*Supporting information for*

# Post-trapping Derivatization of Radical-derived EPR-silent Adducts: Application to Free Radical Detection by HPLC-UV in Chemical, Biochemical, and Biological Systems and Comparison with EPR Spectroscopy

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## 1. Materials

5,5-Dimethyl-1-pyrroline-N-oxide synthesized by Dojindo Molecular Technologies, Inc., Japan was obtained from Cedarlane Laboratories (ON, Canada). It was stored under argon atmosphere at -80°C and was used without further purification. Procarbazine hydrochloride (≥ 99%) was purchased from Santa Cruz Biotechnology, Inc. (CA, U.S.A.). Phenylhydrazine as free base (≥ 97%) or hydrochloride (≥ 99%) was purchased from Sigma (ON, Canada). High purity sodium borohydride ($NABH_4$), L-ascorbic acid, N-acetyl cysteine, and reduced L-glutathione, methyl-, ethyl-, and phenyl-magnesium bromide (1M in tetrahydrofuran), methylmagnesium bromide (1 M in ether), ammonium chloride, granular anhydrous sodium sulfate, 1-hydroxypiperidine, 2,3,5-triphenyltetrazolium chloride, 2-naphthoyl chloride, 1-methylimidazole, anhydrous sodium carbonate, copper (II) acetate monohydrate and chloride, ferric ammonium sulfate hexahydrate and ferric chloride, potassium ferricyanide, ethylenediaminetetraacetic acid disodium and dipotassium salt dehydrate, analytical grade diethylenetriaminepentaacetic acid, anhydrous sodium acetate, anhydrous magnesium sulfate, and trichloroacetic acid for protein precipitation were supplied from Sigma (ON, Canada). All chemicals were used without further purification. Lyophilized, salt-free horseradish peroxidase type VI, 250-330 units/mg solid, sodium salt of microperoxidase-11, lyophilized catalase from bovine serum, 2500-5000 units/mg, *Trace*SELECT® hydrogen peroxide 30% v/v, anhydrous glucose, and lyophilized glucose oxidase type VII from *Aspergillus niger* (> 100,000 units/g solid) were purchased from Sigma, ON, Canada. $H_2O_2$ was assayed using its extinction coefficient (43.6 $M^{-1}$ $cm^{-1}$) at 240 nm. Water and solvents used were HPLC grade except DMSO which was of reagent grade. 0.1M sodium phosphate buffer pH 7.4 (PB) and 0.05 M carbonate buffer pH 10 were prepared and used after metal chelation by incubating overnight with Chelex-100 resin (Bio-Rad Laboratories, ON, Canada). Drug-free human plasma collected from healthy volunteers was obtained from Blood Donor Centre, University of Alberta. Whole blood samples were collected from two different healthy human volunteers upon their written consent and according to the ethical guidelines of the University of Alberta.

## 2. Experimental

### 2.1. Preparation of Methyl, Ethyl, and Phenyl DMPO Adducts as HPLC Standards

Direct synthesis of various carbon-centered DMPO adducts was achieved through a 1,3-addition reaction using the appropriate Grignard reagent (RMgBr, where R= $CH_3$, $CH_2CH_3$, or $C_6H_5$) in THF according to the following general procedure:

1. To a solution of DMPO in THF, 5 molar excess of Grignard salt was added, and the reaction was mixed for 15 min at 50°C.
2. The solution was dried under nitrogen. To hydrolyze the organomagnesium addition compound and to decompose excess Grignard reagent, 50% ethanol was added to the residue. For small volumes (< 1 mL), water is suitable for destruction of excess reagent.
3. The suspension was centrifuged at 5000 × *g* at RT for 3 min, and the clear supernatant was pipetted and diluted with water to the required concentration. The phenyl derivative was dissolved in 50% EtOH. Adducts were at least stable for 3 months without deterioration when kept at 4°C.

Grignard products were identified by their MS and UV absorption spectra as well as EPR spectra of their nitroxyl equivalents. The potency of the standard was determined by comparison to the reported $A_{max}$ values in 95% EtOH.[1, 2, 3, 4]

**2.2. Reduction of Paramagnetic C-centered DMPO Adducts**

Prior to the derivatization reaction, C-centered radicals trapped as DMPO adducts were reacted with equimolar amounts of ASC and GSH in order to confirm their conversion into EPR-invisible HAs. Reduction was verified by both a color reaction (section *4*) and EPR (section *2.6.4.*).

