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NATURALLY OCCURRING QUINOLS AND QUINONES STUDIED AS SEMIQUINONES BY ELECTRON SPIN RESONANCE

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Key Word Index—*Pyrus*; Rosaceae; Juglandaceae; Oleaceae; hydroquinone; hydrojuglone; plumbagin; plastoquinol; phenolic acid; ESR.

Abstract—A simple procedure using electron spin resonance techniques was applied to detect, identify and quantify quinones and quinols in crude plant extracts. Hydroquinone was determined in *Pyrus*, plumbagin in *Drosera* and *Ceratostigma*, and hydrojuglone in Juglandaceae. Hydrojuglone is found in markedly higher concentrations in *Pterocarya* and in *Juglans* than in *Carya*. Plastoquinol has been observed in 500 of 700 plant extracts studied. Esters of phenolic acids are easily detected and distinguished, e.g. chlorogenic and rosmarinic acids. Esters of homoprotocatechuic and of dihydrocaffeic acid occur widely in the Oleaceae. The limitations of the method are discussed.

INTRODUCTION

In this paper we show that electron spin resonance spectroscopy (ESR) can be applied to the detection and identification of *ortho* and *para* quinols or quinones in plant extracts, without prior isolation. The compounds are converted to the corresponding semiquinone radicals, which are subsequently observed by ESR. Radicals of compounds lacking an *ortho* or a *para* dihydroxy grouping, e.g. resorcinols, monohydric phenols or phloroglucinols are not observed due to their short lifetimes. Non-phenolic compounds do not form paramagnetic species and are accordingly not recorded by the spectrometer. Only the semiquinone nucleus is identified by the procedure. For some compounds, however, this gives an absolute proof of identity, e.g. hydroquinone, toluquinone, juglone or methyljuglones. For other compounds, the semiquinone nucleus acts as a label, from which the class of compound present can be identified, e.g. plastoquinols or esters of certain phenolic acids. The identity of the prenyl chain in case of a plastoquinol or the alcohol part in case of an ester must be inferred from the ESR parameters characterizing the label.

RESULTS AND DISCUSSION

Hydroquinone

The simple quinol hydroquinone occurs in many *Pyrus* species [1]. Application of the present method, with aerial oxidation, to alcoholic extracts of leaves from 16 *Pyrus* species gives ESR spectra with five equidistant lines of relative intensity 1-4-6-4-1. Both the hyperfine splitting constant (2.36 Gauss) and the *g*-value (2.00471) are indentical to the values of the semiquinone obtained from an authentic sample of hydroquinone, and we therefore identify the semiquinone in the extracts as derived from hydroquinone. By means of a standard curve we may estimate the hydroquinone concentration from the intensity of the spectrum. For 16 of 17 *Pyrus* species, the concentrations were in the range 0.1 to 1.9 mg hydroquinone/g dry wt. In line with earlier results [1], no hydroquinone was detected in *P. calleryana* var. *faurei*. In addition to hydroquinone, arbutin is found in most *Pyrus* species; our investigation shows that hydrolysis of arbutin does not take place during the experiment. In fact, addition of

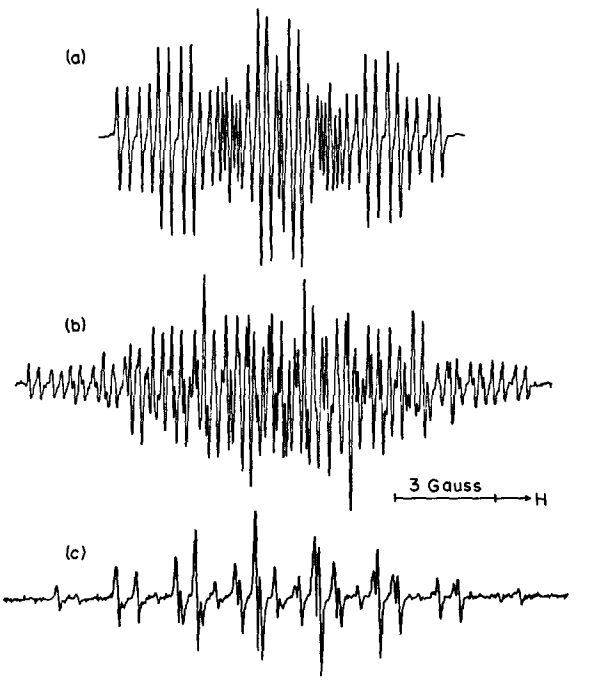
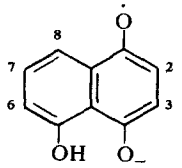


Fig. 1. ESR semiquinone spectra from crude alcoholic leaf extracts. (a) Spectrum of juglone semiquinone from *Pterocarya fraxinifolia* Spach. (b) Spectrum of plumbagin semiquinone from *Drosera binata* Labill. (c) Spectrum of plastosemiquinone from *Aesculus hippocastaneum* L.

