ST 23 - Mueller - Free radicals in mainstream cigarette smoke: methodology and influencing parameters

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Free radicals in mainstream cigarette smoke: methodology and influencing parameters.

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Summary

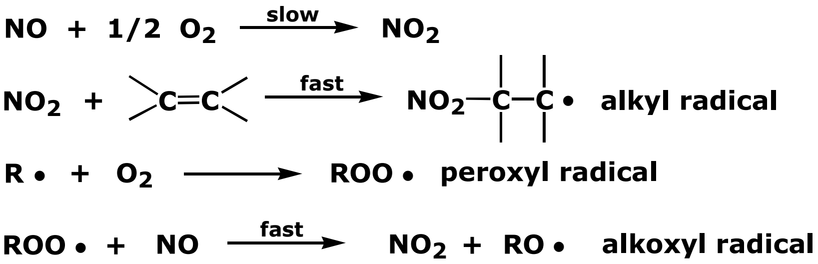
The role of free radicals in smoke and their occurrence and function in biological relevant processes are of major public interest. Thus the presence of long-lived and short-lived free radicals in mainstream cigarette smoke has been reported in numerous studies and toxicological implications have been discussed in the literature. In this work an optimised method for the determination of free radicals in both vapour and particulate phase of mainstream cigarette smoke will be described. The longer-lived particulate phase free radicals (quinone, hydroquinone, semiquinone species) are determined by electron spin spectroscopy (ESR) directly after extraction of the filter pad. Short-lived vapour phase free radicals (alkoxy and alkyl species) are spin-trapped using α-phenyl-*N*-tert-butylnitrone (PBN) and detected by ESR. The application of this methodology will be briefly discussed. The effect of filter design parameters and the influence of different tobacco grades on the amount of free radicals in vapour and particulate phase is demonstrated with two series of prototype cigarettes covering a tar range of 4 – 23 mg. The relation between the occurrence of free radicals in vapour and particulate phase of cigarette smoke and other tobacco and smoke parameters has been studied as well.

Introduction

The presence of long-lived and short-lived free radicals in mainstream cigarette smoke has been reported in numerous studies and toxicological implications have been discussed in the literature [1].

It has been shown in previous work that free radicals are generated in the mainstream smoke of cigarettes and that cigarette smoke can contain up to 10^{16} spins per cigarette. In the vapour phase, both alkoxy (RO•) and carbon-centred alkyl radicals (R•) are present, while the presence of nitric oxide has also been confirmed and quantified [2]. A steady-state mechanism for the continuous formation and destruction of vapour phase radicals has been proposed by Church, Pryor and co-workers, involving nitric oxide oxidation to nitrogen dioxide and subsequent reaction with alkenes in the smoke (Figure 1) [3].

Figure 1: Formation of vapour phase free radicals



Takanami et al. recently have confirmed that this type of mechanism can occur and that isoprene makes a strong contribution to radical formation [4].

The particulate phase radicals are reportedly highly stable and persist for many hours. One of the proposed radical species has been identified as a hydroquinone/semiquinone/quinone system trapped in a polymeric tar matrix. However, this theory has been challenged by Blakley recently since the particulate phase radicals cannot be correlated with cigarette smoke hydroquinone yields [5].

Oxides of nitrogen occur in cigarette smoke at levels up to 300 micrograms per cigarette. The formation of alkyl and alkoxy radicals is suggested to involve the slow oxidation of the relatively unreactive nitric oxide (NO) to nitrogen dioxide (NO_2) and subsequent reaction of NO_2 with reactive substances in smoke (such as olefins and dienes) to form carbon-centred alkyl radicals (Figure 1). These carbon-centred radicals could be scavenged by oxygen to give peroxyl radicals. The high NO concentrations in smoke would be expected to deoxygenate peroxyl radicals to produce oxygen-centred alkoxy radicals. Possible termination reactions that “remove” alkyl and alkoxy radicals from the system are described in Figure 2.

Figure 2

$$RO\bullet + NO_x \longrightarrow RO\text{-}NO_x$$

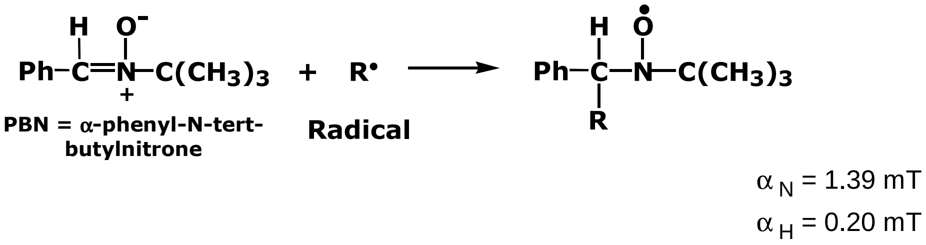
$$R\bullet + NO_x \longrightarrow R\text{-}NO_x$$

Electron spin resonance is clearly the most sensitive and direct method for characterising the presence of long-lived (stable) free radicals.

