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A novel approach to the analysis of spin-trapped free radicals using dimethyl sulfoxide and gas chromatography – mass spectrometry (GC-MS) with both solvent extraction and headspace solid phase microextraction (HS-SPME)

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ABSTRACT

In this study, we have utilized a novel strategy based upon the use of dimethyl sulfoxide (DMSO) and gas chromatography-mass spectrometry (GC-MS) for the detection and identification of spin-trapped free radicals. Hydroxymethyl ($^{\cdot}CH_2OH$) radicals, generated by Fenton-type chemistry, have been trapped by *N-tert*-butyl-α-phenylnitrone (PBN) or one of its derivatives in the presence of DMSO to form a 1,3-diadduct [PBN-(CH_2OH)(CH_3)], which may be detected directly in the reaction mixture following chloroform extraction or in the reaction vial headspace by sampling with SPME. Separation and identification have been carried out by capillary gas chromatography coupled to electron-ionization mass spectrometry (EI-MS). The results demonstrate that using DMSO aids GC-MS analysis of spin-trapped free radicals *via* the formation of radical-methyl diadducts that are sufficiently volatile to be sampled both in the headspace or by an extracting solvent without the need for a derivatization step using silylating agents.

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Gas chromatography-mass spectrometry; solid phase microextraction; spin trapping; free radicals; *N-tert*-Butyl-α-phenylnitrone; Fenton reaction; hydroxymethyl radical

Introduction

Spin trapping using nitrone compounds is a popular technique for the detection of free radicals that are unstable at room temperature and may react rapidly with the nitrone to form a nitroxide. This aminoxyl radical may then be detected using electron paramagnetic resonance (EPR) spectroscopy [1]. However, an alternative approach to the use of EPR spectroscopy is to identify the products of spin-trapped radicals using mass spectrometry-based techniques (for examples, see [2–6]). Since the EPR spin trapping method was first developed in the late 1960s, derivatives of α-phenyl-*tert*-butyl nitrone (PBN; Figure 1) are amongst the most widely synthesized for use in a variety of chemical and biological studies [7–9].

The hydroxymethyl radical ($^{\cdot}CH_2OH$) may be formed from methanol when using Fenton-based chemistry, trapped using PBN or related nitrones, and detected as a stable nitroxide at room temperature by EPR spectroscopy [10] or GC-MS [11]. The latter study identified the PBN-trapped hydroxymethyl radical as the trimethysilylether derivative following derivatization with bis-(trimethylsilyl)-trifluoroacetamide in acetonitrile (1:1). Hydroxymethyl radicals are produced by hydroxyl radicals, formed from the Fenton reaction, abstracting a hydrogen atom from the methyl group of a neighboring methanol molecule (Scheme 1). PBN trapping takes place by addition of the hydroxymethyl radical to the carbon of the C=N forming a nitroxide (first step in Scheme 2). In this study, we have used the PBN trapping of a hydroxymethyl radical as an example to demonstrate a novel approach to detecting and identifying spin-trapped free radical adducts, both in the headspace of the reaction vial and by solvent extraction, using DMSO as an "*in-situ* derivatizing agent" for the aminoxyl radical (second step in Scheme 2).

Extracting volatile compounds from the "headspace" of a sample vial may be carried out using Solid Phase Microextraction (SPME). SPME may be used for extracting a wide variety of analytes from liquid, solid,and gaseous samples [12]. It uses a thin layer of polymeric sorbent, or an immobilized liquid coated on a silica fiber to absorb or adsorb the analyte [13]. In this paper, volatile spin-trapped free radical products have been extracted by SPME from the headspace of a vial containing a Fenton-based reaction mixture with PBN (or one of its derivatives), methanol and DMSO (or their

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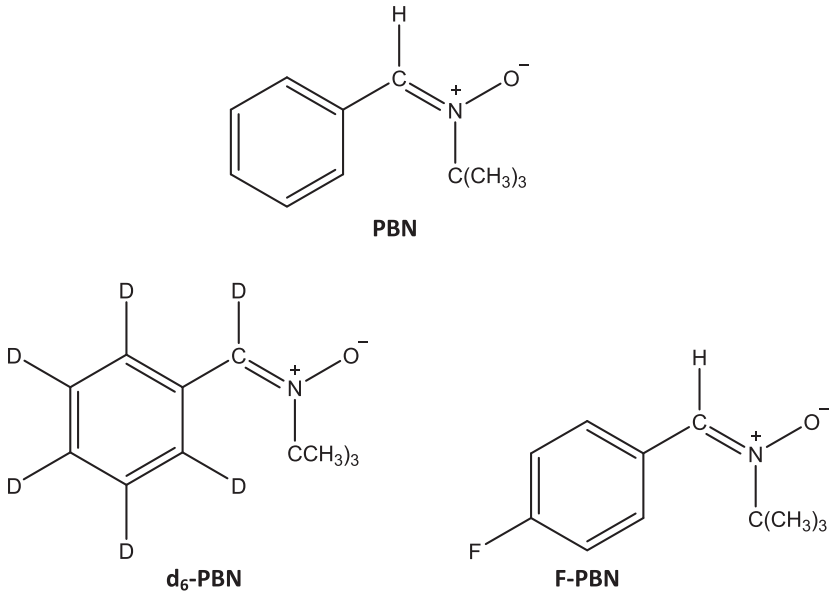
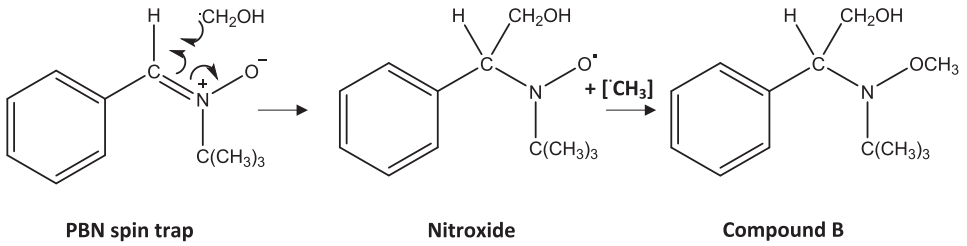


