Methods for Analysis of Free Radicals in Cigarette Smoke

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Abstract: Free radicals in the particulate phase of cigarette smoke were first measured by direct electron paramagnetic resonance (EPR) spectroscopy over 60 years ago. Early efforts to measure free radicals in cigarette smoke were prompted by the theory that radicals could be involved in carcinogenesis. It was thought that free radicals could be either direct acting or produced by other components of cigarette smoke such as polycyclic aromatic hydrocarbons. Even today, it is uncertain which of these routes of action is the most important. Ultimately, the development of a strategy to minimize potential biological damage from free radicals is dependent on the extent to which free radicals delivered in cigarette smoke are directly involved in disease processes. In recent years, the primary instrumental means for identifying and studying free radicals in smoke have been both EPR and mass spectrometry (MS) techniques using spin trapping. The spin trapping technique allows stabilization of short-lived species. When coupled with MS, spin trapping allows complete structural characterization of free radicals. When coupled with EPR, spin trapping allows structural characterization by comparison to EPR spectra of known chemical species. Advances in the development of spin traps and spin trapping techniques, along with EPR and MS methods used for the study of cigarette smoke free radicals are presented in this review.

Keywords: Cigarette Smoke, Electron Paramagnetic Resonance Spectroscopy, Free Radicals, Mass Spectrometry, Spin Trap.

INTRODUCTION

Free radicals in the particulate phase of cigarette smoke were first measured by direct electron paramagnetic resonance (EPR) spectroscopy over 60 years ago [1-3]. Oxidative damage to cellular components producing systemic inflammation has been extensively studied as a potential link between cigarette smoking and the development of a number of diseases including lung cancer, cardiovascular disease, and chronic obstructive pulmonary disease [4, 5]. As early as 1958, researchers considered "the free radical content of cigarette smoke as a parameter in the etiology of lung cancer."[1] More recently, the presence of free radicals in cigarette smoke has been suggested as a potential link between cigarette smoke and cellular oxidative damage because of the ability of free radicals to cause lipid peroxidation, DNA damage, and protein oxidation [6].

Even after five decades, researchers are still uncertain of the extent to which the free radical related cellular damage caused by cigarette smoke is the result of direct exposure to free radicals in the smoke or to the production of free radicals by cells after their exposure to other chemical constituents of cigarette smoke. Interest in assessing the biological relevance of free radicals in cigarette smoke has led to the development and refinement of several instrumental methods for the quantification and structural identification of those free radicals (Table **1**).

ELECTRON PARAMAGNETIC RESONANCE SPECTROSCOPY

Electron paramagnetic resonance (EPR) spectroscopy, also known as electron spin resonance (ESR) spectroscopy [7], has been the method of choice for characterizing free radicals. EPR is highly selective and detects only species that are derived from paramagnetic radicals [7]. EPR is also highly sensitive, allowing detection of small quantities of free radicals. Furthermore, EPR can detect radicals regardless of their physical form, be it gas, liquid, or solid. However, in many cases, the data generated by EPR are not sufficient to structurally identify the radical being detected. It is understood that free radicals in cigarette smoke and biological systems often have half-lives of seconds or less and are too kinetically unstable to be quantified or structurally identified by EPR without the use of other techniques such as spin trapping.

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A common tool to assist in structural identification of kinetically stable free radicals is the use of hyperfine coupling constants (HFCC), usually denoted by the symbol A. HFCCs arise from the interaction of spins of the unpaired electron with the magnetic field of neighboring atomic nuclei. The nuclear magnetic field (magnetic moment) can oppose or add to the magnetic field generated by the instrument magnet resulting in different transition frequencies. From the number and intensity of the lines generated from the hyperfine splitting phenomenon, it becomes possible to determine how many nuclei possessing a magnetic moment interacted with the radical electron. Once the value of the HFCC is determined, it can be correlated with published numbers of the coupling constant for various functional groups. While in some cases identification is possible, in many cases only the general type of radical can be determined (carbon/oxygen/nitrogen centered, alkoxy, peroxyl, alkyl, etc.) [7]. Other techniques such as MS or NMR allow more complete structural identification of free radicals.

DIRECT EPR OF CIGARETTE SMOKE

EPR was the first instrumental method to detect and quantify free radicals in cigarette smoke [1]. Over the five decades since the first publication of EPR data from cigarette smoke, the basic experimental methods have changed only incrementally. Direct EPR methods have been standardized to allow the reproducible quantification of the more stable free radicals in the particulate phase. These methods, however, do not allow quantification of the less stable species in the gas vapor phase [8]. They also do not allow the structural identification of the numerous free radical species present in cigarette smoke, which is needed for a full consideration of the potential biological effects of such free radical species.

Lyons *et al.* first detected radicals in cigarette smoke in 1958 [1]. The authors used solid-state EPR to analyze mainstream smoke collected at liquid oxygen temperatures. The detected radicals were divided into two distinct populations, an unstable population detectable at low temperatures (accounting for 15 to 20% of the total radical population) and a stable portion detectable at room temperature for several days after formation (accounting for 80 to 85% of the total radical population). The particulate phase was estimated to contain 6×10^{15} spins per cigarette.

Lyons and Spence reported the detection of free radicals from dried cigarette sidestream smoke (SSS) by direct EPR [2]. The number or type of cigarettes smoked and the smoking conditions were not specified. The SSS condensate was found to contain 5×10^{14} spins per gram versus 6×10^{15} spins per gram for mainstream

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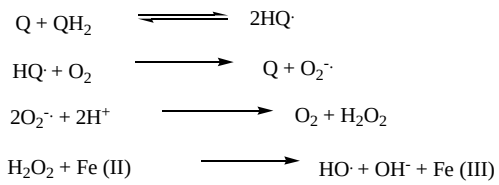
Table 1. Methods Reported for the Study of Free Radicals in Cigarette Smoke and Related Matrices

Method	References; Author (cited in this work)
Direct EPR	Lyons 1958 (1) Lyons 1960 (2) Takeshita 1964 (9) Tully 1969 (11) Bluhm 1971 (39) Pryor 1976 (8) Pryor 1983 (42) Pryor 1983 (10) Pryor 1984 (13) Halpern 1985 (33) Baum 2003 (17) Johnson 2004 (51) Ghosh 2006 (18) Dyakonov 2008 (19) Robinson 2009 (15)
Spin trapping EPR	Bluhm 1971 (39) De Hys1973 (40) Menzel 1976 (41) Pryor 1976 (8) Halpern 1985 (33) Baum 2003 (17) Ghosh 2006, 2007, 2008 (18,14,32) Robinson 2009 (31)
GC/MS	Suezawa 1981 (44)
LC-fluorescence labeling	Kieber 1990 (46) Flicker 1998, 2001 (48,49)
LC/EPR/ESI-MS	Parker 1991 (47) Iwahashi 1992 (34) Johnson 2004 (51)
LC/TSP-MS	Parker 1991 (47)
ESI-MS/MS	Masselot 2002 (50) Johnson 2004, 2005 (51,35) Bartalis 2007, 2009 (53,54) Robinson 2009 (31)
Nano-LC/FTMS	Rolando 2006 (52)
NMR spin trapping	Zoia 2009, 2009, 2010 (57,58,59)

smoke (MSS) and 5 x 10^{18} and 2 x 10^{19} spins per gram for condensate from chimney soot and diesel exhaust, respectively. After column separation with n-hexane, benzene, and acetone, it were found that 35% and 50% of the radicals were present in the benzene and acetone fractions, respectively, while none of the radicals was present in the hexane fraction. The benzene solutions were shown to contain a species, which could decolorize the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical [9]. DPPH is a fluorescent radical that will react with other radicals to produce a non-fluorescent product. By monitoring the colorimetric properties of the solution of DPPH in the presence of radicals, the quantity of radicals can be indirectly determined. The authors proposed that the free radicals were present as electrons delocalized over four- to five-ring aromatic structures. This interpretation would remain unchallenged for more than 20 years until the work by Pryor in the 1980s [10]. Takeshita and Ohe further explored the persistency of the 'tar radical' *via* a colorimetric method that employed DPPH [9]. The authors were able to detect radicals in the condensed whole smoke of flue-cured tobacco 300 hours after combustion.



Scheme 1.

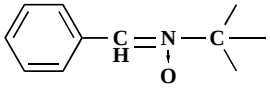
In 1969, Tully *et al.* reported on a study of the free radicals in the gas vapor phase (GVP), trapped at low temperature after removing the particulate phase by passing MSS through a Cambridge filter [11]. The GVP radicals produced no EPR signal at temperatures below -100°C. A three-line spectrum with a nitrogen splitting of 1.26 mT was observed at -100°C. After 1.5 hours at -100°C, the EPR signal reached a maximum. At temperatures above -100°C, the EPR signal disappeared. The authors proposed that the free radicals resulted from participation of nitrogen oxides in radical reactions.