For reactive or transient radicals, with low steady-state concentrations, spin trapping is required in order to trap the radicals and observe the resonance from the stable spin adduct. The methodology of spin trapping involves the addition of a diamagnetic molecule, usually a nitrone or nitroso compound, which reacts with the free radical to form a stable paramagnetic spin adduct which accumulates until it becomes observable by ESR. By analysis of the hyperfine splitting from the spin adduct spectrum, it is often possible to identify the nature of the primary trapped radical, or at least determine the type of radical trapped.

PBN is one of the most applied spin traps mainly used for trapping carbon-centred radicals in non-polar solvents. The hyperfine splitting constants are characteristic for the resulting spin adducts (Figure 3).

Figure 3



$$Ph-CH=\overset{+}{N}(O^-)-C(CH_3)_3 + R^\bullet \longrightarrow Ph-CH(R)-N(O^\bullet)-C(CH_3)_3$$

More recently, a number of groups have started to detect, quantify and identify the free radical adducts by LC-MS/MS [6, 7]. However, great care must be exercised in the spin trap approach for the quantitative analytical investigations of free radicals (either in the ESR or the LC-MS/MS approach) for several reasons.

Experimental

For this study 20 cigarettes were smoked according to ISO in each experiment using a Borgwaldt RM 20/CS smoking machine. The particulate phase has been collected on a 94 mm Cambridge filter pad and the vapour phase radicals have been trapped by passing the vapour phase through a spin trap solution at 10 °C.

ESR quantification procedures on spin trap investigations of free radicals in cigarette smoke have been described recently by Takanami and Baker [4, 8]. It was pointed out that the nature of the spin adduct spectrum and the concentration of the radicals trapped in solution will vary markedly depending on the experimental conditions employed.

Optimised experimental parameters for smoking and spin trapping are summarised. For trapping the vapour phase carbon-centred radicals, PBN in tert.-butyl-benzene has been used for several reasons. Spin-trap and solvent should be readily available so that the methodology can be easily and cheaply applied. The solvent should be non-toxic and - in combination with PBN - non-polar to obtain stable spin adducts. The spin trapping solution needs to be prepared freshly every day.

Each of four liquid traps is filled with a defined amount of glass beads and 5 mL of trapping solution. The liquid traps are kept at 10 °C during smoking using a cooling bath. The glass beads ensure a high exchange rate between vapour phase and trapping solution. Using this trapping system, 98 % of detectable radicals are trapped.

After smoking, 0.7 mL of the combined spin trap solution is transferred into an ESR tube and degassed with Argon for two minutes at a defined flow. This way, significant line-broadening caused by dissolved dioxygen is avoided.

The ESR measurement is started exactly 16 minutes after lighting the first cigarette to ensure the same time frame is analysed every time. This is essential since it is known that PBN spin adducts decay over time.

A Bruker X-band ESR spectrometer was used for acquiring the ESR spectra. A daily instrument check was done using a solid sample of the stable radical Diphenylpicrylhydrazyl (DPPH). High precision ESR quartz tubes have been used. The choice of ESR tube is critical since significant variations in the quantification were found depending on the type of tube used. These variables are likely to be related to the wall thickness of the different tubes. This finding was confirmed by Baker et al. who found high variations depending on the type of tube used but only minimal errors associated with signal integration and tube alignment within the ESR cavity [8].

The calibration was performed using the stable radical TEMPO in tert.-butyl-benzene solution. Spin concentrations were determined by double integration of the first derivative ESR signal, and comparing the integral to a series of TEMPO standards.

Each day, one Monitor cigarette and a blank run have been analysed prior to test cigarettes. For the test cigarettes, each sample set has been smoked on the same day. This procedure was repeated the following days until three replicates per cigarette type were acquired.

For the analysis of particulate phase radicals, the filter pad was extracted using 20 mL of tert.-butyl-benzene and transferred into the ESR tube. The sample was processed without degassing on the same day.

Following these protocols, the measurements for a monitor cigarette varied for the vapour phase radicals (2.36×10^{15} spins/cig.) by ± 4 % within a day and by ± 10 % day-to-day. For the particulate phase radicals (0.198×10^{15} spins/cig.) the replicates varied by ± 4 % within a day and by ± 9 % day-to-day.

