Microchemical Journal 121 (2015) 112–121

Contents lists available at ScienceDirect

Microchemical Journal

journal homepage: www.elsevier.com/locate/microc

Identification and quantification of radical species by ^{31}P NMR-based spin trapping — A case study: NH_4OH/H_2O_2-based hair bleaching

Claudia Crestini [a,*], Jennifer Marsh [b], Giulia Bianchetti [c], Heiko Lange [a]

[a] University of Rome 'Tor Vergata', Department of Chemical Sciences and Technologies, Via della Ricerca Scientifica, 00133 Rome, Italy
[b] The Procter & Gamble Company, Mason Business Center, 8700 Mason Montgomery Road, Mason 45404, USA
[c] Procter & Gamble Italia S.p.A., Via Ardeatina 100, 00144 Pomezia, Italy

ARTICLE INFO

Article history:
Received 9 November 2014
Received in revised form 23 January 2015
Accepted 23 January 2015
Available online 30 January 2015

Keywords:
Radicals
Spin trap
DIPPMPO
^{31}P NMR
Hair bleaching

ABSTRACT

The ^{31}P NMR-spectroscopy-based spin trap technique involving 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO) was used to achieve quantitative analyses of the radical species that are generated in different bleaching solutions. These solutions comprised a mixture of an ammonium salt and hydrogen peroxide. This spin trap-based approach was also applied, with modifications, to the study of bleaching systems of human hair. The obtained results clearly revealed the subtle differences in both the nature and the amounts of the radical species generated in different bleaching solutions, and when allowed to react on hair samples. Generally, the main species involved in the oxidation processes were superoxide and amino radicals. Their amounts, however, showed a significant variation upon the kind of bleaching system and nature of the hair, *i.e.*, virgin or dyed hair.

1. Introduction

The identification, detection and quantification of radical species generated in oxidising processes are challenging tasks due to the extreme reactivity of the species involved. Several analytical techniques have been developed and employed during the last three decades in order to identify such species. The most prominent method is still electron paramagnetic resonance (EPR) or electron spin resonance (ESR) spectroscopy [1,2]. Beside this approach, several spin trapping reagents have been developed for achieving highly resolved detection of a broad variety of radical species in ever refined methods [3], most of these spin trap reagents are on the basis of nitrones [1,4–8]. 'Spin trapping' has been successfully applied to a variety of problems, ranging from environmental studies to *in vivo*-studies [9,10] and improvements including *in-silico* studies are developed in order to extend the applicability [11,12]. More recently, a mass spectrometry-based analysis for the detection of radicals was established, which relies as well on the conversion of reactive radicals into stable adducts for analyses [13,14].

It is, however, always difficult to perform radical detection in complex systems in which very different radical species of varying lifetimes are to be detected and quantitatively analysed over a range of times and at different temperatures. EPR spectroscopy cannot be applied easily or reliably in these cases, and these systems are thus better to be analysed by a combination of mass-spectrometry-based species characterisation and NMR-based spin-trapping experiments [15].

A well-studied spin trap system is the 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO)/^{31}P NMR system; this system is well-known for the analysis of oxygen centred radicals [16,17].

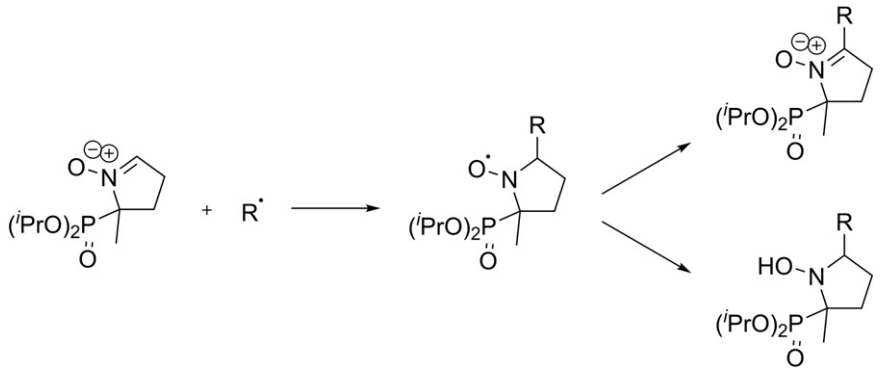
The use of phosphorus-containing spin traps allows for the detection of diamagnetic products by ^{31}P NMR without the complexity of multiple signal overlap spectra usually encountered when common nuclei, such as proton or carbon, are examined. Overall, however, a possible drawback of this technique could be the reduced sensitivity of NMR compared to that of EPR. This is partly overcome by the acquisition of more NMR signals with time [18]. Due to the presence of the phosphorous atom in the DIPPMPO spin trap, ^{31}P NMR spectroscopy can be conveniently used for both qualitative and quantitative analyses, in the presence of a suitable internal standard, benefitting from the fact that different spin trap adducts show different chemical shifts of the ^{31}P atom, depending on the nature of the adduct forming radicals. The basic reactions between DIPPMPO and the various oxygen-based radicals, and the evolving species are shown in Scheme 1.

The shifts of the ^{31}P atom of the different species emerging from various reactions between DIPPMPO and carbon-centred radical species are well documented in the archival literature [14,17,18]. Much less is known, however, concerning the products that emerge from radical recombination reactions between DIPPMPO and nitrogen-centred

Abbreviations: NMR, nuclear magnetic resonance; DIPPMPO, 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide; EPR, electron paramagnetic resonance; ESR, electron spin resonance; EDTA, ethylenediaminetetraacetic acid; GC-MS, gas chromatography coupled with mass spectrometry.

* Corresponding author. Tel.: +39 06 7259 4734.
E-mail address: crestini@stc.uniroma2.it (C. Crestini).

http://dx.doi.org/10.1016/j.microc.2015.01.007
0026-265X/



Scheme 1. General radical combination reaction between DIPPMPO and different radical species, as well as follow-up chemistries of the resulting adducts.

radicals, that occur in, *e.g.*, commercially available ammonia-based alkaline bleaching systems. Among these commercially available bleaching systems, those used in hair colorants are of special interest due to the immediate contact with human hair and skin, since depending on the nature of the dominant oxidative radical species, significant undesired damage of the hair and the skin can occur.

Even though the use of permanent hair colorants containing ammonia-based alkaline bleaching systems is widespread, it comes with certain trade-offs that the consumer has to make if she or he is using these products on a regular basis. One of the main trade-offs is the undesired fibre damage that is sometimes seen over multiple uses [19]. This can lead to the consumer experiencing i) poor hair feel; ii) an increased incidence of split ends; and iii) hair that generally loses some of its healthy appearance and shine. Hence, developing hair colorant systems that allow the consumer to colour on a more regular basis without compromising the natural hair quality is highly desirable.

Two key oxidative chemical processes take place during the colouring process that contribute to the final colouring effect: the first reaction is the oxidation of the natural melanin—based pigmentation and previously deposited artificial dyes, causing a general lightening of the underlying hair colour; the second reaction is the oxidation of the dye precursors to form the coloured chromophores [20,21]. For both processes the oxidant is essential, and in the majority of commercially available retail hair colorants, the oxidant used is a combination of hydrogen peroxide and an ammonia alkalizer that adjusts the final pH in the active mixture to 10. The oxidant is mainly responsible for the damage to the hair fibre and can lead to the loss of the strength and the healthy appearance of hair.