Table 1. Hyperfine splitting constants (in Gauss) and g-values of some juglone derived semiquinones



	a_2	a_3	a_6	a_7	a_8	a_{OH}	g-value†
juglone	3.30	3.05	1.27	0.68	1.27	0.30	2.00429
2-methyljuglone	3.05*	2.33	1.23	0.68	1.34	0.28	2.00420
7-methyljuglone	3.11	3.11	1.10	0.71*	1.39	0.28	2.00426

* Splittings from methyl protons.

† The g-value or g-factor is determined by the ratio between the magnetic field and the microwave frequency. For a free electron $g = 2.00232$.

hydroquinone to the extracts gives an increase of the ESR signal, whereas addition of arbutin has no effect. A somewhat increased hydroquinone concentration, however, is observed in extracts, which were not boiled, indicating the presence of a specific glycosidase converting arbutin into hydroquinone.

Juglone and methyl derivatives

A spectrum of a semiquinone derived from a naturally occurring naphthoquinol is shown in Fig. 1a. The spectrum is obtained from leaf extracts of *Pterocarya fraxinifolia* Spach by oxidation. Spectra identical to this one have been obtained from leaves of a large number of Juglandaceae of the genera *Juglans*, *Pterocarya* and *Carya*. The g-value and the proton hyperfine splittings (Table 1) are indistinguishable from those of an authentic juglone semiquinone spectrum. We therefore interpret the spectrum in Fig. 1a as derived from hydrojuglone. The following concentrations were noted: *Pterocarya fraxinifolia*, *P. hubehensis*, *P. rhoifolia* and *P. stenoptera* from 1.7 to 3.0 mg hydrojuglone/g dry wt; *Juglans cathayensis*, *J. cinerea*, *J. draconis*, *J. nigra*, *J. regia*, *J. rupestris*, *J. sieboldiana* and *J. stenocarpa* from 1.4 to 2.9 mg; *J. hindsii* as well as *Carya laciniosa* and *C. tomentosa* showed a content of about 0.5 mg. From *C. cordiformis*, *C. glabra*, *C. ovalis* and *C. ovata* no juglone semiquinone spectra were observed. Thus, *Pterocarya* and *Juglans* species seem to contain hydrojuglone in markedly higher concentrations than *Carya* species.

A spectrum derived from a naturally occurring methyljuglone is seen in Fig. 1b, obtained from leaf extracts of *Drosera binata* Labill. An identical spectrum is obtained from both the leaves and the roots of *Ceratostigma plumbaginoides* Bunge. However, an alkaline reaction mixture containing the root extract yields the semiquinone spectrum only after addition of sodium dithionite. This indicates that the roots contain a methyljuglone, whereas the leaves contain the corresponding methylhydrojuglone. The methyl group is at C-2, since the semiquinone spectrum of an authentic sample of plumbagin (2-methyljuglone) is identical to Fig. 1b. Similarly, the leaves of *D. capensis* Thunb. give rise to a semiquinone spectrum derived from 7-methylhydrojuglone (Table 1).

Plastoquinol

Leaf extracts of about 500 of 700 plant species yield identical semiquinone spectra (Fig. 1c), exhibiting couplings from a single proton, a methylene group, and two methyl groups, all four substituents attached to a semiquinone nucleus. The splittings found to be 2.0, 2.4, 1.8 and 1.8 Gauss, strongly indicate that the semiquinone is derived from a plastoquinol [2]. Since plastoquinol-9 (2,3-dimethyl-5-nonaprenyl-1,4-dihydroxybenzene) is widely distributed in plants and, moreover, since this compound usually occurs in much larger amount than any other plastoquinol, it seems reasonable to identify the semiquinone of Fig. 1c as derived from plastoquinol-9. We have so far observed only one plastosemiquinone spectrum different from that of Fig. 1c. A reduced methylene splitting indicates the presence of a different prenyl chain in this case. Thus our method appears to have a potential for distinguishing different prenyl chains of plastoquinols.

