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## **► To cite this version:**

Bertrand Vilen, Yannick Port-Lougarre, Elena Giménez-Arnau. Electron paramagnetic resonance and spin trapping to detect free radicals from allergenic hydroperoxides in contact with the skin: from the molecule to the tissue. *Contact Dermatitis*, 2022, 86 (4), pp.241-253. <10.1111/cod.14037>. <hal-03511649v2>

**HAL Id: hal-03511649**

**<https://hal.science/hal-03511649v2>**

Submitted on 11 Jan 2022

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# Electron paramagnetic resonance and spin trapping to detect free radicals from allergenic hydroperoxides in contact with the skin: from the molecule to the tissue

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**Conflict of interest:** The authors have no conflict of interest to declare

## Acknowledgments:

This research was supported by the Chemistry Research Foundation (Fondation ciRFC, Strasbourg, France, Grant Number JL-FRC-0001), the DEFCHEMSKALL Franco-German Collaborative International Research Project financially supported by the ANR (Agence Nationale de la Recherche; project no. ANR-15-CE15-0023-01) and the DFG (Deutsche Forschungsgemeinschaft) and Firmenich SA (Geneva, Switzerland). The authors would like to thank the French Ministry of Research, the University of Strasbourg, the French National Centre for Scientific Research (CNRS) and the Réseau National de Rpe interDisciplinaire (RENARD, Fédération IR-RPE CNRS #3443).

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the [Version of Record](#). Please cite this article as doi: [10.1111/cod.14037](https://doi.org/10.1111/cod.14037)

## Abstract

A major research topic consists of revealing the contribution of radical-mediated reactions in dermatological diseases related to xenobiotic-induced stress, to succeed risk assessment procedures protecting producers and consumers. Allergic contact dermatitis is the clinically relevant consequence of skin sensitization, one of the most critical occupational and environmental health issues related to xenobiotics exposure. The first key event identified for the skin sensitization process to a chemical is its aptitude to react with epidermal proteins and form antigenic structures that will further trigger the immune response. Many chemical sensitizers are suspected to react through mechanisms involving radical intermediates. This review focuses on recent progress we have accomplished over the last few years studying radical intermediates derived from skin sensitizing chemicals by electron paramagnetic resonance in combination with the spin trapping technique. Our work is carried out “from the molecule”, performing studies in solution, “to the tissue”, by the development of a methodology on a reconstructed human epidermis model, very close in terms of histology and metabolic/enzymatic activity to real human epidermis, that can be used as suitable biological tissue model. The benefits are to test chemicals under conditions close to human use and real-life sensitization exposures and benefit from the 3D microenvironment.

**Keywords:** allergic contact dermatitis, hydroperoxides, endoperoxides, radical mechanisms, reconstructed human epidermis, electron paramagnetic resonance

## Abbreviations

ACD, allergic contact dermatitis; AOP, adverse outcome pathway; Asc, ascaridole; C-radical, carbon-centred radical; CD, cluster of differentiation; Citr-OOHs, citronellol hydroperoxides; COCAT, cocultured activation test; Cum-OOH, cumene hydroperoxide; DC, dendritic cells; DEPMPO, 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DIPPMPO, 5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide; DMPO, 5,5-dimethyl-1-pyrroline *N*-oxide; DPRA, direct peptide reactivity assay; EPR, electron paramagnetic resonance; EU, European Union; HaCaT, human keratinocyte cell line; HEPES, 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid; 15-HPA, 15-hydroperoxyabiatic acid; KE, key event; LC-MS, liquid chromatography-mass spectrometry; Lim-OOHs, limonene hydroperoxides; Lim-1-OOH, limonene-1-hydroperoxide; Lim-2-OOH, limonene-2-hydroperoxide; Lin-OOHs, linalool hydroperoxides; Lin-6-OOH, linalool-6-hydroperoxide; Lin-7-OOH, linalool-7-hydroperoxide; MNP, 2-methyl-2-nitrosopropane; NMR, nuclear magnetic resonance; O-radical, oxygen-centred radical; OECD, Organization for Economic Cooperation and Development; PA, photo allergen; PACD, photoallergic contact dermatitis; PB, phosphate buffer; PBN, *N*-tert-butyl- $\alpha$ -phenylnitrone; PCA, 3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl; RHE, reconstructed human epidermis; RNS, reactive nitrogen species; ROOHs, hydroperoxides; ROOR', endoperoxides; ROS, reactive oxygen species; S/N, signal-to-noise ratio; ST, spin trapping; TEMPO, (2,2,6,6-tetramethylpiperidin-1-yl)oxyl; TEMPOL, 4-hydroxy-2,2,6,6-tetramethylpiperidin 1-oxyl; TMIO, 1,1,3,3-tetramethylisindolin-2-yloxy; TMPO-OH, 4-hydroxy-2,2,6,6-tetramethylpiperidin; TTO, tea tree oil; UV, ultraviolet.

## 1. Introduction

Allergic contact dermatitis (ACD) is one of the most important occupational and environmental health issues associated with small chemicals. It is a reaction of the immune system induced by activation of antigen-specific T-cells resulting from skin sensitization to an exogenous chemical after repeated contact.<sup>1</sup> This hypersensitivity reaction occurs through a two-phase process: (i) an initial sensitization phase, *i.e.*, sensitization of the individual acquired after first contact with the allergen, followed by (ii) an elicitation phase triggered after new exposure of the sensitized individual to the allergen (Figure 1). The process of sensitization requires at least 4 days to several weeks, whereas the elicitation phase resulting in the clinical manifestation of ACD is fully developed in 1-4 days. Nowadays, about 27% of the adult population in the European Union (EU) develop ACD reactions to one or more skin allergens present in everyday life, and prevalence continues to rise worldwide.<sup>2</sup> ACD is a long-life condition deteriorating the quality of life of affected individuals drastically. Clinical symptoms are eczema, oedema and vesicles of the skin in the acute phase. Scaling and fissures may develop if exposure remains.<sup>3</sup> There is today no treatment of ACD other than symptomatic. Thus, prevention of the disease relies mainly on early identification of the sensitizing potential of chemicals prior to their introduction in the market and on upstream investigations probing their mechanism(s) of action for risk assessment purposes.