The key chemical species that is reported in the archival literature [22,23] as responsible for both the lightening and damaging processes of keratin fibres is the hydroperoxide anion (HOO^-). This species is present at pH 10 and above, originating from the deprotonation of hydrogen peroxide (Eq. (1)). It is, however, also well known in the archival literature [24] that hydrogen peroxide at high alkaline pH is likely to form reactive radical species which would be an alternative source of fibre damage. Hydrogen peroxide can furthermore readily decompose in the presence of redox-capable metal ions such as copper and iron ions to form both hydroxyl ($HO^\bullet$) and hydroperoxide ($HOO^\bullet$) radicals (Eqs. (2)–(4)). The hydroxyl radical is extremely reactive towards organic substrates, with typical reaction rates that are diffusion controlled ($k = 10^9\ M^{-1}\ s^{-1}$) [25] and would be expected to react instantly with hair polypeptides once it is formed.

$$H_2O_2 \leftrightarrow H^+ + HOO^- \quad pK_a = 11.6 \qquad 1$$

$$H_2O_2 + Cu^{2+} \rightarrow Cu^+ + HOO^\bullet + H^+ \qquad 2$$

$$H_2O_2 + Cu^+ \rightarrow Cu^{2+} + HO + HO^\bullet \qquad 3$$

$$HOO^\bullet + Cu^{2+} \rightarrow Cu^+ + H^+ + O_2 \qquad 4$$

The oxidation of ammonia to amino radicals ($NH_2^\bullet$) via hydroxyl radicals ($HO^\bullet$) is well known and normally of interest in both water-cooled nuclear reactors and atmospheric chemistry. Since, however, ammonia is also used in hair bleaching solutions together with hydrogen peroxide, the formation and the fate of $NH_2^\bullet$-radicals may also be of commercial/cosmetic interest in this sector. For *in vivo* hair bleaching, of course, ammonia and the nitrogen in the hair proteins – both present in high concentration – compete for the $HO^\bullet$-radicals. In all cases the first step involving ammonia is:

$$OH^\bullet + NH_3 \rightarrow H_2O + NH_2^\bullet \qquad 5$$

and the subsequent reaction of $NH_2^\bullet$ with oxygen, amino acids, and melanins are relevant to the balance between hair bleaching and damage [7].

Observed reactions using pulse radiolysis include i) the rather inefficient reactions between the amino radical and amino acids, ii) the bleaching of melanin models initiated by the amino radical, and iii) the efficient reaction between the amino radical and oxygen yielding the amino-peroxyl radical ($H_2NOO^\bullet$). Based on these reactions, it was proposed that during the bleaching of dark hair the ammonia radical $NH_2^\bullet$ oxidises the hair eumelanin, while it is only very slowly reacting with amino acids, and thus only leading to minor damage of hair proteins. In the absence of ammonia $HO^\bullet$-radicals bleach black hair by oxidising eumelanin; additionally, however, they react very efficiently with amino acid residues in the hair proteins, leading to significant hair damage [26].

In order to obtain direct evidence of the radicals emerging during the bleaching process we applied the aforementioned ^{31}P NMR-spectroscopy-based spin trap technique involving 5-diisopropoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DIPPMPO) as spin trap reagent. We seek to achieve a quantitative analyses of the radical species that are generated in different bleaching solutions based on a mixture of an ammonium salt and hydrogen peroxide, before we applied this technique, with suitable modifications, to the study of bleaching systems in the presence of human hair.

2. Materials and methods

2.1. General information

Chemicals were purchased from Sigma-Aldrich in appropriate analysis grades, and were used without further purification if not stated otherwise. The hair samples used in this study were chemically untreated Caucasian source hair purchased from Hair Importers and Products Inc. (Glendale, NY USA). Individual tresses (2 g, ~15 cm) were formed by blending hair from multiple ponytails. Coloured hair was created by treating these hair tresses with an oxidative commercial colorant (Nice N Easy 98 Extra Light Blonde). Three hair samples were used in the

testing. AB69-7 (chemically untreated hair, copper content < 10 ppm), AC7-531 (colour treated hair, copper content = 95 ppm), and AC7-534 (colour treated hair, copper content = 54 ppm). The coloured hair was washed in copper-containing water (0.05 ppm) to achieve the desired copper levels. Copper levels were determined by digesting hair in concentrated nitric acid, followed by analysis via Inductively Coupled Plasma Atomic Spectroscopy (ICP-OES).

2.2. Synthesis of spin-trap reagent 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO)

DIPPMPO was synthesised as described before in a two step procedure [18].

2.2.1. Diisopropyl-(2-methylpyrrolidin-2-yl) phosphonate

2-Methyl-pyrroline was purified by distillation over 5 Å molecular sieves at 40 °C under reduced pressure (27 mbar). To 1 equivalent of 2-methylpyrroline (typically 50 mmol) were added 1.1 equivalents of diisopropylphosphite and a catalytic amount of boron trifluoride diethyl etherate under anhydrous conditions. The reaction mixture was allowed to stir at 20 °C for 3 days. The product-containing mixture was then poured into a suitable volume of 1 M HCl, and extracted with dichloromethane. The aqueous phase was made alkaline by means of saturated sodium carbonate solution and extracted in chloroform. The organic phase was dried over sodium sulphate and evaporated under reduced pressure. Diisopropyl-(2-methylpyrrolidin-2-yl) phosphonate was obtained as an oil and used without further purification.

2.2.2. 5-Diisopropoxy-phosphoryl-5-methyl-1-pyrroline-N-oxide (DIPPMPO)

An aqueous solution of sodium tungstate dihydrate (1 equivalent) was mixed with diisopropyl (2-methylpyrrolidin-2-yl) phosphonate (30 equivalents) at 0 °C. A solution of H_2O_2 (30% m/m, 90 equivalents) was added drop wise during 1.5 h. The reaction mixture was kept at 0 °C for 2 days, before the mixture was extracted with chloroform. The aqueous phase was saturated with brine and extracted again with chloroform. The organic phases were combined and dried over sodium sulphate, before the solvent was evaporated under reduced pressure. The crude product was purified by flash chromatography on silica gel using dichloromethane/methanol as eluent system.

2.3. NMR sequence & spin trap experiments

2.3.1. Experiments in the absence of hair

NMR spectra were recorded on a Bruker 300 MHz spectrometer operated via the software topspin NMR (version 1.3, patch level 8). The spectra were recorded using and inverse gated decoupling sequence, acquiring 4 scans, with a pulse delay of 65 s in order to account for the T1 of the internal standard that is about 13.5 s; overall experiment time for the acquisition of the spectra is thus less than 5 min, allowing kinetic studies without further experimental efforts.

When not differently stated data reported herein are average of three replicate experiments. The relative maximum experimental errors were found to be less than 10% on each measurement.

If not stated otherwise in the full text, spectra were recorded using DIPPMPO solutions in H_2O/D_2O = 1/1 at a concentration of 64 mmol/mL and in the presence of a known amount of trimethyl phosphate as internal standard, typically 10 mmol/mL.

The different reaction media were designed and prepared in order to reach the final concentrations of species that are reported in Table 1 for the variations of the three different basic hair bleaching model systems that were studied: i) ammonium hydroxide–hydrogen peroxide; ii) ammonium carbonate–hydrogen peroxide; and iii) ammonium carbonate–hydrogen peroxide in the presence of glycine sodium salt. As blank control an aqueous solution at pH 10 (adjusted using 1 M NaOH solution) was prepared.

2.3.2. Radical trapping in bleaching systems in the absence of hair

Experiments using the solutions that are detailed in Table 1 were performed in open vials in the dark at an ambient temperature of 20 °C in the presence of spin-trap reagent DIPPMPO and internal standard trimethyl phosphate at concentrations of 64 mmol/mL and 10 mmol/mL, respectively.

For kinetic studies, reaction samplings were performed after 1.5 min, 7.5 min, 12.5 min, 17.5 min, 22.5 min and 27.5 min, respectively.

Bleaching systems tested in this set-up were: i) ammonium hydroxide–hydrogen peroxide; ii) ammonium hydroxide–hydrogen peroxide in the presence of ethylenediaminetetraacetic acid (EDTA) sodium salt; iii) ammonium carbonate–hydrogen peroxide in the presence of EDTA; and iv) ammonium carbonate–hydrogen peroxide in the presence of glycine sodium salt and EDTA.

