Electron paramagnetic resonance of the free radicals in the gas- and particulate phases of cigarette smoke using spin-trapping

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Abstract

Free radicals in cigarette smoke have been studied using spin trapping EPR techniques. 2R4F reference cigarettes were smoked using 35 ml puff volumes of 2 seconds duration, once every 60 seconds. The particulate phase of the smoke was separated from the gas phase by passing the smoke through a Cambridge filter pad. For both phases, free radicals were measured and identified. A range of spin-traps was employed: PBN, DMPO, DEPMPO, and DPPH-PBN. In the gas-phase, short-lived carbon- and oxygen- centered radicals were identified; the ratios between them changed during the smoking runs. For the first puffs, C-centered radicals predominated while for the later puffs, O-centered radicals were mainly observed. The particulate phase and the 'tar' were studied as well.

Keywords: Cigarette smoke, Spin-trap, PBN, DMPO, DEPMPO, DPPH, Free radical, EPR

Introduction

Tobacco smoke is a complex carbon-based dynamic aerosol suspended in an equally complex organic vapor mixture. It is generated from a combination of combustion (up to 950 $^{\circ}$C), pyrolysis and distillation below 600 $^{\circ}$C) of tobacco.[1] Over 4,000 compounds have been identified in cigarette smoke and many are possible participants in radical-generating reactions.[2]

Fifty years ago cigarette smoke was shown to contain free radicals,[3] by condensing it at very low temperature and detecting the radicals by electron paramagnetic resonance (EPR).[3] The study of such radicals smoke has since been important in biology and medicine owing to their potential contribution to cardio-vascular and lung diseases.[4,5] It has been claimed that oxidative damage and oxidative stress in cells might be induced by reactive free radicals.[1-5] The damaging radicals in cigarette smoke include inorganic (NO_2, $^{\bullet}OH$, $^{\bullet}OOH$) and organic carbon-centered ($^{\bullet}R$) or oxygen-centered ($^{\bullet}OR$, $^{\bullet}OOR$) species.[2] However it is important to note that free radicals

are only one of the groups of toxicants in cigarette smoke and their relative toxicities are not fully understood.[1]

Formation of Radicals in Smoke

Free radicals can be generated *via* oxidative processes, and are highly reactive. For example, most primary combustion radicals have lifetimes of less than a microsecond in air.[2] Despite their reactivity, free radicals have been detected in the vapor phase of cigarette smoke for as long as 5 minutes after combustion.[6-10] This result is due to two specific characteristics of cigarette smoke: first, the gas-phase free radicals are stabilized by delocalization and, secondly, the smoke contains high concentrations (up to 700 ppm)[2] of the stable radical nitric oxide (NO$^\bullet$) which can continuously generate short-lived radicals.[10] In this case, the oxidation of NO to NO_2 and the addition of the NO_2 to unsaturated compounds produce carbon-centered radicals which then react with atmospheric oxygen to form oxygen-centered radicals (Scheme 1).
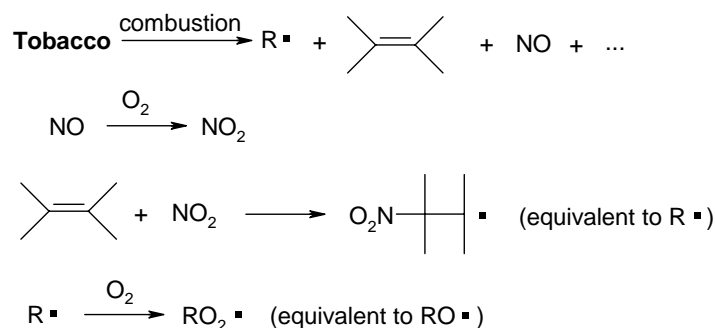
Scheme 1. Formation of C-centered radicals (R$^\bullet$) and O-centered radicals (RO$^\bullet$) in cigarette smoke.[10]

Detection of Radicals in Smoke

It is presumed that the organic radicals formed initially in the cigarette combustion zone are carbon-centered and that they react with O_2 to generate myriad oxygen-centered radical compounds. The high reactivity of most free radicals makes their detection difficult. Nevertheless, detection of oxygen-centered radicals present in smoke is a critical step towards understanding radical chemistry occurring in the system.[2]

Many direct and indirect methods have been employed to detect, quantify and identify short-lived or stable free radicals, including those formed from burning tobacco. They include direct EPR (flow) detection, indirect EPR of spin-trapped species, short-lived radical and stable radical coupling followed by HPLC, LC, fluorescence, GC, MS, or IR methods.[3-11] It can be argued that EPR spectroscopy provides the most information about free radicals in a 'single' experiment. This is because the EPR technique is specific (it detects *only* unpaired electrons),

highly sensitive (it can detect free radicals at less than 10^{-10} *M*) and in many cases may give some structural information such as the nature of the atoms closest to the unpaired electron.