Skin sensitization results from a complex sequence of events. The adverse outcome pathway (AOP) defined by the Organization for Economic Cooperation and Development (OECD) identified four key events (KE) associated to the ACD mechanism: (KE1) binding of chemicals to endogenous proteins in the skin, (KE2) keratinocytes activation, (KE3) dendritic cells (DC) activation and maturation, and (KE4) proliferation of antigen-specific T-cells.<sup>4</sup> KE1 is a chemical event related to the ability of chemical sensitizers, after penetrating the skin through the *stratum corneum*, to modify epidermal proteins and form antigenic structures triggering the immune system. This molecular initiating event, already advanced by Landsteiner and Jacobs many years ago,<sup>5</sup> is now widely accepted by toxicologists and immunologists.

Many chemical sensitizers are suspected to react with epidermal proteins (KE1) through mechanisms involving radical intermediates, instead of the commonly accepted two electrons nucleophile-electrophile mechanism.<sup>6</sup> This applies in particular to hydroperoxides (ROOHs) and endoperoxides (ROOR') derived from autoxidation of natural terpenes broadly used for their odorant properties in perfumes, cosmetic products and occupational settings. Once ROOHs are formed, they can give rise to specific antigens and act as strong skin sensitizers.<sup>7,8</sup> Today, among the most known ROOHs are autoxidation products of limonene (Lim-OOHs), linalool (Lin-OOHs) and citronellol (Citr-OOHs).<sup>9</sup> Patch testing on individuals in a European multicentre study found 5.2% patients

reacting to 3% oxidized limonene in petrolatum (0.33% w/w Lim-OOHs content), and 6.9% to 6% oxidized linalool (1% w/w Lin-OOHs content).<sup>10-13</sup> Repeated open application tests found that repeated exposure to lower concentrations of oxidized linalool and oxidized limonene also induces ACD in sensitized patients.<sup>14,15</sup> Recently, a patch test study on air-oxidized citronellol at several concentrations in petrolatum, from 1% to 6%, detected positive reactions in 0.61%-4.5% of patients tested.<sup>16</sup> The potential and potency of ROOHs derived from limonene, linalool and citronellol to activate DC has been evaluated by measuring upregulation of cluster of differentiation 86 and 54 proteins (CD86 and CD54, respectively) on THP-1 cells upon exposure in the cocultured activation test (COCAT) consisting of HaCaT cells (human keratinocyte cell line) and THP-1 monocytes (as DC surrogate). ROOHs upregulated CD86 and/or CD54 on cocultured THP-1 cells in a concentration-dependent manner, and results were comparable with their sensitization potency ranking (strong to moderate) in predictive animal models such as the local lymph node assay in mice.<sup>17</sup> The ROOR' ascaridole (Asc) is considered to be one of the compounds responsible for ACD to aged tea tree oil (TTO). A study on its elicitation potential in individuals with eczema and suspected fragrance allergy after topical exposure found that every positive reaction to aged oxidized TTO was accompanied by a positive reaction to Asc.<sup>18</sup> A study on the capacity of Asc to react with proteins showed some reactivity toward cysteine- but not lysine-containing peptides in the Direct Peptide Reactivity Assay (DPRA), the regulatory agencies approved *in chemico* method to assess the sensitizing potential of chemicals.<sup>19</sup> Treatment of human monocyte-derived DC supported full DC maturation, and induced significant upregulation of co-stimulatory CD86, CD80, CD40 proteins and the adhesion molecule CD54 in a time-dependent manner.<sup>20</sup>

Very little is known about the formation of antigenic structures through radical mechanisms in the skin allergy field. We believe the first essential step to understand ACD to ROOHs and ROOR' is to identify and characterize radical intermediates issued from the compounds that could potentially react with proteins in the skin and form immunogenic complexes relevant for ACD. Nowadays the best method to identify transient radicals in chemical and biological systems is the use of Electron Paramagnetic Resonance (EPR) in association with the spin trapping (ST) technique.<sup>21</sup> The objective of the present review is to describe recent progress we have accomplished over the last few years studying radical intermediates derived from sensitizing chemicals by EPR-ST. The EPR-ST basics will be first described, together with previous uses in skin and cell cultures in the dermatological context. Our work in the skin allergy field carried out "from the molecule", performing studies in solution, "to the tissue", by the development of an EPR-ST methodology on reconstructed human epidermis (RHE), will then be detailed.

## 2. EPR-ST: the basics

EPR-ST is an indirect technique that takes advantage of EPR sensitivity to detect low yields of short-lived radical species (nano to milliseconds). It was established and developed in the late 1960s<sup>22,23</sup> and gradually became an unavoidable tool in the quest of transient reactive radicals.<sup>21</sup> Not only EPR-ST allows free radicals detection, but it also enables potential identification and characterization. Consequently, EPR-ST found numerous applications in a growing list of research fields ranging from (photo-)biology and chemistry to material and medical sciences.<sup>24-27</sup>