Even as late as 1980, the full identification of specific radicals continued to elude researchers. In 1983, Pryor *et al.* reported studies of the temperature dependence of the EPR signal from cigarette condensate [10]. Twenty 1R1 Kentucky reference cigarettes were smoked under unspecified conditions. The condensate was found to contain at least four types of paramagnetic species with 6 x 10^{14} spins per cigarette (3 x 10^{16} spins per gram of tar), approximately an order of magnitude lower than previously reported by Lyons *et al*. [1]. The authors identified the paramagnetic species as an inorganic phosphorus radical, a graphitic carbon radical (g=2.0028), a polyaromatic hydrocarbon (PAH) radical (g=2.0026), and a quinone-hydroquinone system (g=2.0035) which represented more than 80 percent of the paramagnetism. After extraction into benzene and vacuum evaporation of the benzene, the quinone-hydroquinone system had a half-life of 12 days and a g factor of 2.0025 to 2.0029. Based on these observations, a model with delocalization of the unpaired electron over a large structure containing species similar to a semiquinone radical, was proposed. This model of the "tar" radical remains the most accepted to date. Recently, Dellinger has proposed that Pryor's original data are more consistent with the presence of polymeric or oligomeric phenols with the unpaired electron largely located on an aromatic carbon [12].

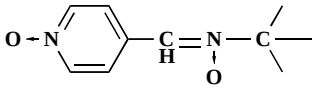
In 1984, Pryor *et al.* [13] published additional findings from EPR analysis of various extracts of tar indicating that the solvents containing phenolic extracts contained radicals. Treatment of the alcohol extracts with base generated radicals that Pryor's group assigned to the oxidation of phenolic and polyphenolic species in tar. The EPR and spin trapping data provided limited structural information not sufficient for unequivocal identification.

The authors defined a series of reactions producing these reactive oxygen species as follows in Scheme **1** [14]. Atmospheric O_2 was reduced by semiquinone (HQ•) to form superoxide, which then disproportionated to form hydrogen peroxide. H_2O_2 then reacted with metals, in particular iron (II), which was present in tobacco, to produce reactive hydroxyl radicals.

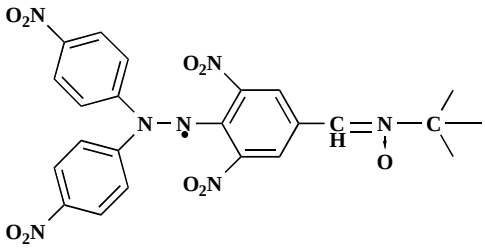
Numerous publications appeared later in the 1980's and 1990's, which reported on the use of direct EPR of cigarette smoke condensate. The basic methods used in these studies remained largely unchanged from those used in the 1960's. Lack of standardization in smoking and collection methods resulted in difficulty in comparing results from one study to another. Changes in smoking conditions recently have been shown to affect the speciation of free radicals in the gas-vapor phase of cigarette smoke [15, 16]. Such changes are likely to affect yield of free radicals. As previously noted, the measured yield of particulate phase free radicals varied over at least an order of magnitude between the initial measurement of Lyons *et al.* (6 x 10^{15} spins per cigarette) and the later measurements by Pryor *et al.* (6 x 10^{14} spins per cigarette) [10].



PBN, N-tert-butyl-a-phenylnitrone



POBN, a-(4-pyridyl-1-oxide)-N-tert-butynitrone



DPPH-PBN

Fig. (1). Nitroso spin traps reported in studies of free radicals in cigarette smoke.

In 2001, Blakley *et al.* [17] reported a lack of correlation between particulate phase free radicals and hydroquinone yield in cigarettes to which nitrate had been added to modulate hydroquinone yield. The authors developed a method which allowed concentrations of particulate phase free radicals to be measured with a relative standard deviation as low as 10%. The method was somewhat complex, involving smoking using Cambridge Filter Pad/ISO puffing parameters followed by extraction of the Cambridge filter with dichloromethane, evaporation of the dichloromethane, and dissolution of the residue in benzene. The yield of free radicals was reported as approximately 10^{13} spins per cigarette, differing substantially from values reported earlier (e.g. 6 x 10^{14} by Pryor *et al.* [10] and 6 x 10^{15} by Lyons *et al.* [1]). No consistent correlation was observed between the yields of hydroquinone and of particulate phase free radicals. Turkish tobacco had a high yield of both hydroquinone and free radicals. Flue cured tobacco had a high yield of hydroquinone but a low yield of free radicals. Burley tobacco had a low yield of hydroquinone and a high yield of free radicals. Interpretation of these results is complicated by the possibility that added nitrate changed the combustion characteristics of the cigarette or nitrated some of the hydroquinone. A small concentration of hydroquinone also may be all that is required to produce particulate phase free radicals. Hence, any changes in hydroquinone yield observed may not result in changes in the free radical yield.

Later that same decade, Baum *et al.* [18] reported on the importance of the solvent used for extraction in the quantification of particulate phase free radicals. Hexane, benzene, isopropanol and mixtures thereof were examined. The particulate phase from 20 1R4F Kentucky reference cigarettes was collected using standard ISO parameters (a 2-second puff of 35 mL with a 1-minute inter-puff period) and then dissolved in the minimum amount of solvent required to dissolve the tar. The volume was then reduced using a rotary evaporator maintained below 30°C in order to minimize any radical decomposition. A small aliquot of this material was placed in an EPR tube and measured directly. No data were reported, but according to the authors, "the reproducibility of the experiments was very poor."

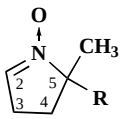
The authors also experimented with placing the entire Cambridge filter pad containing the particulate phase from 20 cigarettes directly into the EPR cavity. The Cambridge pad's background EPR signal was subtracted to obtain the signal from the particulate phase. The radical concentration was estimated to be approximately 10^{14} to 10^{15} spins per cigarette ±15%. There was a good correlation between the intensity of the EPR signal and the number of cigarettes smoked. The signal was inhomogeneously broadened, from which the authors concluded the presence of signals from multiple free radical species.

In 2006, Ghosh *et al.* [19] reported on the direct measurement of particulate phase radicals from a cellulose acetate (CA) filter rod. After smoking using a 2-second puff of 35 mL with a 1-minute inter-puff period, the authors trapped the particulate phase on the CA, and then placed the CA into the EPR cavity. The free radical concentration of the particulate phase was measured to be "on the order of 10^{14} spins per cigarette". Results from smoking four base tobacco blends (Flue-cured, Burley, Turkish, and a 1:1 mix of Flue-cured and Burley) showed the free radical concentration of Burley to be 1.4 times greater than for Flue-cured in the particulate phase. The mixture was intermediate between Flue-cured and Burley results. Results for Turkish were roughly equivalent to those for Flue-cured. These results underscore the importance of combustion kinetics in the formation of smoke free radicals. Changes in combustion processes can potentially alter the concentration and speciation of free radicals.

In 2008, Dyakonov *et al.* [20] reported a modified collection method for the EPR analysis of particulate phase free radicals with a delay of less than one minute between formation and measurement. A 1-mm plug of high-purity quartz wool was inserted into a 4-mm diameter quartz flow-through EPR tube to support approximately 100 mg of ultra pure silica gel selected because it generated no signal under the measurement conditions. The EPR tube was positioned so that the silica gel plug was in the center of the EPR cavity, and one cigarette was smoked through the EPR tube using a 2-second puff of 35 mL with a 1-minute inter-puff period. This collection method allowed the study of particulate phase radicals as they accumulated on the silica gel on a puff-by-puff basis. No values for the concentration of free radicals were reported. Although the cumulative number of free radicals measured increased with puff number, the amount of radicals measured from each puff decreased as the puff number increased. The authors hypothesized that "the radical-containing 'tar', which is deposited on tobacco downstream by smoke, can interact with and destroy the newly formed radicals, so that fewer numbers of them can be detected." It is also possible that relatively short-lived free radicals could decay during the interval between puffs.

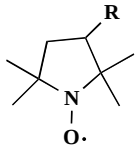
The methods of Ghosh and Dyakonov offer advantages over methods reported in earlier work for the study of particulate phase free radicals. Both offer a simplified means for using direct EPR that does not require collection and dissolution of the condensate. Both methods may allow for better reproducibility and comparison between samples due to lower losses of radicals during the manipulation of samples and the lack of need to control collection and measurement volumes. The Dyakonov method, and potential variants using CA instead of silica gel as a collection medium, offers the ability to study free radicals on a puff-by-puff basis, which can allow the researcher to examine the processes that produce free radicals rather than simply quantifying those radicals.

While direct EPR was the first widely used method for detecting and quantifying free radicals from cigarette smoke, it has a number of shortcomings that have limited its continuing utility for studying cigarette smoke. Direct EPR allows detection of only those species in smoke stable enough to remain unreacted for minutes to tens of minutes. Those species not sufficiently stable to remain unreacted within the time-scale of the measurement will be undetected. This lack of stability limits measurement by direct EPR to the long-lived species present in particulate phase; to date, no



Spin Trap	R=
BMPO	—C(=O)—OC(CH3)3
DMPO	—CH3
DEPMPO	—PO(OCH2CH3)2
DIPPMPO	—PO(OCH(CH3)2)2

Fig. (2). Nitrone spin traps reported in studies of free radicals in cigarette smoke.



