Development of a HPLC-ESR Spin-trapping System for Post-column On-line Detection of Superoxide Radical Scavenging Ability of Column Eluates

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A new HPLC-ESR spin-trapping system for post-column on-line detection of the superoxide radical scavenging activity of column eluates was developed. HPLC-ESR analysis are performed on a mixture of three phenolic compounds, gallic acid (Gal), 4-hydroxycoumaric acid (Cou), and caffeic acid (Caf), by monitoring ESR signal intensity of the superoxide radical adduct of 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO/O_2).

ESR spin-trapping technique has been applied for detection of short-lived free radicals using spin-trapping reagents, which react with radicals to form relatively stable radicals, called spin adducts.[1] On the basis of the hyperfine coupling constants (hfcc) of the spin adducts, the structure of the original radical is deduced.[2] DMPO is a spin-trapping reagent that is widely used in the detection of reactive oxygen radicals. In addition, DMPO has frequently been applied for screening of superoxide radical scavengers based on competitive reaction kinetic treatments.[3]

Recently, a flow-injection ESR system was used in the spin-trapping ESR measurements for the estimation of the second-order rate constants of the reaction between hydroxyl radical and scavengers.[4] In the present study, a HPLC-ESR system is developed for post-column screening of superoxide radical scavengers. A flow UV-irradiation cell is employed for the photoexcitation of riboflavin (Rf) to generate superoxide radical in the presence of an appropriate electron donor.[5] In order to estimate the scope and reliability of this system, HPLC-ESR analysis is performed on mixtures of three phenolic compounds, gallic acid (Gal), 4-hydroxycoumaric acid (Cou), and caffeic acid (Caf). The xanthine/xanthine oxidase reaction[3] which is widely used as a superoxide radical-generating system, is unsuitable for the present system, because phenolic derivatives are known to exhibit inhibit the enzymatic reaction.[6]

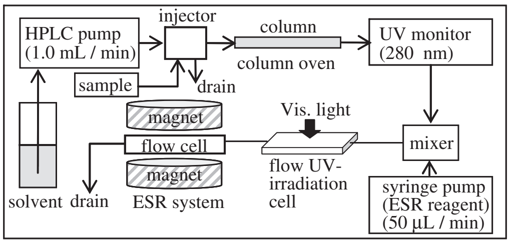

Figure 1. Instrumentation of the post-column on-line HPLC-ESR spin-trapping system.

As illustrated in Figure 1, the HPLC-ESR system comprises an X-band ESR spectrometer (JEOL, TE-30, operated by IPRIT-tsv. software) and a HPLC system (TOSOH 8020, monitored by absorbance at 280 nm). Sample solutions were injected through a 6-multiport valve (Rheodyne) equipped with a 20-μL injection loop. Post-column infusion of the ESR reagent was carried out using a magnetic-stirring mixer (Kyoto-spinlabo, inner volume 100 μL) and a syringe pump (Harvard Apparatus, HPD3000) equipped with a gas-tight syringe (Hamilton, 10 mL). The flow UV-irradiation cell (Kyoto-spinlabo, TFUV1, 4.0 mm × 50.0 mm × 0.2 mm i.d.) was installed between the mixer and a quartz flat-ESR cell (Wilmad). A halogen lamp (LA-100USW, Hayashi Watch-Works Co., Ltd.) was used for photoexcitation of Rf. HPLC analysis was conducted under isocratic conditions to keep the sensitivity of the ESR spectrometer constant. A TSK-gel-size exclusion column (TOSOH, G3000PW, 7.5 mm i.d. × 30 cm) was chosen because by using this column it is possible to separate phenolic molecules on the basis of their interaction with the column material.[7]

The following chemicals were used without further purification: ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA), Rf, Gal, Cou, and Caf (Wako Pure Chemical), DMPO (Labotec), and 4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPOL, Sigma Aldrich). NaH_2PO_4–Na_2HPO_4 buffer (0.1 M, pH 7.4) prepared with Milli-Q grade water (Millipore) was used for preparing the samples and the carrier solution. The concentrations of DMPO[8] and Rf[9] were verified by extinction coefficient 7800 $M^{-1}\,cm^{-1}$ at 228 nm and 12200 $M^{-1}\,cm^{-1}$ at 450 nm, respectively.

Under continuous flow of the carrier solution (1.0 mL min^{-1}), a solution of ESR reagent containing DMPO (210 mM), Rf (100 μM), and EDTA (10.5 mM) was infused (0.05 mL/min) to the micromixer. The flow-rate at the output port of the mixer was increased to 1.05 mL/min, and the final concentrations of DMPO, Rf, and EDTA were set to 10.0 mM, 4.8 μM, and 500 μM, respectively. The stream of the reaction solution passed through the UV-irradiation cell in 2.4 s and reached the ESR cell after 0.7 s. The column-separated compound reached the ESR cell from the UV monitor after approximately 0.2 min. Before visible light irradiation, it was observed that no detectable amounts of radicals were derived from the DMPO. When the UV-irradiation cell was exposed to visible light, an ESR spectrum composed of 12-hyperfine splitting was recorded under a continuous flow condition, as shown in the inset of Figure 2.[10] This species, on the basis of its hfcc values, is ascribed to be the superoxide radical adduct of DMPO ($g = 2.0058$, $a^{N} = 1.40$, $a^{H\beta} = 1.13$, and $a^{H\gamma} = 0.13$ mT).[2,3,8]