The basis in ST relies on the use of a diamagnetic molecule *a.k.a.* the spin trap, that reacts with transient reactive radicals forming a paramagnetic spin adduct (Figure 2). Hence by nature, spin adducts are EPR active as electronic transition(s) are enabled when the degeneracy of the electronic level is lifted through the Zeeman effect: the cornerstone principle of EPR spectroscopy.<sup>28</sup> The most commonly used spin traps are nitroso or nitron based molecules that lead to stable nitroxide-based radical adducts such as: MNP (2-methyl-2-nitrosopropane),<sup>29</sup> PBN (*N-tert*-butyl- $\alpha$ -phenylnitron),<sup>30</sup> DMPO (5,5-dimethyl-1-pyrroline *N*-oxide),<sup>31</sup> DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide),<sup>32</sup> and DIPPMPO (5-(diisopropoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide)<sup>33</sup> (Figure 3). The choice of the spin trap is of utmost importance and is strongly dependent of both experimental conditions (polarity of the solvent, temperature, irradiation...) and the nature of the targeted radicals (*e.g.*, carbon- or oxygen-centred). While nitroso-based compounds such as MNP exhibit good results in organic solvents, their cytotoxicity precludes any *in vivo* applications.<sup>34</sup> Also, their application in polar solvents such as aqueous solutions is complicated by the presence of parasitic stable dimeric species. Finally, the stability of oxygen-mediated spin adducts is often limited and *e.g.*, the spin trap is sensitive to both light and temperature.<sup>35</sup> Consequently, most of the EPR-ST bio-related investigations are performed nowadays using nitron based compounds. Indeed, such spin traps are known to be well tolerated *in vivo* at relatively high concentrations and may be then employed safely for *in vivo* EPR investigations.<sup>36</sup> These spin traps are prone to detect both oxygen- and carbon-centred radicals in a large range of pH, temperature and/or under illumination and are compatible with various organic solvents. In this regard, PBN is a very efficient spin trap, yet the resulting spin adducts are not very specific to the trapped radical, which is however the case for DEPMPO and DIPPMPO. Worthy of note, the (commercial) availability of the spin trap which often goes hand in hand with the abundance of relevant literature can also be a criterion of choice to rely on while analysing the spin adducts and identifying free radicals. On this basis, such spin traps appear pertinent for bio-related investigations.

Ideally, the EPR fingerprint of a given spin adduct is strongly influenced by the trapped radical. Like any other radical in EPR, the spin adduct is defined by its *g* factor,<sup>28</sup> the relative amplitudes,



linewidths and the pattern of EPR lines that are governed by the hyperfine coupling. The  $g$  factor (of *ca.* 2.0055 when the spin adduct is a nitroxide-based molecule in aqueous solutions whatever is the trapped molecule) defines the absolute position of the centre of the EPR spectrum and is of little information in EPR-ST investigations. The linewidth of the EPR line can depend on the nature of the buffer, molecular oxygen concentration and on the size of the trapped molecules (*e.g.*, protein radicals) giving rise to distinctive spectra.<sup>37,38</sup> Finally, the most informative parameters in EPR-ST studies rely on the presence and strength of hyperfine coupling resulting from interactions between the unpaired electron and surrounding nuclei that possess nuclear spin  $I \neq 0$ . Supplementary electronic transitions are thus enabled, splitting an EPR line into  $2I+1$  lines, described by the hyperfine fine coupling constants (*hfccs*, see Fig. 2c for illustration).<sup>39</sup>

Deconvolution of the contributions of the different spin adducts observed in the EPR spectra might be mandatory if more than one radical is trapped. Thus, radical(s) identification can be obtained by resolving numerically their *hfccs* and comparing them with existing literature,<sup>40</sup> database<sup>41</sup> or theoretical calculations.<sup>42</sup> EPR-ST investigation coupled to mass spectrometry is also a powerful approach to identify the nature of the radicals involved while evaluating the mass of the spin adduct.<sup>43,44</sup>

EPR-ST has been successfully employed in the detection of various free radicals centred *e.g.*, on oxygen, carbon, sulphur or boron atom.<sup>45,46</sup> Considering our research topic on ACD, the two first mentioned are the most frequently trapped radicals. Even though DMPO was the first cyclic nitron successfully used in EPR-ST history for detecting ROS, intrinsic limits restrict its use in biological investigations. Mostly, the weak persistence of the superoxide ( $\cdot\text{O}_2$ ) DMPO spin adduct ( $< 1$  minute) that rapidly converts into hydroxyl ( $\cdot\text{OH}$ ) radical's one and their prompt degradation by the cells, often prevent gaining a comprehensive picture.<sup>47</sup> Electron-withdrawing groups at position 5 as in DEPMPO were then introduced.<sup>48</sup> DEPMPO is able to trap O- and C-centred radicals<sup>49</sup> with formed adducts having a half-life superior to those formed by DMPO, particularly for the spin adduct formed with the  $\cdot\text{O}_2$  radical<sup>50</sup> and no spontaneous decomposition to the  $\cdot\text{OH}$  adduct is observed. Another advantage of DEPMPO over DMPO is its ability to both trap and identify peroxy radicals unambiguously. Indeed, for DMPO former EPR studies wrongly attributed DMPO-peroxy radical adducts based on their close similarity to DMPO-superoxide spin adduct, and were finally reassigned to alkoxyl radical adducts.<sup>51</sup>

Despite EPR-ST obvious potential, it is of primary importance to be aware of few intrinsic limitations that lead to biased conclusions when not considered. Firstly, it is important to keep in mind that EPR-ST is an indirect method, and what is observed is the product of the reaction between the spin trap and the probed radical, not the radical itself. Secondly, if the EPR amplitude and intensity



are directly related to the probed number of paramagnetic centres, the absolute quantification of the probed radicals is hardly achievable. Indeed, the reaction kinetics of the spin trap with the reactive species is known to be slow and spin adducts can be degraded when considering the surrounding milieu. Relative quantification (*i.e.*, one spin adduct *vs.* another one) is accessible under certain conditions. Thirdly, nitroxide generation can be achieved following other reactions than spin trapping leading to false positive results. Several acknowledged processes such as inverse spin trapping, the Forrester-Hepburn reaction and the ene-reaction are known to form spin adducts in the absence of radicals. For more details about EPR-ST and especially its good practice guide, an exhaustive review has been very recently published by Lauricella and Tuccio.<sup>21</sup>