Spin Trap	R=
3AP	NH2
3CP	CN

Fig. (3). Proxyl spin traps reported in studies of free radicals in cigarette smoke.

published results have identified GVP radicals based on direct EPR. Direct EPR also does not allow unambiguous identification of smoke radicals since the signal from the particulate phase tends to be quite broad and contains signals from multiple species. Without the identification of cigarette smoke free radicals, it is difficult to assess fully the potential role of such free radicals on the biological damage observed from smoke exposure in *in vitro* and *in vivo* systems. Over the last two decades, new techniques, particularly spin-trapping methods, have provided insight into the structural components and dynamic equilibrium of the radical population of cigarette smoke that, to date, has not been possible using direct EPR.

SPIN TRAPPING

The spin trapping technique was first suggested in the late 1960s as an approach to detecting free radicals in situations where concentration is below the limit-of-detection by EPR [21]. Since that time, a large number of spin trapping reagents have been developed and studied. Most of the commonly used species are nitroso compounds (Fig. **2**) or nitrones (Fig. **3**). The spin adducts of these two classes of compounds differ in the structural information that can be determined from their free radical adducts. With nitroso compounds, the HFCCs are relatively small and insensitive to changes in the free radical; with nitrones, the HFCCs are larger and more sensitive to the nature of the adducted free radical [22].

Free radicals in cigarette smoke, particularly in the GVP, are transient species that undergo fast reactions and can have half-lives of much less than a second [13]. Because of this transient nature, it can be difficult to identify or quantify free radicals without a means of stabilizing them [13]. Even direct injection into a mass spectrometer for identification allows sufficient time for substantial decay of the free radicals. The development of spin trapping techniques allowed researchers to overcome this problem. In the spin trapping technique, a diamagnetic compound capable of reacting by addition with transient free radical species was added to a sample to produce a stabilized free radical (a spin adduct) which persisted long enough to be detectable. Measurement of free radicals with spin traps, however, may underestimate radical concentrations depending on spin-trapping efficiency and the decay rate of spin adducts.

Historically, there has been no systematic approach to the development of spin traps, with variously substituted nitrones and nitroxides being synthesized without any solid hypotheses for how changes in substitution would affect reactivity, stability, and selectivity. The first nitrone spin trap to be used widely was 5,5-dimethyl-1-pyrroline-n-oxide (DMPO) with the first report of detection of a superoxide adduct in 1974 [23]. In recent years, there have been tremendous advances in the development of new nitrone spin traps, particularly by the Zweier and Villamena groups at the Ohio State University, using a density functional theory approach to predict the effect of substitution on reactivity, stability, and selectivity [24-30]. As shown in Table **2**, the half-life for the superoxide adduct (used by the authors simply as a reference ROS for ranking spin traps) of DMPO is more than an order of magnitude lower than the half-life for two of more recently developed spin traps such as 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-n-oxide (DEPMPO), and 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-n-oxide (DIPPMPO). Villamena also has found that the rate of superoxide adduct formation can be increased by the addition of electron withdrawing groups at the C-5 position of the nitrone (see Fig. **3**), that produce a positive charge on the nitronyl carbon. Increasing the rate of adduct formation allows free radicals to be trapped before they decompose. The presence of hydrogen-bonding interactions can also increase the rate of spin adduct formation and the stability of the spin adduct. These H-bond interactions can be increased through the incorporation of spiro functionality into the nitrone spin trap [29]. All of these factors contribute to a two order of magnitude difference in the rate of formation of the superoxide adduct between 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-n-oxide (DEPMPO) and 5-carbamoyl-5-methyl-1-pyrroline N-oxide (AMPO). This difference in rates is likely to hold for other oxygen-centered free radicals.

As shown by Villamena, "[f]unctional groups provide versatility and can potentially improve spin-trap reactivity, adduct stability, and target specificity." [29] Various alkyl, amide, and phosponate substituted nitrone species have been synthesized for use as spin traps. Table **2** illustrates the limited use of these spin traps for the study of cigarette smoke. To date, only 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline-n-oxide **(**BMPO), [16, 28, 31, 32] 5,5-dimethyl-1-pyrroline-n-oxide (DMPO), [8, 15, 16, 18, 31, 33-37] 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-n-oxide (DEPMPO), [16, 32, 33, 36] and 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-n-oxide (DIPPMPO) [32, 36] have been reported for the study of cigarette smoke. Spin traps that may have promise but have yet to be studied for trapping cigarette smoke free radicals include 5-carbamoyl-5-methyl-1-pyrroline N-oxide (AMPO), and 5-(ethoxycarbonyl)-5-methyl-1-pyrroline N-oxide (EMPO), which have higher rate constants for spin adduct formation with superoxide than either DEPMPO or DMPO [25, 29].

EPR SPIN TRAPPING OF CIGARETTE SMOKE RADICALS

In 1971, Bluhm *et al.* first applied spin trapping to the short-lived radicals (half-lives of tens of seconds [38, 39]) in cigarette smoke by collecting smoke in a benzene solution of phenyl t-butylnitrone (PBN) [40]. The resulting samples exhibited two EPR

Table 2. Spin Traps and Labels Reported in Studies of Cigarette Smoke Free Radicals. See Figs. 2-5 for Structures

Spin Trap	Type of Radical Trapped	$t_{1/2}$ (sec), Superoxide Adduct	Cigarette-Related References: Author (Cited in this Work)
3-AP	carbon		Flicker 1998, 2001(48,49) Johnson 2005 (35) Bartalis 2007, 2009 (53,54)
3-CP	carbon		Bartalis 2007, 2009 (53,54)
PBN	carbon		Bluhm 1971 (39) De Hys 1973 (40) Pryor 1976 (8) Menzel 1976 (41) Pryor 1984 (13) Halpern 1985 (33) Iwahashi 1992 (34) Baum 2003 (17) Johnson 2004 (51) Ghosh 2006, 2008 (18,32) Johnson 2009 (51)
DPPH-PBN	carbon		Ghosh 2008 (32)
POBN	carbon		Iwahashi 1992 (34) Baum 2003 (17) Johnson 2005 (35) Robinson 2009 (31)
BMPO	oxygen	23	Robinson 2009 (15) Robinson 2009 (31)
DMPO	oxygen	55	Pryor 1976 (8) Halpern 1985 (33) Iwahashi 1992 (34) Baum 2003 (17) Johnson 2005 (35) Ghosh 2007, 2008 (18,32) Robinson 2009 (15) Robinson 2009 (31)
DEPMPO	oxygen	930	Johnson 2005 (35) Ghosh 2008 (32) Robinson 2009 (15) Robinson 2009 (31)
DIPPMPO	oxygen	1296	Johnson 2005 (35) Ghosh 2008 (32) Robinson 2009 (31)

signal patterns: a triplet (A=8.01G) and a triplet of doublets (A^N=13.76±0.12G and A^H=1.99±0.10G). The triplet was assigned to the α–benzoyl-N-t-butylnitroxide formed from oxidation of PBN. The authors attributed the second signal to a spin-adduct formed from an alkoxy radical but specific structural assignment was not possible.

De Salles de Hys *et al.* reported on free radicals spin trapped from the filtered MSS of 1R1 Kentucky reference cigarettes smoked into a 0.2 M solution of PBN in benzene using standard ISO smoking parameters [41]. After aging for 24 hours, a weak EPR signal could still be observed from the spin-trapped radicals. Holding the smoke in the syringe for 15 seconds before bubbling through the PBN spin trap solution yielded no change in the shape or intensity of the EPR spectrum. However, holding the smoke in the syringe for 30 seconds resulted in a signal decrease of approximately 50%. For both experiments, the obtained spin-adduct spectra were broad and did not exhibit any proton hyperfine splitting in the EPR spectra. The authors proposed several explanations for the lack of hydrogen hyperfine splitting: electron or chemical exchange between the spin adducts and PBN that caused collapse of the hyperfine structure; a β-proton splitting too small to be resolved; or attachment of the dominant free radical in the spectra to the spin trap by a nitrogen atom. Nitrogen dioxide was subsequently bubbled through a PBN solution in an attempt to identify the free radical. The resulting spectrum was much sharper and more intense than that observed with cigarette smoke. The authors concluded that nitrogen dioxide was not the dominant free radical.

In 1976, Menzel *et al.* also reported on spin trapping of free radicals from 1R1 Kentucky reference cigarettes into a 0.2 M solution of PBN in benzene [42]. Yet another variant smoking procedure was used with one puff of 23 mL and 1.5-second duration per minute. The authors estimated the efficiency of spin trapping to be about 47%. From spin adduct spectral line broadening, the concentration of free radicals in MSS was estimated to be about 1×10^{18} spins per puff (about 8×10^{18} spins per cigarette. A single free radical species seemed to dominate the spectra at low concentrations with one or more additional species suggested at higher concentrations. The half-life of the free radicals in smoke was estimated to be 30 seconds. Aging of the spin adduct samples for several days resulted in at least two long-lived species. This half-life estimate is consistent with that reported by other researchers [38, 39].