2.4. Experiments of radical trapping in the presence of hair

Experiments were carried out in an open vial at 20 °C in the presence of a suitable amount of bleaching system and hair to reach a ratio of bleaching system/hair = 4/1 (m/m) in the presence of spin-trap reagent DIPPMPO and internal standard trimethyl phosphate at concentrations of 64 mmol/mL and 10 mmol/mL, respectively. After 5 and 25 min, respectively, the hair was physically separated from the solutions before these were transferred into an NMR tube and analysed by ^{31}P NMR spectroscopy.

For kinetic studies, reaction times were fixed at 5 min and 25 min.

Bleaching systems tested in this set-up were: i) ammonium hydroxide–hydrogen peroxide; ii) ammonium carbonate–hydrogen peroxide; and iii) ammonium carbonate–hydrogen peroxide in the presence of glycine sodium salt.

3. Results and discussion

The study of radical species involved in bleaching systems is particularly relevant in order to evaluate both reactivity and selectivity of different processes. From the commercial point of view a relevant interest

Table 1
Compositions (%) and pH of the bleaching solutions used in this study.

Components	Concentrations (% v/v)					
	NH_4OH/H_2O_2	NH_4OH/H_2O_2/EDTA	$(NH_4)_2CO_3/H_2O_2$/EDTA	$(NH_4)_2CO_3/H_2O_2$/EDTA/glycinate	$(NH_4)_2CO_3/H_2O_2$	$(NH_4)_2CO_3/H_2O_2$/glycinate
H_2O_2 (35%)	8.57	8.57	8.57	8.57	8.57	8.57
NH_4OH (30%)	5.00	5.00	–	–	–	–
EDTA	–	0.10	0.10	0.10	–	–
$(NH_4)_2CO_3$	–	–	5.00	5.00	5.00	5.00
sodium glycinate	–	–	–	2.05	–	2.05
H_20[a]	86.43	86.33	86.33	84.28	86.43	84.38
pH[b]	10	10	9	9	9	9

[a] Di-ionised water.
[b] Adjusted with acetic acid.

is focused on the development of specific bleaching systems for hair bleaching and laundry. In these cases the detailed knowledge of specific radical species that are generated, their kinetics and selectivity constitute the fundamental scientific base for the development of more efficient and safe products for the market. In this effort we focused on the study of hair bleaching systems and evaluated by the aforementioned ^{31}P NMR-based spin trap technique the generated radical species, their relative amounts, and the reaction kinetics. We used three alkaline oxidative systems: i) ammonium hydroxide–hydrogen peroxide; ii) ammonium hydroxide–hydrogen peroxide in the presence of ethylenediaminetetraacetic acid (EDTA) sodium salt; iii) ammonium carbonate–hydrogen peroxide in the presence of EDTA; and iv) ammonium carbonate–hydrogen peroxide in the presence of glycine sodium salt and EDTA. For achieving the desired comparative analyses, we decided to work on a two-step process: We first investigated the radical species generally generated in the different systems in order to get a detailed overview and to determine their abundances. In the second step, the experiments were repeated in the presence of different hair samples. We ran selected experiments both in the absence of, and in the presence of EDTA; the latter with the aim of avoiding any effects of trace metal contaminations coming from the reagents used, and to reveal the effects of this often-added complex-building preservative often added in cosmetics on the formation and equilibrium concentration of the expected radical species. In order not to eliminate the effect of the different copper concentrations in the hair samples, however, all hair-bleaching experiments were conducted without the addition of EDTA, since this complex builder is not present in commercially available hair bleaching solutions.

3.1. Radical trapping in H_4NOH/H_2O_2 solution

Before studying the first test system, a blank sample was analysed as control. In an aqueous solution at pH 10, only a small amount of hydroxyl-based spin-adducts could be detected, proofing that only a negligible background reaction exists.

The H_4NOH/H_2O_2 system was studied first, and assignment of the peaks in the ^{31}P NMR spectra to the nature of the DIPPMPO-adducts listed in Table 1 was accomplished for this model system. A representative spectrum is shown in Fig. 1.

As stated above, the shifts of the ^{31}P atom of the different species emerging from various reactions between DIPPMPO and carbon-cantered radical species are well documented, but adducts of DIPPMPO and nitrogen-cantered radicals are not; careful interpretation of the signals seen in the ^{31}P NMR spectra in light of the known facts concerning both oxygen and carbon-cantered radicals allowed, however, an identification of most of the DIPPMPO/nitrogen radical adducts. The presence of these adducts as signal-causing species are further proven by mass-spectrometric analyses after gas-chromatographic separation. The identified fragmentation patterns are given in Table 2 together with the characteristic chemical shifts that are present in ^{31}P NMR spectra obtained for this oxidative system H_4NOH/H_2O_2.

Based on this assignment, kinetics and potential equilibrium concentrations of the radical species of interest were firstly evaluated at 20 °C over a time span of 30 min. The results are reported in Fig. 2A. Generally, the determinations of hydroxyl radicals and nitrogen containing species were found more accurate and reproducible than the corresponding determination of superoxide radical species, since the latter appear only in form of two distinct but broad NMR peaks.

The formation of the radical species that could be detected as well as their equilibration occur during the first minutes of the reaction; a steady state situation is quickly reached, with the exception of the superoxide radical-spin trap adduct, that decreases over time (Fig. 2A). Noteworthy the most abundant radical species detected was the superoxide radical, trapped in the amount of about 85 mmol/L after ca. 1.5 min of treatment, and decreasing to about 55 mmol/L after ca. 30 min. Hydroxyl radicals were present in a much lower amount of 10 mmol/g during the first 10 min and their amount increased up to 30 mmol/g between ca. 20 and 30 min of treatment. The peak at 26.6 ppm was supposedly related to the formation of DIPPMPO adducts with nitrogen containing radical species $NH_2^{\bullet}$. It is well known that hydroxyl radicals generate $NH_2^{\bullet}$ upon reaction with ammonia with a diffusion controlled kinetic. A previously unassigned peak was also detected in the reaction mixture at 11.0 ppm; this peak is reminiscent to the formation of a DIPPMPO adduct with a peracetic acid radical that could be formed by reaction of hydroxyl radical species with the acetic acid added to the reaction mixture to buffer the pH. This assignment was confirmed by the fact that in the additional GC-MS-based analysis a m/z-signal corresponding to this adduct is absent in case the pH was adjusted by sulphuric acid rather than acetic acid. A resonance signal at

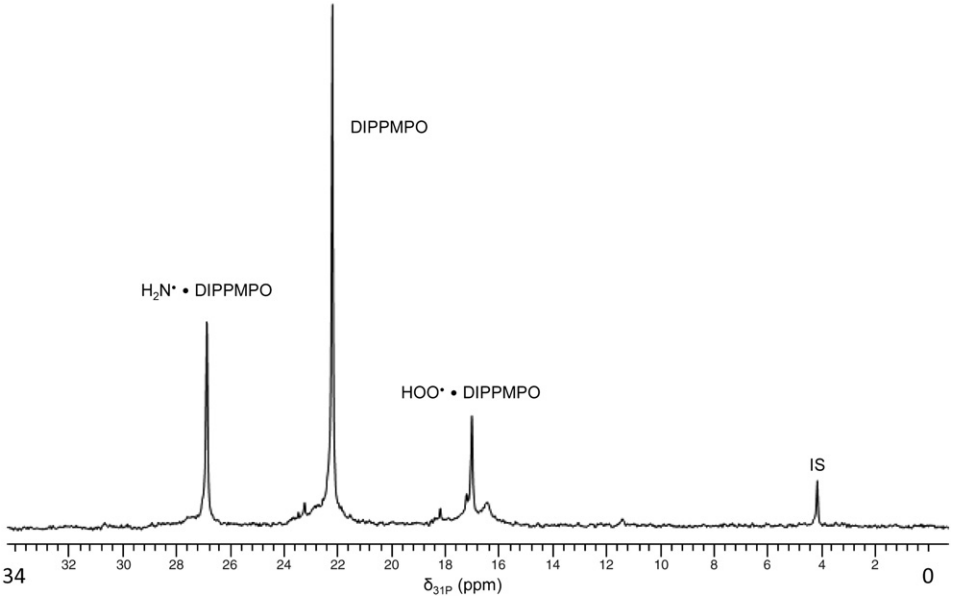


Fig. 1. Representative ^{31}P NMR spectrum of a spin trapping experiment showing the most important species trapped in the system under investigation. DIPPMPO = 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide; IS = internal standard trimethyl phosphate.