Noteworthy, approaches complementary to genuine EPR-ST prevail in specific cases: (i) EPR spin scavenging consists in investigating free radical generation by following reduction of nitroxide radicals (EPR active) to hydroxylamines, or one-electron oxidation to *N*-oxoammonium cations, both EPR silent.<sup>52</sup> Although known as stable radicals, such nitroxide probes are prone to be degraded especially in intracellular reductive milieu<sup>53,54</sup> or upon UV light irradiation. However, EPR spin-scavenging is not specific to the probed radical; (ii) reactive species can be characterized even if there is no spin to trap. For example, TMP-OH (4-hydroxy-2,2,6,6-tetramethylpiperidin) is a precursor of the paramagnetic TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidin 1-oxyl) upon reaction with singlet oxygen, the first excited state of molecular oxygen.<sup>55,56</sup>

### 3. EPR and ST, important tools in skin research

The skin is, with a surface area about 1.5 m<sup>2</sup>, one of the most important organs.<sup>57,58</sup> Covering the entire surface of the human body, it is the first interface with the outside world, protecting it from ultraviolet rays and shocks while keeping the body temperature constant by retaining or eliminating water. Its permeability, constant renewal and elasticity make it a great natural physical barrier between the body and external aggressions of a physical, biological or chemical nature. Not to mention its metabolic capacity to detoxify foreign molecules that can cross the *stratum corneum* and get into the epidermis.<sup>59</sup>

The skin is a living organism where one-electron redox processes and free radical mechanisms are part of its survival (*e.g.*, mitochondrial respiration). Also, some biomolecules in the skin such as melanin, at the origin of skin pigmentation, are paramagnetic biopolymers revealing strong and stable paramagnetism. Though, such endogenous free radicals are also responsible of oxidative stress leading to serious pathologies. The skin is thus a perfect target for EPR studies and the development of medical applications. EPR is increasingly used for many years now in skin research, in applications going from the study of redox metabolism and detection of reactive oxygen and nitrogen species (ROS, RNS), to the comprehension of the barrier function, drugs release and distribution in human

skin, or the diagnosis of skin diseases such as melanoma, among others. While a recent review on the EPR latest achievements devoted to the specific field of dermatology can be found in the literature,<sup>60</sup> for the concerns of the present review we will focus on applications based in the use of nitroxides and spin traps.

A major research topic is the identification of reactive free radicals and ROS in human skin exposed to UV radiation, strongly involved in skin damages like cancer and aging. While the UV induced ascorbate radical is directly measurable by EPR in skin at room temperature,<sup>61</sup> EPR-ST has been employed successfully in the detection of various short-lived radicals centred on oxygen and carbon atoms, in keratinocytes, epithelial cells, skin homogenates, mouse skin or human skin biopsies exposed to UV irradiation.<sup>62,63</sup> The spin scavenging approach is also used to study these reactions in human skin. The piperidine-nitroxide TEMPO (2,2,6,6-tetramethylpiperidin-1-yl)oxyl has been employed to study the role of ROS in *ex vivo* measurements on irradiated skin biopsies. As a result of the scavenging process, the loss of TEMPO signal was attributed to both UV-mediated ROS and enzymatic activity of the skin (Figure 4).<sup>64</sup> Also, PCA (3-carboxy-2,2,5,5-tetramethylpyrrolidine-1-oxyl) was used for EPR imaging spatial distribution of UV generated free radicals and ROS, being then possible to estimate the penetration of UVA and UVB irradiation.<sup>64</sup> PCA is EPR active, becoming EPR inactive after reaction with a radical. It is thus possible to assess free radical production by measuring PCA decay as it has been reported on UV irradiated porcine skin.<sup>65</sup>

Much more related to our research topic on ACD, several EPR-ST studies are found in the literature on the identification of free radicals derived from organic hydroperoxides widely used in the polymer, cosmetics, pharmaceutical and chemical industries. Among them, *tert*-butyl hydroperoxide and cumene hydroperoxide (Cum-OOH). There was substantial indirect evidence that free radicals are involved in stages of their related skin tumour promotion, skin irritation and skin sensitization. The use of different spin traps allowed indeed to characterize oxygen and carbon centred radicals derived from these hydroperoxides in isolated mouse keratinocytes<sup>66</sup>, human carcinoma skin keratinocytes<sup>67</sup>, normal human keratinocytes<sup>68</sup>, samples of murine skin<sup>69</sup> and lipid extracts from skin of mice fed with vitamin E sufficient or deficient diets<sup>70</sup>. Finally, because of ethical considerations and European regulations banning animal testing in the cosmetics area, models of RHE and full thickness skin substitutes have started to be used this last decade.<sup>71,72</sup>

#### **4. EPR-ST and skin sensitizers: from the molecule to the tissue**

##### **4.1 Chemical sensitizers and radical intermediates**

During the last decades a big step forward has been given related to the autoxidation of non-allergenic compounds to form potent contact allergens. Compounds with oxidizable allylic positions can autoxidize on air exposure. This autoxidation is a free radical chain reaction in which hydrogen

atom abstraction with addition of oxygen forms ROOHs as primary oxidation products, that may be skin sensitizers. Karlberg *et al.* were the first authors to describe a full picture of skin sensitizers, mainly natural terpenes, concerned by this phenomenon (Figure 5).<sup>73</sup> Original studies date back to the middle of the 20<sup>th</sup> century with the highly allergenic turpentine, a complex mixture of terpenes extracted from coniferous trees, and the hydroperoxide of  $\Delta^3$ -carene showed to be “the eczematogenic factor” in the Scandinavian population.<sup>74,75</sup> Later on, 15-hydroperoxyabietic acid (15-HPA), issued from abietic acid autoxidation, was shown to be the responsible for ACD to colophony, a non-volatile resin exuding from coniferous trees.<sup>76</sup> But the interest on sensitizing ROOHs has considerably increased these last years with the rising prevalence of fragrance allergy due to the worldwide use of fragranced products and odorant terpenes.<sup>2</sup> It has been revealed that ROOHs from commonly used fragrance terpenes that autoxidize on storage and handling are frequent causes of ACD. Sharing the spotlight, there are those derived from limonene, linalool and citronellol.

