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AN EPR SPIN-TRAPPING STUDY OF FREE RADICALS IN CIGARETTE SMOKE

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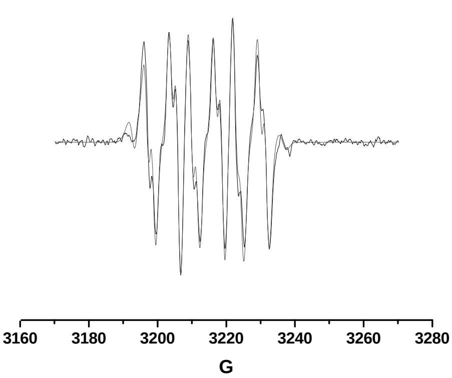
Tobacco smoking represents a source of exposure of humans to the reactive radicals which are considered responsible for chronic diseases. In this work we present the evidence of reactive radicals formation in tobacco smoke using the electron paramagnetic resonance (EPR) spin-trapping method. Four types of commercially available cigarette were used. In each case the cigarette was smoked a quarter, half and totally, and the smoke was passed through a filter into a toluene solutions of the spin traps α-phenyl-N-*t*-butylnitrone (PBN) and 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The analysis of the spin-adducts spectra evidenced the formation of different free radicals with short lives (C•, CO•, O• and HO•). The ratio between these radical species depends on the cigarette characteristics (tar and nicotine) and the cigarette length consumption. It was found that the cigarette tar gives also an EPR signal, corresponding to quinone type radicals.



INTRODUCTION

Tobacco smoke represents a major health hazard to humans being classified as carcinogenic.[1] Cigarette smoke is a complex mixture of organic compounds, and thus far has been identified approximately 8000 compounds.[1,2] The cigarette smoke has two components, one gaseous component represented by the steam and one solid component identified as the tar. The two components can be completely separated with a filter.

The research initiated behind five decades has demonstrated the presence of free radicals in the cigarette smoke, both in the solid and in the gaseous component. Detailed measurements showed that the solid phase contain several exceptionally long-lived radical species, being mainly a mixture of semiquinone, hydroquinone and quinine type radicals. In the gas-phase of the cigarette smoke are present only short-lived free radicals, principally O-centered and C-centered free radicals.[3] Exposure to the cigarette smoke can generate the chronic diseases through perturbation of the balance between pro-oxidative stimuli and oxidant defense of the human organisms due to the presence of short-lived free radicals in high quantity.

The formation of free radicals in the cigarette smoke follows a complicated mechanism, supported by a series of chemical reactions in which the temperature and the presence of air (oxygen) lead to the formation of an entire range of free radicals.

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$$ST + A \longrightarrow ST\text{-}A$$

spin-trap (diamagnetic) | unstable radical from cigarette smoke | persistent radical (paramagnetic)

Fig. 1– Formation of a persistent free radical (an adduct) in the spin-trapping method.

For understanding the chemical composition of cigarette smoke, is necessary to take into account various factors such as the methods of generating cigarette smoke, the choice of the smoking system and the approaches used to capture the smoke.[4]

In the cigarette combustion zone are firstly formed organic free radicals, mainly carbon-centered radicals. In a second step they react with oxygen from air and generate a multitude of oxygen-centered radicals. Detection and quantification of these free radicals formed in the gaseous component of a cigarette represent a difficult task being characterized by short-life; therefore, the spin-trapping EPR method is usually employed. Nevertheless, detection of free radicals present in smoke is a critical step towards understanding the system of a cigarette burning.[5]

EPR spectroscopy is the most suitable method to detect the short-lived radicals or stable radicals generated in the two components of a cigarette smoke.[6] This method is very sensitive and specific for paramagnetic species. In the same time, the analysis of EPR spectra provides certain structural information. In addition, an EPR spectrum provides information about both the type of the free radical (using the giromagnetic factor, *g*) and the number of paramagnetic centers.[7] Short-lived radicals and stable radicals have also been quantified using high-performance liquid chromatography (HPLC) and gas – chromatography (GC) coupled with EPR.[8-10]

In essence, the short-lived radicals, which are difficult to detect directly by EPR spectroscopy are captured by a spin-trap and the persistent spin-adduct (paramagnetic) formed is analyzed by EPR (Fig. 1). The spin-trap method allows the detection of free radicals with a concentration which exceed the limit of detection of electron spin resonance spectroscopy.[6,11]

Compounds used as spin-trap belongs to the nitrone and nitroso classes. The most used spin-traps found in literature for evidencing of short-lived radicals are *N*-*t*-butyl-α-phenylnitrone (PBN), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) and 2-methyl-2-nitrosopropane (MNP). In this work we used PBN and DMPO with the aim to obtain detailed information about the presence of short-lived radicals in cigarette smoke of four cigarette types and to correlate the results with cigarettes characteristics (content in nicotine, tar, the presence of additives).