The intensity of the semiquinone spectra (Fig. 1c) varies in a random fashion from extract to extract, reflecting variation of the plastoquinol concentrations in the plants. Since plastoquinol-9 functions in the electron transport system of chloroplasts in consort with its quinone counterpart, such variation is expected. Experiments with authentic plastoquinone-9 samples have revealed that the quinone part of the quinone-quinol system is not converted to the semiquinone state unless a reducing agent is added. The present method thus constitutes a technique for assaying plastoquinols in plants.

Phenolic acids and their esters

Universal amoung the angiosperms are many phenolic acids with a di- or a trihydroxy grouping, e.g. caffeic acid, protocatechuic acid, gentisic acid and gallic acid. They seldom occur as free acids but are esterified or bound as glycosides. The free acids and their esters are quinols and thus detectable by our procedure. Naturally occuring esters may be released from plant tissues by alkaline or acid hydrolysis, and the acidic and alcoholic parts detected separately. By our method, the esters or the free acids can be detected immediately in the extracts. The semiquinones of the free acids give rise to spectra easily distinguishable by their unique sets of coupling constants. These constants change when an acid is esterified. Accordingly, from a given spectrum we can identify the acid and are further informed whether the acid is esterified or not. For some acids, e.g. gallic acid, gentisic acid and protocatechuic acid, it is possible to distinguish esters of primary, secondary or tertiary alcohols, since the spectra exhibit varying numbers of lines. For other esterified acids, e.g. homoprotocatechuic acid and dihydrocaffeic acid, each alcohol gives rise to characteristic shifts of the semiquinone parameters making an indirect determination of the alcohol moiety possible. From an investigation of

60 species of Oleaceae, we have in this way observed and distinguished esters of homoprotocatechuic acid in *Olea*, *Phillyrea* and *Ligustrum*, and esters of dihydrocaffeic acid in *Fontanesia*, *Forsythia* and *Jasminum*; *Fraxinus*, *Syringa* and *Osmanthus* contain esters of both acids in appreciable amounts.

Some phenolic acids (esters) have a wide distribution throughout the plant kingdom, whereas others are confined to few families, e.g. chlorogenic acid as an example of the first kind and rosmarinic acid one of the second. The possibility that rosmarinic acid (caffeic esterified to 3,4-dihydroxyphenyl-lactic acid) has been mistaken for caffeic acid during chromatographic surways has been indicated [3]. Caffeic acid yields an ESR spectrum completely different from any of its esters, and chlorogenic and rosmarinic acids are unambiguously distinguished in our procedure. Fig. 2a shows a spectrum obtained from an extract of *Lycopus europaea* L. We identify the semiquinone as derived from rosmarinic acid since an identical spectrum is obtained from an authentic sample of this compound. Spectra identical to Fig. 2a have further been observed in extracts of one Apiaceae (*Sanicula europaea*), 11 Boraginaceae (*Anchusa*, *Borago*, *Echium*, *Lycopsis*, *Myosotis*, *Omphalodes*, *Pulmonaria*, *Symphytum*) and 7 Lamiaceae (*Glechoma*, *Lycopus*, *Mentha*, *Prunella*) our finding being in line with the detection of rosmarinic acid in these families [3, 4].

Fig. 2b shows a spectrum obtained from *Asperula odorata* L. Spectra identical to this have been observed from extracts of several plant species (35 genera of 16 families). Since an authentic sample of chlorogenic acid gives rise to a spectrum indistinguishable from Fig. 2b the many extracts, including the one giving rise to Fig. 2b, may contain this acid. The absolute identity of the compounds giving rise to the seemingly identical spectra are questionable, however, since compounds closely related to chlorogenic acid, e.g. cynarin (1,4-dicaffeylquinic acid) also yield semiquinone spectra similar to the one of Fig. 2b. For the cynarin semiquinone only a slight change in the lineshape of the spectrum can be recognized.

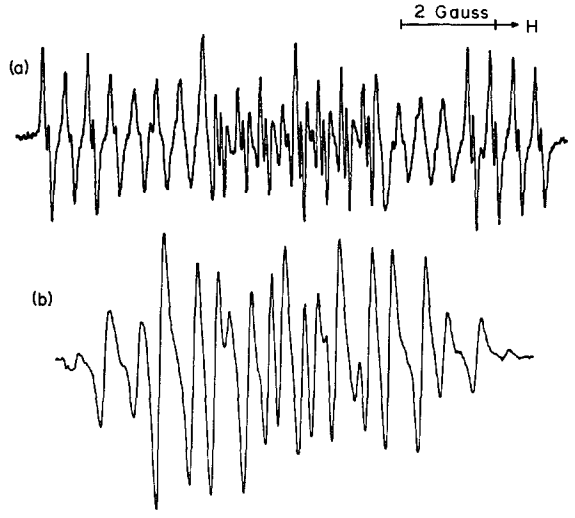


Fig. 2(a) ESR semiquinone spectrum of rosmarinic acid obtained from an alcoholic leaf extract of *Lycopus europaea* L. (b) ESR spectrum from an alcoholic leaf extract of *Asperula odorata* L. The spectrum is from a caffeic acid ester in the leaves presumably chlorogenic acid.