ROOHs reactivity starts with the cleavage of the O-O bond of low dissociation energy (*ca.* 175 kJ.mol<sup>-1</sup>). This cleavage being a simple process gives rise to unstable alkoxyl (RO<sup>\*</sup>) radicals. Because of their instability and the presence of double bonds in the terpene chemical structure, they can undergo radical rearrangements (*i.e.*, intramolecular cyclization, allylic hydrogen abstraction,  $\beta$ -scission) and form more stable C-radicals (R<sup>\*</sup>). An example is shown in Figure 6 for Lim-1-OOH.

To understand how ROOHs can form immunogenic structures in the epidermis, we have carried out reactivity studies with amino acids known to participate in radical processes. These studies, conducted in solution (buffer, semi organic) and followed by nuclear magnetic resonance (NMR) and/or liquid chromatography combined to mass spectrometry (LC-MS), proved that C-radicals issued from ROOHs react with amino acids such as cysteine, histidine and tryptophan when using Fe(II)/Fe(III) redox systems to trigger radical initiation.<sup>77</sup> This was important information as it revealed that several reactive radical intermediates can derive from these compounds depending on their chemical structure, and thus different chemical modifications of proteins could potentially lead to sensitization. It seemed therefore essential to investigate if the formation of free radicals from ROOHs happens truly in the skin. With this objective, our efforts are focused from many years now on the use of EPR-ST to identify and characterize those radicals, first in solution, then in a 3D model of reconstructed human epidermis (RHE).

## 4.2 EPR-ST studies in solution

Our very first studies in solution began with the identification of radicals derived from 15-HPA following preliminary investigations on model mono and bicyclic tertiary allylic hydroperoxides.<sup>78-80</sup> At that time, we took advantage of the ability of TMIO (1,1,3,3-tetramethylisoindolin-2-ylloxyl) to trap C-radicals giving stable diamagnetic alkoxyamine products, which can be characterized by using

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a full range of spectroscopic techniques. The formation of C-centred radicals  $R^\bullet$  derived from alkoxyl and peroxy radicals as a consequence of the hydroperoxide scission in presence of Fe(II)/Fe(III) systems was confirmed, and structures of the adducts identified by mono and bidimensional NMR (Figure 7). TMIO being able to trap only C-radicals, we started to use EPR-ST to investigate the potential formation of O- and C-centred radicals coexisting together in organic-aqueous media by triggering also radical initiation with Fe(II)/Fe(III) redox systems. Using a monocyclic model and spin traps DMPO and DEPMPO, it was demonstrated that C-radicals  $R^\bullet$  were in solution simultaneously with O-radicals (*i.e.*, allyloxyl  $R-O^\bullet$ ) (Figure 7). Mechanistic interpretations of radical intermediates formation were suggested, and it was proposed that both C- and O-radicals might be considered as potential haptens of 15-HPA.

In the last few years our studies have focused mainly in the study of ROOHs derived from autoxidation of fragrance terpenes. Early studies with a linalyl hydroperoxide derivative still were founded on the use of TMIO,<sup>81</sup> but EPR-ST quickly gained the upper hand as it is more sensitive detecting a much larger picture of intermediates and is commonly employed in biological media. A complete identification by EPR-ST of radicals issued from Lim-2-OOH and Lin-7-OOH, described among the main responsible compounds for skin sensitization to limonene and linalool respectively, was first reported.<sup>82</sup> In these early studies DMPO and DEPMPO were used, in water-acetonitrile (1:1 v/v) or acetonitrile media. Alkoxyl and C-centred free radicals were trapped by DMPO, whereas peroxy radicals were characterized by spin trapping with DEPMPO.<sup>48</sup> However, given the above-mentioned advantages of using DEPMPO (section 2), this spin trap was favoured in our studies from that time on.

Though, our final aim was to develop a method to detect and identify all kinds of radical intermediates issued from skin sensitizers in a RHE model of epidermis, a cellular keratinocytes matrix. In this context we always carry out EPR-ST investigations in solution prior to the RHE investigations, in an attempt to get a preliminary overview of the reactive radicals potentially arising from ROOHs in an over simplified model, and to determine optimal experimental conditions (sensitivity limit) while providing a decent signal-to-noise ratio (S/N). As a general procedure, the target ROOH and the spin trap are dissolved in a semi-organic solvent or buffer. In such approach, *i.e.*, in absence of biological systems, radical initiation in solution setup is triggered by a Fenton-like reaction through addition of Fe(II) sulfate. At this level, HEPES is preferred to phosphate buffer (PB) to avoid formation of insoluble Fe(III) by Fe(II) oxidation catalysed by PB, decreasing the Fe(II) content available.<sup>83</sup> The reaction mixture is subjected to stirring and directly transferred within an EPR submillimeter glass capillary (20  $\mu$ L) sealed on both ends and further placed into the cavity of an EPR X-band spectrometer (Figure 8). EPR spectra are then recorded. Computer simulation and

deconvolution of the experimental spectra are achieved by using labmade scripts based on EasySpin toolbox under Matlab environment.<sup>84</sup> Examples are shown in Figure 9. A mixture of Lin-OOHs (Lin-7-OOH/Lin-6-OOH ratio 2:3) gave a spectrum showing the spin adduct corresponding to  $\cdot\text{OH}$  radicals (DEPMPO-OH), together with species testimony of C-centred radicals (DEPMPO-R1 and DEPMPO-R2 spin adducts) and potential alkoxyl radicals (DEPMPO-OR spin adduct), all with characteristic hyperfine coupling constant (*hfccs*) values (Table 1).<sup>85</sup> In the case of Lim-OOHs, both Lim-1-OOH and Lim-2-OOH gave very similar EPR signatures pointing to the formation of DEPMPO spin adducts corresponding to the trapping of two distinct C-centred radicals, together with  $\cdot\text{OH}$  and other radicals (Table 1).<sup>86</sup>

Such simplistic model although informative is relatively far from real-life situations. Hence, the RHE approach has been developed to model allergenic-mediated radicals close to actual exposure to ROOHs and xenobiotics.