Pryor *et al.* [8] also applied spin-trapping with PBN, DMPO, and 3,5-di-tert-butyl-4-hydroxyphenyl)-N-tert-butylnitrone (OHPBN) to the study of "grade R" non-commercial research cigarettes smoked with a modified ISO method: one 35 mL puff of 2 sec duration taken every 20 seconds. The concentration of free radicals was measured to be 4×10^{14} per puff (approximately 3×10^{15}

per cigarette), about 3.5 orders of magnitude lower than Menzel's 1976 estimate using 1R1 cigarettes [42]. Three types of signals were identified by EPR. The first type of signal was interpreted to indicate the presence of oxygenated radicals, identified as a mixture of alkoxy radicals (RO.) and aroyloxy ($ArCO_2$.) radicals. The nature of the R or Ar groups in these oxy radicals was not identified, but the authors stated that considerations based on half-lives suggested that the R group is tertiary. It was suggested that the other two types of signals resulted from reaction of smoke and smoke radicals with the PBN spin trap and indicated that smoke had the ability to affect one-electron oxidations. The intensity of the signal from the alkoxy adduct increased with path length that the filtered smoke must travel from the smoking machine before being spin trapped up to a critical distance of 60cm; at longer distances the intensity decreased. This seems to indicate formation of free radicals subsequent to each puff.

In 1983, Pryor *et al.* applied spin trapping with PBN to both the mainstream and sidestream smoke of 1R1 cigarettes [43]. Cigarettes were smoked using standard ISO puffing parameters. Mainstream and sidestream smoke GVP were collected in 0.10 M PBN in tert-butylbenzene after filtering the smoke through a Cambridge filter. Mainstream and sidestream GVP radicals both were present at about 1 x 10^{16} spins per cigarette. This value was about three times greater than Pryor's earlier report [8] from 1976 and two orders of magnitude lower than Menzel's report [42], also from 1976. The GVP radicals were much more stable than expected; they could still be spin trapped 5 minutes after smoking. Based on this observation, the authors hypothesized a "steady state" mechanism in which GVP radicals were continuously formed and destroyed in gas phase reactions involving NO and NO_2. In these reactions, NO was oxidized to NO_2 which then reacted with olefins in the smoke to produce radicals.

Pryor *et al.* showed that GVP from 1R1 Kentucky reference cigarette smoke contained alkyl and alkoxy radicals using three collection methods: bubbling filtered smoke through a 0.1 M solution of PBN; passing GVP through a Cambridge filter coated with 100 mg PBN; and passing GVP through a solid spin trapping system of 5 g of silica gel coated with 300 mg of PBN [13]. In all cases, a nonstandard procedure with constant airflow through the cigarette of 500 mL/ min was used to smoke the cigarettes. No concentration of free radicals was reported. In accord with previous work from the Pryor group, the authors found the GVP radicals to be much longer-lived than expected for oxygen and carbon-center radicals. A NO/air/isoprene model system gave essentially the same types of radicals as cigarette smoke. Results were consistent with the previously proposed hypothesis of a "steady state" mechanism for formation of GVP radicals

Halpern and Knieper [34] used PBN and DMPO as spin traps to study the GVP free radicals. A non-standard smoking procedure was used with a 5-second puff of 25 ml volume drawn every 20 seconds into 2ml of a 0.05M solution of the spin trap. Three cigarettes of unspecified type were smoked. With PBN, spectra were indicative of the presence of two spin adducts. On aging, the triplet of doublets (A^N=13.63G) remained the prevailing signal but the intensities of the two signals changed in favor of the triplet signal (10.25G) which had been assigned by Bluhm [40] and by Pryor [8] as an alkoxy spin adduct. It was concluded that the triplet of doublets was likely to be a superposition of lines due to both an alkoxy and an alkyl adduct. From their DMPO studies, the authors observed a triplet (A^N=13.5G) split into a doublet by a β–proton (A_β^H=8.0G) and another doublet by a γ-proton (A_γ^H=2.2G) which was attributed to a t-butoxy radical.

In 2003, Baum *et al.* [18] published a study in which a series of experiments was conducted to determine the optimal conditions for maximum signal intensities and reproducibility of results for spin trapping experiments on 2R4F cigarette smoke using PBN, 4-POBN (α-(4-pyridyl-1-oxide)-N-tert-butylnitrone); Fig. **2**), and DMPO. The variables studied were solvent, spin trap, instrument physical parameters (double integration routine, cell type, and cell positioning), analysis volume, and collection volume. Standard smoking conditions of one 35 mL puff of 2-second duration per minute were used.

While no spin adducts were found in polar solvents, a triplet of doublets was observed in benzene, toluene, and hexane. Significant differences in the quantities of free radicals were observed depending on the quality (quartz purity) of tube used. The authors attributed this to changes in wall thickness from tube to tube and indicated the need to use high quality quartz tubes and, preferably, a single tube for a set of experiments. Interestingly, errors associated with repositioning of the EPR tube within the EPR cavity were minimal.

From systematic studies of the analysis volume, the optimum volume for measurements with a standard 4mm outer diameter tube was found to be 0.4 cm^3. With lower volumes, maximal signal intensity was not reached, while with higher volumes, lower concentrations of radicals per cigarette were recorded due in part to the sample being placed outside the more sensitive part of the cavity. The optimum 'collection volume' was 4–5 cm^3. The authors noted that higher volumes produced less intense signals while lower volumes gave rise to higher concentrations of oxidized spin adducts. The 4-5 cm^3 volume maximized the signal while keeping the level of oxidized species low. Since it gave a single spectrum that could be reproducibly integrated, PBN was determined to be the best spin trap for quantifying free radicals from cigarette smoke. DMPO allowed the observation of at least three species, including an alkoxyl radical, a carbon-centered radical and a breakdown product of the spin trap. For both spin traps, a 0.1M solution in any of the studied solvents gave the maximum trapping efficiency.

In 2006, Ghosh *et al.* [19] reported further work on the development of a method for quantifying cigarette smoke free radicals in the GVP of 2R4F cigarettes by EPR. Free radicals were collected in a 0.01M PBN solution in benzene after filtration with a Cambridge pad to remove the particulate phase. Smoking used ISO puffing parameters. This report and subsequent Ghosh work followed the standardized parameters defined in the 2003 Baum work: a 5 mL collection volume, a 0.4 mL analysis volume, and a standard 4 mm quartz EPR tube. The reported hyperfine coupling constants for the spectrum were indicative of carbon-centered free radicals. A close examination of the experimental spectra, however, indicates a hyperfine coupling constant typical of an oxygen-centered radical. [44] The authors found the concentrations of gas phase free radicals to be about 10^{15} spins per cigarette. This value is within an order of magnitude of that previously reported by Pryor [8, 43] but still three orders of magnitude lower Menzel's estimate using 1R1 cigarettes [42]. These large differences in measured free radicals underscore the importance of using standardized methods for quantitation and the potential for changes in combustion processes, such as when changing cigarette type, to influence radical concentration or speciation.

In 2007, Ghosh and Ionita [15] also reported on the analysis of oxygen-centered free radicals from GVP with a 0.01M solution of DMPO in benzene using the smoking and collection parameters described in their 2006 work. The authors found concentrations of free radicals to be 10^{14} -10^{15} spins per cigarette. This concentration compares to values of 3 x 10^{15} and 1 x 10^{16} spins per cigarette reported by Pryor [8, 42, 43] and 1 x 10^{18} reported by Menzel [42]. By EPR simulation, it was determined that there were signals from two oxygen-centered species and a species that was tentatively identified as a decomposition product of the spin-adducts. A stability study showed that the oxygen-centered species decayed faster than the carbon-centered species. The authors concluded that this

was support for Pryor's steady-state mechanism for production of free radicals in cigarette smoke.

In 2008, Ghosh and Ionita [33] published additional data on the speciation of free radicals in the GVP using several different spin traps (PBN, DEPMPO, and DMPO) and a hybrid species (DPPH-PBN; Fig. **2**) containing both a stable hydrazyl-type free radical (DPPH) and a nitrone-type spin trap (PBN). Standard smoking conditions (one 35 mL puff of 2-second duration per minute) were used to examine the difference in EPR spectra between the 2nd and 10th puffs. The 10th puff appeared to contain more oxygen-centered free radicals. This difference between puffs indicated changes in either the formation or decomposition of free radicals as the cigarette was smoked. Oxygen-centered free radicals decayed faster than the carbon-centered free radicals. Unfortunately, the implications of these observations for biological systems are still unknown

In 2009, Robinson and Dyakonov [16] reported on an examination of changes in GVP free radical speciation with changes in cigarette smoking parameters using both EPR and MS. They smoked 2R4F Kentucky reference cigarettes using modified ISO puffing parameters in which puff interval was varied from 2 seconds to 120 seconds. They trapped free radicals using a 0.02 M solution of BMPO in methanol. EPR demonstrated that decreases in the puff interval caused a change in speciation of oxygen-centered radicals. Neither identification of individual free radical species nor determination of their concentration was possible because of weak EPR signals. These results, together with those reported by Ghosh and Ionita [32], illustrate the importance of the dynamic aspects of the smoking process on the yield and speciation of free radicals. The impact of these observations on human smoke exposure are unknown, but it does appear likely that both concentration and speciation of delivered free radicals will vary as a function of changes in smoking behavior. This will be a fruitful area for further research, particularly using whole smoke rather than separated smoke phases.

