Reversed-Phase High-Performance Liquid Chromatography of Prenylquinones in Green Leaves Using an Electrochemical Detector

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Ubiquinone-9, -10, plastoquinone-A, -B, -C, phylloquinone and α-tocopherolquinone in spinach leaf extract were separated and determined by reversed-phase high-performance liquid chromatography using an electrochemical detector. These prenylquinones were eluted with a mixture of ethanol and methanol containing 50 mM NaClO₄ and 2 mM HClO₄ from an octadecyl silica column. The electrochemical detector could selectively detect the quinones in the eluate, and enabled to determine even the minor quinones such as PQ-B and PQ-C which had not been evaluated by HPLC with an optical detector. The method is simple and sensitive to the degree that amounts of prenylquinones could be determined as low as 0.1 nmol.

Key words: HPLC — Phylloquinone — Plastoquinone — Prenylquinone — Tocopherolquinone — Ubiquinone.

Cells of green leaves contain several quinones, such as PQ, TQ and KI in the chloroplasts, and Q in the mitochondria (Barr and Crane 1971, Morton 1971). These quinones have usually been determined spectrophotometrically after separating each quinone by ordinary liquid column, thin layer, or paper chromatographies. Recently, Lichtenthaler and Prenzel (1977), Prenzel and Lichtenthaler (1979, 1982), and Lominski and Rienits (1981) reported the separation of PQ-A, KI and TQ by HPLC. They determined the quinones by the absorbance at 254 nm. However, there are too many compounds in the extracts of chloroplasts which strongly absorb ultraviolet light to detect the minor quinones such as PQ-B and PQ-C by the optical method. Ikenoya et al. (1979) reported the application of an electrochemical detector to HPLC of TQ, Q and KI in blood. The detector reveals only electroactive or redox substances, and so is highly specific for quinones. Okayama (1983) applied the electrochemical detector for the analysis by HPLC of quinones in Photosystem II particles.

In this paper, details of the method for the quantitative separation of Q-9, Q-10, PQ-A, PQ-B, PQ-C, TQ and KI extracted from spinach leaves using HPLC equipped with an electrochemical detector will be presented.

Materials and Methods

Prenylquinones for the HPLC-analysis were extracted from 5 g of spinach leaves with 50 ml of acetone for 5 min in a Waring blender. Homogenates were centrifuged at 1,000 × g for

Abbreviations: HPLC, high-performance liquid chromatography; KI, phylloquinone; PQ, plastoquinone; TQ, α-tocopherolquinone; Q, ubiquinone.
5 min. Residues were successively extracted with 50 ml each of acetone, diethyl ether and petroleum benzine (boiling range between 50 and 90°C). Twice the volume of distilled water was added to the combined extracts. The lower water phase was discarded, and the upper ether-benzine phase was washed thoroughly with distilled water and then evaporated to dryness at 35°C in vacuo. Dried extracts were dissolved in 5 ml of petroleum benzine and applied on an alumina column (1.5 × 4 cm), which was built with Merck's acidic aluminium oxide deactivated with 8% distilled water and washed thoroughly with petroleum benzine before use. Quinones together with carotenoids were eluted successively with 20 ml each of 10, 20 and 30% diethyl ether in petroleum benzine (v/v). Combined eluates were evaporated to dryness at 35°C, and then dissolved in 5 ml of ethanol.

The prenylquinones used as authentic standards were extracted with acetone and then petroleum benzine from spinach leaves (1 kg). The extracts were purified by chromatography on the deactivated alumina column (4 × 15 cm) with mixtures of ether and petroleum benzine (from 0 : 1 to 1 : 3, v/v) as eluents, and also on a Lobar column (LiChrophrep RP-8 type, Merck) with methanol, or a mixture of methanol and ethanol (4 : 1, v/v) as eluents.

HPLC was run on a Waters Assoc. Model 294 compact type instrument with a Radial-PAK Cartridge C-18 column. The ultraviolet detector was set at 254 nm. A Yanaco VMD-101 electrochemical detector (Yanagimoto Manufacturing Co., Japan) was set at −0.25 or −0.30 V against an Ag/AgCl reference electrode. The electrochemical detector was connected to the outlet of the ultraviolet detector. The mobile phase was a mixture of methanol and ethanol (3 : 1 or 3 : 2, v/v) containing 50 mM NaClO4 and 2 mM HClO4 at a flow rate of 2.0 ml min⁻¹.

**Results and Discussion**

Fig. 1 shows the profile of HPLC of quinones purified from spinach leaves. TQ (peak 1), KL (peak 2), PQ-C1–4 (peak 3), PQ-C5,6 (peak 4), PQ-B (peak 5), PQ-A (peak 7), and Q-10 (peak 8) are well separated from each other. Curves a and b in Fig. 1 are the chromatograms obtained using the electrochemical detector and the optical detector, respectively. At the measuring wavelength of the optical detector (254 nm), the absorbance coefficients are lower for ubiquinones (peaks 6 and 8 in curve b) than for other quinones (cf. Barr and Crane 1971). The potential of the working electrode of the electrochemical detector was set at −0.3 V against Ag/AgCl reference electrode. The calibration curves constructed by plotting the peak currents or the absorbances against the amount of the quinones were linear over the tested range from 0.1 to 10 nmol.