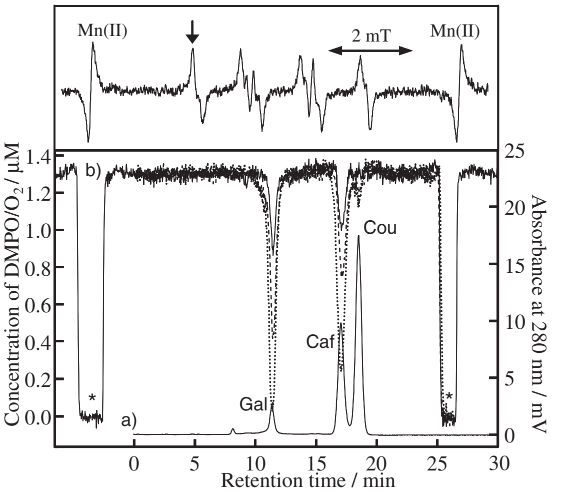

Figure 2. a) UV chromatogram of the solution of Gal, Caf, and Cou (25 μM), observed at 280 nm. b) ESR chromatograms of 25 (solid line), 50 (broken line), and 150 μM (dotted line) of the same solution. The concentrations obtained correspond to the initial concentration just after mixing. The retention time of ESR was adjusted to that of UV. The asterisks corresponds to the zero level of $[DMPO/O_2]_t$ recorded by shutting off the halogen lamp. **Inset**, ESR spectrum of DMPO/O$_2$ radical. The downward arrow indicates the static field for the time-sweep ESR measurements.

Using TEMPOL as a standard, the concentration of the DMPO/O$_2$ radical (denoted as $[DMPO/O_2]_0$) after duplicate integration was estimated to be 1.3 μM. It is well known that the excited state of DMPO (DMPO*) readily changed to the hydroxyl radical adducts of DMPO (DMPO/OH).[11] In the present system, DMPO/OH was formed probably due to the energy transfer occurring between DMPO and photoexcited Rf or phenolic compounds. Similar ESR measurements were conducted in the presence of Caf, Gal, and Cou (100 μM); however, the intensity of the ESR signal attributed to be the DMPO/OH radical was negligibly weak (data not shown).

The magnetic field was adjusted at the peak top of the first low-field line of the DMPO/O$_2$ radical (Figure 2, inset), and the concentration of the radical ($[DMPO/O_2]_t$) was monitored in the time-sweep ESR mode. A mixed solution (20 μL) containing the three phenolic compounds Gal, Cou, and Caf (25 μM after mixing) was injected. The chromatogram, which was monitored at 280 nm (Figure 2a), showed three peaks at 11.4, 17.3, and 18.5 min; these peaks were reliably assigned to Gal, Caf and Cou (Table 1). On the other hand, the ESR chromatograms obtained in the time-sweep ESR mode exhibited two negative peaks at 11.6 and 17.5 min, as shown in Figure 2b.[10] The apparent signal intensity of these peaks showed concomitant growth depending on the concentration of the samples (50 and 150 μM). A weak negative peak was observed at 18.7 min. The negative peaks appearing in the ESR chromatograms were reliably assigned to Gal, Caf, and Cou, respectively, on the basis of the retention times.

Table 1. The retention times (min), relative intensity of ESR negative peaks, and k_s values of phenolic compounds

	r.t.[a]	r.t.[b]	Relative intensity[c]			k_s/M^{-1} s^{-1}	ref
			25[d]	50[d]	150[d]		
Gal	11.4	11.6	1.0	1.8	3.8	3.4×10^5	12
Caf	17.3	17.5	0.8	1.7	3.6	4.0×10^5	13
Cou	18.5	18.7	n.d.	ca. 0.2	0.4	5.5×10^4	14

[a]Retention time observed by UV and [b] by ESR, [c] The integrated signal intensity were normalized by that of Gal at 25 μM, [d] Initial concentration of phenolic compounds (μM) obtained just after mixing.

The signal intensities of these negative peaks were evaluated by integration, and the relative intensities were normalized by the signal intensity recorded for Gal at 25 μM as a standard (Table 1). The relative intensities of Gal and Caf, observed at the same concentration, were comparable with each other (Table 1). On the contrary, the relative intensity of Cou recorded at 150 μM was only about 10% of the relative intensities of Gal and Caf. The second-order rate constants (k_s), defined for the reaction between the superoxide radical and these phenolic compounds, have been evaluated by means of pulse radiolysis[12,13] or chemiluminescence measurements.[14] As summarized in Table, the k_s value of Cou was almost one order smaller than those of both Gal and Caf. On comparing the k_s values and the relative intensities (Table 1), we found that the intensity of the negative peaks depended not only on the k_s value but also on the concentration of the phenolic compounds. This means that the minimum detectable concentration of the scavengers is affected by their k_s values. The detection limit of the present HPLC-ESR system for scavengers having a k_s value equivalent to that of Caf (4.0×10^5 M^{-1} s^{-1}) is expected to be approximately 10 μM; this value is sufficient for the primary selection of the potent antioxidants present in column eluates. Thus, we can infer that this system is suitable for screening superior superoxide radical scavengers. Further efforts on the possibilities are in progress.

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