EXPERIMENTAL

Materials. The spin trapping agents *N*-*t*-butyl-α-phenylnitrone (PBN) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Sigma. The solvent used, toluene, was supplied by ChimReactiv. Four brands of commercially available cigarettes from two different manufactures were used to generate the cigarette smoke. The first type of cigarette (A) is characterized by a low mass of tar and nicotine (sample A); sample B were menthol cigarettes, while samples C and D have a relatively large mass of tar and high nicotine content.

The EPR spectra were recorded on X-band spectrometer JEOL FA100. The general EPR parameters were: samples were charged into NMR tubes in deoxygenated toluene, frequency 8.99 GHz, field 3330 G, sweep width 100 G, sweep time 60 s, time constant 30 ms, modulation frequency 100 kHz, modulation amplitude 1G, room temperature (22-25^{o} C). The EPR spectra recorded were simulated using the software WinSim.[12] In each figure, in black is presented the experimental spectrum and in red is presented the spectrum obtained by simulation.

Sample preparation. For each measurement 10 mL of toluene containing the used spin-trap with a concentration of 2×10^{-2} M was used. The cigarettes were smoked by a vacuum system and the smoke obtained (after passing a filter) was bubbled into the solution of the spin-trap. Next, the solution was deoxygenated by bubbling argon and the EPR spectra were recorded immediately.

RESULTS AND DISCUSSION

The spin-trapping agents are characterized by different selectivity towards radical capture, therefore is recommended to use at least two spin-traps to distinguish different free radicals from a complex system, such as cigarette smoke. As has been mentioned before, PBN and DMPO (Fig. 2) were used throughout this work.

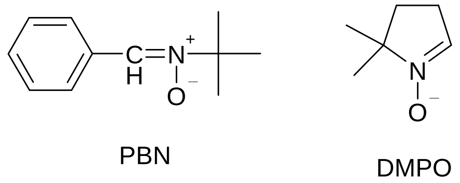

Fig. 2 – Chemical structure of the used spin-traps.

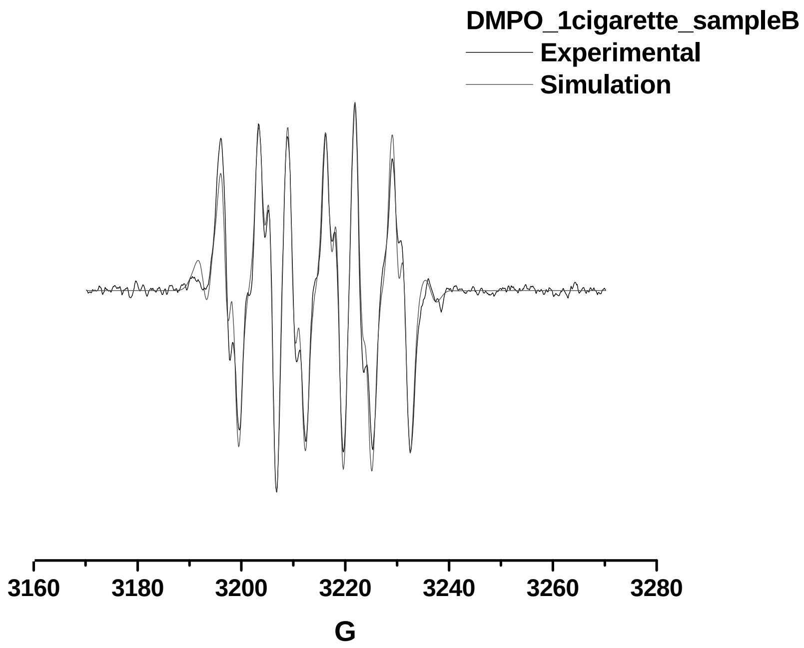

Fig. 3 – EPR spectrum of DMPO adducts corresponding to a whole type B cigarette.