Figure 1. The structures of the spin traps used in this study.

$$CH_3OH + {\cdot}OH \longrightarrow {\cdot}CH_2OH + H_2O$$

Scheme 1. Generation of hydroxymethyl radicals by hydrogen atom abstraction from the methyl group of methanol.



Scheme 2. The mechanism of formation of compound B. The first step involves the addition of the hydroxymethyl radical to the spin trap PBN to generate a nitroxide. The second step involves the addition of a methyl radical, derived from DMSO, to give the di-adduct {PBN-(CH_2OH)(CH_3)}.

isotopically labeled analogues) as secondary sources of free radicals.

Materials and methods

L-ascorbic acid, di-potassium hydrogen phosphate (K_2HPO_4), ethylene diaminetetraacetic acid (EDTA), methanol, and N-*tert*-butyl-α-phenylnitrone (PBN) were obtained from Sigma-Aldrich (Suffolk, UK). Methanol-d_3 (CD_3OH) and deuterated dimethyl sulfoxide {$(CD_3)_2SO$} were obtained from CDN Isotopes (Dunmow, UK). Ammonium ferrous sulfate hexahydrate {$Fe(NH_4)_2(SO_4)_2.6H_2O$} was obtained from Fluka Biochemika (Loughborough, UK). Hydrogen peroxide (30% w/v) and dimethyl sulfoxide (DMSO) were purchased from Alfa Aesar (Lancashire, UK). N-*tert*-butyl-α-4-fluorophenylnitrone (F-PBN; Figure 1) and N-*tert*-butyl-α-phenylnitrone-d_6 (PBN-d_6; Figure 1) were synthesized from the respective benzaldehydes using the method of Hinton and Janzen [14]. SPME fibers were purchased from Merck Life Sciences ltd. (Dorset, UK).

Generation of spin-trapped free radicals

A standard method reported previously was used throughout the experiment to generate and spin trap free radicals with PBN or one of the derivatives [15]. The Fenton-based reaction mixture (10 cm^3) was made up at room temperature, as follows: potassium phosphate (50 $mmol.dm^{-3}$) buffer (pH = 7.4); EDTA (1 $mmol.dm^{-3}$); spin trap compound {PBN/F-PBN/d_6-PBN} (10 $mmol.dm^{-3}$); ascorbic acid (10 $mmol.dm^{-3}$); ferrous ammonium sulfate (1 $mmol.dm^{-3}$); H_2O_2 (0.3% v/v); DMSO/d_6-DMSO (100 $mmol.dm^{-3}$); and methanol {methanol-d_3} (100 mmol dm^{-3}). The Fe^{2+} compound was added last to the mixture to initiate the reaction.

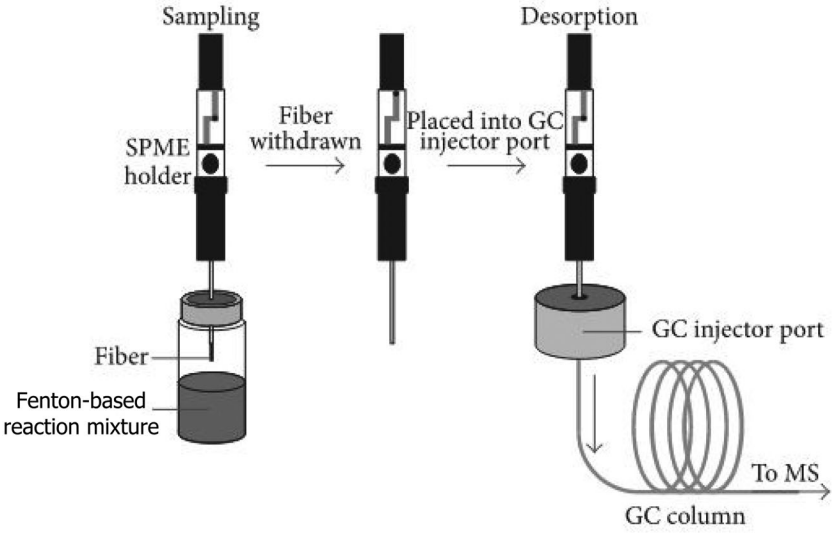


Figure 2. Diagram of analysis of volatile compounds in the reaction vial headspace using solid phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) (Adapted from: 16].

