation of plant acids and to act as a grinding medium to obtain a fine leaf powder. The frozen, ground powder was lyophilized over-night in a refrigerator at 4° (in later experiments in a deep freeze at a temperature of —6°). The powder was easily extracted with cold acetone to remove most of the lipids and pigments. Chlorophyll determinations and column chromatography were then carried out as described under materials and methods.

Preliminary experiments were carried out, using a gradient eluting system, of increasing concentration of diethyl ether in hexane. From the results of these exper-

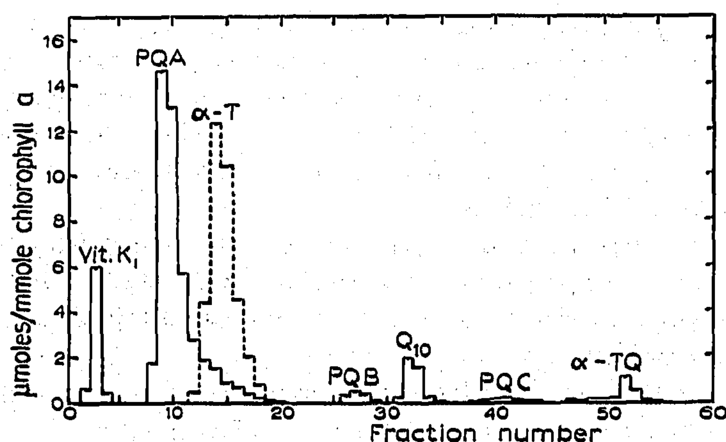

Fig. 1. Histogram showing typical elution pattern of quinones and α-tocopherol eluted from Kieselgel G–Celite column with diethyl ether in hexane solutions. Solid lines represent quinones determined by changes in U.V. absorption after reduction; dashed lines represent α-tocopherol determined by the α,α-dipyridyl–$FeCl_3$ method. Vit. K_1 = vitamin K_1; PQA, PQB and PQC = plastoquinones A, B, and C respectively; α-T = α-tocopherol; Q_{10} = coenzyme Q_{10}; α-TQ = α-tocopherylquinone.

TABLE I

QUINONES AND α-TOCOPHEROL IN FULLY MATURE LEAVES

Experiment	*Quantity applied to column*	*Quantity of each quinone and α-tocopherol eluted from column in µmoles/mmole chlorophyll "a"*							*Molar ratios α-T and α-TQ over PQA*	
	Chlorophyll "a" (mmoles)	*PQA*	*PQB*	*PQC*	*α-TQ*	*α-T*	Q_{10}	*Vit.* K_1	*α-T/PQA*	*α-TQ/PQA*
1	0.0170	46.03	1.95	6.15	9.16	—	1.03	—	—	0.192
2	0.0163	38.12	1.78	3.40	3.54	82.95	1.96	—	2.17	0.093
3	0.0166	47.03	7.03	6.34	3.01	64.15	4.06	2.32	1.36	0.064
4	0.0170	60.25	1.34	5.82	1.95	77.67	2.58	—	1.28	0.032
5	0.0146	63.38	3.75	4.81	2.98	63.81	3.04	4.39	1.00	0.044
6	0.0118	51.48	3.16	4.34	3.07	56.86	5.59	8.39	1.10	0.054
7	0.0174	46.30	3.53	2.47	1.34	40.14	4.33	7.40	0.86	0.028
8	0.0165	54.63	4.20	4.29	2.83	40.11	5.80	8.31	0.73	0.051
9	0.0172	52.80	3.96	4.62	3.08	52.97	4.17	6.86	1.00	0.058
Average	0.0160	51.11	3.38	4.69	3.44	59.83	3.62	6.28	1.06	0.068

TABLE II

QUINONES AND α-TOCOPHEROL IN YOUNG LEAVES

Experiment	*Quantity applied to column*	*Quantity of each quinone and α-tocopherol eluted from column in μmoles/mmole chlorophyll "a"*							*Molar ratios α-T and α-TQ over PQA*	
	Chlorophyll "a" (mmoles)	*PQA*	*PQB*	*PQC*	*α-TQ*	*α-T*	Q_{10}	*Vit.* K_1	*α-T/PQA*	*α-TQ/PQA*
10	0.0156	52.94	1.39	1.52	1.79	33.67	4.14	7.58	0.63	0.033
11	0.0144	43.62	1.36	0.60	2.14	34.97	4.09	7.13	0.80	0.049
12	0.0173	35.47	0.84	1.16	5.07	29.87	3.78	2.54	0.84	0.114
17	0.0175	43.40	0.55	1.60	4.15	42.00	4.98	5.68	0.99	0.095
Average	0.0162	43.86	1.04	1.22	3.29	35.13	4.25	5.76	0.82	0.073