DMPO as spin-trap. Using the type **A** cigarettes and DMPO as spin-trap, no EPR signals were detected. Therefore, in this case the free radicals obtained from cigarette smoke cannot be identified. This might be correlated with the low amount of tar and nicotine in the light cigarette smoke.

For the smoke collected from a whole **B** type cigarette (menthol cigarette), an EPR spectrum with a high intensity was recorded which correspond to adduct of DMPO. This spectrum show a dominant component characterized by six lines (Fig. 3). Simulation of the EPR spectrum obtained for B type cigarette revealed the presence of O-centered radicals in a large proportion. The data extracted from simulations are compiled in Table 1 and Table 2.

The EPR spectrum corresponding to the DMPO adducts obtained after consumption of a whole cigarette of **C** type shows a major component characterized by three intense lines (Fig. 4).

By simulation of the spectrum obtained for **C** sample have been identified commonly O-centered and C-centered free radicals. Besides the two types of quantified radicals, it can be noticed the presence in high percentage (40 %) of products arising from the decomposition of the spin-adduct (see Table 2 for exact values) and a relative small amounts of -CO• type free radicals. Similar results were obtained in the case of **D** type cigarette. Fig. 5 shows the experimental and simulated spectra of the spin adducts radicals formed by the spin-trap DMPO in this case (**D**).

The values of coupling constants a_N and a_H corresponding to the spin adducts identified for the cigarettes C and D (characterised by high tar content) are similar (Table 1). This outcome may be a consequence of the close tar content and the same amount of nicotine and carbon monoxide, as showed by the manufacturer.

The presence of menthol in the **B** type cigarettes is responsible for the differences observed between the spectra obtained for the **B** type cigarette on one side and **C** and **D** types on the other side. During combustion of cigarettes type **B** the O-centered free radicals are generated in larger quantities (menthol cigarette has an average of 0.5 – 2% content of menthol by weight) and this leads to an intense signal of the corresponding the spin-adduct with DMPO.

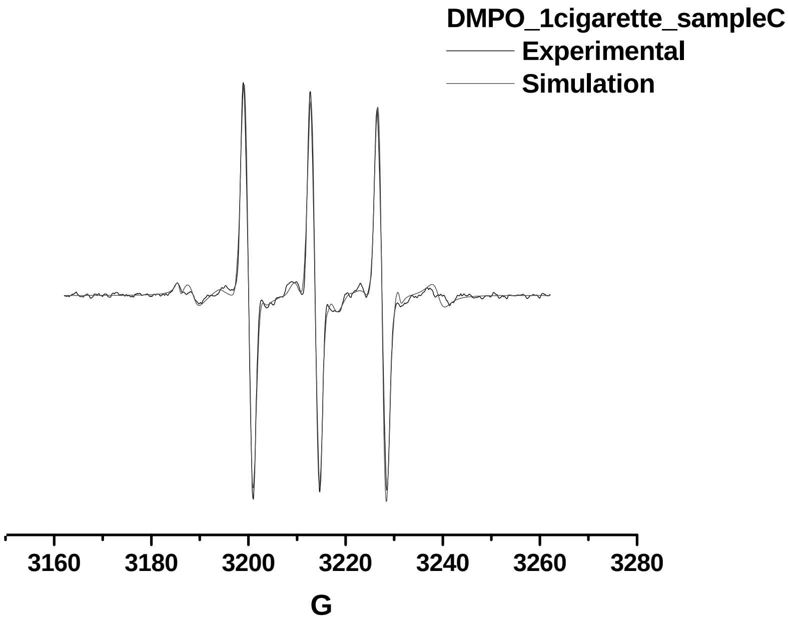

Fig. 4 – EPR spectrum corresponding to DMPO adducts obtained after consumption of a whole type **C** cigarette.