Table 2
DIPPMPO-based adducts of radical species and their characteristic chemical shifts in ^{31}P NMR spectroscopy (for references see main text).

Entry	Species	Chemical shift (δ (ppm))	m/z [c]
1	DIPPMPO/$OOC(O)CH_3$–adduct[a]	11.0	(337, 339); 259; 220; 178; 162; 99; 98; 80; 43; 41
2	DIPPMPO	22.2	262; 221; 179; 162; 123; 100; 99; 98; 82; 81; 80; 65; 55; 43; 41
3	DIPPMPO/OOH–adduct	16.9, 17.1	295; 236; 212; 193; 165; 147; 123;99; 84; 83; 65; 55; 43; 41
4	DIPPMPO/intermediate radical species–adduct	18.0, 18.3	Not assigned
5	DIPPMPO/OH–adduct	25.3	279, 281; 259; 236; 195; 114; 99; 98; 86; 81; 43; 42; 41
6	DIPPMPO/NH_2–adduct [b]	26.6	278, 280; 260; 235; 220; 218; 207; 194; 193; 176; 165; 154; 137; 123; 113; 99; 96; 68; 43; 41
7	DIPPMPO/$OOC(O)O^-$–adduct[a]	28.3	Not detected
8	DIPPMPO/NH_2OO–adduct[b]	30.7	310, 312; 262; 219; 203; 185; 139; 123; 82; 43; 41

[a] Tentative assignment based on literature data.
[b] Assigned based on literature and GC-MS analysis.
[c] Major m/z-peaks (intensity > 5%) are listed; numbers in brackets represent M^+ peaks that are not directly detectable.

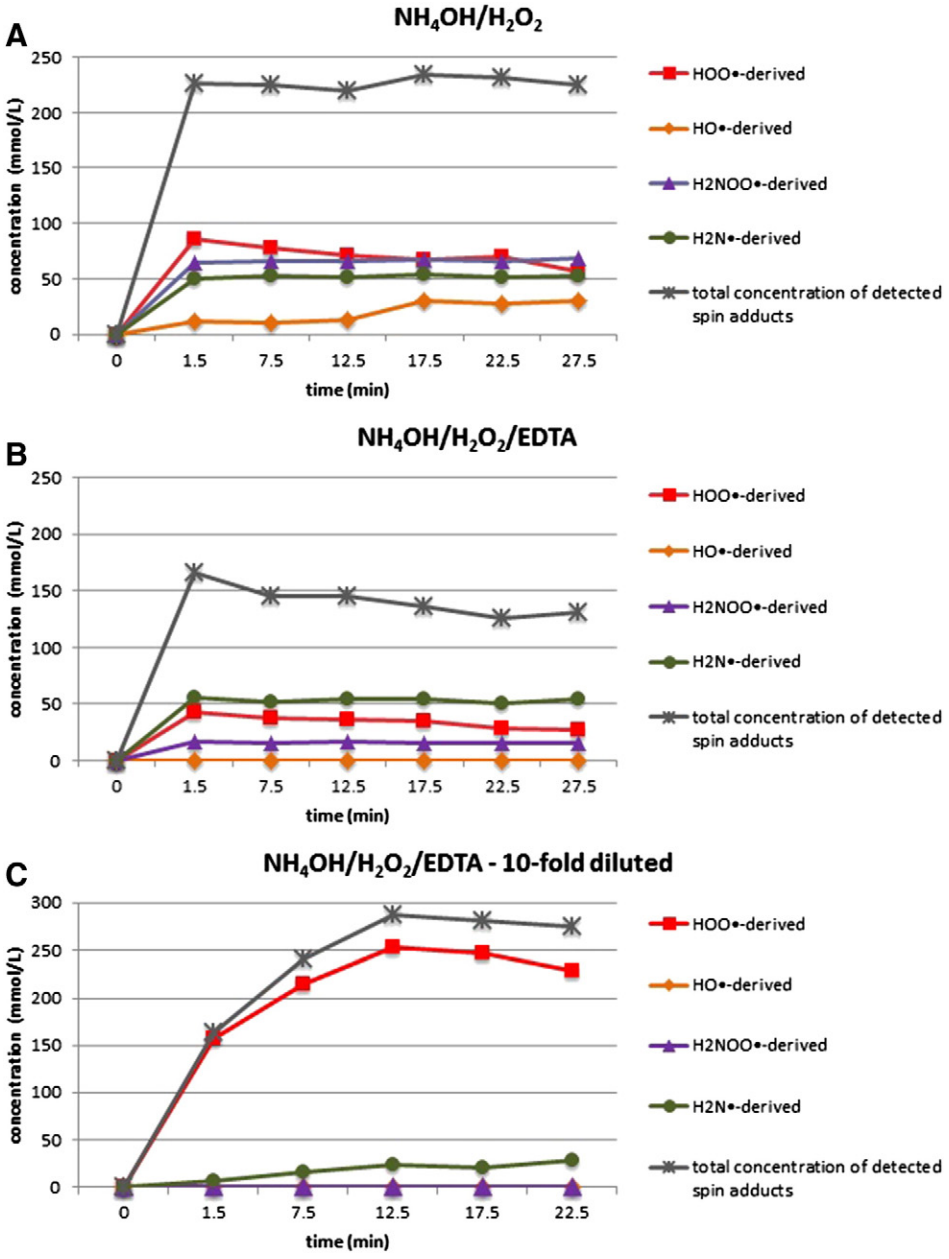


Fig. 2. DIPPMPO radical adducts (concentration in mmol/L) trapped at 20 °C in NH_4OH/H_2O_2 systems: A) NH_4OH/H_2O_2 (64 mmol/mL for DIPPMPO) B) NH_4OH/H_2O_2/EDTA (64 mmol/mL for DIPPMPO); and C) NH_4OH/H_2O_2 in 10-fold diluted conditions (6.4 mmol/mL for DIPPMPO).

30.7 ppm has to be assigned to a DIPPMPO adduct, which has not been identified before: our attempts to characterise all adducts via additional GC-MS analysis of the reaction mixtures finally allowed us to assign this signal to the adduct of DIPPMPO with the $H_2NOO^{\bullet}$ radical species that is known to be formed upon reaction of the amino radical with oxygen.

Addition of metal complexing EDTA to the bleaching solution had an effect on both formation and stability of the radical species over time (Fig. 2B), as expected. The superoxide-based species is no longer the dominant one, but DIPPMPO-$NH_2^{\bullet}$. This seems to indicate that fully depressing the Fenton side-reaction in the medium allows the predominance of amino-based radicals over pure superoxide ones. Hydroxy radical-based spin adducts are no longer present in the reaction mixture. It can be assumed that all radical species formed are readily trapped by ammonia. In general, generation of radical species drops over time at a rate faster than that seen in the absence of EDTA.