EPR spin trapping has allowed quantification and identification of broad categories of free radicals from cigarette smoke, for example, alkyl and alkoxy radicals [18, 45]. EPR spin trapping has also allowed the influence of changes in puffing parameters and of puff number on the generated free radicals to be studied. The method, however, has a major limitation in that it does not allow the unambiguous structural identification of individual species of free radicals; identification of free radicals by this method relies on "fitting" of simulated spectra to experimental spectra that can allow coupling constants to be derived. From these coupling constants, inferences about radical speciation can be made based on comparisons to empirical data for synthesized free radical species.

ALTERNATIVE METHODS

Although EPR methods have been used in the study of cigarette smoke free radicals for over 50 years, these methods do not allow the structural identification of free radical species that is necessary for a complete consideration of their potential biological impact.

Scientists outside of the tobacco industry presented the first examples of alternative instrumental techniques for assignment of radical structures. For example, in 1981 Suezawa *et al.* [46] published a gas chromatography- mass spectrometry (GC-MS) method for the identification of various radicals trapped with nitrones and concluded that their "method will provide a sure and widely applicable way to identify spin trapped radicals when used together with EPR spectroscopy". The following year Watanabe [47] and colleagues reported on a GC-MS method for sampling of hydroxyl radicals in the atmosphere. Kieber and Blough [48] published an HPLC-Fluorescence method in 1990 in which they described the use of a water soluble nitroxide spin trap ((3-(aminomethyl)-2,2,5,5-tetra-methyl-1-pyrrolidinyloxy free radical (3-AMP)) and fluorescamine to detect micromolar to subnanomolar levels of photochemically generated carbon-centered radicals in aqueous systems. The authors noted that while the method is very sensitive (exceeding EPR for the analytes studied) there is a risk of non-specific detection of other primary amines due to the reactivity of fluorescamine and that positive identification of analytes would likely require a mass spectrometric detector.

In 1991, Parker *et al.* [49] demonstrated the use of high performance liquid chromatography (HPLC) coupled with EPR and mass spectrometry (MS) as a tool for detection and identification of short lived radicals. Ethyl and pentyl radicals produced from the decomposition of ethyl hydrazine and pentyl hydrazine were stabilized using the spin trap 4-POBN. HPLC coupled with EPR and electrospray ionization mass spectrometry (ESI-MS) or HPLC coupled with thermospray (TSP) - MS were used for detection and identification. The authors showed the detection of quasi-molecular ions for each of the four spin-trapped adducts and clearly rationalized the observed base peaks due to expected fragmentation, associated with the mass analysis, within the radical adducts. The use of MS allowed for the unequivocal assignment of structures of the detected spin-adducts based on both mass and fragment information.

In a subsequent publication, Iwahashi *et al.* [35] described the coupling of HPLC, EPR, and MS for the analysis of phenyl and substituted phenyl-adducts of PBN, 4-POBN, MNP (2-methyl-2-nitrosopropane), and DMPO [17]. Coupling of these instruments allowed structural determination of free radical adducts of PBN and 4-POBN that was not possible by EPR alone due to the small differences in hyperfine coupling observed for these spin traps.

In 1998, Flicker and Green [50] published the details of an experimental method adapted from the previous work of Kieber [48] for analysis of gas phase systems of diesel exhaust and cigarette smoke. Carbon-centered radicals from cigarette GVP and diesel exhaust were trapped by passing the gas stream over a bed of 15 g of glass beads coated with 5.5 mg of the spin-trap, 3-amino-2,2,5,5-tetramethyl-1-pyrrolidinyloxy (3-AP; Fig. **3**) The resulting spin adducts were derivatized with naphthalenedicarboxaldehyde (NDA) to produce the fluorescent products which were injected onto an HPLC. Standard smoking conditions were used in which 35 mL puffs of 2-second duration were drawn from the cigarette.

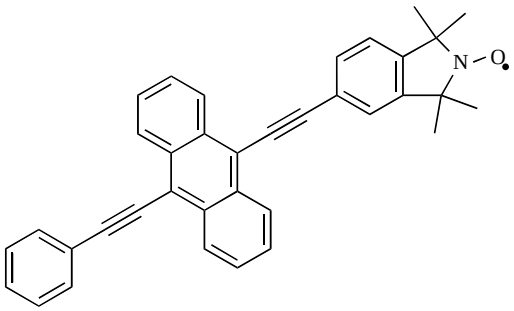


Fig. (4). BPEAnit fluorescent labeled spin trap.

An important aspect of Flicker's work was the trapping of radicals in the gas phase *via* the solid supported spin trap. Trapping of the radicals in a gas phase environment minimized the occurrence of secondary radicals such as those potentially formed when reactive radicals interact with solvent molecules during solution phase trapping. The authors detected a variety of carbon-centered radicals but did not determine a specific number of radicals. They concluded by noting that "radicals were found in tobacco smoke at somewhat higher concentrations than previously reported" [50].

In 2001, Flicker and Green [51] used their previously reported method [50] to study model gasses and various tobacco based products in an effort to identify specific radicals, compare radical suites between the populations of different smoking articles such as cigars

and cigarettes, and study the formation mechanisms of radicals in smoke. The authors used a different smoking procedure from that reported in their previous work, increasing the puff volume to 45 mL. Both similarities and differences were apparent between the radical populations of cigarettes, clove cigarettes, and cigars. The dominant peaks in each of the chromatograms appeared to be the same between the three samples based on the observed retention times. However, they observed differences in the types and quantities of minor peaks indicating differences in the types of radicals produced between the three products. It was also noted that the retention times for the radicals derived from smoke were typically shorter than those determined for $^{\cdot}CH_3$ and $^{\cdot}C(O)CH_3$ indicating that the radical population of smoke may be quite polar in nature (containing significant quantities of hydroxyl, amino, and nitro functionalities).

While the separation and specificity of the fluorescence tagging methods of Flicker and Green have added to the knowledge of the numbers and types of radicals in cigarette smoke, the authors recognized the importance of assigning structure to the radicals [19]. Not only could knowing the structures and quantities of the various radical types be important in understanding their toxicological significance in a smoke environment, but it would also be important in understanding the role of radicals in smoke chemistry.

At the 2002 Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA) Congress meeting, Masselot and colleagues presented a new method using MS for the characterization and quantification of radicals produced in cigarettes [50]. They tested a variety of radical scavengers for compatibility with ESI-MS. They noted that one-dimensional MS techniques did not provide sufficient sensitivity and could be overly complicated when analyzing cigarette smoke. When coupled with liquid chromatography, tandem MS methods allowed both identification and quantification, and provided superior results to standard MS analysis. They reported two radical species in cigarette smoke, hydroxyl and methyl radicals.

In 2004, Johnson and Chapman reported on the use of tandem MS methods to study spin trapping of cigarette free radicals generated with a nonstandard smoking procedure (a 35 mL puff over 2 seconds with a 10 second puff interval) and trapped in 20 mM solutions of PBN or DMPO in water [51]. The authors also demonstrated the detection of PBN and DMPO spin trapped phenyl radicals derived from phenyl hydrazine. Tandem MS methods were sensitive to the fragmentation of spin adducts which allowed structural identification of the spin trapped smoke radicals. The authors used precursor ion scans to look for the loss of masses associated with the spin-trap/scavenger from the adducted radical. After identification of those masses, they used product ion scans to generate fragmentation spectra for structural assignment/confirmation. The assignment of a series of oxygen centered (ethoxy thru pentoxy) radicals differentiated the potential of tandem MS from less specific methods in its ability to detect and provide important mass and fragmentation information necessary for the assignment of structures to the numerous radicals produced during tobacco combustion [36].

In 2005, Johnson presented a continuation of his original 2004 work by expanding the MS techniques to a variety of spin traps including DMPO, DEPMPO, DIPPMPO, POBN, and 3-AP [35]. He used the same smoking parameters and spin trap concentrations as reported in the 2004 work. He also presented an adaptation of Flicker's work [48, 49] using 3-AP coated onto a solid supported spin trap for the analysis of gas phase radicals. He identified approximately 30 spin adducts over the various spin traps and analytical techniques including eight oxygen centered radicals that were identified and assigned structures based on fragmentation data from tandem MS experiments. This technique ultimately allowed identification of the spin trapped adducts based on the liberation of fragments resulting from collisional dissociation. Product ion scans were developed to differentiate spin adducts from other smoke compounds. Four carbon-centered radicals were detected based on solid phase spin trapping with 3-AP, but no structural assignments were made. Of the spin traps studied, DIPPMPO and DEPMPO allowed detection of the greatest variety of spin trapped radicals [35].

Rolando *et al.* presented a new method for identifying and quantifying cigarette derived radicals based on nano-liquid chromatography Fourier Transform Mass Spectrometry (FT-MS) at the 2006 CORESTA Conference [52]. The authors noted that an advantage this method is its ability to detect EPR silent analogues. Two additional advantages were the shorter analysis times associated with nano-LC and the high mass accuracy of Fourier transform mass spectrometers.