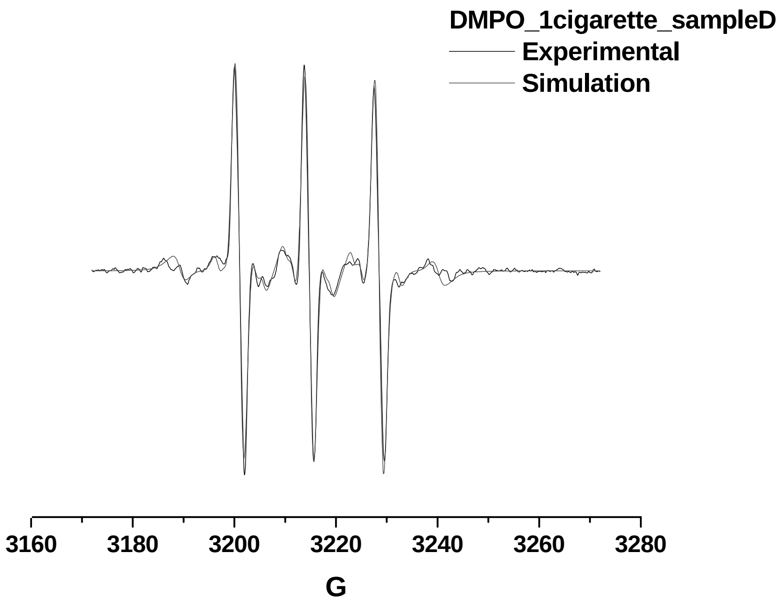

Fig. 5 – EPR spectrum for a whole type D cigarette obtained with the DMPO spin-trap.

Table 1

Hyperfine coupling values and g factors for the free radicals detected (G) using DMPO as spin-trap

Free radical	a_N	a_H	g
-C•	13.81-14.62	21.18-22.02	2.0056-2.0063
-CO•	12.71-13.8	16-16.9	2.0062-2.0073
-O•	12.37-13.8	6.39-7.6 1.72-1.85	2.0058-2.0066
HO•	14.9	14.9	2.0072-2.0077
decomposition products	13.71-13.75	-	2.0057-2.0074

The shape of EPR spectra corresponding to the samples obtained after the smoking of a half or a quarter of cigarette led are similar for all cigarette types. After the complete burning of cigarettes, the EPR spectra are different compared with the partial burning stages. This observation demonstrates that the cigarette smoke composition varies during the smoking period.

The simulation of experimental EPR spectra shows that cigarettes types can be characterized by different composition of radicals mixtures generated during combustion. Table 2 shows the ratio of the species identified by simulation in the cigarette smoke using DMPO as spin-trap. Furthermore, it reveals the change in composition of the mixture of spin adducts in all three stages of burning cigarette. Thus, in the case of type **D** cigarette the C-centered free radicals reached about 30% after burning the entire cigarette, while in the case of **C** type the ratio increases to about 35%, for the same stage of burning cigarettes.

By comparing the ratio for various radical species for all types of cigarettes used with cigarettes with menthol, the species identified are significantly different (see Table 2). The C-centered radicals were not detected in the case of **B** type cigarettes using DMPO spin trap. However, the O-centered radicals have higher reactivity and can generate in subsequent steps other types of radicals.

PBN as spin-trap. Using the spin trap agent PBN in the same experimental conditions as previous, were obtained very similar spectra for all types of cigarettes. The shape of the EPR spectra consist mainly in a triplet of doublets, as is shown in Fig. 6 (for type **C** cigarette). This is typical for C-centered free radicals, in agreement with the EPR coupling constants. The hyperfine coupling constants were the following: a_N= 13.61-13.66 G, a_H= 1.9-2.05 G, g= 2.0063-2.0066.

Table 2

The ratio (%) of free radicals in various stages of combustion of the cigarette

Type ***A***	1 cigarette	½ of cigarette	¼ of cigarette
No radicals detected			
Type ***B***	1 cigarette	½ of cigarette	¼ of cigarette
-C•	-	-	-
-CO•	9.82	15.27	11.28
-O•	74.28	61.97	49.85
HO•	5.25	5.33	1.5
decomposition products	10.66	17.44	37.38
Type ***C***	1 cigarette	½ of cigarette	¼ of cigarette
-C•	34.77	27.78	27.89
-CO•	2.61	3.01	7.46
-O•	21.18	29.03	20.66
decomposition products	41.43	40.18	43.66
Type ***D***	1 cigarette	½ of cigarette	¼ of cigarette
-C•	30.61	31.17	32.17
-CO•	1.14	8	2.88
-O•	23.45	26.19	34.29
decomposition products	44.8	34.64	34.29

