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IDENTIFICATION OF PLASTOQUINONE-C IN SPINACH AND MAPLE LEAVES BY REVERSE-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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Key Word Index—*Spinacia oleracea*; Chenopodiaceae; *Acer pseudoplatanus*; Aceraceae; HPLC; plastoquinone; thylakoid.

Abstract—To reinvestigate whether the plastoquinone-C (PQ-C) identified in chloroplasts in the 60's is a natural component or an isolation artefact as suggested by some authors, we used a gentle and fast extraction procedure followed by direct RP–HPLC separation of the whole leaf extract in a solvent system which enables separation of both the reduced and oxidised forms of plastoquinone-A (PQ-A, PQ-9) and PQ-C in the presence of photosynthetic pigments. We found that PQ-C in spinach occurs at 3–4% of the amount of PQ-A and in maple at 10% of the PQ-A amount both in illuminated as well as dark-adapted leaves. However, after oxidation of the extract with ferricyanide, the amounts of PQ-C increased to 31.5% and 18.5%, respectively. These results indicate that, regardless of the light conditions, PQ-C is present in chloroplasts mostly in the reduced state. PQ-A was found predominantly in the reduced form in dark-adapted leaves which was attributed to the dark-reduction of PQ-A by chloroplast ascorbate. The possible source of discrepancies existing in the literature about the PQ-C content in chloroplasts is discussed.

INTRODUCTION

Apart from plastoquinone A (PQ-A, PQ-9), whose role in photosynthesis is well characterised [1, 2], other plastoquinones (PQ-B and C) were found in chloroplasts in the 60's mainly by Barr and Crane [3, 4] and several papers were devoted to their taxonomic distribution [5–7], concentration in chloroplasts [8, 9], function in photosynthetic electron transport [10] and structure [11]. It was found that PQ-C is a PQ-A derivative with a hydroxyl group in the isoprenyl side chain [11] and PQ-B is a fatty acid ester of PQ-C. Both plastoquinones could be resolved into six isomers (PQ-B_{1-6} and PQ-C_{1-6}) by thin-layer chromatography [8], differing probably by the position of the hydroxyl group in the isoprenyl side chain. However, Lichtenthaler suggested [12, 13] that PQ-B and PQ-C are not regular components of a functional photosynthetic apparatus, but are artefacts arising during lipid extraction and chromatography or showing up only in extracts of old leaf tissue. Since the development of HPLC methods, only a few papers have been published on the prenylquinone content of chloroplasts [14–16]. These studies reported that plastoquinones B and C were undetectable in leaf extracts or were detected in lower amounts than originally determined in the 60's. To reinvestigate this discrepancy, we prepared young (Spring) leaf extracts using fast and gentle extraction method, without solvent partition steps where some polar compounds could be lost, and analysed the total extract by HPLC without earlier preseparation which, potentially, could also be the reason for polar prenyllipid losses. The solvent system used permitted simultaneous determination of both the reduced and oxidised forms of PQ-A, as well as the oxidised form of PQ-C with good resolution from the pigments and enabled application of large amounts of the extract without losing resolution. Since the plastoquinones could be present in the reduced form, we also performed the HPLC analysis after prior oxidation of the extract with ferricyanide (FeCy).