**2.3. Preparation of Plasma and Whole Blood Samples**

**2.3.1. Extraction from Plasma/HRP Matrix**

Placebo human plasma samples were left to thaw at room temperature, then vortexed for 5-10 s. Aliquots of 200 µL plasma, to which was added 50µL of an HRP solution (100 µM) in 0.1 M phosphate buffer pH 7.4 (PB), were spiked with DMPOH/$CH_3$ as an HPLC standard to provide final concentrations of 1 mM. The final matrix volume contained DMPO (100 mM) and $H_2O_2$ (0.4 mM) as well. Following a brief vortex-mixing of the samples for 5-10 s, 300 µL of ice-cold acetonitrile (ACN) was added followed by centrifugation for 3 min at 10,000 × *g* at RT. The clear supernatant was separated and shaken with 1 mL of methyl *t*-butyl ether for 10 min. The ethereal (upper) layer was carefully separated into a dry clean polypropylene Eppendorf® microtube and subsequently dried under a gentle stream of $N_2$ gas. The residue was dissolved in 200 µL $H_2O$/ACN (1:1, v/v) and derivatized according to section *2.4*.

**2.3.2. Whole Blood Collection, Incubation, and Processing**

Blood samples were drawn by a specialized phlebotomist from the antecubital vein of 2 healthy, non-smokers, middle-aged male volunteers. 20-30 mL whole blood volumes were collected into sterile plastic tubes containing 100 µL of ethylenediaminetetraacetic acid dipotassium (100 mM) as

anticoagulant and stored at 4°C for not more than 40 days. To 1 mL of whole blood containing 1mM diethylenetriaminepentaacetic acid, 10 μL of 5-ethyl DMPO hydroxylamine (10 mM) and 100μL aqueous DMPO (1 M) were added as the internal standard and the spin trap, respectively. Afterwards, samples were incubated for 5 min with phenylhydrazine to result in final matrix concentrations of 1, 5, and 10 mM. Whole blood samples not containing the xenobiotic (blank) and those containing DMPOH/$C_6H_5$ (HPLC standard at 0.8 mM) were run in parallel. Whole blood samples were then deproteinized by vortex-mixing for 30 sec with 2 mL of ACN at 0.4 % formic acid. Next, equal weights of premixed magnesium sulfate and sodium acetate (ea. 250 mg) were dropped into each sample and shaken vigorously for 2 min. Samples were then centrifuged at 5,000 × *g* for 5 min at RT. Aliquots of 250 μL of the clear supernatant were derivatized according to the following section.

### 2.4. Derivatization of C-centered-trapped or Synthetic DMPO Hydroxylamine Adducts

A general optimized post-trapping derivatization procedure is described below:

1. Immediately to 200-250 μL aqueous standard (DMPOH/$CH_3$, 0.5-10 mM; DMPOH/$C_6H_5$, 0.15-0.9 mM), free radical chemical reaction mixture (section *2.6.1.*), peroxidase reaction mixture (section *2.6.2.*), or plasma/HRP extract (section *2.3.1.*), ASC and GSH were sequentially added to yield a final concentration of 10 mM for each.
2. 100 μL of each 1-methylimidazole and 2-naphthoyl chloride (100 mM in ACN) were added and the mixture was agitated at 1300 rpm for 5 min at 4°C or RT using an Eppendorf Thermomixer® R dry heat block and cooling shaker (Eppendorf Canada).
3. 100 μL of 10% aqueous $Na_2CO_3$ was added followed by 700 μL of *n*-hexane and the bilayer was allowed to mix at RT for 5 min at 1000 rpm.
4. The upper hexane layer was separated and dried under a gentle stream of nitrogen leaving a residue which was dissolved in 150 μL of a reconstitution solution of $H_2O$/ACN (1:1, v/v).
5. The reconstituted sample was pipetted into polypropylene inserts (Target PP Polyspring, National Scientific, TN, USA) which were placed in autosampler vials (Amber 10-425 Screw Thread, Fisher Scientific, U.S.A.). Sample vials were then positioned in the autosampler compartment and 50 μL aliquot volumes were injected into the chromatograph.

### 2.5. Effect of Different Factors on the Labeling Reaction

Major factors that might interfere with the labeling reaction were studied using standard DMPOH/$CH_3$ and a model system in which procarbazine is enzymatically catalyzed to generate $CH_3$ radicals. At different concentrations, metal ions ($Fe^{3+}$ or $Cu^{2+}$), peroxides (neat $H_2O_2$ or indirectly produced via glucose/glucose oxidase), reducing agents (ASC, GSH, or $NABH_4$), the spin trap (DMPO), and diverse

peroxidases (HRP, MP-11) were added, and their effects on the peak area of the derivative was monitored.