The particulate phase smoke (or Total Particular Matter, TPM), contains several exceptionally long-lived (greater than 24 hours) radical species, principally a mixture of quinone, semiquinone and hydroquinone functionalities held in a polymeric matrix. These can be detected directly by EPR.

In contrast, gas-phase tobacco smoke contains carbon- and oxygen-centered radicals that are too reactive to be detected directly.[6-12] To enable detection, these are reacted with a diamagnetic molecule, called a spin-trap, in order to form a persistent spin-adduct. This increases the lifetime to minutes–hours, which allows the radical to be seen easily and identified by EPR spectroscopy (Scheme 2). The most common spin-traps are nitrones and nitroso- derivatives, both of which contain a double bond which reacts with the free radical (Figure1).
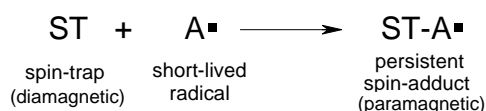
Scheme 2. Formation of a persistent radical by spin-trapping.

Oxygen-centered free radicals such as alkoxyls and peroxyls have been identified in cigarette smoke by spin-trapping or by using a non-reductive scavenging method.[6,7,10] However, the EPR spectra for O-centered radicals presented in the literature are usually weak and complex, because they are mixed with C-centered radicals which are formed first and are more stable. It is well known that the best spin-trap for O-centered radicals is 5-diethoxyphosphoryl-5-methyl-1-pyrroline N-oxide (DEPMPO, Figure 1), which increases the life-time of the trapped peroxyl radicals by a factor of more than 15.[13,14]
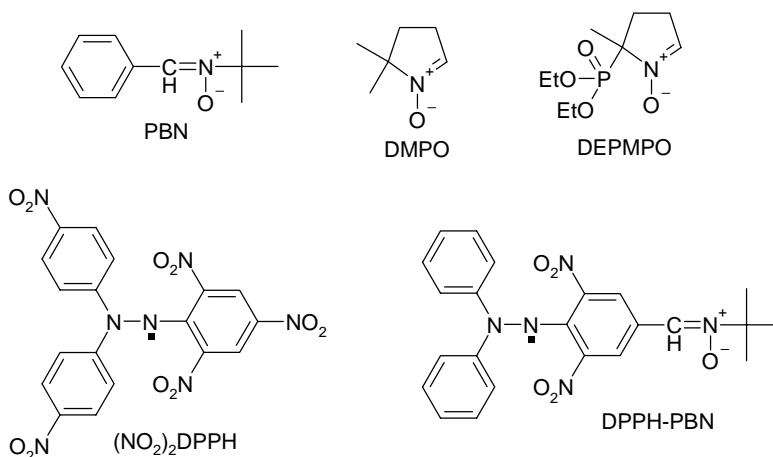
Figure 1. Chemical structures of the spin-traps and hydrazyl radicals.

Different spin-traps have different selectivities towards radical capture, hence a range of spin-traps is recommended for the study of a complex system such as cigarette smoke. In the present study we used several nitrones (*N-t*-butyl-α-phenylnitrone PBN, 5,5-dimethyl-1-pyrroline-*N*-oxide DMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide DEPMPO), as well as a stable hydrazyl free radical 2,2-*p*-nitrophenyl-1-picrylhydrazyl $(NO_2)_2$DPPH (Figure 1) as an oxidant. The hybrid compound DPPH-PBN (Figure 1), which contains a hydrazyl moiety and a nitrone moiety, was also used.[15,16] In most studies on spin-trapped gas-phase free radicals, the oxidation product derived from the corresponding spin-trap can also be seen clearly by EPR spectroscopy.[6]

Results and Discussion

Gas-phase study. For preliminary screening purposes, four spin traps were evaluated – PBN, DMPO, DEPMPO, and DPPH-PBN (Figure 1).