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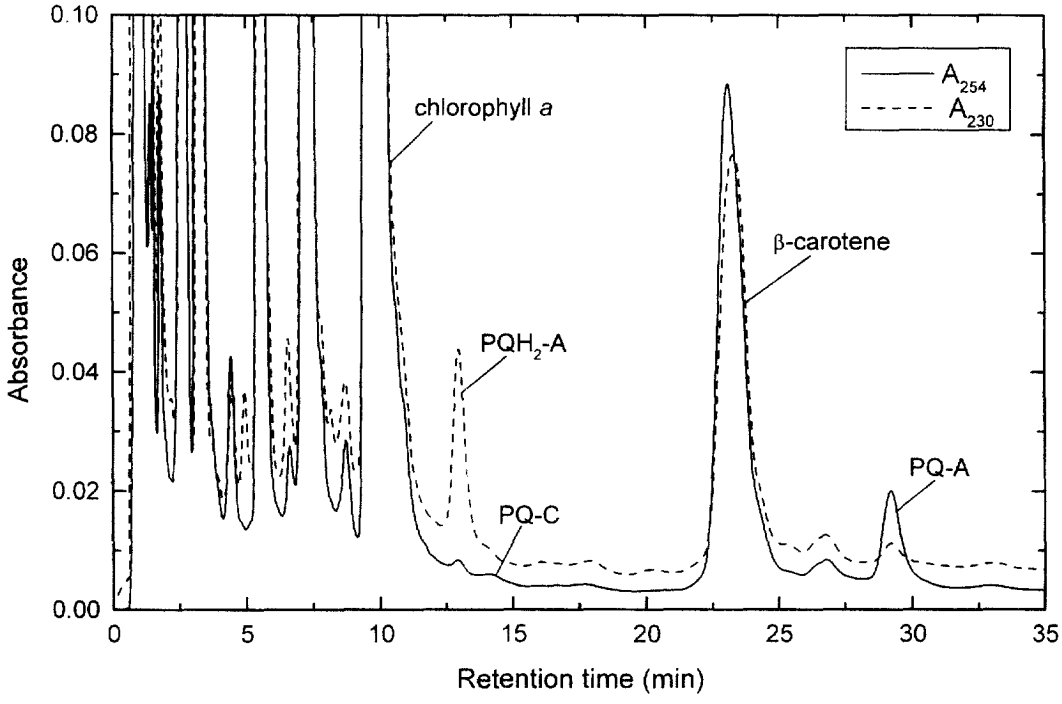

Fig. 1. RP–HPLC of the acetone extract from dark-adapted spinach leaves.

RESULTS AND DISCUSSION

The solvent system used enables detection and resolution in a single run of the photosynthetic pigments (chlorophylls and carotenoids) as well as the reduced and oxidised forms of PQ-A (Figs 1 and 2), so direct determination of the ratio of PQ-A to chlorophylls (Table 1) is possible, knowing the molar absorption coefficients: $\epsilon = 17.94\ mM^{-1}\ cm^{-1}$ for PQ-A [17] and $23\ mM^{-1}\ cm^{-1}$ for chlorophyll *a*, both at 254 nm. The values obtained in Table 1 agree with the literature data [8, 9, 14]. To our