### 2.6. Electron Paramagnetic Resonance Experiments

#### 2.6.1. Chemical Systems

##### 2.6.1.1. Methyl Radicals

A Fenton's reaction was carried out in Chelex-100 treated PB (0.1 M, pH 7.4) and contained 2 mM $H_2O_2$, 5 mM ethylenediaminetetraacetic acid disodium, 100 mM DMPO, 2.8 or 0.35 mM DMSO, and 50 μM ferric ammonium sulfate which was added last to trigger the reaction. DMSO was added to scavenge the generated hydroxyl radicals and produce equivalent amounts of methyl radicals.

##### 2.6.1.2. Phenyl Radicals

The reaction was carried out in carbonate buffer (0.05 M, pH 10) and contained 37 mM phenylhydrazine, 100 mM DMPO, and 0.5 mM $CuCl_2$ which was added last to trigger the reaction.

#### 2.6.2. Biochemical Systems

##### 2.6.2.1. Methyl Radicals

The reaction was carried out in Chelex-100 treated PB (0.1 M, pH 7.4) and contained 1 mM procarbazine, 100 mM DMPO, 20 μM HRP or 100 μM MP-11, and 400 μM $H_2O_2$ which was added last to trigger the reaction.

##### 2.6.2.2. Phenyl Radicals

Same as above but phenylhydrazine was used instead of procarbazine and the reaction was enzymatically catalyzed using the same concentration of HRP.

#### 2.6.3. Phenyl Radicals in Whole Blood

200 μL of whole human blood containing 1 mM final concentration of diethylenetriaminepentaacetic acid was incubated with 100 mM DMPO and 1, 5, or 10 mM phenylhydrazine which was added last to initiate phenyl radicals.

#### 2.6.4. Effect of Various Reductants on DMPO Adducts

In order to select the most appropriate reductant or reducing mixture. Several reducing reagents were tested, namely aqueous $NABH_4$, ASC, GSH, NAC, and ASC/GSH mixture. DMPO-trapped $^{\bullet}CH_3$ adducts produced from procarbazine/MP-11 system were chosen to investigate the effect of various reducing agents by recording the corresponding EPR spectra before and after reduction. Results obtained out of this biochemical reaction were extrapolated to other systems. Likewise, fresh standard solutions of methyl- and phenyl-DMPO HA adducts (DMPOH/$CH_3$ and DMPOH/$C_6H_5$) prepared by Grignard addition and diluted with PB to a final concentration of 5 mM were flushed with $N_2$ and

subjected to EPR before and after reduction. Results obtained from this experiment were extrapolated to other synthetically prepared DMPO adducts.

## 3. Instrumentation

### 3.1. EPR measurement

All reactions for EPR were carried out at ambient temperature using a final volume of 200 µL. Controls not containing the substrate (procarbazine or phenylhydrazine) or DMSO were run in parallel. After a brief vortex-mixing, the reaction mixture was transferred to a flat cell for data acquisition. An Elexsys E500 EPR spectrometer was used with the following instrumental parameters: microwave frequency = 9.78 GHz; incident microwave power = 20 mW; center field = 3485 G, scan range = 100 G, modulation amplitude = 1 G; receiver gain, 60 dB; and time constant = 163.84 ms. After the data was obtained, spectra were simulated and deconvoluted using WinSim® version 0.98 obtained from the Public EPR Software Tools (National Institute of Environmental Health Sciences, NIH).

### 3.2. High Performance Liquid Chromatography and UV Detection

Naphthoylated DMPO hydroxylamine adducts derived from $^{\bullet}CH_3$ and $^{\bullet}C_6H_5$ radicals or synthetically prepared via Grignard reaction were analyzed using a Shimadzu® chromatography system composed of SCL-10A *VP* system controller, DGU-14A vacuum degasser, LC-10AD and 10AT *VP* liquid chromatography pumps, SPD-10AV UV-VIS detector and SIL-10AD *VP* autosampler connected to a computer loaded with Clarity® software for data acquisition and manipulation. The separation was performed throughout the experiments on an Ultra II Aromax (150 × 4.6 mm, 5 µM) column purchased from Restek Inc. U.S.A. The corresponding guard columns Ultra II Aromax (20 × 4.6 mm, 5 µm) supplied by Restek, were replaced after ~ 120 injections. All analyses were carried out isocratically with a mobile phase consisting of acetonitrile-water (55:45, v/v) unless otherwise specified at a flow-rate of 1 ml/min. As required and according to the application, deliberate changes of the ratio between the mobile phase components were made either to enhance peak resolution or to examine the robustness of the separation. The mobile phase was freshly prepared twice weekly, membrane-filtered and degassed by sonication prior to circulating into the system. The UV detector was monitored at 230 nm unless otherwise stated.