PBN as spin-trap. The PBN spin trap has been widely used to trap radicals in cigarette smoke, and our experiments with it yielded no new information. The recorded EPR spectra and the hyperfine coupling constants of a_N=0.142 mT and a_H=0.205 mT indicate the presence of a carbon-centered radical.[3,6]

DMPO as spin-trap. DMPO spin-trap has also been used previously to trap O-centered free radicals[6] but the EPR spectra obtained were complex and had low intensities. In the earlier study, the EPR spectra were assigned to a mixture of at least three radicals – an O-centered radical (alkoxyl type), an unidentified C-centered radical, and a breakdown product from the DMPO spin-trap. In this work, the EPR spectra obtained were different and had higher intensities and better resolutions (probably due to the shortening of the distance from the cigarette to the spin-trap solution).

To study the formation and decay of the short-lived radicals during the smoking period, EPR measurements were performed on smoke collected after different numbers of puffs had been completed: the first sample was taken after 2 puffs and the second one after 10 puffs. The two EPR spectra are shown superimposed in Figure 2. The spectra were assigned to two dominant oxygen-centered radicals and one unidentified by-product. The ratios between the radicals were different for the two samples, indicating the dynamic changes that occurred throughout the smoking period.
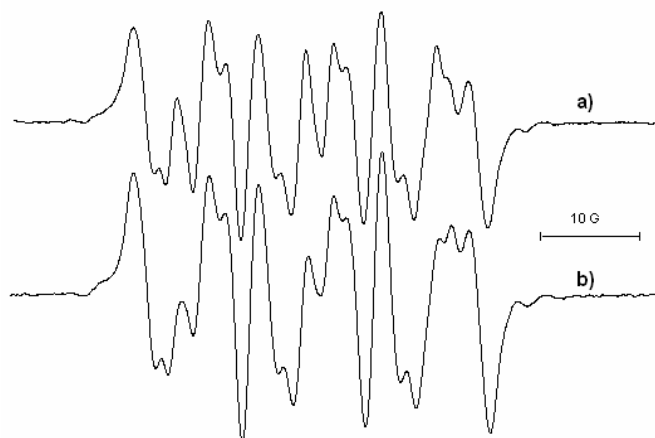
Figure 2. EPR spectra recorded with DMPO as spin-trap, (a) after 2 puffs, and (b) after 10 puffs.

Figure 3 displays the computer-simulated spectrum (in red) together with the experimental EPR spectrum (in black). The fitting was achieved with the following simulation parameters based on a mixture of three species: two oxygen-centered radicals (~90% of the total, with hyperfine coupling constants a_N =1.295 mT, a_H =0.668 mT, and a_H = 0.176 mT for the first O-centered radical, and a_N =1.322 mT, a_H=0.751 mT, and a_H = 0.183 mT for the second O-centered radical), and an unidentified radical (~10%) with a_N =1.350 mT, probably resulting from the decomposition of the spin-adducts. The coupling constants reported in the literature for the methoxyl radical in benzene (1.360 mT, 0.760 mT and 0.185 mT) are very close to those of the oxygen-centered radicals trapped by DMPO.[6] Any mismatch between the simulation and the experimental spectra in Figure 3 may be due to the complex nature of the mixture.[17,18]
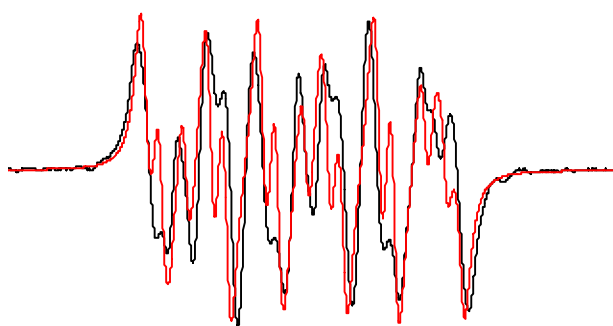

Figure 3. Simulation (in red) of the EPR spectra recorded after 2 puffs (DMPO used as spin-trap).

The difference in the EPR spectra between the smoke collected after the 2nd and after the 10th puffs may be caused by the different ratios of the radicals trapped. The 10th puff appeared to

contain more O-centered radicals. The composition of cigarette smoke changes on a puff-by-puff basis, due to the gradual reduction of the tobacco rod length, which, in turn, changes the environment where the smoke is formed.[1] Despite the fact that puff-resolved free radical measurements are rarely reported in the literature, the phenomenon should perhaps not come as a total surprise.