For analysis, either 1 cm^3 of the Fenton-based reaction mixture was extracted into 2 cm^3 chloroform or the vial headspace was sampled using solid phase microextraction – see below. Where chloroform extraction was used, the extract was left for 5 minutes, the aqueous layer removed, and 1 μL of the organic layer was injected into the gas chromatograph.

Headspace solid phase microextraction (HS-SPME)

Manual SPME was carried out to extract the volatile compounds from the headspace (see Figure 2). Four different SPME fibers were tested to determine which gave optimum extraction of the di-adduct from the headspace, as follows: carboxen/polydimethylsiloxane (CAR/PDMS); polyethylene glycol (PEG); polyacrylate; polydimethylsiloxane/divinylbenzene (PDMS/DVB). 5 cm^3 of the Fenton-based reaction mixture was transferred into a sampling vial (10 cm^3) and extraction of the di-adduct was done by exposing the fiber to the headspace of the vial (heated at 40 °C) for 10 minutes. For GC-MS analysis, the di-adduct was desorbed from the fiber by placing it in the injection port of the GC at 250 °C for 5 minutes.

Gas chromatography-mass spectrometry (GC-MS)

A Varian 3800 GC coupled to a 1200 triple quadrupole mass spectrometer was used for the sample analysis with the mass spectrometer in single quadrupole mode. Data handling was carried out using a Varian workstation. Separation of PBN-derived products, including di-adducts, was carried out by using a capillary column coated with poly(dimethylsiloxane) (Rtx-5; Thames Restek, UK). The capillary column was 30 m in length with an internal diameter of 0.25 mm and the film coating thickness of the stationary phase was 0.25 μm. Helium (BOC, UK) was used as the carrier gas with a flow rate of 1 cm^3/min. The sample was introduced into the injector port using the split-less mode with the injector temperature at 250 °C. The purge activation time was set for 3 minutes after injection. The oven temperature program was initially held at 100 °C for one minute (for direct sample injection) or 50 °C for 5 minutes (for HS-SPME sampling) and then set to increase at a rate of 15 °C per minute (for direct sample injection) or 25 °C per minute (for HS-SPME sampling) until a final temperature of 320 °C where it was then held for 2 minutes. The transfer line temperature and the ion source temperature were maintained at 250 °C. The ionization energy was set at 70 eV and the photoelectron multiplier at 1300 volts with a scan range of 50–500 m/z.

Results

GC-MS analysis of the Fenton-based reaction mixture containing spin trap, methanol, and DMSO following solvent extraction

Figure 3 shows the total-ion chromatogram (TIC) generated by the GC-MS analysis of the Fenton-based reaction mixture containing PBN, methanol, and DMSO, following solvent extraction with chloroform – see materials and methods for further details. The peaks in the chromatogram have been assigned from interpretation of their electron-ionization mass spectra (EI-MS) when using either PBN or one of its derivatives as the

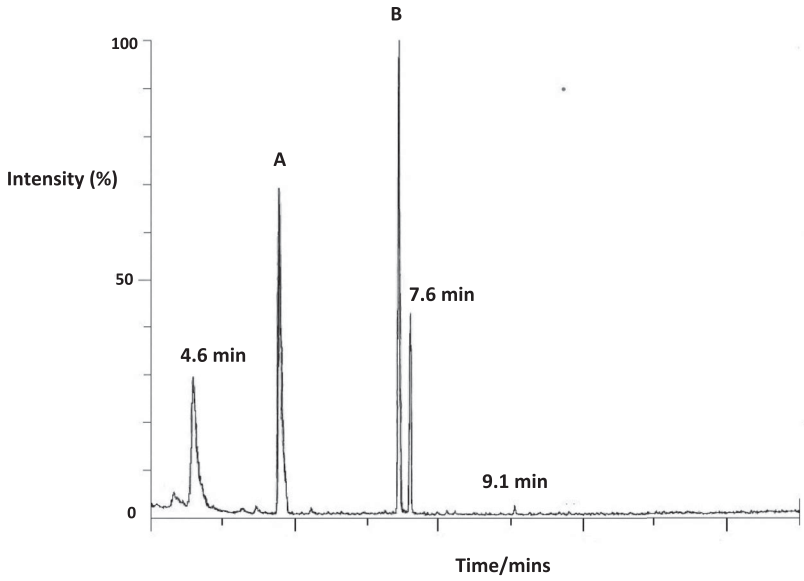
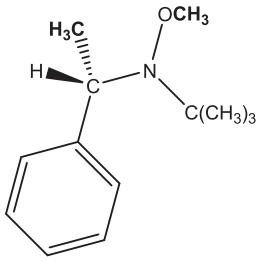
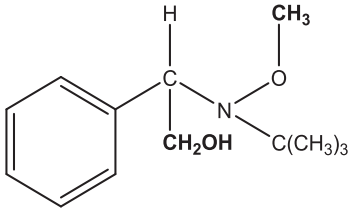


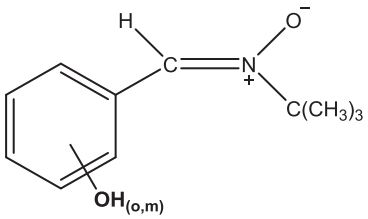
Figure 3. Total ion chromatogram (TIC) obtained from GC-MS analysis of the Fenton-based reaction mixture containing PBN, methanol and DMSO following extraction by chloroform. The peaks labeled A and B correspond to di-adducts of the spin trap PBN.