At the 2009 Tobacco Science Research Conference (TSRC), Robinson and Dyakonov [15] reported an extension of Ghosh's 2008 work using both EPR and MS. 2R4F Kentucky reference cigarettes were smoked under modified ISO conditions in which the puff interval was varied. The speciation of GVP radicals was determined as a function of puff number and puff interval by trapping with a 0.02 M solution of BMPO in methanol. Both EPR and MS demonstrated that decreases in the puff interval caused an increase in the concentration of higher molecular weight oxygen-centered radicals. Absolute concentrations were not measured, but several fold changes in relative concentration were observed. The authors also reported on changes in free radical speciation as a function of cigarette puff number. Mass spectrometry showed a shift of later puffs to lower mass oxygen-centered radicals. The authors suggested that changes in the residence time at high temperature allowed more decomposition of radical precursors. They also hypothesized that changes resulted from differences in combustion of tobacco and of tobacco with deposited tar in later puffs and that deposited tar-quenched radicals in later puffs. It appears likely that both concentration and speciation of delivered free radicals will vary to at least a small degree as a function of changes in smoking behavior. As noted earlier, however, the implications of these changes in free radical concentration and speciation for human health are unknown.

Also at the 2009 TSRC, Robinson and Johnson [31] presented work using a series of spin traps (PBN, POBN, BMPO, DMPO, DEPMPO, and DIPPMPO) to identify free radicals in GVP. Smoking 2R4F Kentucky reference cigarettes using ISO puffing parameters, they identified seven alkoxides, one amide, one amine, and two phenoxide radicals. They pointed out the importance of using multiple spin traps to allow detection of more species because of spin trap specificity. They also noted that the use of a series of functionalized nitroxide spin traps allowed confirmation of alkoxide radicals.

Bartalis *et al.* also employed high resolution MS, as well as a unit resolution triple quadrupole platform, for their identification of 7 acyl and 11 alkylaminocarbonyl radicals using 3-AP and the 3-cyano-proxyl free radical (3-CP; Fig. **4**) [53, 54]. They reported the combined abundance of the identified radicals in fresh whole smoke as $1.4x10^{17}$ radicals per cigarette (225 nmol), almost an order of magnitude higher than values reported for GVP by Pryor *et al. via* EPR [42]. They also identified two classes of carbon-centered radicals that had previously not been identified in cigarette smoke. In agreement with Johnson's original precursor work [35], Bartalis *et al.* detected several suites of radicals that occurred in a homologous series (acyl and alkylaminocarbonyl). The authors pointed out that the newly identified radicals could not be explained using the long accepted steady state mechanism associated with NO_2 and alkenes; they detected no radicals containing NO_2 groups. They also reported on the sensitivity of acyl radicals to the presence of a Cambridge filter pad noting that acyl radical content decreased by 96%

for a 2R4F cigarette when the smoke was exposed to a Cambridge filter pad [53].

Bartalis also demonstrated that separation of the phases using a Cambridge pad could result in profound changes in radical speciation. Since the reaction of GVP smoke constituents with filtered particulate phase species appears to be an important factor in these observed changes, it seems possible that high filtration cigarettes will produce similar results, especially at low ISO tar yields. If this is the case, free radical speciation, in addition to yield, may depend strongly on degree and type of filtration. Previous work had focused on reference cigarettes such as the 1R1 and 2R4F Kentucky reference cigarettes and on cigarettes produced with single blend types. There has been no systematic examination of free radical yield as a function of ISO tar level. Additional work will be necessary to confirm the broad application of Bartalis' results.

If proven correct, Bartalis' [53, 54] work illustrates the importance of using caution when interpreting data from separated gas and particulate phases. The work also points out the fact that analysis of free radicals from whole smoke may be more relevant than analysis of radicals from the individual phases. Whole smoke is increasingly the route of exposure for *in vitro* studies of cigarette smoke and is the route of exposure for *in vivo* inhalation studies. To the extent that separation of the phases alters their composition, the focus on whole smoke is a practical one. The ultimate concern is understanding the biological effect of exposure to free radicals in whole smoke.

However, the focus on whole smoke does not necessarily invalidate the several decades of data collected on the separated particulate and GVP that preceded Bartalis' work. The carbon-centered free radicals on which the Bartalis conclusion rests had not been previously identified, and Bartalis' work did not consider the previously identified oxygen-centered free radicals. Consequently, while Bartalis' work suggests that Pryor's hypothesized mechanism [13] for the steady-state production of GVP free radicals may need refinement, without further work it is uncertain if the focus on separated phases in the previous work on oxygen-centered free radicals introduced artifacts or errors.

Recently, Miljevic *et al.* reported on the development of what they termed a "profluorescent nitroxide" for the quantification of cigarette smoke free radicals by fluorescence spectroscopy [55]. The authors synthesized 9-(1,1,3,3-tetramethylisoindolin-2-yloxyl-5-ethynyl)-10-(phenylethynyl)anthracene (BPEAnit) which contains a 9,10-bis(phenylethynyl)anthracene fluorophore bound to a 5-membered nitroxide-containing ring (Fig. **4**). This compound has low fluorescence due to quenching by the nitroxide group. On trapping of a radical, the molecule becomes strongly fluorescent.

The authors tested BPEAnit with MSS and what they called "sidestream smoke", which because of substantial aging and dilution is more properly termed environmental tobacco smoke. For MSS, 3R4F Kentucky reference cigarettes were smoked into 40 mL of a 40 μM solution of BPEAnit in dimethyl sulfoxide (DMSO) using standard ISO puffing parameters. Both whole smoke and a GVP samples were collected. Aliquots of the BPEAnit were withdrawn from the impinger for sampling after every two puffs and diluted to 1 μM for fluorescence measurement. A linear increase in fluorescence intensity was observed with an increasing number of collected puffs. An average of 101.4 nmol of free radicals per cigarette was reported with 40.7 nmol in the GVP and 60.7 nmol in the particulate phase.

For "sidestream smoke" experiments, the authors allowed a cigarette to smolder in a 3 m^3 environmental chamber for one minute with mixing by a fan mounted in the center of the chamber. Starting 3 minutes after the cigarette was introduced into the chamber, the chamber was sampled at 1 L per minute for 20, 40, and 60 minutes by drawing the aerosol into an impinger containing 22 mL of a 4 μM solution of BPEAnit in DMSO. Both a whole smoke and a GVP sample were analyzed. A high efficiency particulate air (HEPA) filter was used to separate the GVP from the particulate phase. Fluorescence intensity was much lower for the whole smoke sample than for the GVP. Total free radical concentrations were 50 $\pm$ 2 nmol/ mg, 32 $\pm$ 9 nmol/ mg, and 25 $\pm$ 12 nmol/ mg, respectively, for the 20, 40, and 60-minute samples.

Miljevic's method offers an advantage over the technique reported earlier [48, 49, 53, 54], using spin trapping by 3-AP and subsequent fluorescent derivatization. The Miljevic single step method allows faster detection of the spin-adduct, thus enhancing the detection of those spin adducts which still undergo rapid decay This leads to a more accurate measurement of the total radical population. However, a drawback to this method is the fact that it measures total radical concentration by fluorescence, which does not allow identification and measurement of individual free radical species. The authors noted a potential source of error in their assumption that all fluorescent products generated would have the same fluorescence quantum yield. Interestingly, they also noted that DMSO as a solvent can react with hydroxyl radicals to produce methyl radicals, which readily react with the nitroxide moiety in BPEAnit. While the method has been tested only for bulk fluorescence measurements, it could be coupled with HPLC or MS techniques to allow identification of individual species of free radicals.

METHODS NOT YET APPLED TO CIGARETTE SMOKE

In 2006, Argyropoulos *et al.* reported on the use of ^{31}P nuclear magnetic resonance (NMR) spectroscopy of free radicals spin trapped with DIPPMPO [56]. The presence of phosphorous in the spin trap allows the use of ^{31}P NMR to detect and quantify free radical adducts and to follow the reactions leading to the decomposition of the radical adducts or their conversion into diamagnetic forms. Differences in chemical shifts for various adducted radical species can allow unambiguous detection and quantification – a distinct advantage over EPR. The use of ^{31}P avoids the complexity of multiple overlapping signals that would occur when monitoring carbon or proton nuclei. For example, the chemical shifts for the $^{\cdot}CH_3$, $^{\cdot}CH_2OH$, $^{\cdot}CH(OH)CH_3$, and $^{\cdot}C(O)CH_3$ spin adducts were 23.1, 22.6, 27.3, and 30.2 ppm, respectively. The authors noted that a disadvantage of this technique might be the lower sensitivity of NMR compared to EPR. The acquisition of multiple scans over a time period shorter than the half-life of the radical adducts may overcome this drawback. While there has been no application of this method to cigarette smoke free radicals, the method holds promise as an additional means of identifying those free radicals because of its specificity and ability to resolve multiple species without the use of chromatography.