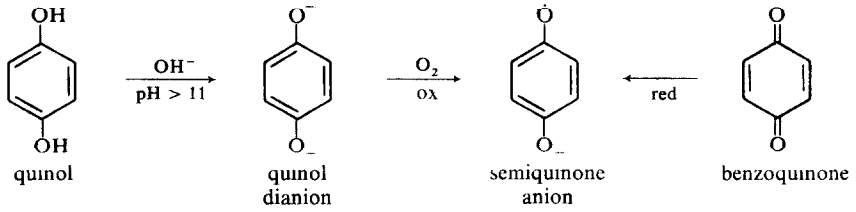
In studies of phenolic acids or their esters, two or three spectra often occur simultaneously and a thorough investigation requires careful spectra simulations as well as comparison with spectra of a large number of synthetic esters.

The present communication describes a rapid and simple procedure to study *ortho* and *para* quinols or quinones as contained in crude extracts. The modest requirement of material (50 μl of extract required for each run) makes the procedure suitable for phytochemical studies. The gentle treatment of the tissue, involving an extraction only, increases the possibility of observing genuine compounds, and errors in concentration measurements from loss of compounds during isolation are eliminated. However, the role of the alkali for the fate of the compounds must be carefully examined. Thus, hydroxylations of catechol compounds yielding products according to the scheme reported previously [5] have been observed. In case of absence of a particular compound, the role of contaminants in the extract must be considered. Furthermore, a strong signal may be masking a weaker one when compounds to be detected occur in concentrations at very different levels. The lower limit for detecting and confidently identifying a compound is in the μg range, the actual value being dependent on the compound in question. The limit may be improved a hundred fold by careful instrument settings.

The number of compounds usually identified in crude extracts ranges from 0 to 5. By a proper choice of the pH and an adjustment of the solvent composition the radicals normally can be made stable one at a time. It should be emphasized that observation of a semiquinone spectrum primarily yields parameters, by which a semiquinone nucleus is identified, and that this identification not automatically means an absolute proof of identity of the corresponding compound in the extract. It appears, however, that relatively few, closely related compounds, e.g. the different esters of a phenolic acid, give rise to spectra of a similar pattern. Thus, this semiquinone method may become a useful tool for the rapid detection and immediate identification, by pattern recognition, of quinols or quinones, whether contained in crude extracts or being available pure.

EXPERIMENTAL

The leaves were sampled from 1 August to 27 October 1976. Seasonal variations in the compounds studied were not considered. The fr. leaf material (0.1 to 5 g) was chopped, covered with EtOH, boiled for 30 sec and then left in the cold. ESR spectra were sometimes obtainable after 10 min of extraction, but extraction for 2 to 24 hr was the rule. The semiquinones were formed from quinols in alkaline solution by oxidation, the oxidizing agent being molecular oxygen. The process involves a prior dissociation of the hydroxyl protons and subsequent formation of the semiquinone radical (Scheme 1). The stabilization of the anion radical is determined by the presence of the alkali. For details of the mechanism see [5]. The procedure used was as follows: 50 μl of the extract was mixed with a reagent of a preselected composition. For plastoquinols addition of 5–10 μl of alkaline water (1 M in NaOH) usually produced the corresponding semiquinone. Other quinols were observed as semiquinones after addition of 20–30 μl of alkaline water (0.1 M in NaOH). Optimum stability of a particular semiquinone was found by varying the solvent composition (alcohol–water) and/or the NaOH concn about the above figures. For quinone detection, sodium dithionite was added to the alkaline water. The



Scheme 1.

spectra were run on a Varian E-3 or an E-15 ESR spectrometer in less than one min from mixing. The reaction mixture was contained in a microcapillary. Computer simulations were performed of all spectra as a check of the analysis. An account of these results was given in May 1977 at the VIth International Symposium on Magnetic Resonance, Banff, Alberta, Canada.

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