### 3.3. Mass Spectrometric Analysis (MS)

Peak volumes isolated from HPLC were subjected to ESI-MS (Micromass ZQ 4000 spectrometer; Waters, Milford, MA). The nebulizer gas was obtained from an in-house high-purity nitrogen source. The temperature of the source was set at 140°C, and the voltages of the capillary and the cone were 3.6

kV and 20 V, respectively. For each application, the mass spectrometer was operated using single-ion recorder (SIR) acquisition in positive ionization mode to monitor and confirm the carbon-centered DMPO adduct in question modified post-trapping using derivatization with 2-naphthoyl chloride.

### 3.4. Spectrofluorometry

Fluorescence spectra of DMPO-phenyl hydroxylamine adducts were acquired via a SpectraMax® M5 Multi-Mode microplate reader (Molecular Devices, Inc.) in the cuvette read-mode using 50% EtOH at $\lambda_{ex}$ and $\lambda_{em}$ of 270 and 310 nm, respectively. The instrument was interfaced with an external computer loaded with SoftMax® Pro software for data display and statistical data analysis.

## 4. Confirmatory Color Reactions

### 4.1. Test for a Complete Grignard Reaction

10 μL of a solution of 100 mM $FeCl_3$ was added to an aliquot of 50 μL of the supernatant from the aforementioned Grignard addition. Absence of violet color after 5 min confirmed the complete conversion of DMPO into a mixture of HAs (major products) and nitroxides (minor products) of C-centered adducts.

### 4.2. Test for Reduction of Spin Adducts

To 50 μL of the tested solution, an equal volume of triphenyltetrazolium chloride 1% solution in water is added followed by a similar volume of aqueous NaOH (1 M). Appearance of a faint pink color after 2-3 min indicates the presence of a cyclic hydroxylamine in the solution being tested. On standing, the color intensifies and after ≈ 20 min a reddish-purple precipitate forms. Analogously, this test can be used to confirm the reduction of various C-centered radical-derived DMPO spin adducts into the corresponding HAs. 1-Hydroxypiperidine was used as a positive control.

## References


(1) Bonnett, R.; Brown, R. F. C.; Clark, V. M.; Sutherland, I. O.; Todd, A. *J. Chem. Soc.* **1959**, 2094-2102.

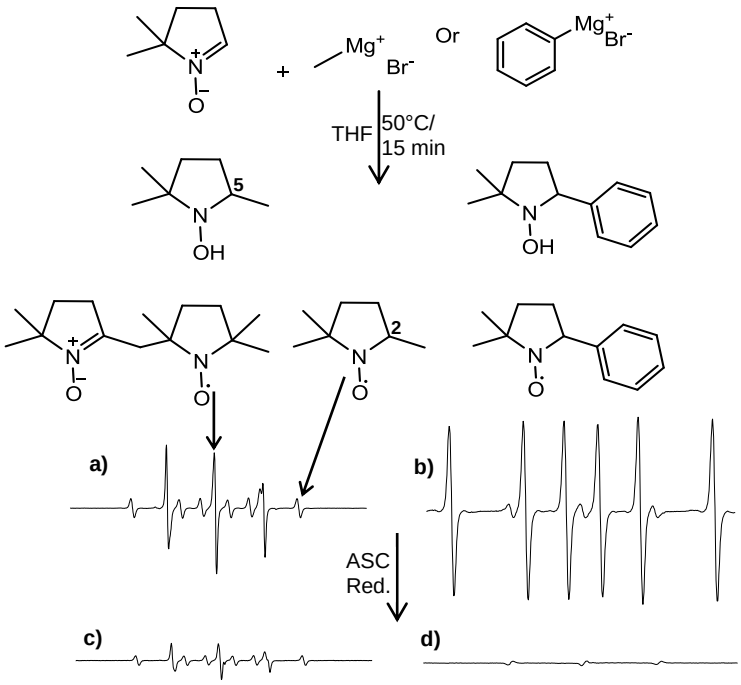
(2) Black, D. S. C.; Boscacci, A. B. *Aust. J. Chem.* **1976**, *29*, 2511-2524.

(3) Thesing, J.; Sirrenberg, W. *Chem. Ber.* **1958**, *91*, 1978-1980.