#### 4.3 EPR-ST in reconstructed human epidermis

EpiSkin<sup>TM</sup> RHE (EpiSkin, Lyon, France) is an *in vitro* reconstructed epidermis from normal human keratinocytes cultured on a collagen matrix at the air-liquid interface (13 days stratification) very similar to human tissue in terms of morphology and lipid composition.<sup>87</sup> Therefore, RHE such as EpiSkin<sup>TM</sup> are a very good alternative for testing phototoxicity, irritancy and corrosivity of chemicals in contact with the skin before use in commercial products.

Our interest in the study of radicals derived from ROOHs in contact with the skin led us to develop an EPR-ST methodology to investigate *in situ* in the EpiSkin<sup>TM</sup> 3D model their formation and behaviour. EPR had never been used before for the study of radicals derived from skin sensitizers in RHE, complex system closer to human epidermis simulating what may happen *in vivo*. No additional Fe(II) radical initiation was used for all *in situ* approaches since the epidermis itself might be able to initiate the reactions as in ordinary skin exposure to ROOHs.

Cum-OOH, reported to be a skin sensitizer in guinea-pigs with individual positive responses in cross-reactivity studies with allergenic cyclohexene ROOHs,<sup>7</sup> was used as proof of concept for the development of the methodology.<sup>88</sup> RHE (0.38 cm<sup>2</sup> format) were kept at arrival in 2 mL supplied maintenance medium (37 °C, 5% CO<sub>2</sub>, saturated humidity atmosphere). After 24 h, the maintenance medium was replaced by 2 mL of assay medium provided by EpiSkin. The very first studies consisted of incubating Cum-OOH and DEPMPO in the assay medium to avoid the epidermal barrier present in RHE (although less developed than that of normal skin) and quickly assess by using absorption from RHE bottom if hydroperoxide-mediated radicals could be formed in the epidermis. Cum-OOH (200  $\mu\text{L}$ , 5.4 mM HEPES/acetonitrile 9/1 solution) and DEPMPO (100  $\mu\text{L}$ , 500 mM HEPES solution)

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were added to 300  $\mu$ L assay medium and 1400  $\mu$ L HEPES. The RHE was then placed in a well containing this mixture. After 2 h incubation, the RHE was positioned in an EPR tissue cell (Willmad, Vineland, NJ, USA) and spectra recorded in a X-band spectrometer. Although being noisy, a signal was observed rising with time. The major peak was assigned to a C-centred radical DEPMPO spin adduct, minor peaks to O-radical spin adducts. For control purposes, assay medium aliquots (20  $\mu$ L) were collected regularly, inserted into a capillary and EPR registered showing no signal. Though, the OECD guideline on *in vitro* skin irritation assessment using RHE specifies that test chemicals must be applied topically.<sup>89</sup> Thus, to get closer to real-life sensitization, we based next experiments on a topical application procedure. These radicals are of short-life (nano to milliseconds) which goes hand in hand with their great reactivity, but also meaning that available time to trap them with DEPMPO for identification is particularly short. Hence, in order to give us the best possible chance of detecting all radical intermediates in RHE, we decided to beforehand load the skin samples with DEPMPO to ensure that it is present at the site of radical generation before application of the hydroperoxide. This spin trap preload was previously described for detection of free radical production in murine skin treated with tumour promoting peroxides using DMPO as spin trap.<sup>69</sup> It was adapted here for use with DEPMPO. This needed to further optimize the concentrations of products used in order to achieve the best S/N ratio.<sup>90</sup> RHE were topically treated first with DEPMPO (20  $\mu$ L, 250 mM in HEPES) and post-incubated 15 min (37 °C, 5% CO<sub>2</sub>). After the incubation time, the RHE were placed in the EPR tissue cell and Cum-OOH (20  $\mu$ L, 50 mM in acetone) was added on RHE top and spectra registered. Control experiments with (i) single DEPMPO and (ii) single Cum-OOH gave no signal, indicating that radicals trapped were derived from Cum-OOH radical decomposition and not from biomolecules composing the skin. Despite a weak S/N ratio, EPR fingerprints were compatible to those obtained in solution studies, thus indicating similar spin adducts formed in RHE, testimony of C- and O-centred radicals. The general procedure for this finally adopted methodology based on topical application is shown in Figure 10.

To identify radical intermediates potentially involved in skin sensitization processes, this methodology has been further successfully applied to skin sensitizers such as endoperoxide Asc,<sup>91</sup> and ROOHs derived from autoxidation of linalool<sup>85</sup>, limonene<sup>86</sup> or citronellol (publication in progress). We quickly noticed that concentrations of spin trap and ROOH needed in the case of EPR-ST studies in RHE are in general at least 10-fold higher than those giving a good S/N ratio signal in solution, yet still within the typical range of clinical ACD diagnostic tests.<sup>85</sup> This is possibly due to the fact that RHE are complex 3D structures including a *stratum corneum*, thus skin penetration concerns need to be considered, as well as the content in phospholipids and ceramides that may influence RHE barrier properties.<sup>87</sup> Another explanation stems from the fact that spin adduct's



lifetime is lowered in the epidermis environment where reducing agents are present (*e.g.*, ascorbate and glutathione are prone to reduce nitroxides in the skin<sup>92</sup>). Worthy of note, in certain cases spin adducts can be resolved albeit of low S/N, based on comparison with the investigations in solution. It is central to stress here that no additional Fe(II) is added in RHE experiments, indicating that radical initiation is supposed to be driven by endogenous skin constituents (*i.e.* electron donors, metals, amino acids or porphyrins). In Figure 11 experimental spin adducts and subsequent simulations/deconvolution obtained for Asc are shown for parallel studies in solution and RHE.<sup>91</sup> Both investigations highlight the potential involvement of mostly C-centred radicals in Asc-induced ACD. Slight differences are observed between the detected *hfccs* (see Table 2) when compared in solution experiments and RHE respectively and can be explained by the substantial difference within the spin adduct neighbourhood in each configuration.