Fig. 2 shows the hydrodynamic voltammograms for PQ-A (curve a), PQ-C1–4 (curve b) and TQ (curve c), which were constructed by plotting the peak reductive currents against the potentials applied on the working electrode. The voltammograms for Q-9, Q-10 and PQ-B were almost the same as the one for PQ-A. PQ-C5,6 and KL showed similar voltammograms for PQ-C1–4 and TQ, respectively. The peak currents, or the maximum responses of the detector occurred at −0.25 or −0.30 V.

The reductive half-wave potentials obtained hydrodynamically for the quinones are summarized in Table I together with their typical retention times. The retention times for quinones could be adjusted by changing the ratio of methanol and ethanol depending on the condition of the HPLC-column. After a column is used many times, a better separation can be obtained by increasing the ratio of methanol. Addition in the mobil phase of more HClO4 than used in this study shifted the reduction potentials to more positive values. At concentrations of HClO4 higher than 2 mM, however, some components, possibly carotenoids, in the extracts of leaves were degraded to give substances which interfered the separation and detection of the quinones.
HPLC of prenylquinones in green leaves

Fig. 1 HPLC of a mixture of quinones purified from spinach leaves. Quinones (1 nmol each) were eluted from a Radial-PAK Cartridge C-18 column with a mixture of methanol and ethanol (3:2, v/v) containing 50 mM NaClO₄ and 2 mM HClO₄ at a flow rate of 2 ml min⁻¹, and detected either with the electrochemical detector set at −0.3 V vs. Ag/AgCl electrode (curve a), or with the optical detector set at 254 nm (curve b). (1) TQ, (2) K, (3) PQ-C₁₋₄, (4) PQ-C₅₋₆, (5) PQ-B, (6) Q-9, (7) PQ-A, (8) Q-10.

Fig. 2 Hydrodynamic voltammograms for quinones purified from spinach leaves. PQ-A (curve a), PQ-C₁₋₄ (curve b) and KI (curve c), 0.2 nmol each, were eluted from a Radial-PAK Cartridge C-18 column with the mixture of methanol and ethanol (3:1, v/v) containing 50 mM NaClO₄ and 2 mM HClO₄ at a flow rate of 2 ml min⁻¹.

Fig. 3 shows the chromatograms of the extracts of spinach leaves before (A) and after (B) the pretreatment with the deactivated alumina column. Curve b in Fig. 3 A and B are the chromatograms obtained using the optical detector. Peaks 7 and 10 in curve b were identified as PQ-A and β-carotene, respectively. Other peaks (not numbered) were composed of several substances which strongly absorbed ultraviolet light, and concealed the peaks of Q-9, PQ-B, PQ-C, KI and TQ. Thus it is practially impossible to determine these quinones by the optical method. In contrast, when the electrochemical detector was used (curve a in Fig. 3), quinone in each peak was able to identify. The component in peak 9 was identified to be chlorophyll a which showed a reductive half-wave potential of −0.3 V. After the pretreatment with the deactivated alumina column (curve a, Fig. 3 B), a better separation of KI and PQ-C₁₋₄ was
Table 1  Retention times and reductive half-wave potentials of quinones purified from spinach leaves

<table>
<thead>
<tr>
<th>Quinone</th>
<th>Retention time (min)</th>
<th>Half-wave potential (V vs. Ag/AgCl electrode)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TQ</td>
<td>3.4</td>
<td>-0.15</td>
</tr>
<tr>
<td>K</td>
<td>5.9</td>
<td>-0.17</td>
</tr>
<tr>
<td>PQ-C_{4,5}</td>
<td>8.0</td>
<td>-0.09</td>
</tr>
<tr>
<td>PQ-C_{1-4}</td>
<td>6.9</td>
<td>-0.08</td>
</tr>
<tr>
<td>PQ-B</td>
<td>10.9</td>
<td>-0.05</td>
</tr>
<tr>
<td>PQ-A</td>
<td>15.2</td>
<td>-0.05</td>
</tr>
<tr>
<td>UQ-9</td>
<td>11.9</td>
<td>-0.06</td>
</tr>
<tr>
<td>UQ-10</td>
<td>17.0</td>
<td>-0.06</td>
</tr>
</tbody>
</table>

Retention times and half-wave potentials were determined under the same conditions as in Fig. 2.

obtained by the removal of chlorophyll a, and the responses of the electrochemical detector for quinones, especially for TQ, was increased by the autooxidation of the quinones in extracts during the chromatography on deactivated alumina.

Incubation with ferric chloride at a final concentration of 1 mM for 20 min at 25°C in the ethanolic solution of spinach extracts did not affect on the responses of the electrochemical detector for the quinones other than TQ (data not shown). The response for TQ might be increased by conversion from a-tocopherol with ferric chloride (Gaunt and Stowe 1967). These facts suggest that any quinol or quinone in reduced form was autooxidized during the extraction.

Fig. 3  HPLC-chromatograms obtained using the optical detector (a) and the electrochemical detector (b) of quinone extracts from spinach leaves before (A) and after (B) pretreatment with an alumina column (see Materials and Methods). Other conditions were the same as in Fig. 1. (1) TQ, (2) K, (3) PQ-C_{1-4}, (4) PQ-C_{5,6}, (5) PQ-B, (6) Q-9, (7) PQ-A, (8) Q-10, (9) chlorophyll a, (10) β-carotene. Other peaks were not identified.
HPLC of prenylquinones in green leaves

and the pretreatment before HPLC. Thus the amount of the quinone determined by the present method indicates the contents of the total oxidized and reduced quinones in leaves.

In conclusion, the method described herein is a useful technique for the rapid separation and selective determination of prenylquinones in complex plant extracts.

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References


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