Compound A



Compound B



Compound C

Figure 4. The structures of compounds identified by GC-MS analysis of a Fenton-based reaction mixture containing PBN, methanol, and DMSO. The groups in bold are derived from the following: methyl from DMSO (compounds A and B); hydroxymethyl from methanol (compound B); hydroxyl from hydrogen peroxide (compound C). For compound C, the exact position of the OH group in the ring is not known but is either at the ortho (o) or meta (m) position.

trapping agent and using methanol (or CD_3OH) and DMSO {or $(CD_3)_2S{=}O$} as secondary sources of free radicals.The chromatogram (Figure 3) shows five peaks, of which two may be assigned as di-adducts to PBN: a dimethyl adduct of the spin trap {PBN-$(CH_3)_2$; 5.8 minutes; compound **A**, Figure 4}, and a methyl and hydroxymethyl di-adduct to the spin trap {(PBN-$(CH_2OH)(CH_3)$; 7.5 minutes; compound **B**, Figure 4}. One of the peaks may be assigned to unreacted PBN (7.6 minutes), and the smallest peak to a hydroxyl

adduct to the phenyl ring of the spin trap {PBN-OH; 9.1 min; compound **C**}, (Figure 4). The broad peak at 4.6 minutes is seen in control experiments in the absence of either Fe^{2+} or hydrogen peroxide (data not shown). It appears to consist of two overlapping peaks with one of the compounds having an EI-MS spectral pattern consistent with benzaldehyde oxime. This compound has been observed previously by GC-MS when using PBN as a spin trap [17,18].

As mentioned previously, the peak at 7.6 minutes in the chromatogram (Figure 3) corresponds to unreacted PBN. Its EI-mass spectrum is well characterized [8] and will not be discussed further. It is worth noting, however, that whilst PBN was observed by GC-MS following extraction of the Fenton-based reaction mixture into chloroform, it was not observed in the vial headspace when SPME was used as the sampling method, irrespective of the type of fiber used. This agrees with a previous study where PBN spin-trapped products were sampled in the headspace using thermal desorption [3].

GC-MS analysis of compound A

Table 1 shows the m/z values of key ions, including the molecular ion ($M^{+\cdot}$), found in the EI mass spectrum of compounds **A**, **B**, and **C** observed when the Fenton-based reaction mixture containing equimolar amounts of methanol and dimethyl sulfoxide was sampled either by (a) SPME of the vial headspace, or (b) liquid-liquid (solvent) extraction using chloroform as the extracting solvent. The identity of **A** has been confirmedby its EI-mass spectrum as the dimethyl adduct of the PBN spin trap (PBN-Me_2; one methyl adding to the carbon atom of the C=N and the other adding to the oxygen). Replacing DMSO with DMSO-d_6 in the Fenton-based reaction mixture containing both methanol and PBN gives rise to an increase in the m/z value of the molecular ion of **A** of 6 units, whereas, replacing methanol, but keeping both PBN and DMSO, with its deuterated analogue has no effect on the m/z value of the molecular ion (Table 1). This demonstrates that the methyl groups are both derived from DMSO and not methanol. The EI-mass spectrum of this compound and its interpretation has been published previously by Janzen et al. [19], and PBN-Me_2 has also been observed previously when ethanal was used as a secondary source of free radicals in a Fenton-based reaction [3]. In addition, a similar adduct (POBN-Me_2) has been identified when *N-tert*-butyl-α-(4-pyridyl)nitrone *N'*-oxide (POBN) was used as the spin trap and either GC-MS or matrix-assisted laser

Table 1. Molecular ion ($M^{+\cdot}$) m/z values for compounds A, B, and C, detected by GC-MS analysis of the Fenton-based reaction mixture containing PBN, methanol and DMSO.