(4) House, H. O.; Manning, D. T.; Melillo, D. G.; Lee, L. F.; Haynes, O. R.; Wilkes, B. E. *J. Org. Chem.* **1976**, *41*, 855-863.

## Scheme S1

**Scheme 1.** Grignard synthesis of 5-substituted DMPO hydroxylamines as HPLC standards and their paramagnetic reaction byproducts [a]




[a] Traces (a and c): EPR spectra of paramagnetic synthetic traces of DMPO$^{\bullet}$/$CH_3$ and dimer aminoxyl and their reduction using ascorbate (5 mM). Traces (b and d): EPR spectra of paramagnetic synthetic traces of DMPO$^{\bullet}$/$C_6H_5$ and their reduction using ascorbate (5 mM).

**Scheme S2**

**Scheme S2.** Labeling reaction and major characteristics of derivatized DMPO hydroxylamine adduct

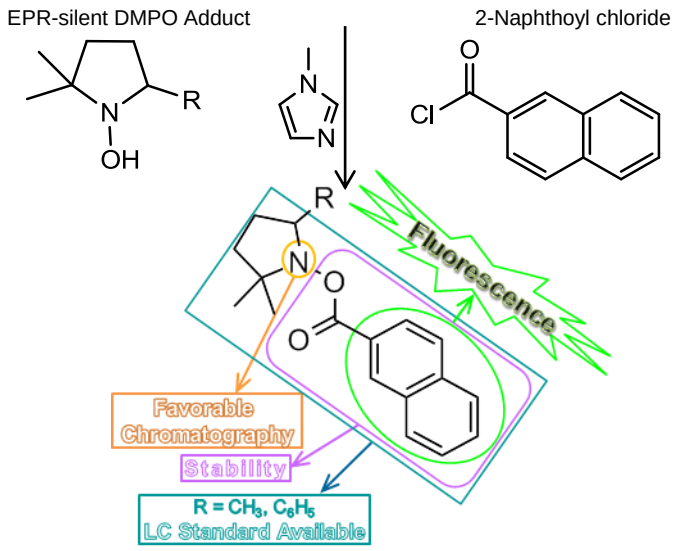


**Figure S1**

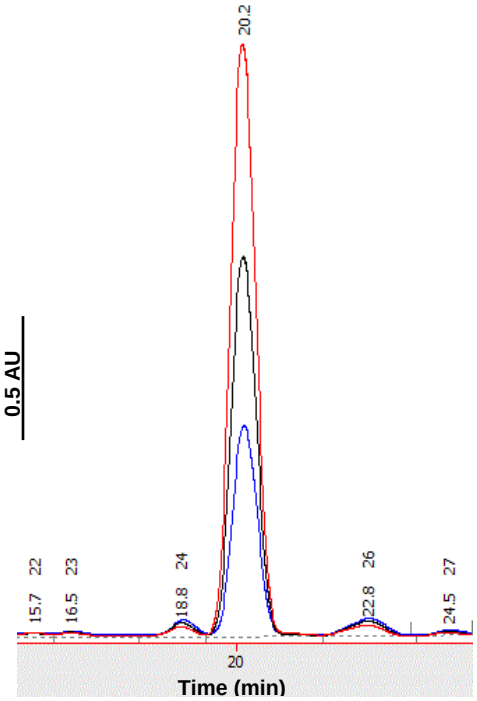



**Figure S1.** Linearity of the optical response for labeled DMPOH/$C_6H_5$ at 300, 600, and 900 μM ($t_R$ ≈ 20 min) in 60:40 acetonitrile:water, v/v at 254 nm.