Experimental results on the stability of the spin-adducts as a function of time are given in Figure 4. In contrast to a previous study,[6] it was found that O-centered radicals decayed faster than C-centered radicals, as shown in Figure 4, probably due to the greater stability of the carbon-centered radical.
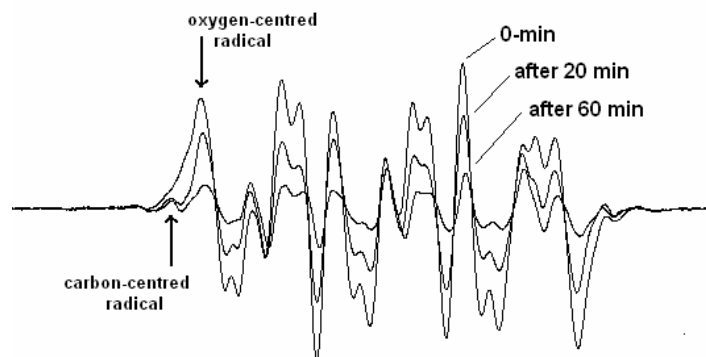

Figure 4. Change in the EPR spectrum with time: After 1 h, the peaks corresponding to the carbon-centered radical become more visible.

DEPMPO as spin-trap. The DEPMPO spin-adducts of O-centered radicals (especially peroxides) have a very long life-time compared with the DMPO congener.[12,13] Therefore, it seemed reasonable to use this spin-trap for the short-lived O-centered free radicals. Similar experiments were performed after 2 and 10 puffs. These spectra are shown in Figure 5. The spectra consisted mainly of C- and O- centered radicals. The carbon-centered radicals appeared to dominate after 2 puffs, whilst the oxygen-centered radicals became dominant after 10 puffs. The values of the hyperfine coupling constants obtained from this work are close to those reported in the literature for C-centered radicals: a_N =1.372 mT, a_H =2.319 mT, and a_P = 4.623 mT; for O-centered radical: a_N =1.382 mT, a_H =0.820 mT, and a_P = 4.511 mT. The exact coupling constants are difficult to extract due to the complexity of the mixture and to the fact that DEPMPO forms two different spin-adducts.[13,19]

The change in the ratio between C- and O- centered radicals may be the result of several factors. Firstly, the C-centered spin-adduct radicals are more stable in the mixture. Secondly, there is a genuine difference in gas-phase composition on a puff-by-puff basis.[20-25] Literature shows that in aged smoke the concentration of the free radicals is unexpectedly increased; this

has been explained by the radical reactions associated with NO and NO_2.[10, 18] More work is needed to address the contributions of these factors.
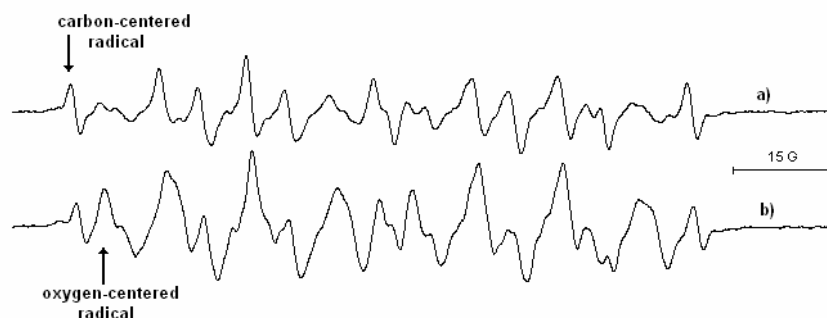
**Figure 5.** EPR spectra recorded with DEPMPO as spin-trap, after 2 puffs (a), and after 10 puffs (b).