Although the protocol used so far delivered reliable results, the high consumption of DIPPMPO – the final concentration of DIPPMPO at the end of the kinetic studies in this system was 64 mmol/mL – renders the protocol un-practical for broader screening efforts. We therefore re-run the initial tests using a ten-fold dilution of the initially used H_4NOH/H_2O_2 system (Fig. 2C). It is immediately apparent that the superoxide-derived spin-adduct is the dominant species again, as it was in the undiluted system without EDTA under otherwise unchanged conditions. Interestingly, the total amount of radical species is found to be four-fold increased. The DIPPMPO-$NH_2^{\bullet}$-adduct is still present in amount comparable to the original experiment. The dilution of the system has, however, apparently an effect on both the amount on amino-based radicals as well as, more strongly, on the amount of superoxide radical species. In connection with the change in the dominant radical species, this points at a generally higher efficiency of the diluted system based on a reduced number of undesired radical re-combination reactions. In the presence of EDTA, hydroxyl radicals cannot be detected in any case.

In order to evaluate the influence of temperature on the kinetics of formation of the radical species, as well as their equilibriums of the different radical species, a 10-fold diluted (final concentration in DIPPMPO = 6.4 mmol/mL) H_4NOH/H_2O_2/EDTA system was studied over 30 min at different temperatures. Qualitatively, the general trends with respect to formation and stability of the different radical species do not differ at different temperatures. Quantitatively, the overall amount of radical species increases with increasing temperatures based on an increase in each radical species. The superoxide-derived DIPPMPO-adduct is the dominant species at all temperatures; above 30 °C the amount of the $NH_2^{\bullet}$-derived species increases significantly, but remains at a maximum of ca. 25% of the concentration of the superoxide radical-derived adduct. The concentrations of the DIPPMPO-adducts derived from the $HOO^{\bullet}$-radical and the $NH_2^{\bullet}$-radical at different temperatures are shown in Fig. 3A and 3B, respectively.

3.2. $(H_4N)_2CO_3/H_2O_2$/EDTA and $(H_4N)_2CO_3/NaC_2H_4NO_2/H_2O_2$/EDTA

The $(H_4N)_2CO_3/H_2O_2$ system was studied next, in the presence of EDTA, and results were compared to those obtained for an analogous system that additionally contained sodium glycinate. The introduction of glycine has allowed this system to achieve a bleaching without previously observed negative aspects connected to a loss of tensile strength [27]. It is proposed that the glycine acts as a scavenger of the carbonate radical that can be formed in this system, and that is known to cause undesired damages to hair fibres. Assignment of the peaks in the ^{31}P NMR spectra to the nature of the DIPPMPO-adducts was performed according to the results and experiences gathered with the H_4NOH-based system. Hydroxyl-derived spin-adducts cannot be detected at all in this system. Fig. 4 shows the concentrations of the DIPPMPO-adducts detected: the $HOO^{\bullet}$-radical, the $NH_2^{\bullet}$-radical, and a low amount of a new radical species indicated by a newly emerging peak at 28.3 ppm, that can be assigned to the DIPPMPO-adduct of a percarbonate-radical.

The superoxide-derived DIPPMPO species were present once more in at least two-fold abundance compared to any other detectable species.

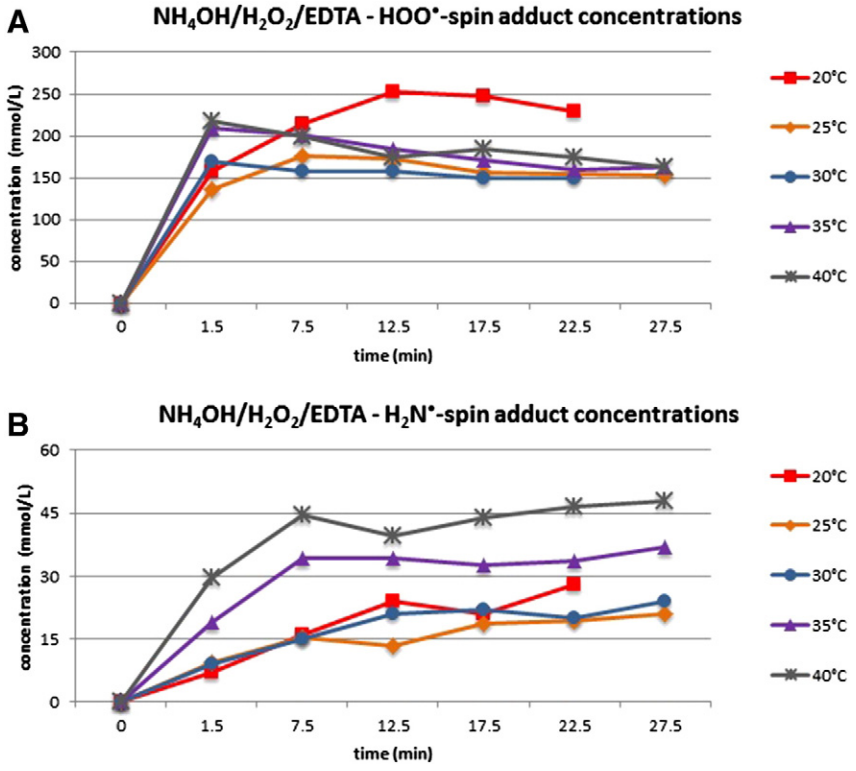


Fig. 3. Radical species (concentration in mmol/L) trapped at different temperatures in the system NH_4OH/H_2O_2/EDTA in 10-fold diluted conditions: A) $HOO^{\bullet}$-derived spin adducts; B) $NH_2^{\bullet}$-derived spin adducts.

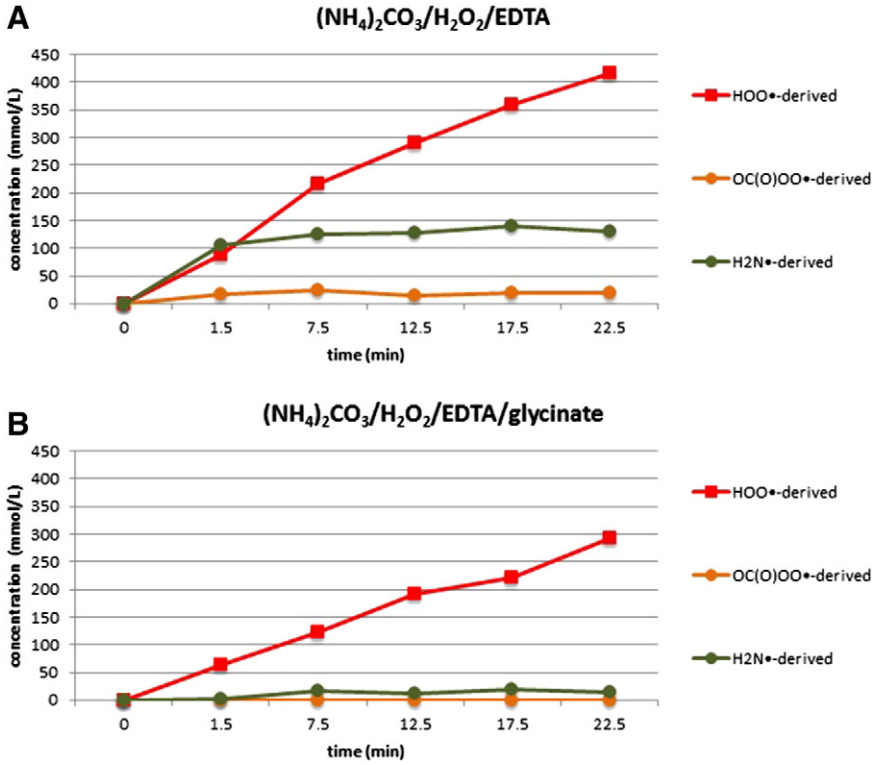


Fig. 4. Comparative qualitative and quantitative (concentrations in mmol/L) analyses of the radical species present in the $(H_4N)_2CO_3/H_2O_2$/EDTA system (A) and the $(H_4N)_2CO_3/NaC_2H_4NO_2/H_2O_2$/EDTA system (B).