Regarding the stability of trapped radicals, a solvent dependent decay of PBN adducts was observed over time. That means that a reasonable comparison of different cigarette types can only be made on the basis of the same experimental protocol (same spin trap, solvent, time frame for ESR analysis, etc.).

A typical PBN radical adduct ESR spectrum in tert.-butyl-benzene, obtained from smoking 20 Monitor cigarettes is shown. The hyperfine coupling constants measured from the spectrum were $\alpha_N = 1.39$ mT and $\alpha_H = 0.20$ mT with a line-width of 0.10 mT. These couplings are indicative for a carbon-centred radical.

A typical particulate phase spectrum is shown obtained after extraction of the filter pad using tert.-butyl-benzene. As described by Pryor, a broad asymmetric resonance was observed centred at $g = 2.003$ [1, 2]. It has been suggested that this signal is inhomogeneously broadened, indicating the presence of several overlapping signals. As a result, an accurate assignment of this spectrum to one or a number of radicals is virtually impossible.

Results and discussion

For this study, the protocols developed for the analytical testing of free radicals in mainstream smoke were applied to two sets of prototype cigarettes with different characteristics.

The first series focussed on cigarettes of the same blend but different filter ventilation and different filter material covering a tar range from 4 to 23 mg/cig (Table 1). The respective filter material was cellulose acetate, paper and charcoal. Filter retention for tar was adjusted to obtain comparable levels for WTPM.

Table 1: Design parameters

Cigarette prototype	Filter	Ventilation	Puffs per cig.	Tar yield [mg/cig.]	Nicotine [mg/cig.]
1	Cellulose acetate	0%	7.6	10.9	0.76
2	Cellulose acetate	30%	8.0	8.4	0.66
3	Cellulose acetate	60%	9.4	4.4	0.38
4	Tube	0%	7.5	22.7	1.36
5	Paper	0%	7.5	9.0	0.52
6	Charcoal	0%	7.6	10.3	0.72

The second series of prototype cigarettes is characterised by different grades of raw tobaccos including five flue-cured, five air-cured and one oriental blend type (Table 2). For all cigarettes of this series, a cellulose acetate filter without ventilation has been used. The tar yield is ranging from 10 to 19 mg/cig. covering nicotine levels between 0.5 and 2.5 mg/cig. The level of tobacco nitrate is generally higher for the air-cured types than for flue-cured and oriental blend.

Table 2: Blend parameters (CA filter, 0% ventil.)

Cigarette prototype	Type / Blend	Puffs per cig.	Tar yield [mg/cig.]	Nicotine [mg/cig.]	Tobacco nit [% d. b.]
7	flue cured	9.5	15.6	1.95	n.d.
8	flue cured	7.3	10.2	1.04	0.36
9	flue cured	9.0	12.8	1.38	n.d.
10	flue cured	9.6	17.3	2.51	0.11
11	flue cured	9.7	19.3	2.07	n.d.
12	air cured	6.5	11.3	0.73	1.58
13	air cured	5.4	10.3	0.53	0.63
14	air cured	6.0	11.0	0.82	2.12
15	air cured	8.1	13.4	2.20	1.90
16	air cured	6.5	11.7	1.56	1.79
17	Oriental blend	10.9	17.6	0.80	0.12

For the first set of prototype cigarettes, concentrations up to 2.5×10^{15} spins/cig. have been found for vapour phase radicals and a clear effect of filter ventilation was observed. Regarding the different filter material, no significant differences in vapour phase radicals concentration were found for cellulose acetate, empty tube, paper and charcoal within the limits of variation. The concentration of particulate phase radicals varies between 0.13 and 0.29×10^{15} spins/cig. and reflects the retention properties of the filter material for tar (Figure 4). However, only a weak correlation between WTPM and particulate phase radicals was observed (linear correlation coefficient of $R^2 = 0.63$).

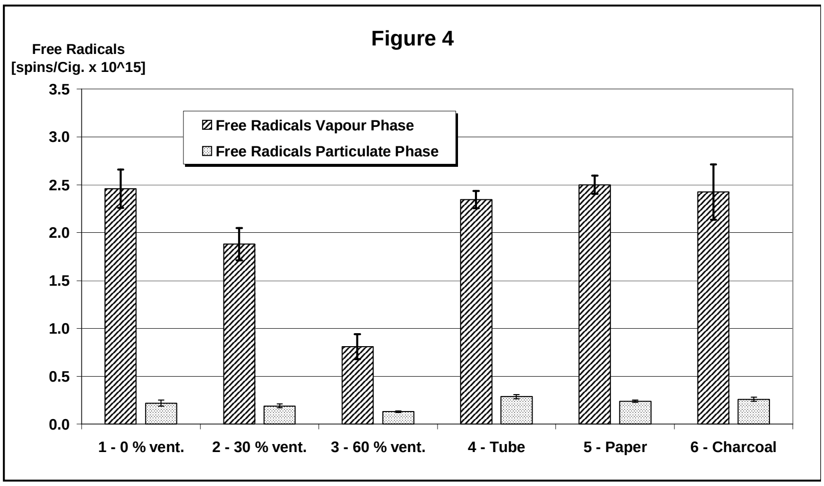


Figure 4

Calculating the particulate phase radicals per mg WTPM, increasing ventilation results in increasing radical yields. In this tar-specific approach, no significant difference between charcoal and paper was found. The lowest radical yield per mg WTPM was observed for the tube filter.