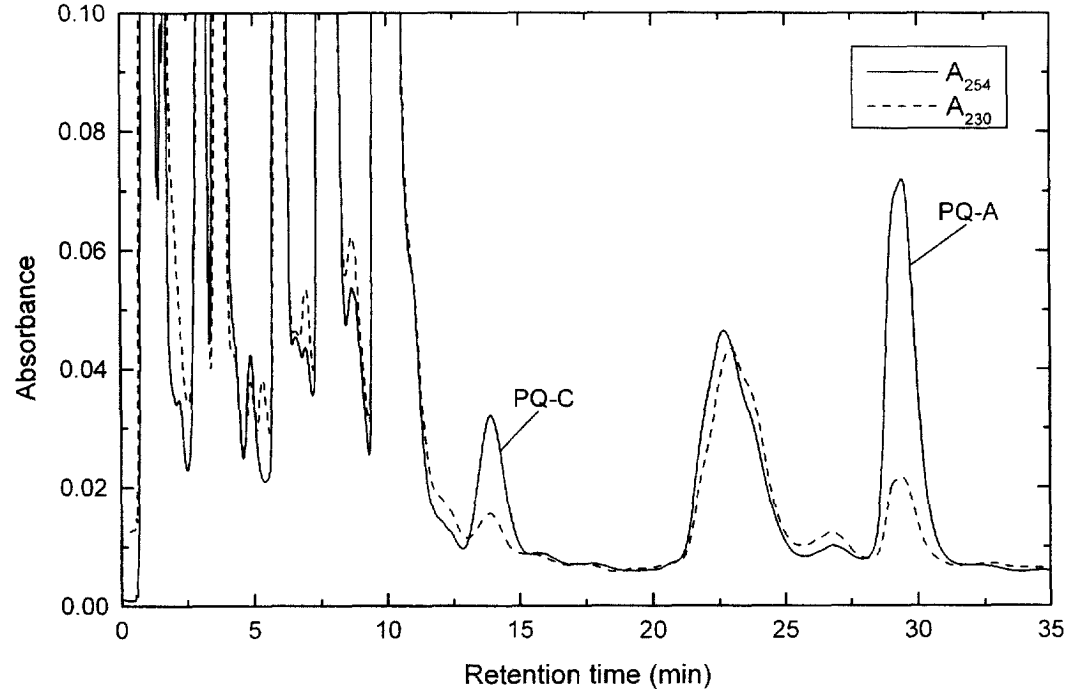

Fig. 2. RP–HPLC of the acetone extract from dark-adapted spinach leaves oxidised with FeCy.

Table 1. Molar ratios of PQ-A (total = oxidised + reduced) and chlorophyll (total = a + b), and chlorophyll a/b ratios in spinach and maple leaves.

	$chl_{tot}/PQ\text{-}A_{tot}$ (mol/mol)	$PQ\text{-}A_{tot}/chl_{tot}$ (mol/100 mol)	chl a/b
Spinach	17.3	5.8	2.95
Maple	16.5	6.1	2.22

knowledge, this is the first system where both forms of PQ-A could be detected simultaneously by an optical detection method. Their ratio (reduction state) could be calculated using $\epsilon = 17.94\ mM^{-1}\ cm^{-1}$ for PQ-A and $4.91\ mM^{-1}\ cm^{-1}$ for PQH_2-A at 230 nm (Table 2). The use of detection at 254 and 230 nm enables identification of prenylquinone and prenylquinol compounds in a complex mixture and estimation of the purity of the separated compounds since the prenylquinones show a high A_{254}/A_{230} ratio (e.g., 4.6 for PQ-A standard sample), and prenylquinols show a high A_{230}/A_{254} ratio (e.g., 11.8 for PQH_2-A standard sample), while chlorophylls or carotenoids show similar absorbance values at these two wavelengths. In our pigment separations, the A_{254}/A_{230} ratio for PQ-A was 4.5–4.6 and the A_{230}/A_{254} ratio for PQH_2-A was 4.5 or higher (Figs 1 and 2).

The PQ-C peak was identified by comparing the R_fs of the peaks obtained for leaf extracts with the R_f of a standard PQ-C sample. In addition, the high A_{254}/A_{230} ratio of the PQ-C peak also confirms its prenylquinone character (Fig. 2). The R_fs of PQ-C and other standard prenyllipids in the HPLC system are given in Table 3. We have found that the R_fs of all compounds increased slightly in FeCy treated samples probably because of trace amounts of FeCy present in the extract applied on the column. FeCy is slightly soluble in acetone and ethanol and when present in the extract it may increase the polarity of the solvent. Our PQ-C standard sample ran on HPLC as a single band without resolving into the individual PQ-C isomers, while when run on TLC plates (bezene–heptane, 3:2) it separated into two bands ($PQ\text{-}C_{1\text{–}4}$ and $PQ\text{-}C_{5,6}$).

Table 2. PQ-C and PQ-A molar ratio, the redox state of PQ-A and PQ-C in light-adapted (LA), dark-adapted (DA) and DA spinach and maple leaves after oxidation of the extract with FeCy.

	PQ-C/PQ-A (%PQ-C)	$PQ\text{-}A/PQ\text{-}A_{tot}$ (% oxidised)	$PQ\text{-}C/PQ\text{-}C_{tot}$ (% oxidised)
Spinach—LA	3–4	11.5	1.3–2.2
Spinach—DA	3	17.2	1.5
Spinach—DA + FeCy	31.5	100	100
Maple—LA	10.2	29	22
Maple—DA	9.8	41.5	24
Maple—DA + FeCy	18.5	100	100

Table 3. R_fs of chloroplast prenyllipids on LiChrosorb RP-18 column for the solvent system used (see Experimental).