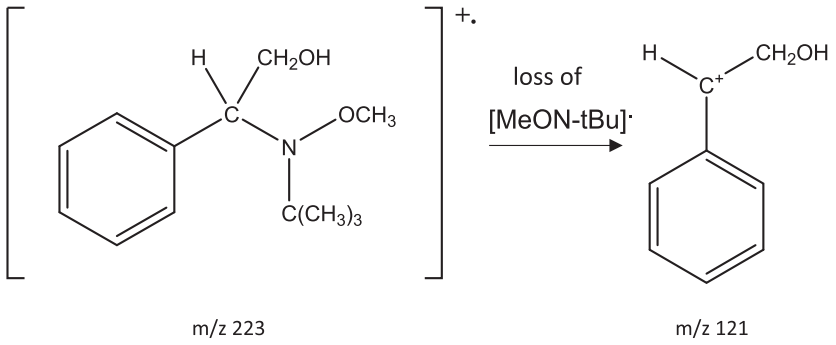
Compound	Sampling method	The Fenton System	Compound name or formula	$M^{+\bullet}$ (m/z)	Other significant ions (m/z)
A*	Chloroform extraction and SPME	PBN + CH_3OH + DMSO	PBN-Me_2	207	192, 151, 105 (bp**)
		PBN + CD_3OH + DMSO	PBN-Me_2	207	192, 151, 105 (bp)
		PBN + CH_3OH + d_6-DMSO	PBN-$(CD_3)_2$	213	198, 157, 108 (bp)
		PBN + CD_3OH + d_6-DMSO	PBN-$(CD_3)_2$	213	198, 157, 108 (bp)
B	Chloroform extraction and SPME	PBN + CH_3OH + DMSO	PBN-$(CH_2OH)(CH_3)$	223	See Table 2
		PBN + CD_3OH + DMSO	PBN-$(CD_2OH)(CH_3)$	225	
		PBN + CH_3OH + d_6-DMSO	PBN-$(CH_2OH)(CD_3)$	226	
		PBN + CD_3OH + d_6-DMSO	PBN-$(CD_2OH)(CD_3)$	228	
		d_6-PBN + CH_3OH + DMSO	d_6-PBN-$(CH_2OH)(CH_3)$	229	
		F-PBN + CH_3OH + DMSO	F-PBN-$(CH_2OH)(CH_3)$	241	
C	Chloroform extraction only	PBN + CH_3OH + DMSO	PBN-OH	193	137 (bp), 120, 119, 91
		PBN + CD_3OH + DMSO	PBN-OH	193	137 (bp), 120, 119, 91
		PBN + CH_3OH + d_6-DMSO	PBN-OH	193	137 (bp), 120, 119, 91
		PBN + CD_3OH + d_6-DMSO	PBN-OH	193	137 (bp), 120, 119, 91
		d_6-PBN + CH_3OH + DMSO	PBN-d_6-OH	198	142 (bp), 125, 124, 96
		F-PBN + CH_3OH + DMSO	F-PBN-OH	211	155 (bp), 138, 137, 109

*For the EI-mass spectrum and its interpretation see (19).
**bp: base peak.

Table 2. Key m/z values from the EI-mass spectra for the di-adduct (compound B).

		m/z values			
The Fenton System	Di-adduct formula	M-15	M-31 or M-33	Base peak	Fragment D*
PBN + CH_3OH + DMSO	PBN-$(CH_2OH)(CH_3)$	208	192	136	121
PBN + CD_3OH + DMSO	PBN-$(CD_2OH)(CH_3)$	210	192	136	123
PBN + CH_3OH + d_6-DMSO	PBN-$(CH_2OH)(CD_3)$	211	195	139	121
PBN + CD_3OH + d_6-DMSO	PBN-$(CD_2OH)(CD_3)$	213	195	139	123
d_6-PBN + CH_3OH + DMSO	PBN-$d_6(CH_2OH)(CH_3)$	214	198	142	127
F-PBN + CH_3OH + DMSO	F-PBN-$(CH_2OH)(CH_3)$	226	210	154	139

*See Scheme 3.



m/z 223 m/z 121

Molecular ion of compound B **Fragment D**

Scheme 3. The structure of fragment D formed in the ion source of the mass spectrometer from the molecular ion of compound B (a methanol and methyl radical di-adduct). Compound B and fragment D provide clear evidence for the PBN spin trapping of a $^{\cdot}CH_2OH$ radical.

desorption/ionization – time of flight (MALDI-TOF) mass spectrometry was used for its detection [15,20].