**Figure S2**

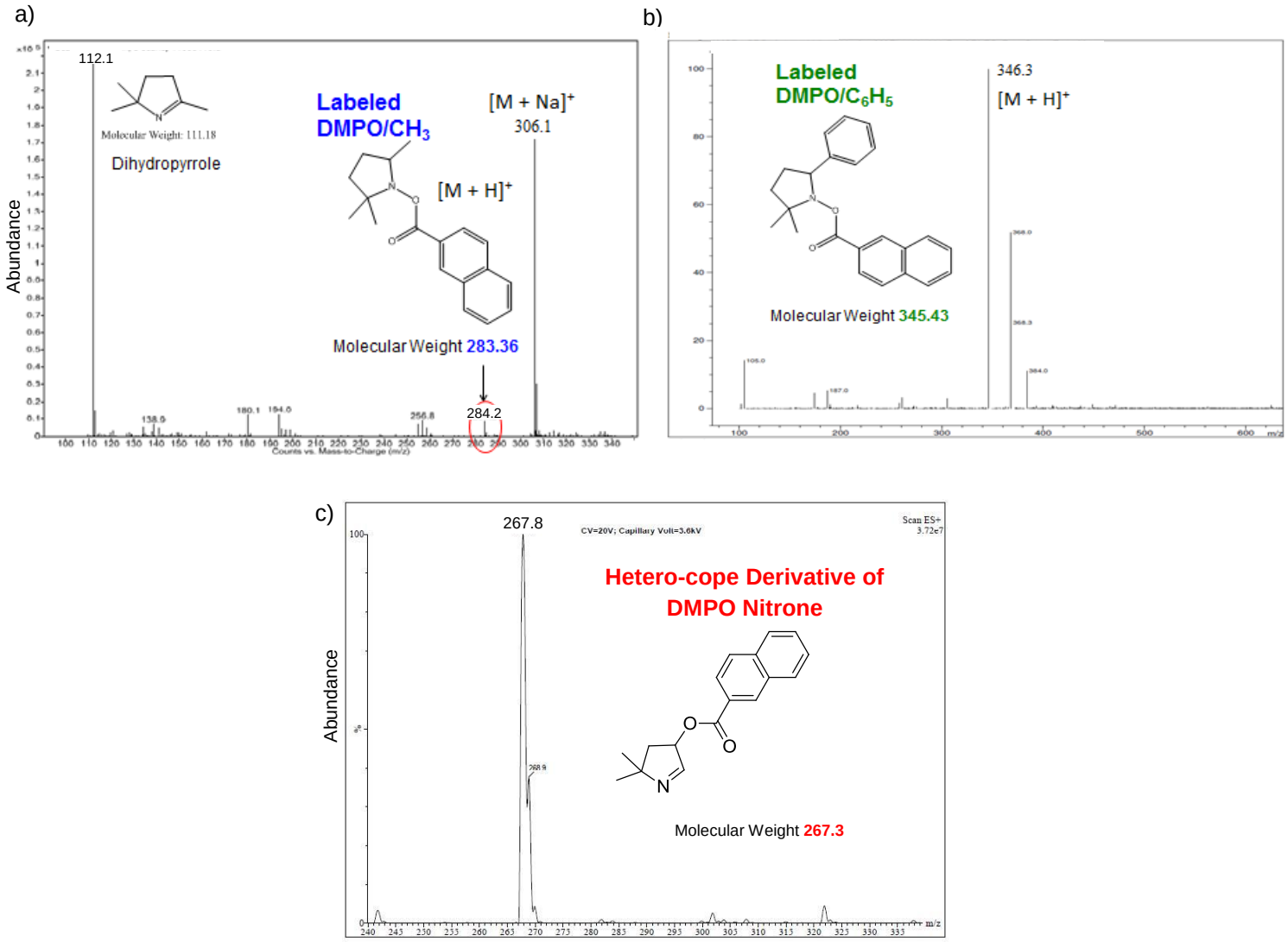



**Figure S2.** MS Characterization of (a) N-naphthoyloxy DMPOH/$CH_3$,(b) N-naphthoyloxy DMPOH/$C_6H_5$, and (c) 3-Naphthoyloxy-1-pyrroline, the hetero-cope rearrangement product of DMPO nitrone (the spin trap).

**Figure S3**

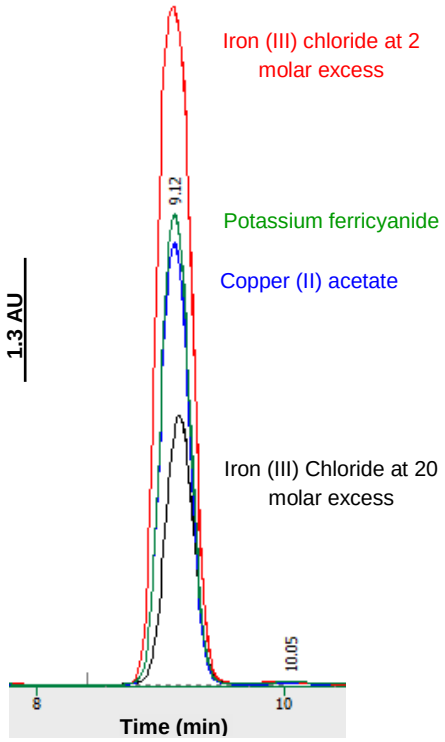



**Figure S3.** Effect of different metal ions on the hydroxylamine adduct (DMPOH/$CH_3$): Ferric chloride (at 2 and 20 molar excess); potassium ferricyanide and copper (II) acetate (each at 2 molar excess). Detector response in presence of 2 molar excess ferric chloride represents control value.