Second-most species was the $NH_2^{\bullet}$-derived adduct, whereas the putative percarbonate species is only detectable in small amounts. Overall, the most abundant species are the same as before in the H_4NOH/H_2O_2 system, as well as the general kinetic trends for the built-up of the equilibrium concentrations. Absolute abundances, however, are significantly different; it can be speculated that the presence of the carbonate leads to an obvious increase of overall abundances of the radical species: when sodium glycinate is added to this system under otherwise unchanged conditions, general abundances are essentially halved, and the signal caused by the putative percarbonate adduct is barely detectable, which is in accordance with current knowledge concerning the effect of glycinate in this system [27]. The superoxide-derived DIPPMPO species remains being the major species in the presence of glycinate. Generally, the $(H_4N)_2CO_3H_2O_2$/EDTA system seems less complex than the H_4NOH/H_2O_2/EDTA in terms of the number of generated radical species.

3.3. Evaluation of radical species in the presence of samples of natural hair

In order to have a realistic picture of the generation of radical species in *in vivo* systems, a different experimental set-up was developed to

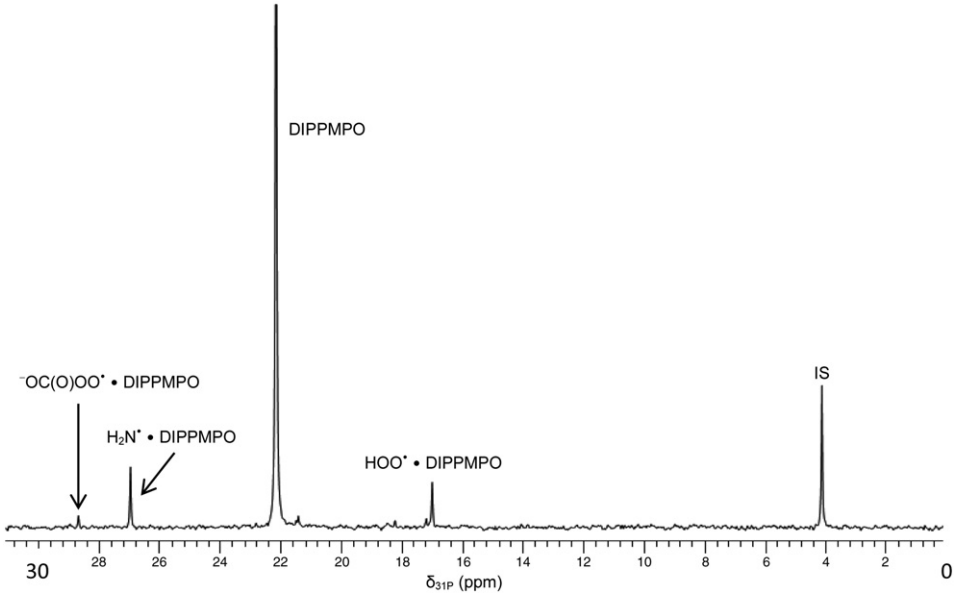


Fig. 5. Representative ^{31}P NMR spectrum of a spin trapping experiment showing the most important species trapped in the $(H_4N)_2CO_3/H_2O_2$/EDTA system. DIPPMPO = 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide; IS = internal standard trimethyl phosphate.

account for the presence of solids, *i.e.*, the hair, in this system. The experiments were carried out in an open vial at 20 °C in the presence of a suitable amount of hair, spin trap and internal standard. After 30 min the hair was physically separated from the solution that was analysed by ^{31}P NMR.

This necessary change in set-up and procedure, however, caused an increased experimental error. Generally speaking the spectra of experiments conducted in the presence of hair were found less resolved and more complex than the corresponding spectra of the bleaching solutions lacking a natural substrate. Nevertheless, accurate quantification of the main radical species trapped was possible. Fig. 5 shows a typical ^{31}P NMR spectrum of the DIPPMPO-trapped radical species in the presence of hair.

The selection of hair bleaching systems studied as discussed above set the background for the evaluation of the radical species that are present when natural hair is present in these systems. Although most of the studies were performed in the presence of EDTA for masking any metal species present so that we could compare the results of the different systems more systematically on an essentially metal-free basis by eliminating any influences of impurities of the reagents applied, we did not want to purposefully exclude the possibility to see any influence of metal traces that are present in the hair samples. We used three distinctively different hair samples, named AB69-7 (virgin hair, copper content: <10 ppm), AC7-531 (coloured hair, copper content: 95 ppm), AC7-534 (coloured hair, copper content: 54 ppm). All three hair samples were treated under identical conditions using the three bleaching systems i) ammonium hydroxide–hydrogen peroxide; ii) ammonium carbonate–hydrogen peroxide; and iii) ammonium carbonate–hydrogen peroxide in the presence of glycine sodium salt. Fig. 6 shows a typical spectrum of an experiment conducted in the presence of hair. The different concentrations of the $HOO^{\bullet}$-derived, $NH_2^{\bullet}$-derived and $H_2NOO^{\bullet}$-derived DIPPMPO-adducts for the different hair samples treated with the different systems are summarised in Fig. 7.

Again, superoxide-derived and amino-derived radical species are present in the systems. As can be seen in Fig. 7A, the different copper concentrations have a clear effect on the abundances of the main radical species detectable as DIPPMPO spin adducts using the H_4NOH/H_2O_2 system: an increase in the metal content leads to an increase of the superoxide-derived spin adduct, whereas the intensity of the $NH_2^{\bullet}$-derived adduct decreases with increasing metal content. This shows that, in principle, virgin hair undergoes less damage than dyed hair upon bleaching.

Less clear trends are observed for the concentrations of the two main radical spin adducts originating from $HOO^{\bullet}$- and $H_2N^{\bullet}$-radicals in the $(H_4N)_2CO_3/H_2O_2$ system (Fig. 7B); their amount cannot be directly correlated with the different concentrations of copper in the hair samples (Fig. 7B). It can be stated, however, that the concentration of $HOO^{\bullet}$-radicals is significantly lower overall in the $(H_4N)_2CO_3/H_2O_2$ system for copper-containing hair samples, but initially very high for essentially copper-free virgin hair. The concentration level of $NH_2^{\bullet}$-radicals is invariant for hair samples with different copper concentrations; for virgin hair, however, that is characterised by a very low copper content, the concentration of $NH_2^{\bullet}$-radicals is significantly lower. Overall, higher concentration of $NH_2^{\bullet}$-radicals are detected compared to the H_4NOH/H_2O_2 system. Last but not the least, whereas the H_4NOH/H_2O_2 system was essentially stable over the observed time-span, the $(H_4N)_2CO_3/H_2O_2$ system undergoes noticeable changes.

The $(H_4N)_2CO_3/NaC_2H_4NO_2/H_2O_2$ system shows a behaviour comparable to that observed in the corresponding glycinate-free system: $HOO^{\bullet}$- and $NH_2^{\bullet}$-radical-derived spin adducts are the most abundant species, but concentrations do again not correlate with the copper content in the hair samples. The different copper concentrations of the hair sample do not affect the initial and the equilibrium concentrations of the $NH_2^{\bullet}$-radicals in the system beyond the uncertainty of the measurement. Significant copper contents do affect, however, the formation and the stability of the superoxide-derived DIPPMPO-adducts, and these findings for the $(H_4N)_2CO_3/H_2O_2$ system do not depend on the presence of glycinate under the standardised conditions we tested here. The presence of glycinate does have a reducing effect on the overall concentration of radical species in the solutions when the hair contains significantly amounts of copper (>10 ppm) (Fig. 7B, C). This finding is in accordance with the orienting experiment on the $(H_4N)_2CO_3/NaC_2H_4NO_2/H_2O_2$ system described above, in which EDTA was used to exclude metal effects.