DPPH-PBN as spin-trap. The stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) has been used for quantitative analysis of peroxy radicals in cigarette smoke *via* its non-reductive scavenging properties.[8] The hybrid compound (DPPH-PBN, Figure 1) contains a stable free radical moiety of the hydrazyl type and a nitrone spin-trap moiety, which allows it to be used as a sensor to probe free radical chemistry.[14] In this experiment, the objective was to identify *only* the O-centered radicals (as a mixture of alkoxyl and peroxyl radicals). Ideally, the spin-trapping moiety of DPPH-PBN will trap the O-centered radicals whilst the C-centered radicals would be trapped by the hydrazyl moiety. The experimental EPR spectra ((a)-before smoking, (b)-after 5 puffs, (c)-after 10 puffs) are presented in Figure 6. The results in Figure 6 only indicate the disappearance of the initial hydrazyl moiety in the EPR signal, i.e., no spin-adducts were formed. The absence of the spin-trapped O-centered radicals in this case cannot be explained at this stage. However, the amount of free radicals detected is similar to the literature data.[6]
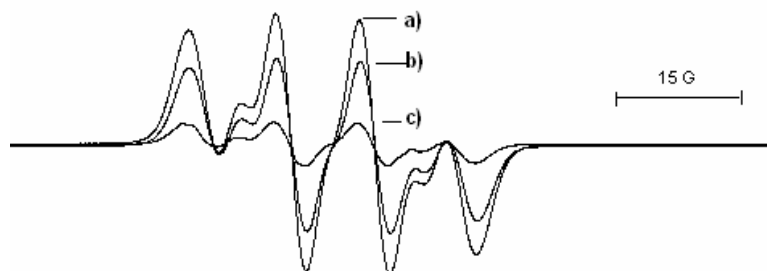
Figure 6. Decrease of the EPR signal from DPPH-PBN hybrid compound during the smoking: (a)-initial signal, (b)-after 5 puffs, (c)-after 10 puffs.

Particulate-phase study. The total particulate matter (TPM) collected on a Cambridge filter pad (from ten 2R4F cigarettes) was extracted in benzene. This was followed by the experiment as described below.

TPM oxidation by a hydrazyl stable radical and their spin-trapping by DMPO. Hydrazyl stable free radicals are well known as organic oxidants. They can generate C-, O-, N-, S-, and P-centered short-lived radicals *via* electron- or hydrogen- abstraction.[14,15] To generate short-lived radicals from TPM, a mixture of DMPO and $(NO_2)_2$DPPH hydrazyl radical (as an oxidant) was added to the TPM extracted in benzene. An intense EPR signal from the mixture was recorded (Figure 7). This spectrum consisted mostly of oxygen-centered radicals but it also contained a few new features, which were tentatively assigned to nitrogen-centered radicals. The argument against a mixture of three different C-centered radicals with the same concentration and coupling constants is based on the assumption that it is more difficult for the hydrazyl radical to abstract an H-atom from a C-H bond (strong bond) than from an N-H bond.[14]

possible splitting due to a
nitrogen-centred radical
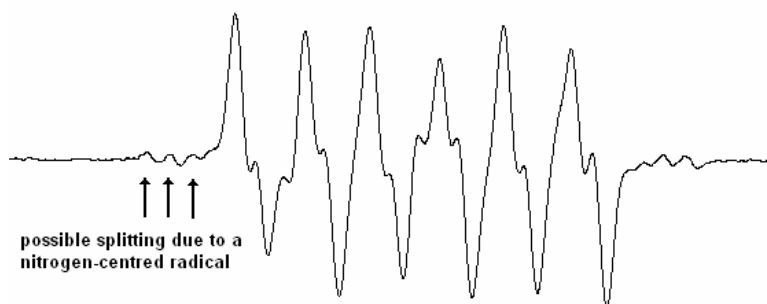
Figure 7. EPR spectrum obtained by TPM oxidation with a hydrazyl radical in the presence of DMPO spin-trap.

The computer simulation (in red) superimposed on the experimental EPR record is presented in Figure 8. The simulation was based on a mixture of three species. The first species is an oxygen-centered radical (~90%) with hyperfine coupling constants of $a_N = 1.295$ G, $a_H = 0.668$ mT, and $a_H = 0.176$ mT. The second species is an unidentified radical (~5%) with $a_N = 1.350$ mT, probably formed from the decomposition of the spin-adducts. The last species is a nitrogen-centered radical with the hyperfine coupling constants $a_N = 1.489$ mT, $a_H = 1.806$ mT, and $a_N = 0.217$ mT (similar values have been reported in the literature).[14,19]