Prenyllipid	R_f (min)
α-Tocopherol quinone	5.5
α-Tocopherol	7
Vitamin K_1	10
PQH_2-A	13
PQ-C	14
PQ-B	22–25
PQ-A	29

By applying larger amounts of the extract to the HPLC column, we could increase the detection limit of PQ-C in our preparations to about 1 PQ-C/3500 chlorophyll molecules, equal to 1 PQ-C/200 PQ-A molecules which corresponded to 45 ng PQ-C in the sample. Since the absorption spectrum of PQ-C (and also PQ-B) is identical with that of PQ-A, and all the plastoquinones have the same chromophore, it was assumed that the extinction coefficient of PQ-C was the same as for PQ-A.

The relative amount of PQ-C (PQ-C/PQ-A ratio) in both dark-adapted (DA) (Fig. 1, Table 2) and light-adapted (LA) (Table 2) spinach leaves was small and accounted for 3–4% the amount of PQ-A. In DA (Table 2) and LA (Table 2) maple leaves this proportion was higher (~10%). However, the PQ-C content in the oxidised extracts of DA spinach and maple leaves (Fig. 2 and Table 2) accounted for 31.5% and 18.5% of the PQ-A amount, respectively (Table 2). These values represent the ratio of the total (reduced and oxidised) amounts of both plastoquinones in the extracts. The evidence that FeCy treatment completely converted the reduced forms of the prenylquinones to their oxidised forms was provided by the absence of PQH_2-A peak in the FeCy oxidised samples (Fig. 2). At the same time, the amount of PQ-A increased quantitatively. The low PQ-C content in the LA and DA samples as compared to their contents in FeCy treated samples indicated that most of the PQ-C occurs in the reduced form in thylakoids, especially in spinach and its redox state is more or less independent of the light or dark conditions. PQ-A was present mostly in the reduced state even in DA samples, where it was expected to be mostly in the oxidised state. Exposure to light caused a partial photoreduction of the PQ-A pool (7–10%), as expected (Table 2). The reason for the high level of PQ-A reduction in DA leaves may be due to the possibility that, in intact chloroplasts, it is kept in the reduced state by ascorbate which occurs in chloroplasts in high concentrations and might directly reduce the PQ-A pool. The fast extraction procedure used gives the redox state of prenyllipids as it is in chloroplasts, while the extraction of isolated thylakoids in the absence of ascorbate, the method used very often, could result in pronounced

oxidation of the PQ-A pool during this step, which is usually carried in the dark, before extraction. The reduction of PQ-C by ascorbate in chloroplasts could explain the high proportion of PQH_2-C in our determinations.

The highly reduced state of PQ-C might be the main reason for its apparent absence or its low concentration found by many authors. Without prior oxidation of the extract with FeCy, the PQ-C level is very low and hard to detect. In many solvent systems, it is probably masked by the chlorophylls because of its similar polarity and when the pigments are preseparated from the prenylquinones by column chromatography, PQ-C is probably lost, at least partially, together with the pigments. The reduced form of PQ-C, because of its high polarity migrates in the pigment region and it would be very difficult to detect it directly on a chromatogram. The frequently used organic solvent/water partitioning of the extract preparations could be also the source of losses of polar compounds. It is known that prenylquinols are sensitive to oxidation and it is often assumed that during the isolation procedure most of the reduced forms are converted to the oxidised forms. However, it is very often not the case. In another experiment, we followed a longer extraction procedure, using non-deaerated solvents [14] and found that not more than 10% of the starting PQH_2-A was oxidised during the procedure. The occurrence of PQ-C, mostly in the reduced form, indicates that it cannot be an artefact formed from PQ-A during the extraction/separation procedure. We have also checked if the FeCy treatment of PQH_2-A gave any products other than PQ-A. On HPLC, the only detectable product was PQ-A (data not shown). It is known that the prenyllipids, apart from being present in the thylakoid membrane, are found in osmophilic globuli [18] which are more abundant in older leaves. Since our extracts were obtained from whole leaves, there exists the possibility that part of the plastoquinones could come from the plastoglobuli. However, the PQ-A/chlorophyll ratios obtained in our study, which are in the range of the values obtained by others for thylakoids [14, 18] and the fact that the extractions were made on relatively young leaves, makes such a possibility rather unlikely.