GC-MS analysis of compound B

Compound **B** is another di-adduct of the spin trap containing both a hydroxymethyl and methyl group {PBN(CH_2OH)(CH_3)} (Figure 4). When using DMSO and methanol as secondary sources of free radicals, the resulting mass spectrum shows a weak peak corresponding to the molecular ion at m/z 223 (Table 1; Figure 5(A) inset). Replacing either DMSO and/or methanol with deuterium analogues in the Fenton-based reaction mixture produces mass spectra with the molecular ion m/z value increasing by 3, 2, or 5 units, respectively, for the $(CD_3)_2SO/CH_3OH$, $(CH_3)_2SO/CD_3OH$,or $(CD_3)_2SO/CD_3OH$ systems. In addition, when the alternative spin traps are used, the molecular ion m/z value increases by 6 and 18 units, respectively, for d_6-PBN and F-PBN, to m/z 229 and m/z 241 (Table 1). It should be noted, however, that these molecular ions are very weak and so identification of other ions is required to provide unequivocal proof of the trapping of the hydroxymethyl radical by the spin trap. The inset of the mass spectrum given in Figure 5(A) also shows a weak ion at m/z 208 corresponding to a fragment ion whereby a methyl radical has been lost from the molecular ion (M-15) in the ion source. The equivalent ion is observed in all systems using deuterium analogues as secondary sources, or alternative spin-traps (Table 2) demonstrating that the methyl has been lost from the *tert*-butyl group of the di-adduct. Figure 5(A) also shows the presence of ions at m/z 192, 136, and 121. The ion at m/z 192 is formed in the ion source by loss of a $^{\cdot}CH_2OH$ radical from the molecular ion (M-31) and supports the identification of the structure of compound **B** as a hydroxymethyl adduct. Additional evidence is provided by the experiments using deuterated methanol as a secondary source, in which $^{\cdot}CD_2OH$ is lost from the molecular ion (M-33) (Figure 5; Table 2). The ion at m/z 136 is the base peak and is likely formed in the ion source of the mass spectrometer by loss of 2-methyl-2-propene from the ion at m/z 192. Again, this is supported by experiments using deuterium-labeled secondary sources and/or alternative spin-traps (Figure 5; Table 2). Finally, the ion at m/z 121 is formed in the ion source from the molecular ion by the breaking of the C–N bond (fragment ion D; Scheme 3). This fragment provides clear evidence as to the position of the CH_2OH group in **B**, and thus the mechanism of trapping of radicals by PBN. The structure of fragment ion **D** is confirmed by experiments where methanol has been replaced by CD_3OH as a secondary source and/or PBN by alternative spin traps in the Fenton-based reaction mixture. The m/z value for fragment ion **D** increases by 2 units (Figure 5(B,D)) due to the presence of CD_2OH, and by 6 or 18 units, respectively, when d_6-PBN or F-PBN have been used as spin traps (Table 2).

GC-MS analysis of compound C

The weak peak shown in the chromatogram in Figure 3 at 9.1 minutes corresponds to a hydroxyl adduct of PBN, with a molecular ion at m/z 193 and the base peak at m/z 137 (PBN-OH; compound **C**; Figure 4; Table 1). The base peak is formed directly from the molecular ion in the ion source of the mass spectrometer by loss of 2-methyl-2-propene [8]. The base peak ion (m/z 137) may then fragment in the ion source losing either a hydroxyl radical or a water molecule to form the ions at m/z 120 and m/z 119, respectively. The ion at m/z 91 is characteristic of a tropylium ion ($C_7H_7^+$) formed by a rearrangement. The hydroxyl radical, formed in the Fenton-based reaction mixture, has clearly added to the