TABLE III

QUINONES AND α-TOCOPHEROL IN MATURE LEAVES ESTIMATED AFTER ELUTION FROM A WIDE (2 CM) COLUMN

Experiment	*Quantity applied to column*	*Quantity of each quinone and α-tocopherol eluted from column in μmoles/mmole chlorophyll "a"*							*Molar ratios α-T and α-TQ over PQA*	
	Chlorophyll "a" (mmoles)	*PQA*	*PQB*	*PQC*	*α-TQ*	*α-T*	Q_{10}	*Vit.* K_1	*α-T/PQA*	*α-TQ/PQA*
13	0.0414	55.79	2.82	3.46	4.69	37.19	4.63	9.50	0.67	0.084
14	0.0613	52.00	1.58	1.80	0.83	52.10	5.07	7.12	1.00	0.015
15	0.0928	44.00	1.36	1.42	0.98	59.01	3.60	4.35	1.11	0.022
16	0.0783	41.39	2.25	—	3.28	60.28	3.60	6.80	1.46	0.079
Average	0.0685	48.30	2.00	2.23	2.42	52.15	4.38	6.94	1.06	0.050

iments four concentrations of diethyl ether in hexane were chosen: 0.5 %, 3.0 %, 10 % and 20 %. The optimum volume of these, size of fractions and size of column were chosen to give the best and most rapid separation of the quantity of extract to be determined.

Rechromatography of PQA and α-tocopherol using this system has resulted in recoveries of greater than 90 %, suggesting that there is very little, if any, loss by breakdown on the column.

Fig. 1 is a histogram of a typical separation using these methods. Although PQA and α-tocopherol overlap, they are determined by different techniques which do not interfere with each other. Clear separation of vitamin K_1, PQA, PQB, Q_{10}, PQC and tocopherylquinones has been obtained. No separation of the individual tocopherylquinones has been obtained, but as α-tocopherylquinone occurs in higher concentrations than β- and γ-tocopherylquinones, the tocopherylquinones will be referred to as α-TQ.

Table I lists a number of determinations made on leaves extracted and assayed in this way. All of the leaves are from 3–4 week-old broad bean plants and are fully mature. Samples containing approximately the same amounts of chlorophyll were applied to the columns. The values of PQA, PQB and PQC, while varying, are within the variation that may be expected of heterogeneous samples of leaves harvested at different times. It should also be noted that any variation in chlorophyll content will be reflected throughout the determinations.

Table II lists the results of experiments conducted on young leaves which were just fully expanded. The PQA concentrations show a slight increase in older leaves. The differences in concentrations of α-TQ, Q_{10} and vitamin K_1 are not consistent, suggesting that the variations in concentration of these are not directly related to age. α-Tocopherol, however, increases considerably with the age of the leaf.

Table III represents the results obtained in attempting to increase the yield of quinones by using wider columns. The results indicate that it is possible to use larger columns and apply larger quantities of material while still obtaining results comparable with the smaller columns. Mature leaves were used in these separations. With wide columns, however, larger quantities of eluting solvent and an increase in eluting time are necessary, making the method less suitable for quantitative determinations.

DISCUSSION

The extraction and column chromatography methods presented, represent new methods of quinone and α-tocopherol separation capable of use for quantitative determination of these lipids. The accuracy of all methods of separation and determination seem, at the moment, to be questionable. Many chemical changes may occur during extraction and manipulation before, during and after chromatography. This method, it is hoped, is an improvement on the existing techniques while not claiming to provide a perfect system.

In the present studies, using the final method presented, 3 plastoquinones were separated, each with similar absorption spectra before and after reduction. These plastoquinones are presumably identical to PQ's identified by BUCKE AND HALLAWAY[24] and BARR AND CRANE[25] in *Vicia faba*.

Table IV contains a summary of some of the results of PQ, α-TQ and α-tocopherol contents of *Vicia faba* leaves so far published. The results are compared with those found in the present study.

The levels of PQA found in this study are similar to those found by BUCKE *et al.*[24,26], whereas those of BARR AND CRANE are approximately five times greater. While these differences may be due to growth conditions, variety of broad bean and age differences, this would seem unlikely.

TABLE IV

PQ'S, α-TQ AND α-TOCOPHEROL FROM *Vicia faba* LEAVES

All values converted to μmoles/mmole chlorophyll from original published figures where necessary.

Reference	*PQA*	*PQB*	*PQC*	*α-T*	*α-TQ*
BUCKE *et al.*[24,26]	29	—	0–18	trace	22
BARR AND CRANE[25]	135	9	3.8	—	—
Present study	29–34	0.7–2.3	0.8–3.1	20–62	0.5–6.1

The levels of PQB and PQC in *Vicia faba* are extremely low resulting in difficulties during assay. The levels for PQB reported by BARR AND CRANE are maximal and may be much lower. In the case of PQC the variation may be due to growth conditions. BUCKE AND HALLAWAY report an inverse relationship between PQC and α-TQ grown throughout the year. During the winter months the PQC content fell to levels difficult to detect. However, BARR AND CRANE found little variation under different light and temperature growth regimes. PQC contents during the present study increased considerably with age.

Leaves used by BUCKE *et al.* contained only traces of detectable α-tocopherol whereas in the present study fairly large quantities of α-tocopherol were detectable at all times. The quantities of α-TQ, however, detected by BUCKE *et al.* are considerably higher than the quantities reported here. Since α-TQ may be formed by the oxidation of α-tocopherol, it is possible that interconversion of these two compounds may occur either prior to or during extraction and separation.

While it is possible that the variations in quinone and α-tocopherol levels may be due to differences in variety of bean, growth conditions and age, they are also likely to be caused by differences in experimental method. In evaluating the function of these lipids in the chloroplast and photosynthesis, it is essential that accurate estimates of quantities present are known and that more accurate and reliable methods of determining these are available.

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