**Figure S4**

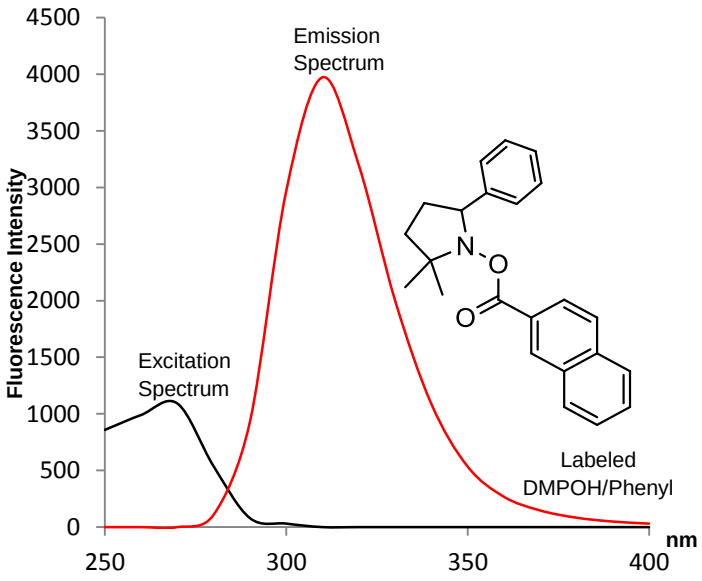



**Figure S4.** Excitation and emission fluorescence spectra of labeled DMPOH/$C_6H_5$

**Figure S5**

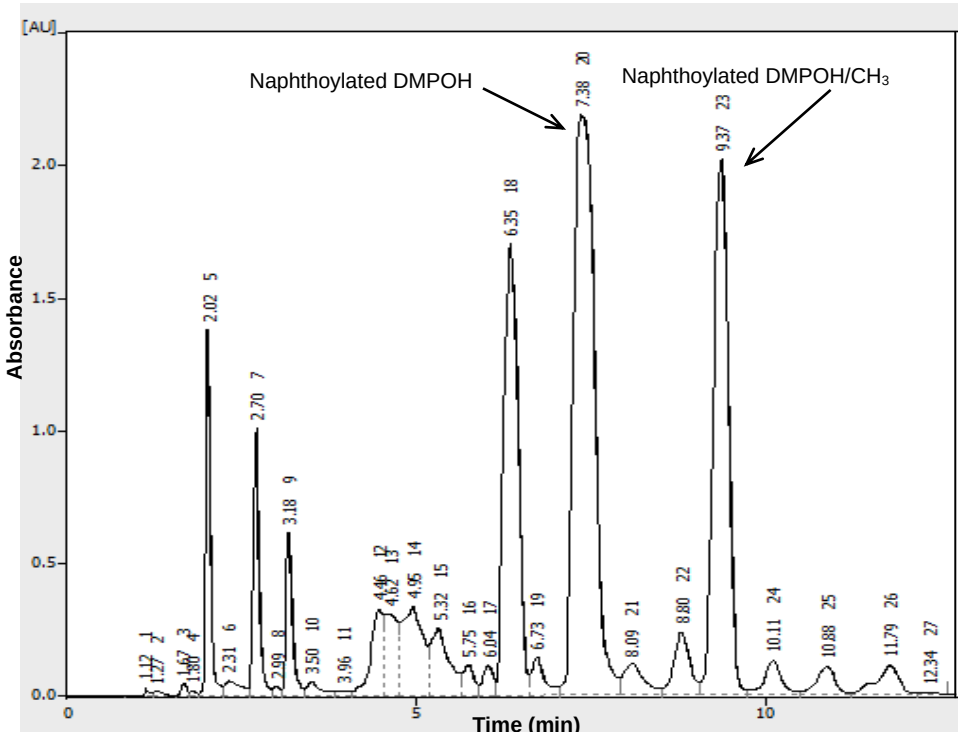



**Figure S5.** HPLC-UV chromatogram of $NaBH_4$-reduced DMPO, the spin trap ($t_R$ ≈ 7.4 min), and 1 mM DMPO/methyl adduct ($t_R$ ≈ 9.4 min) labeled as naphthoate esters in a plasma/HRP compound matrix after protein precipitation and extraction. The mobile phase, acetonitrile-water (60:40, v/v), was monitored at 254 nm.