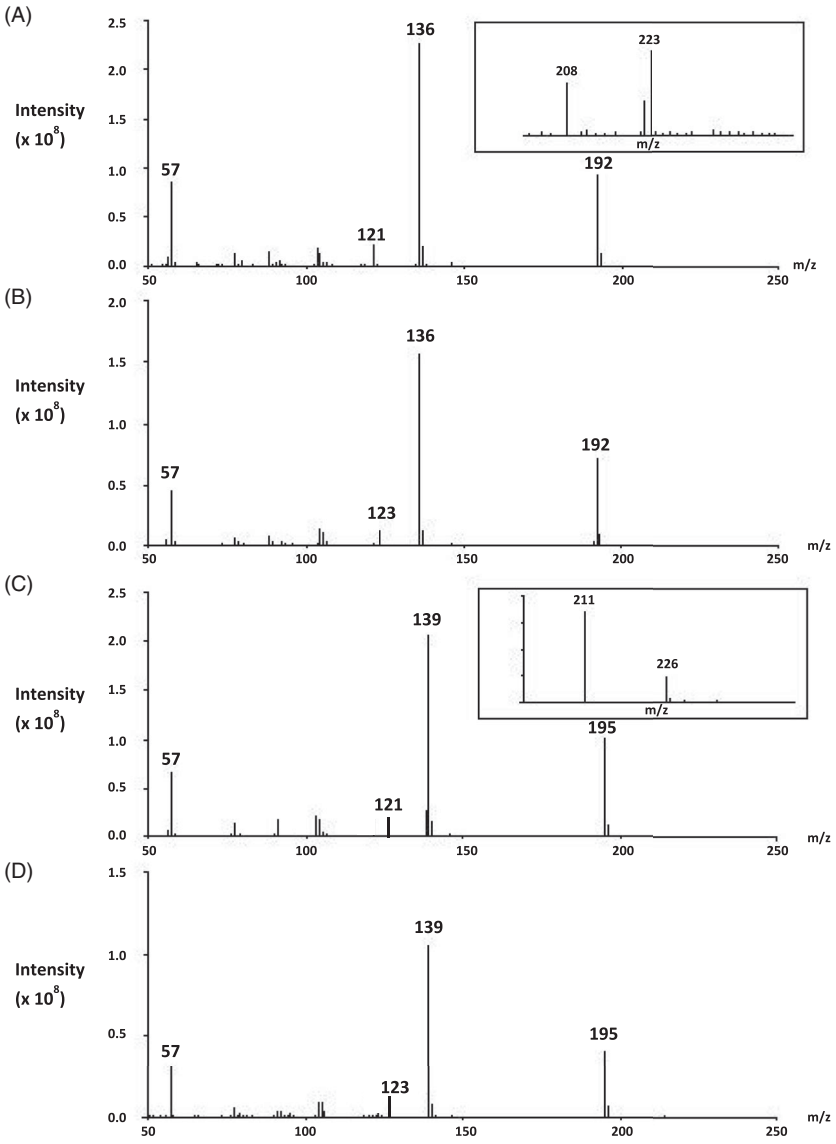


Figure 5. The EI-mass spectra for the methyl and hydroxymethyl di-adduct of the spin trap PBN (compound B) when using the following as secondary sources of free radicals: (A) DMSO and methanol {$PBN(CH_2OH)(CH_3)$}, (B) DMSO and d_3-methanol {$PBN(CD_2OH)(CH_3)$}, (C) d_6–DMSO and methanol {$PBN(CH_2OH)(CD_3)$}, and (D) d_6-DMSO and d_3-methanol {$PBN(CD_2OH)(CD_3)$}. The insets for (A) and (C) show the mass spectra with the intensity scale expanded for the region 200–240 m/z.

phenyl ring of PBN and replaced one of the hydrogen atoms; the m/z values of the molecular ion and fragment ions listed in Table 1 increase by only 5 units (rather than 6) when d_6-PBN is used instead of PBN in the Fenton-based reaction. The site of addition is not entirely clear although replacing PBN by F-PBN gives a nearly identical chromatogram and with the EI-mass spectrum of **C** now having a molecular ion at m/z 211, indicating that the hydroxyl radical has added either to the ortho or meta position on the phenyl ring. Although only a single peak is observed for PBN-OH in the chromatogram (Figure 3), previous studies have observed the formation of several isomers (hydroxyl adducts to the phenyl ring of PBN) from Fenton-type chemistry when using either HPLC with electrochemical detection [21] or derivatization with a silylating agent followed by GC-MS [22].

GC-MS analysis of compound B following HS-SPME

In a previous study, we demonstrated that free radicals trapped by PBN may be detected and identified by sampling the reaction vial headspace using thermal desorption followed by gas chromatography with mass spectrometry (TD-GC-MS). Here, a slightly different approach to sampling the headspace has been used,

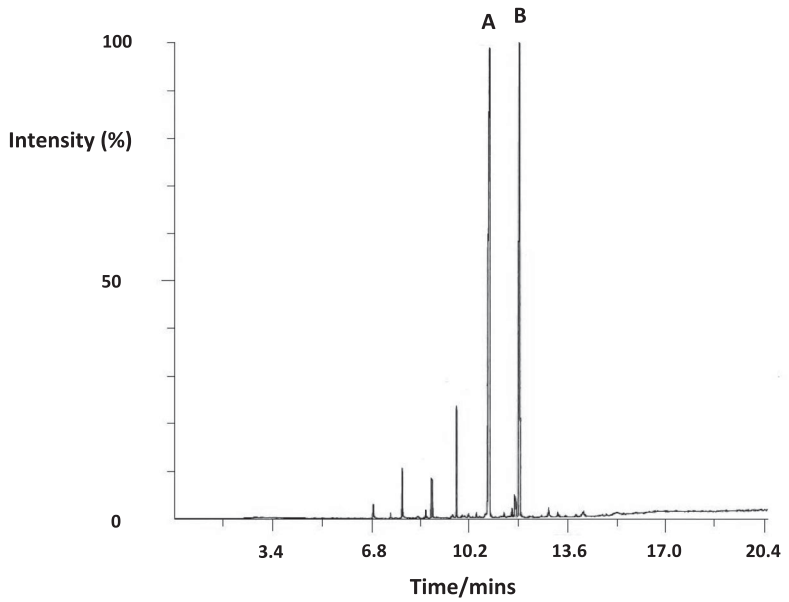


Figure 6. Total ion chromatogram (TIC) obtained from GC-MS analysis of the Fenton-based reaction mixture containing PBN, methanol and DMSO following SPME sampling of the vial headspace. The peaks labeled with letters have been identified from their EI-mass spectra as PBN-Me_2 and PBN(CH_2OH)(CH_3), compounds A and B, respectively.

$$\cdot CH_2OH + O_2 \longrightarrow \cdot OOCH_2OH$$

Scheme 4. Generation of the hydroxymethyl peroxyl radical by addition of oxygen to the hydroxymethyl radical.

$$\cdot OOCH_2OH \longrightarrow \cdot OOH + H_2C{=}O$$

Scheme 5. Dissociation of hydroxymethyl peroxyl radical to hydroperoxyl radical and formaldehyde [27,28].

i.e. SPME. Figure 6 shows the total-ion chromatogram (TIC) generated by the GC-MS analysis of the Fenton-based reaction mixture containing PBN, methanol, and DMSO, following sampling of the reaction vial headspace using SPME with a carboxen/polydimethylsiloxane (CAR/PDMS) fiber. Both PBN-Me_2 (compound **A**) and PBN(CH_2OH)(CH_3) (compound **B**) are observed and their identities confirmed by using alternative spin-traps (d_6-PBN and F-PBN) and isotopically labeled sources of secondary free radicals (d_6-DMSO and CD_3OH) – see Tables 1 and 2. **A** and **B** are detected, with varying intensity, by HS-SPME irrespective of the type of fiber used to sample the reaction vial headspace, however, the intensity of the peak for **B** is highest when CAR/PDMS is used for extraction (data not shown). It is well-known that extraction of more polar analytes can often be enhanced by combining polar and non-polar materials within the fiber [23].