We tried to identify PQ-B on the chromatograms. However, its R_f in the solvent system used (broad band between 22–25 min) is in the range of the R_f of β-carotene (Fig. 1, Table 3). For its separation, another solvent system is required or the use of normal phase chromatography. Okayama [15], using a solvent system similar to ours and electrochemical detection, was able to detect PQ-B in spinach extracts, although in smaller amounts than PQ-C.

The results of our studies show that, by using a gentle extraction method and direct HPLC separation of the total pigment extract, it is possible to detect the presence of PQ-C in spinach and maple leaves which amounts to 31.5% and 18.5% of the PQ-A amount, respectively. Moreover, PQ-C was found to be mostly in the reduced state.

The presence of significant amounts of PQ-C in thylakoid membranes raises the question of its possible function in the photosynthetic electron transport. The older data suggested its considerably higher activity as an electron acceptor compared to PQ-A in some systems [10, 19]. Our finding that PQ-C occurs mostly in the reduced form suggests that it might be the product of photoreduction.

EXPERIMENTAL

Spinach and maple leaves (*Acer pseudoplatanus*) were collected in May/June and extracted with cold Me_2CO with or without addition of solid $CaCO_3$. The extraction was performed both on dark-adapted (2–3 hr) and light-adapted leaves (2 hr, white fluorescent light, 100 $\mu E/m^2/s$). The extracts were evaporated on a rotatory evaporator. The remaining water in the extracts was removed by the addition of small amounts of EtOH and evaporation. Then the dry residue was dissolved in a small vol of Me_2CO, the chlorophyll concentration determined and an aliquot evaporated to dryness and dissolved in 0.5 ml MeOH–aq. 95% EtOH (3:2) to give a chlorophyll concentration of 2 mM. Subsequently, 100 μl of this soln was applied to the HPLC column. Samples treated with potassium ferricyanide to oxidise the reduced forms of prenylquinones were prepared as follows. After evaporation of the Me_2CO extract, 20 ml EtOH was added and 5 ml of 5 mM FeCy soln was gradually added to the ethanolic pigment soln with stirring, to give a final FeCy concentration of 1 mM. The mixture was then evaporated to dryness and treated as before. All the solvents used for the extraction were bubbled with N_2 before use and the extraction and sample preparation was performed under dim light.

The extracts were separated on a reverse phase LiChrosorb column RP-18 (Merck, 250 × 4 mm, 7 μM) at the flow rate of 2.5 ml/min, using the following solvent gradient: MeOH–aq. 95% EtOH (3:2) (solvent A) containing decreasing amounts of water (solvent B), 0–5 min, 10% H_2O to 3%; 5–20 min, 3% H_2O; 20–25 min, 3% H_2O to 0.5%; 25–35 min, 0.5% H_2O to 0%.

Optical detection was performed simultaneously at two wavelengths, 230 and 254 nm (Pharmacia LKB instrument).

Standard samples of PQ-A, PQ-B and PQ-C were isolated from lyophilised maple leaves (*A. pseudoplatanus*) by petrol (b.p. 40–60°) extraction, cc on silica gel (Merck) in benzene–heptane (17:3) or benzene, followed by TLC on silica gel plates (Merck) in $CHCl_3$ or $CHCl_3$–MeOH (100:0.5) for

PQ-C separation, and benzene–heptane (3:2) for PQ-A and PQ-B purification.

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