In 2009, Zoia and Argyropoulos reported on the detection of phenoxy radicals and ketyl radicals using ^{31}P NMR [57, 58]. In both studies the radicals were derived synthetically and multiple radicals were detected and spectrally resolved. In 2010, Zoia and Argyropoulos extended the technique by coupling of ^{31}P NMR with MS to characterize spin adducts of DIPPMPO [59]. By examination of the fragmentation pattern of the spin adducts by GC-MS, structures could be assigned to the ^{31}P NMR signals. The authors noted that the coupling of the two methods allowed unambiguous identification of short-lived, low-molecular weight free radicals.

CONCLUSIONS

Much of the work on free radicals in cigarette smoke relied on direct EPR methods to quantify number of spins (concentration). Direct EPR, however, cannot structurally identify individual radical species, which limits its ability to provide information needed to clarify the role of free radicals in biological damage. Over the past several decades, there have been substantial advancements in the development of spin traps and spin trapping techniques for use with EPR and MS. The analysis of spin-trapped radicals by EPR allows

the identification of classes of chemicals from smoke but still cannot provide the structural information needed for identification. The coupling of MS with spin trapping has allowed detailed study of the speciation of free radicals and their identification in the gas and particulate phases as well as in whole smoke. Today, the majority of work on cigarette free radicals involves the spin trapping of those radicals and their subsequent analysis by EPR or MS techniques.

As demonstrated by Baum *et al.* [17], both the spin adduct spectrum and the concentration of the radicals trapped are strongly dependent on the experimental conditions used, including EPR tube type and diameter, collection solvent and volume, and measurement volume. Ghosh *et al*. [18] observed that changes in the tobacco blend making up the cigarette could also affect the free radical concentration in smoke. Ghosh and Ionita [32] and Robinson and Dyakonov [15] also have shown that changes in puffing parameters can influence speciation and concentration of free radicals. The influence of changes in puffing parameters on the speciation or concentration of free radicals is not well understood. Consequently, it is difficult to compare data from early publications, many of which used nonstandard puffing parameters or unspecified experimental conditions.

There has been intense interest recently in the influence of smoking parameters on the yield and speciation of free radicals in smoke [15]; changes in free radical yield and speciation as a function of puff number [14, 15]; and on the possible introduction of artifacts through the separation of the particulate and gas phases versus the analysis of whole smoke [53, 54]. In particular, the recent work of Bartalis *et al.* caused many to question the validity of earlier methods relying on the separation of GVP from particulate. Future research will be necessary to determine the validity of data collected using these earlier methods.

An additional promising method using ^{31}P NMR [56-59] to characterize free radicals trapped by fluorinated spin traps has not yet been applied to cigarette smoke. This method may allow the structural characterization of additional radical species. The collection of such data may allow the formulation of a refinement of Pryor's steady state theory of free radical formation [13] that recently was called into question by the work of Bartalis [53, 54].

Despite the considerable number of publications on free radicals in cigarette smoke and on the ability of free radicals to cause cellular damage, the biological relevance of smoke free radicals remains unknown. Observations that tobacco blend, puffing parameters, and, potentially, filtration means can impact the yield of free radicals in smoke suggest that it may be possible to reduce that yield. A reduction of more than a few orders of magnitude for a conventionally burning cigarette appears unlikely. If free radicals are found to be major factors in disease causation, it is unknown whether such a reduction will have an impact on reducing the risk associated with cigarette smoking. Further research to characterize free radicals in smoke and their effect on biological endpoints will be necessary to determine whether their removal from smoke should be highly prioritized.

REFERENCES

[1] Lyons, M. J.; Gibson, J. F.; Ingram, D. J. E. Free-Radicals produced in cigarette smoke. *Nature* **1958**, *181*, 1003-1004.

[2] Lyons, M. J.; Spence, J. B. Environmental free radicals. *Br. J. Cancer* **1960**, *14*, 703-708.

[3] Makino, K.; Hagiwara, T.; Murakami, A. A mini review: Fundamental Aspects of Spin Trapping with DMPO. *Int. J. Radiat. Appl. Instrum. Part C. Radiat. Phys. Chem.* **1991**, *37* (5-6), 657-665.

[4] Hecht, S. S.; Hoffmann, D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* **1988**, *9* (6), 875-884.

[5] Singh, U.; Jialal, I. Oxidative stress and atherosclerosis. *Pathophysiology* **2006**, *13* (3), 129-142.

[6] Valko, M.; Leibfritz, D.; Moncol, J.; Cronin, M. T. D.; Mazur, M.; Telser, J. Free radicals and antioxidants in normal physiological functions and human disease. *Int. J. Biochem. Cell Biol.* **2007**, *39* (1), 44-84.

[7] Corvaja, C. Introduction to electron paramagnetic resonance. In *Electron Paramagnetic Resonance: A Practioner's Toolkit*, Brustolon, M.; Giamello, E., Eds. John Wiley and Sons: Hoboken, New Jersey, **2009**.

[8] Pryor, W.; Terauchi, K.; Davis, W. J. Electron spin resonance (ESR) study of cigarette smoke by use of spin trapping techniques. *Environ. Health Perspect.* **1976**, *16*, 161-76.

[9] Takeshita, T.; Ohe, H. *Aging of Cigarette Smoke and its Relation to Radical Concentration.* Scientific Papers, Central Research Institute, Japan Monopoly Corporation **1964**, vol. *106*, pp. 163-167.

[10] Pryor, W.; Hales, B.; Premovic, P.; Church, D. The radicals in cigarette tar: their nature and suggested physiological implications. *Science* **1983**, *220* (4595), 425-427.

[11] Tully, G. W.; Briggs, C. D.; Horsfield, A. E.P.R. absorption by the vapor phase of cigarette smoke, condensed and examined at low temperatures. *Chem. Ind. (London)* **1969**, (7), 201-3.

[12] Maskos, Z.; Dellinger, B. Formation of the secondary radicals from the aging of tobacco smoke. *Energy Fuels* **2008**, *22* (1), 382-388.

[13] Pryor, W. A.; Tamura, M.; Church, D. F. ESR spin-trapping study of the radicals produced in nitrogen oxide (NOx)/olefin reactions: a mechanism for the production of the apparently long-lived radicals in gas-phase cigarette smoke. *J. Am. Chem. Soc.* **1984**, *106* (18), 5073-9.

[14] Ghosh, M.; Ionita, P. *Investigation of Free Radicals in Cigarette Mainstream Smoke*. Proceedings of the European Combustion Meeting **2007**.

[15] Robinson, E. A.; Dyakonov, A. J. Formation of Radicals from Cigarettes under Different Smoking Conditions. *Abstracts of Papers.* 63rd Tobacco Science Research Conference, Amelia Island, Florida, **2009**.

[16] Blakley, R. L.; Henry, D. D.; Smith, C. J. Lack of correlation between cigarette mainstream smoke particulate phase radicals and hydroquinone yield. *Food Chem. Toxicol.* **2001**, *39* (4), 401-406.

[17] Baum, S. L.; Anderson, I. G. M.; Baker, R. R.; Murphy, D. M.; Rowlands, C. C. Electron spin resonance and spin trap investigation of free radicals in cigarette smoke: development of a quantification procedure. *Anal. Chim. Acta* **2003**, *481* (1), 1-13.

[18] Ghosh, M.; Cunningham, F.; McGrath, C. J.; Baker, R. R.; McAughey, J. Development of a quantitative method for free radicals in cigarette smoke by electron spin resonance. *Abstracts of Papers.* 39th International Conference on Advanced Techniques & Applications of EPR, Edinburgh, Scotland, **2006**.

[19] Dyakonov, A. J.; Walker, R. T.; Brown, C. A.; Perini, F. R.; Passer, D. S.; Guan, J.; Robinson, E. A. Studies of the formation of smoke phenols *Beitrage Zur Tabakforschung Int. Contrib. Tobacco Res.* **2008**, *23* (2), 68-84.

[20] Janzen, E. G.; Blackburn, B. J., Detection and identification of short-lived free radicals by an electron spin resonance trapping technique. *J. Am. Chem. Soc.* **1968**, *90* (21), 5909-5910.

[21] Janzen, E. G. Spin Trapping. In *Foundations of Modern EPR*, Eaton, G. R.; Eaton, S. S.; Salikhov, K. M., Eds. World Scientific: New Jersey, **1998**.

[22] Harbour, J. R.; Chow, V.; Bolton, J. R. An electron spin resonance study of the spin adducts of OH and HO_2 radicals with nitrones in the ultraviolet photolysis of aqueous hydrogen peroxide solution. *Can. J . Chem* . **1974**, *52*.

[23] Villamena, F.; Hadad, C. M.; Zweier, J. L. AMPO spin traps. US Patent 7,598,400, 2006.

[24] Villamena, F. A.; Hadad, C. M.; Zweier, J. L. Comparative DFT study of the spin trapping of methyl, mercapto, hydroperoxy, superoxide, and nitric oxide radicals by various substituted cyclic nitrones. *J. Phys. Chem. A* **2005**, *109* (8), 1662-1674.