Among the different blend prototype cigarettes, vapour phase radical concentrations between 1.6 and 7.0 × 10^{15} spins/cig. were found. All sample cigarettes gave similar ESR spectra with different intensities for the radical adducts formed in PBN solution. The particulate phase radical concentration range between 0.15 and 0.26 × 10^{15} spins/cig reflecting the different tar levels (Figure 5).

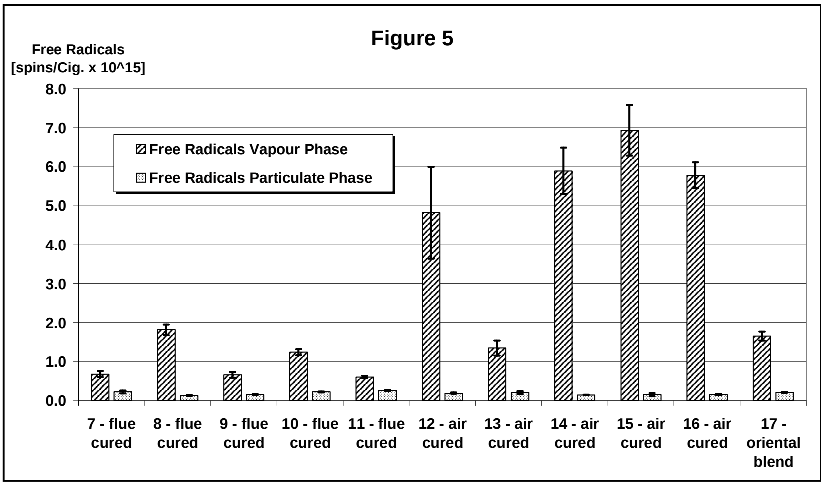
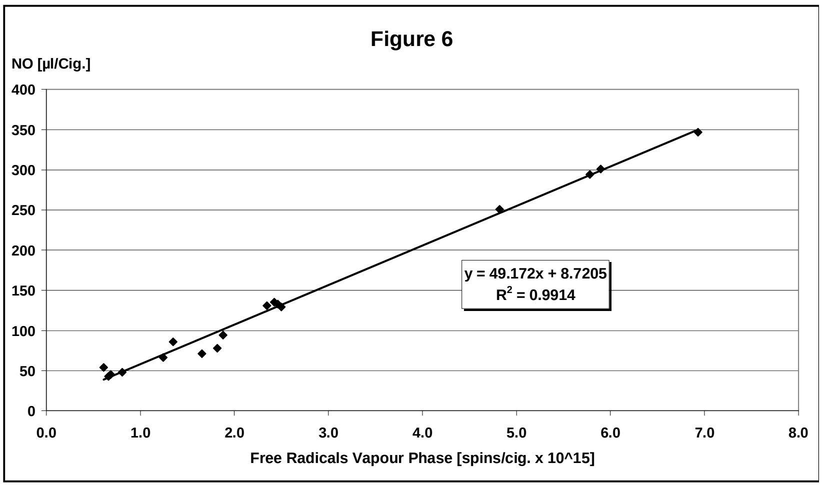


Figure 5

For all prototype cigarettes, a strong dependence between vapour phase radicals and NO in mainstream smoke was found. Within the first set of prototype cigarettes, the NO level in mainstream smoke is mainly affected by filter ventilation while the amount of tobacco nitrate obviously determines the NO level in smoke within the second series of prototype cigarettes.

These findings support the strong contribution of NO in vapour phase radical formation as described by Pryor and Takanami [3, 4]. A linear correlation between mainstream NO and vapour phase free radicals result in a correlation coefficient of $R^2 = 0.991$ (Figure 6).



Conclusion

To summarise the conclusion of a methodology for the quantification of free radicals in mainstream smoke, it needs to be pointed out that a rigorous set of experimental parameters must be applied. Following a strict experimental protocol using optimum experimental conditions, relative comparisons between cigarettes are possible.

Each methodology implies certain limitations. These limitations must be taken into account when discussing the “amount of free radicals” in cigarette smoke.

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