Discussion

Methanol is an industrial solvent used in many household products which, on absorption, is metabolized by alcohol dehydrogenase to give formaldehyde, which may then be further metabolized by enzymes such as formaldehyde dehydrogenase to formic acid [24]. Previous nitrone spin trapping studies have demonstrated the formation and capture of the hydroxymethyl radical from rat liver microsomal and nuclear activation of methanol [11] or in the bile and urine of male Sprague Dawley rats following administration of methanol [25]. In the latter, POBN was used as the spin trap and injected into the rats following methanol administration, and the resulting radical adduct (POBN-CH_2OH) was detected by EPR spectroscopy. The hydroxymethyl radical may also be generated chemically using Fenton reagents and methanol, with the hydroxyl radical, produced by the Fenton reaction, abstracting a hydrogen atom from either the methyl or hydroxyl group, the former being more energetically favorable (Scheme 1; [26]). Once formed, $\cdot CH_2OH$, in the presence of oxygen, may be converted into the corresponding peroxyl radical (Scheme 4) which is known to eliminate $\cdot HO_2$ to give formaldehyde (Scheme 5; [27,28]). In the presence of PBN, $\cdot CH_2OH$, adds to the C=N carbon to form a nitroxide PBN-CH_2OH (first step in Scheme 2)

which is stable at room temperature and thus may be detected by EPR spectroscopy [10] or GC-MS [11]. However Castro et al. [11] only observed the nitroxide following derivatization of the dried solvent extract with a silylating agent to give a trimethylsilyl ether derivative. Indeed, many previous studies involving identification of PBN trapped radicals using GC-MS have required treatment of the resulting nitroxide with a derivatizing agent, which adds a trimethylsilyl or related group to the N-O moiety {for examples, see [18,29,30]}. Furthermore, in a previous study, when trapping methyl radicals with PBN, we did not observe a nitroxide in the reaction vial headspace using thermal desorption GC-MS [3]. The potential need for an extra step in the experimental approach to GC-MS detection and identification of PBN spin adducts is also supported in the current work where, in the absence of DMSO, the PBN-CH_2OH nitroxide was not observed in either the headspace or by extraction with chloroform (data not shown). However, the inclusion of DMSO in the Fenton-based reaction mixture led to the formation of PBN(CH_2OH)(CH_3) which was easily detected by GC-MS.The mechanism of formation of this di-adduct is likely to be *via* methyl radical addition to the PBN-CH_2OH nitroxide (second step in Scheme 2). Boyd and Boyd [31] have demonstrated through computational work that PBN di-adduct formation is energetically more favorable when compared to formation of only the mono-adduct, although the C=N carbon is the most favored site for initial radical addition. Thus, the presence of DMSO, or its isotopically labeled analogue, in the Fenton-based reaction mixture effectively allows an "*in-situ* derivatization" by methylation of the nitroxide making the trapped radical sufficiently volatile to be detected *via* solvent extraction or in the headspace of the reaction vial. This approach avoids the need for a time-consuming silylation step, which also potentially complicates the interpretation of the mass spectrum when more than one group from the derivatizing agent is added to the nitroxide. Also, SPME offers an alternative to thermal desorption (TD) for extracting products of free radical trapping [3]. Potentially, it is more suited to the extraction of volatiles with polar groups, as a range of fibers are available for selection.

An alternative to the use of GC-MS for separating and identifying spin-trapped free radicals is high performance liquid chromatography – mass spectrometry (HPLC-MS). This has been used successfully in previous studies to separate out the spin-adducts and detect them using electrospray ionization mass spectrometry (ESI-MS) (for examples, see [4,5]). This approach potentially allows the detection in aqueous solution of the nitroxide directly without the need for derivatization. It is also more suited to the detection of less volatile spin-adducts than GC-MS. However, due to the nature of ESI-MS, which gives molecular species ($M+H^+$) and mostly little fragmentation in the ion source, it is generally less useful for structural analysis of the PBN spin adducts and di-adducts than EI-MS. Whilst electrospray ionization with tandem mass spectrometry (ESI-MS/MS) may improve structural identification, it is potentially more time-consuming and requires more expensive equipment. In addition, GC-MS may be used directly for the analysis of volatile compounds in the headspace.

In conclusion, we have demonstrated a novel approach to detecting PBN spin trapped free radicals. DMSO, or an isotopically labeled analogue, may be used as an "*in-situ* derivatizing agent" for the nitroxide formed by radical trapping at the C=N carbon. The resulting di-adduct is then sufficiently volatile to be analyzed by GC-MS, both in the sample vial headspace and by liquid-liquid extraction. For the former, SPME may be used, providing a simple solvent-free extraction step. This methodology may potentially be applied to many nitrone spin traps and for a variety of chemical, biochemical and biomedical applications involving free radicals, thereby providing unequivocal identification of the radical that has been trapped.

Disclosure statement

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