[25] Villamena, F. A.; Locigno, E. J.; Rockenbauer, A.; Hadad, C. M.; Zweier, J. L. Theoretical and experimental studies of the spin trapping of inorganic radicals by 5,5-dimethyl-1-pyrroline N-oxide (DMPO). 1. Carbon dioxide radical anion. *Theor. J. Phys. Chem. A* **2006**, *110* (49), 13253-13258.

[26] Villamena, F. A.; Merle, J. K.; Hadad, C. M.; Zweier, J. L. Superoxide radical anion adduct of 5,5-dimethyl-1-pyrroline N-oxide (DMPO). 2. The thermodynamics of decay and EPR spectral properties. *J. Phys. Chem. A* **2005**, *109* (27), 6089-6098.

[27] Villamena, F. A.; Rockenbauer, A.; Gallucci, J.; Velayutham, M.; Hadad, C. M.; Zweier, J. L. Spin trapping by 5-carbamoyl-5-methyl-1-pyrroline N-oxide (AMPO): theoretical and experimental studies. *J. Org. Chem.* **2004**, *69* (23), 7994-8004.

[28] Villamena, F. A.; Xia, S.; Merle, J. K.; Lauricella, R.; Tuccio, B.; Hadad, C. M.; Zweier, J. L. Reactivity of superoxide radical anion with cyclic nitrones: role of intramolecular H-bond and electrostatic effects. *J. Am. Chem. Soc.* **2007**, *129* (26), 8177-8191.

[29] Villamena, F. A.; Zweier, J. L. Detection of reactive oxygen and nitrogen species by EPR spin trapping. *Antioxidants Redox Signal.* **2004**, *6* (3), 619-629.

[30] Rhodes, C. J.; Morris, H.; Paul, W.; Alan, T.; Colin, P. Electron Spin Resonance Spectroscopy: Specialized Techniques. In *Encyclopedia of Analytical Science*, Elsevier: Oxford, **2005**; pp. 337-343.

[31] Robinson, E. A.; Johnson, J. D. *Identification of Gas-Phase Free Radicals By Tandem Mass Spectrometry (MSMS)*. Abstracts of Papers. Tobacco Science Research Conference, Amelia Island, Florida, **2009**.

[32] Ghosh, M.; Ionita, P.; McAughey, J.; Cunningham, F. Electron paramagnetic resonance of the free radicals in the gas- and particulate phases of cigarette smoke using spin-trapping. *ARKIVOC* **2008**, *xii*, 74-84

[33] Halpern, A.; Knieper, J. Spin trapping of radicals in Gas-Phase cigarette smoke. *Zeitschrift für Naturforschung. Teil b, Anorganische Chem.* **1985**, *40B* (6), 850-852.

[34] Iwahashi, H.; Parker, C. E.; Mason, R. P.; Tomer, K. B. Combined liquid chromatography/electron paramagnetic resonance spectrometry/electrospray ionization mass spectrometry for radical identification. *Anal. Chem.* **1992**, *64* (19), 2244-2252.

[35] Johnson, J. D. *Analysis of Gas Phase Smoke Radicals Via Spin Trapping and Tandem Mass Spectrometry*. Abstracts of Papers. 59th Tobacco Science Research Conference, Stone Mountain, Georgia **2005**.

[36] Johnson, J. D.; Little, C. A., Unpublished Results.

[37] Pryor, W. A.; Stone, K.; Zang, L.-Y.; Bermudez, E. Fractionation of aqueous cigarette tar extracts: fractions that contain the tar radical cause DNA damage. *Chem. Res. Toxicol.* **1998**, *11* (5), 441-448.

[38] Smith, C. J.; Fischer, T. H. Particulate and vapor phase constituents of cigarette mainstream smoke and risk of myocardial infarction. *Atherosclerosis* **2001**, *158* (2), 257-267.

[39] Bluhm, A. L.; Weinstein, J.; Sousa, J. A. Free radicals in tobacco smoke. *Nature* **1971**, *229* (5285), 500-500.

[40] de Hys, L. d. S.; Francis, H. E.; Seebach, G. L.; Wasson, J. R. *Electron Spin Resonance Spin Trapping Studies of Cigarette Smoke*. Tobacco Health Workshop Conference Proceedings **1973**, *4*, 474-484.

[41] Menzel, E. R.; Vincent, W. R.; Wasson, J. R. Dipolar broadening of electron spin resonance lines of free radicals: Spin-trapping studies of free radicals in cigarette smoke. *J. Magn. Reson.* **1976**, *21*, 321-330.

[42] Pryor, W. A.; Prier, D. G.; Church, D. F. Electron-spin resonance study of mainstream and sidestream cigaret smoke: nature of the free radicals in gas-phase smoke and in cigaret tar. *Environ. Health Perspect.* **1983**, *47*, 345-55.

[43] Halpern, H. J.; Pou, S.; Peric, M.; Cheng, Y.; Barth, E.; Rosen, G. M. Detection and imaging of oxygen-centered free radicals with low-frequency electron paramagnetic resonance and signal-enhancing deuterium-containing spin traps. *J. Am. Chem. Soc.* **1993**, *115* (1), 218-223.

[44] Suezawa, H.; Afge, K.; Hirota, M.; Ishil, T. Mass spectrometric determination of spin adducts in spin trapping technique. *Chem. Lett.* **1981**, *10*(7),1049-1052.

[45] Watanabe, T.; Yoshida, M.; Fujiwara, S.; Abe, K.; Onoe, A.; Minoru, H.; Igarachi, S. Spin trapping of hydroxyl radical in the troposphere for determination by electron spin resonance and gas chromatography/ Mass Spectrometry. *Anal. Chem.* **1982**, *54* (14), 2470-2474.

[46] Kieber, D. J.; Blough, N. V. Determination of carbon-centered radicals in aqueous solution by liquid chromatography with fluoresence detection. *Anal. Chem.* **1990**, *62*, 2275-2283.

[47] Parker, C. E.; Iwashi, H.; Tomer, K. B. Spin-trapped radicals: determination by LC-TSP-MS and LC-ESI-MS *J. Am. Soc. Mass Spectrometry* **1991**, *2*, 413-418.

[48] Flicker, T. M.; Green, S. A. Detection and separation of gas-phase carbon-centered radicals from cigarette smoke and diesel exhaust. *Anal. Chem.* **1998**, *70* (9), 2008-2012.

[49] Flicker, T. M.; Green, S. A. Comparison of gas-phase free-radical populations in tobacco smoke and model systems by HPLC. *Environ. Health Perspect.* **2001**, *109* (8), 765-771.

[50] Masselot, D.; Emami, I.; Rolando, C.; Le Gac, S. A Newa nd Efficient Method for Detecting Free Radicals by LC-MS/MS. Application to the Optimization of a Cigarette Filter which Remove Free Radicals in Cigarette Smoke. Abstracts of Papers. CORESTA Congress, New Orleans, **2002**.

[51] Johnson, J. D.; Chapman, J. J. *Identification of Radicals in Whole Smoke: Alternative Methods.* Abstracts of Papers. 58th Tobacco Science Research Conference, **2004**; p. 42.

[52] Rolando, C.; Cantais, F.; Emami, I. *Precise Identification of Free Radicals in Cigarette Smoke By Exact Mass Measurment Using nano-LC, nanoESI FT-MS/MS*. Abstracts of Papers. CORESTA Congress, Paris, **2006**.

[53] Bartalis, J.; Chan, W. G.; Wooten, J. B. A new look at radicals in cigarette smoke. *Anal. Chem.* **2007**, *79* (13), 5103-5106.

[54] Bartalis, J.; Zhao, Y.-L.; Flora, J. W.; Paine, J. B.; Wooten, J. B. Carbon-centered radicals in cigarette smoke: Acyl and Alkylaminocarbonyl radicals. *Anal. Chem.* **2009**, *81* (2), 631-641.

[55] Miljevic, B.; Fairfull-Smith, K. E.; Bottle, S. E.; Ristovski, Z. D. The application of profluorescent nitroxides to detect reactive oxygen species derived from combustion-generated particulate matter: Cigarette smoke - A case study. *Atmos. Environ.* **2010**, *44* (18), 2224-2230.

[56] Argyropoulos, D. S.; Li, H.; Gaspar, A. R.; Smith, K.; Lucia, L.; Rojas, O. J. Quantitative 31P NMR detection of oxygen-centered and carbon-centered radical species. *Bioorg. Med. Chem.* **2006**, *14*, 4017-4028.

[57] Zoia, L.; Argyropoulos, D. S. Phenoxy radical detection using P NMR spin trapping. *J. Phys. Org. Chem.* **2009**, *22* (11), 1070-1077.

[58] Zoia, L.; Argyropoulos, D. S. Detection of ketyl radicals using 31P NMR spin trapping. *J. Phys. Org. Chem.* **2009**, *23*(6), 505-512.

[59] Zoia, L.; Argyropoulos, D. S. Characterization of free radical spin adducts of the DIPPMPO using mass spectrometry and (31)P NMR. *Eur. J. Mass Spectrometry* **2010**, *16*(2),175-85.

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