**Figure S6**

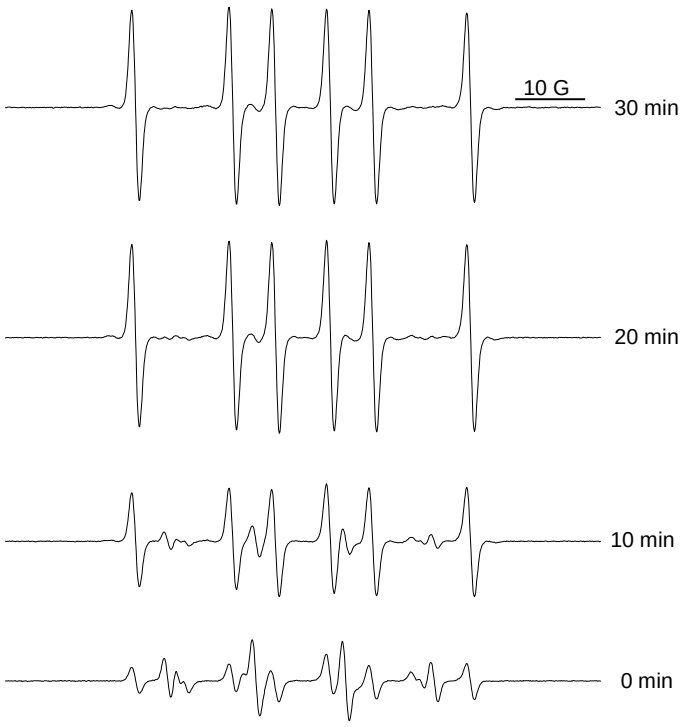



**Figure S6.** EPR spectra of procarbazine-derived radicals at different time points catalyzed by 20 μM HRP.

**Figure S7**

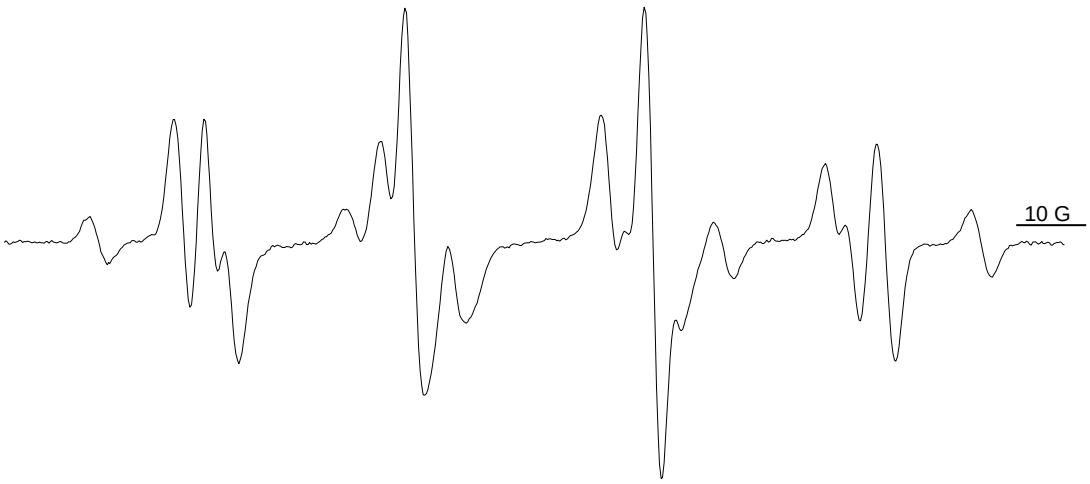



**Figure S7.** EPR spectrum of procarbazine-derived radicals at zero time catalyzed by 2 μM HRP.

**Figure S8**

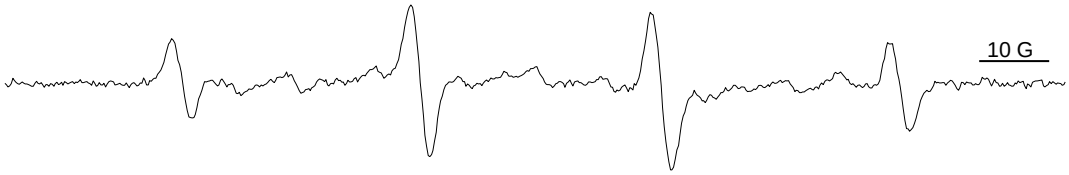



**Figure S8.** EPR spectrum of phenylhydrazine-derived radicals at zero time catalyzed by 0.5 μM HRP