DEPMPO has been shown thus to be very convenient to trap C-radicals derived from skin sensitizers in RHE leading to particularly stable spin adducts. These C-radicals seem to be of major importance in skin sensitization to ROOHs and ROOR'. Theoretically, these sensitizers could either be specific haptens or form non-specific antigens. In the case of a non-specific action, structurally different compounds would form the same immunogenic protein by acting as oxidizing agents able to oxidize functional groups in proteins. Indeed, in many experiments with structurally different ROOHs we have also identified O-centred radicals such as  $\cdot\text{OH}$  that might act as oxidizing agents. However, no general cross-reactivity has been observed in sensitization studies in guinea pigs with different ROOHs, suggesting that the non-specific mechanism is unlikely.<sup>73</sup> Thus, the activity of ROOHs as haptens is likely to involve radical reactions implicating specific structure-related C-radicals for the formation of covalent bonds with proteins by radical coupling.

Though, nitrones hardly distinguish between different C-radicals, the coupling patterns observed on the spectra being always very similar. Thus, unambiguous assignment of C-centred radicals formed in RHE among the different options resulting from radical rearrangement processes is difficult. To overcome this and obtain evidence for the precise assignment of C-radicals in RHE, we conduct additional spin trapping studies with target compounds substituted with  $^{13}\text{C}$  at specific positions precursors of potentially reactive radicals. The introduction of  $^{13}\text{C}$  (nuclear spin  $1/2$ ) gives specific EPR spectra showing the hyperfine coupling with the  $^{13}\text{C}$  nucleus. If the trapped radical is centred on a  $^{13}\text{C}$ , there is indeed an additional hyperfine coupling on the spectrum due to its non-zero nuclear spin. As an example, our EPR-ST studies in RHE with Cum-OOH with  $^{13}\text{C}$ -substitution at methyl positions confirmed the presence of methyl radicals issued from decomposition of the hydroperoxide in the epidermis model (Figure 12).<sup>90</sup>



## 5. Conclusions and perspectives

Current approaches for predicting the sensitizing potential of chemicals are based on the different key events of the pathophysiological process leading to skin sensitization and the subsequent ACD inflammatory response. Reactivity studies to understand the first chemical event leading to the modification of proteins by allergenic compounds are necessary in order to cover all categories of molecules for risk assessment. In this context, skin sensitizers reacting through radical intermediates are an important chapter of these studies. With the desire to get closer to what may happen *in vivo*, we have developed an EPR-ST methodology with the potential to investigate *in situ* the formation and behaviour of free radicals derived from these xenobiotics in a RHE 3D model. Experiments are based on a topical application procedure to get closer to the real-life sensitization, and O- and C-centred radicals issued from several skin sensitizers have been identified in this complex environment. Our approach is thus in line with today ethical guidelines which forbid the use of animal testing for skin sensitization risk assessment of environmental chemicals.

This approach is a step forward in dermato-allergy research and now opens up a wide range of research opportunities. Considering prospective EPR investigations, the next step is EPR imaging techniques together with both EPR spin trapping and scavenging to address the spatial localization of the radicals formed within the epidermis. That will allow deciphering whether the reactive species are specifically localized in the outer or deeper layers of the epidermis. Also, should the radicals be formed inside or outside the cells, this shall be studied using chemically modified spin traps able to penetrate or not skin cells.<sup>93,94</sup> Though, we also believe this approach can be adapted to investigate other skin pathologies involving ROS and free radicals such as photoallergic contact dermatitis (PACD) and photo irritation, critical photosensitivity diseases related to exposure of the skin to xenobiotics.<sup>95</sup> Both are adverse reactions caused by combined exposure to photoreactive chemicals in contact with the skin and light (UVA, UVB and/or visible). While photo irritation is mainly caused by immediate generation of ROS inducing cellular damage, PACD is a well-organized immunological reaction just as ACD. Upon uptake of the photo allergen (PA) into the epidermis, excitation by light of the appropriate wavelength gives rise to reactive species which bind to skin proteins through the formation of stable covalent bonds. The conjugate so formed acts then as an antigen triggering an adaptive immune response. The mechanism of PA-protein photo binding is thought to proceed via the formation of highly reactive free radicals produced upon UV irradiation of the PA, though the link between free radicals and ROS actually formed in the epidermis and their mechanism of action is far from being unravelled.

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## Tables

**Table 1.** Hyperfine coupling constants (*hfccs*) of spin adducts identified in solution for Lin-OOHs and Lim-OOHs

Spin adducts	<i>hfccs</i> (G)		
	$a_N$	$a_H$	$a_P$
<b>Lin-OOHs</b> <sup>85</sup>			
DEPMPO-OH	14.1	13.3	47.6
DEPMPO-R1	15.0	22.5	47.2
DEPMPO-R2	14.4	20.9	47.1
DEPMPO-OR	13.6	8.5	48.2
<b>Lim-OOHs</b> <sup>86</sup>			
DEPMPO-OH	14.0	14.1	47.4
DEPMPO-R1	14.6	21.6	47.3
DEPMPO-R2	14.5	21.9	52.4
DEPMPO-7*	14.6	14.6	53.6

\* Unassigned signal

**Table 2.** Hyperfine coupling constants (*hfccs*) of spin adducts identified in solution and in RHE for Asc and (<sup>13</sup>C-Me)<sub>2</sub>CumOOH