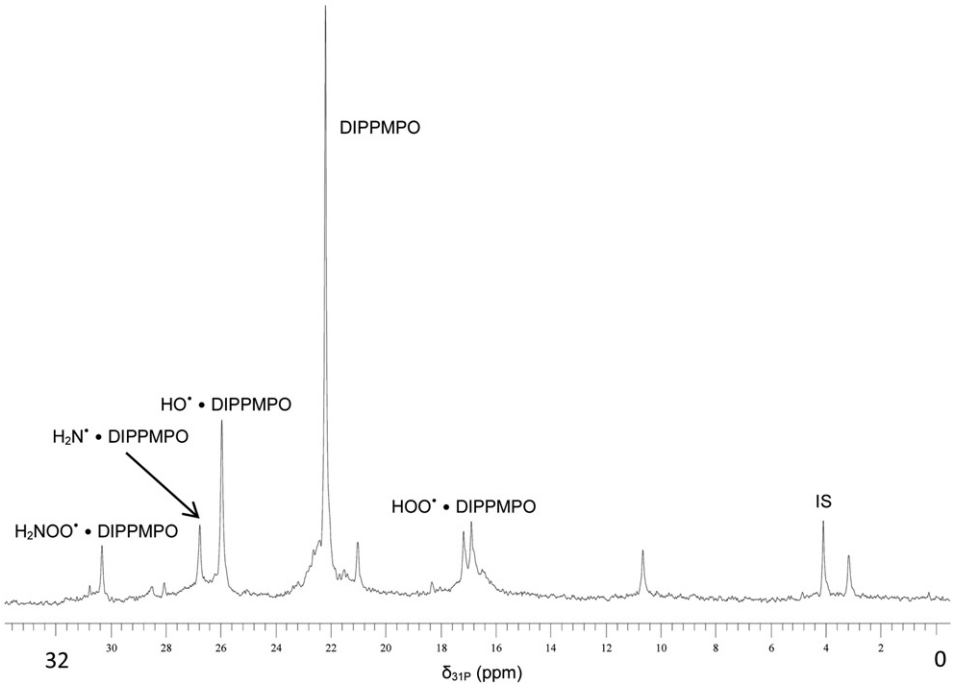


Fig. 6. Representative ^{31}P NMR spectrum of a spin trapping experiment in the presence of hair: AB69-7 (virgin hair, copper content: <10 ppm) treated with H_4NOH/H_2O_2. DIPPMPO = 5-diisopropoxy-phosphoryl-5-methyl-1-pyrroline-*N*-oxide; IS = internal standard trimethyl phosphate.

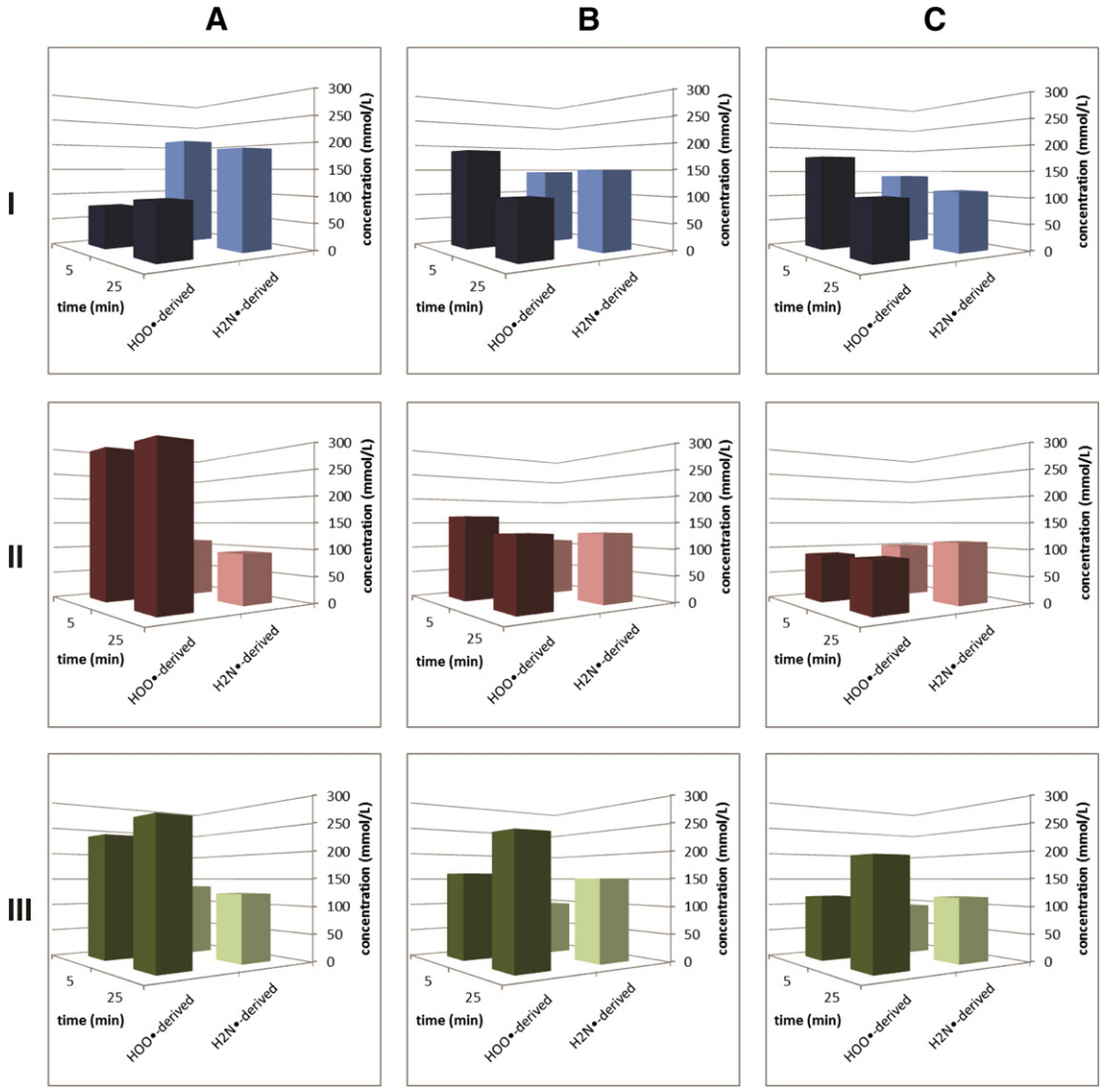


Fig. 7. Radical species detected in the three different ammonium-containing hydrogen peroxide solutions in the presence of different hair samples. A) H_4NOH/H_2O_2 system; B) $(H_4N)_2CO_3/H_2O_2$ system; and C) $(H_4N)_2CO_3/NaC_2H_4NO_2/H_2O_2$ system. I) AB69-7 (virgin hair, copper content: <10 ppm); II) AC7-534 (copper content: 54 ppm); and III) AC7-531 (copper content: 95 ppm).

The glycinate presence does have a specific effect in case of the virgin hair sample: the decrease of superoxide radicals goes together with an increase in the amount of the melanin bleaching amino radicals. This effect is not evident in the presence of copper containing hair samples. These findings are generally supporting earlier observations on the beneficial effects of glycine addition with respect to the sanity of the hair fibres during the bleaching process [27].