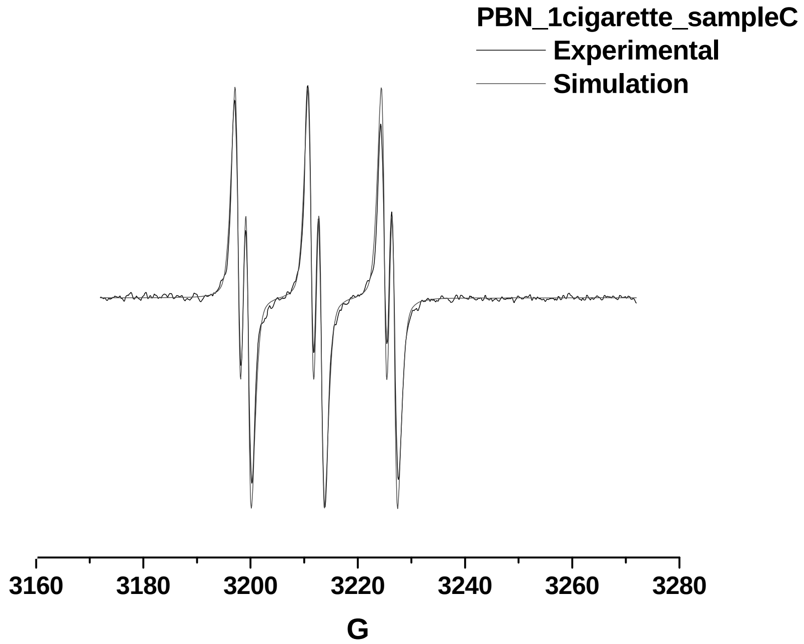

Fig. 6 – Typical EPR spectrum obtained using PBN as spin-trap.

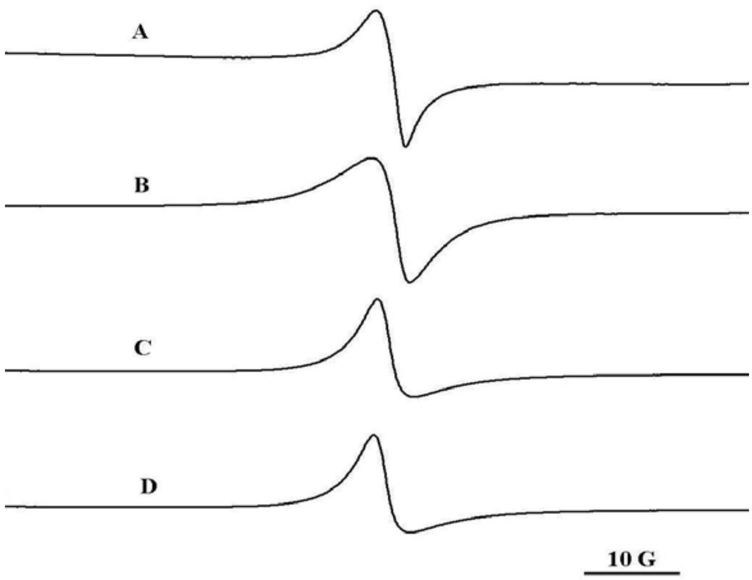

Fig. 7 – EPR spectra recorded for the tar of cigarettes **A**-**D**.

Identification of free radicals from the tar. Whereas the spin-trapping experiments demonstrated that the cigarette smoke contains a high concentration of reactive radicals in the gas phase, in the tar of each cigarette types was evidenced the presence of stable organic radicals.

Fig. 7 shows the EPR spectra of the tar collected for the cigarettes consisting in a single broad band with *g* values of about 2.0034-2.0035. This is a typical case of semiquinone radicals embedded into a solid matrix, as literature data shows.[13]

The EPR spectra obtained for the spin adducts with reactive radicals present in the cigarette smoke of the spin traps used suggest that DMPO is more efficient than PBN. DMPO can simultaneously evidence the presence of C-, O- or CO- centered radicals.

CONCLUSIONS

The spin trapping experiments performed on the gas phase of the cigarette smokes resulted from four types of cigarettes demonstrate the presence of a variety of the reactive radicals. The presence of C- and O-centered short lived radicals in the gas phase was demonstrated by using of DMPO as spin-trap, whilst PBN only C-centered radicals. The composition of radical mixture generated in the gas phase of the cigarettes smoke depends on the type of cigarette, the burning stage of the cigarette and the presence of other additives. During the cigarette smoking the radical species are the same, although the composition of the smoke might change.

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