The simulation fits well with the experimental spectrum. The presence of the radical adducts can be separately seen by deconvolution of the simulated spectra. The presence of the same radicals trapped from the gas-phase and from the oxidized TPM may suggest that both the gas phase and the particulate phase smoke contain, besides other species, some common class of radical-generating compounds such as polyphenols.[26]
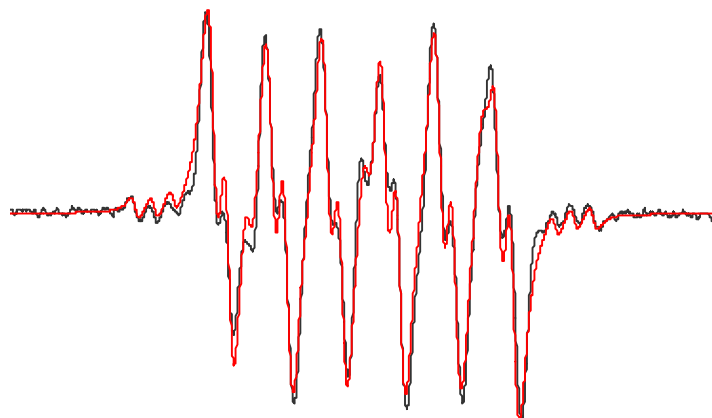
Figure 8. EPR spectrum from Figure 7 (in black), together with the simulated one (in red). For EPR parameters see text.

Conclusions

The predominant short-lived radicals in the gas phase are C- and O- centered radicals. PBN trapped mainly C-centered radicals whilst O-centered radicals were detected with DMPO and DEPMPO traps. Differences in the composition of gas-phase free radicals were found between smoke collected at the beginning and at a later stage of smoking. More C- centered radicals were detected at the start, while O- centered radicals became dominant towards the end. Experiments revealed short-lived radicals formed by oxidation of TPM with a hydrazyl radical. These were mostly O-centered radicals, with small amounts of C- and N- centered free radicals.

Experimental Section

General Procedures. All the chemicals and solvents were from Aldrich and used as received. DPPH-PBN and $(NO_2)_2$DPPH were synthesized as before.[15,16] The EPR spectra were recorded at ambient temperature on a JEOL FR30EX spectrometer using the following settings: centre field 336 mT, sweep field 10 mT, frequency 9.42 GHz, power 4 mW, sweep time 60 s, time constant 0.1 s, modulation frequency 100 kHz, gain 200 and modulation width 0.1 mT. The reference cigarettes used in this work, 2R4F, were supplied by the University of Kentucky. Ten cigarettes were smoked simultaneously using a standard 20-port Borgwald RM 200 smoking machine under ISO smoking conditions: 35 ml puffs of 2 seconds duration, every 60 seconds. The smoke was passed through a standard Cambridge filter pad which separated the particulate phase (retained by the filter pad) from the gas-phase (bubbled through the spin-trap solution).

The standard spin-trapping procedure involved trapping the free radicals from the gas-phase in 5 mL of a $0.01M$ solution of the spin-trap dissolved in benzene. The conditions were based on

those described by Baum *et al.*,[6] except that the distance between the Cambridge pad holder and spin-trap solution was considerably shortened (*ca.* 5 cm) to minimize free-radical losses. The spin-trap solution was placed in a 25 ml glass three-necked pear-shaped flask, and the gas phase smoke was bubbled through the solution via a PTFE tube connected to the Cambridge pad holder. After smoking, the volume of the solution was adjusted to 5 mL (some benzene was evaporated during the experiment) and 0.4 mL was placed into a standard EPR cylindrical quartz tube (4 mm inner diameter). The sample was deoxygenated using the freeze-pump-thaw technique at a pressure of 10^{-3} mm Hg. This step was essential in order to get sharp EPR lines as the presence of atmospheric oxygen dramatically reduced the intensity and broadened the peaks. The concentration of the radicals was estimated by comparing the values of the double integral of the EPR experimental spectrum with the double integral of a standard stable radical (Tempol) at a known concentration.

The particulate-phase retained by the Cambridge filter was extracted with 5 mL of benzene and analyzed by adding DMPO spin-trap (final concentration 0.01 M), followed by the $(NO_2)_2DPPH$ oxidant. The sample was analyzed using the same procedure as for the gas-phase.

The experimental recorded spectra were digitalized using software supplied by JEOL. Spectra were simulated using WinSim software.[17]

Acknowledgements

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