4. Conclusions

The relative ratios of superoxide to amino radicals in ammonium hydroxide/hydrogen peroxide alkaline bleaching systems have been determined by the DIPPMPO/^{31}P-NMR spin trap system. This study confirmed the occurrence of these species during such bleaching processes and allowed for the quantification of their relative abundance as a function of time and temperature. This facile methodology displays a wide versatility for use in the analysis of heterogeneous bleaching systems offering and revealing subtle differences with regard to both the nature and the amounts of the radical species generated when different bleaching solutions are used on human hair samples. The main radical species involved in the process are the superoxide ($HOO^{\bullet}$) and the amino radical $H_2N^{\bullet}$ Amino radicals are known to react directly with melanin and display slow kinetics with hair proteins. On the contrary superoxide radical species are known to show a low reactivity towards polypeptides. Their relative amount tunes the efficiency of bleaching *vs* hair damage.

Acknowledgements

The authors would like to thank Procter & Gamble for financial support.

References

[1] B.C. Gilbert, M.J. Davies, D.M. Murphy (Eds.), Electron Paramagnetic Resonance, Royal Society of Chemistry, Cambridge, UK, 2006.
[2] G. Bačić, I. Spasojević, B. Šećerov, M. Mojović, Spin-trapping of oxygen free radicals in chemical and biological systems: new traps, radicals and possibilities, Spectrochim. Acta A 69 (5) (2008) 1354–1366. http://dx.doi.org/10.1016/j.saa.2007.09.047.

[3] F.A. Villamena, J.L. Zweier, Detection of reactive oxygen and nitrogen species by EPR spin trapping, Antioxid. Redox Signal. 6 (3) (2004) 619–629. http://dx.doi.org/10.1089/152308604773934387.
[4] G.I. Likhtenshtein, J. Yamauchi, S. Nakatsuji, A.I. Smirnov, R. Tamura (Eds.), Nitroxides: Applications in Chemistry, Biomedicine, and Materials Science, Wiley, Weinheim, Germany, 2008.
[5] Y. Han, B. Tuccio, R. Lauricella, A. Rockenbauer, J.L. Zweier, F.A. Villamena, Synthesis and spin-trapping properties of a new spirolactonyl nitrone, J. Org. Chem. 73 (7) (2008) 2533–2541. http://dx.doi.org/10.1021/jo702434u.
[6] S. Goldstein, G.M. Rosen, A. Russo, A. Samuni, Kinetics of spin trapping superoxide, hydroxyl, and aliphatic radicals by cyclic nitrones, J. Phys. Chem. A 108 (32) (2004) 6679–6685. http://dx.doi.org/10.1021/jp048441i.
[7] H. Zhoa, J. Joseph, H. Zhang, H. Karoui, B. Kalyanaraman, Synthesis and biochemical applications of a solid cyclic nitrone spin trap: a relatively superior trap for detecting superoxide anions and glutathiyl radicals, Free Radic. Biol. Med. 31 (5) (2001) 599–606. http://dx.doi.org/10.1016/S0891-5849(01)00619-0.
[8] M.J. Turner III, G.M. Rosen, Spin trapping of superoxide and hydroxyl radicals with substituted pyrroline 1-oxides, J. Med. Chem. 29 (12) (1986) 2439–2444. http://dx.doi.org/10.1021/jm00162a004.
[9] (E.g.). E.A. Robinson, J.D. Johnson, Methods for analysis of free radicals in cigarette smoke, Mini-Rev. Org. Chem. 8 (4) (2011) 401–411. http://dx.doi.org/10.2174/157019311797440362.
[10] (E.g.). C. Sánchez-Moreno, Review: methods used to evaluate the free radical scavenging activity in foods and biological systems, Food Sci. Technol. Int. 8 (3) (2002) 121–137. http://dx.doi.org/10.1106/108201302026770.
[11] F.A. Villamena, C.M. Hadad, J.L. Zweier, Theoretical study of the spin trapping of hydroxyl radical by cyclic nitrones: a density functional theory approach, J. Am. Chem. Soc. 126 (6) (2004) 1816–1829. http://dx.doi.org/10.1021/ja038838k.
[12] T. Koto, R. Michalski, J. Zielonka, J. Joseph, B. Kalyanaraman, Detection and identification of oxidants formed during •NO/O_2•-reaction: a multi-well plate CW-EPR spectroscopy combined with HPLC analyses, Free Radic. Res. 48 (4) (2014) 478–486. http://dx.doi.org/10.3109/10715762.2014.886774.
[13] M. Karonen, H. Mattila, P. Huang, F. Mamedov, S. Styring, E. Tyystjärvi, A tandem mass spectrometric method for singlet oxygen measurement, Photochem. Photobiol. 90 (5) (2014) 965–971. http://dx.doi.org/10.1111/php.12291.
[14] L. Zoia, D.S. Argyropoulos, Characterization of free radical spin adducts of the DIPPMPO using mass spectrometry and 31P NMR, Eur. J. Mass Spectrom. 16 (2) (2010) 175–185. http://dx.doi.org/10.1255/ejms.1062.
[15] V.V. Khramtsov, T.L. Clanton, NMR spin trapping: insight into the hidden life of free radical adducts, Appl. Magn. Reson. 41 (2–4) (2011) 305–323. http://dx.doi.org/10.1007/s00723-011-0274-9.
[16] L. Zoia, D.S. Argyropoulos, Phenoxy radical detection using 31P NMR spin trapping, J. Phys. Org. Chem. 22 (11) (2009) 1070–1077. http://dx.doi.org/10.1002/poc.1561.
[17] D.S. Argyropoulos, H. Li, A.R. Gaspar, K. Smith, L.A. Lucia, O.J. Rojas, Quantitative 31P NMR detection of oxygen-centered and carbon-centered radical species, Bioorg. Med. Chem. 14 (12) (2006) 4017–4028. http://dx.doi.org/10.1016/j.bmc.2006.02.009.
[18] L. Zoia, R. Perazzini, C. Crestini, D.S. Argyropoulos, Understanding the radical mechanism of lipoxygenases using 31P NMR spin trapping, Bioorg. Med. Chem. 19 (9) (2011) 3022–3028. http://dx.doi.org/10.1016/j.bmc.2011.02.046.
[19] M.L. Tate, Y.K. Kamath, S.B. Ruetsch, H.-D. Weigman, Quantification and prevention of hair damage, J. Soc. Cosmet. Chem. 44 (6) (1993) 347–372.
[20] K.C. Brown, S. Pohl, A.E. Kezer, D. Cohen, Oxidative dyeing of keratin fibers, J. Soc. Cosmet. Chem. 36 (1) (1985) 31–37.
[21] J.F. Corbett, An historical review of the use of dye precursors in the formulation of commercial oxidation hair dyes, Dyes Pigments 41 (1–2) (1999) 127–136. http://dx.doi.org/10.1016/S0143-7208(98)00075-8.
[22] C.R. Robbins, Bleaching human hair, in: C.R. Robins (Ed.), Chemical and Physical Behavior of Human Hair, Springer, New York, USA, 2002, pp. 102–121.
[23] J. Marsh, J. Flood, D. Domaschko, N. Ramji, Hair coloring systems delivering color with reduced fiber damage, J. Soc. Cosmet. Chem. 58 (5) (2007) 495–503.
[24] C.W. Jones, Activation of hydrogen peroxide in the presence of inorganic and organometallic species, in: C.W. Jones (Ed.), Applications of Hydrogen Peroxide and Derivatives, Royal Society of Chemistry, Cambridge, UK, 1999, pp. 40–60.
[25] J. Fossey, D. Lefort, J. Sorba (Eds.), Free Radicals in Organic Chemistry, Wiley, New York, USA, 1997.
[26] K. Clarke, R. Edge, V. Johnson, E.J. Land, S. Navaratnam, T.G. Truscott, Direct observation of NH_2• reactions with oxygen, amino acids, and melanins, J. Phys. Chem. A 112 (6) (2008) 1234–1237. http://dx.doi.org/10.1021/jp076395r.
[27] J. Marsh, R.M. Dahlgren, C. Clarke, J. Stonehouse, C. Nunn, A new oxidant for hair coloring, J. Cosmet. Sci. 60 (2) (2009) 205–215.