Spin adducts	<i>hfccs</i> (G)			
	$a_N$	$a_H$	$a_P$	$a_{13C}$
<b>Asc – Solution</b> <sup>91</sup>				
DEPMPO-R1	14.7	21.5	47.2	
DEPMPO-R2	15.0	25.8	47.7	
<b>Asc – RHE</b> <sup>91</sup>				
DEPMPO-R1	14.8	21.6	47.4	
DEPMPO-R2	14.8	22.8	50.5	
<b>(<sup>13</sup>C-Me)<sub>2</sub>CumOOH-Solution</b> <sup>90</sup>				
DEPMPO-OH	14.2	13.4	47.4	
DEPMPO- <sup>13</sup> CH <sub>3</sub>	15.4	22.6	48.3	6.4
<b>(<sup>13</sup>C-Me)<sub>2</sub>CumOOH- RHE</b> <sup>90</sup>				
DEPMPO-OH	13.1	14.0	46.4	
DEPMPO- <sup>13</sup> CH <sub>3</sub>	15.1	21.4	48.2	6.4

## Figure legends

**Figure 1.** Mechanism of ACD: (1) penetration of the allergen into the epidermis; (2) binding to endogenous proteins; (3) internalization of modified proteins by DC; (4) DC migration to lymph nodes and maturation; (5) presentation of allergen-protein antigen to naïve T-lymphocytes; (6) proliferation of antigen-specific memory T-cells; (7) antigen-memory T-cells leaving the lymph nodes and entering the circulation; (8) re-internalization of another modified protein by a new DC and its maturation after re-exposure to the allergen; (9) presentation of the antigen to memory T-lymphocytes leading to release of inflammatory agents and ACD clinical response.

**Figure 2.** (a) Schematic description of the EPR-ST mechanism with (b) an example on DMPO reacting with methyl radical, where the hyperfine coupling between the unpaired electron and  $^{14}\text{N}$  ( $I_{\text{N}}=1$ ) and  $^1\text{H}$  ( $I=1/2$ ) nuclei lead to (c) a specific fingerprint characterized by the values of the so resulting hyperfine coupling constants (noted  $a_{\text{N}}$  and  $a_{\text{H}}$  respectively).

**Figure 3.** Classical EPR nitroso ( $\text{MNP}^{29}$ ) and nitron ( $\text{PBN}^{30}$ ,  $\text{DMPO}^{31}$ ,  $\text{DEPMPO}^{32}$ ,  $\text{DIPPMPO}^{33}$ ) based EPR spin traps with highlighted atoms involved in electron-nuclei hyperfine coupling, hydrophilicity character and biocompatibility.

**Figure 4.** Nitroxides react with free radicals (induced for example by UV radiation of the skin and/or cutaneous enzymatic activity) to form EPR silent diamagnetic compounds.

**Figure 5.** Examples of sensitizing ROOHs issued from autoxidation of natural terpenes.

**Figure 6.** Radical decomposition of Lim-1-OOH.

**Figure 7.** Identification of radicals derived from 15-HPA by i) trapping C-radicals with TMIO and ii) trapping O- and C-radicals with DEPMPO. NMR and EPR were complementary techniques to identify carbon ( $\text{R}^\bullet$ ) and oxygen ( $\text{RO}^\bullet$ ) radicals.

**Figure 8.** Schematic description of the experimental procedure for EPR-ST in solution and subsequent data analysis (Exp for experimental spectrum, Sim for computer simulation).

**Figure 9.** EPR-ST in solution with DEPMPO. Identification of radicals derived from Lin-OOHs (1 mM)/DEPMPO (25 mM)/Fe(II) (0.1 mM) (experimental spectrum (Exp), computer simulation (Sim) and deconvolution affording spectra of spin adducts DEPMPO-OH, DEPMPO-R1, DEPMPO-R2 and DEPMPO-OR) and Lim-OOHs (1mM)/ DEPMPO (50 mM)/Fe(II) (0.1 mM) (experimental spectrum (Exp), computer simulation (Sim) and deconvolution accounting for spin adducts DEPMPO-R1, DEPMPO-R2, DEPMPO-OH). Values of  $hfccs$  are shown in Table 1. Control experiments with sole ROOHs or DEPMPO gave no signal.

**Figure 10.** General topical application procedure developed for studies with RHE by EPR-ST.

**Figure 11.** Parallel EPR-ST investigation in solution (left) and RHE (right) of Asc with DEPMPO. Experimental EPR spectra (Exp) were simulated (Sim) and deconvoluted pointing to C-centred radicals  $\bullet$ R1 and  $\bullet$ R2 (see Table 2 for *hfccs*).  $\bullet$ R1 and  $\bullet$ R2 are, upon the mechanism of radical degradation of Asc, methyl or isopropyl C-centred radicals. Concentrations were Asc (1 mM)/DEPMPO (25 mM)/Fe(II) (0.1 mM) in solution and Asc (50 mM)/DEPMPO (250 mM) for RHE investigations. All experiments were performed in 10 mM HEPES, pH 6.8. Control experiments with sole presence of Asc or DEPMPO gave no signal.

**Figure 12.** EPR-ST studies using  $^{13}\text{C}$ -substitution: confirmation of formation of methyl radicals from Cum-OOH in RHE. (A) EPR spectrum in solution ( $^{13}\text{C-Me}$ )<sub>2</sub>Cum-OOH (0.54 mM)/DEPMPO (25 mM)/Fe(II) (0.1 mM). Two spin adducts were mainly detected attributed to DEPMPO- $^{13}\text{CH}_3$  and DEPMPO-OH (see Table 2 for *hfccs*). (B) Comparative spectrum in RHE ( $^{13}\text{C-Me}$ )<sub>2</sub>CumOOH (50 mM)/DEPMPO (250 mM). Spin adducts were assigned despite low S/N based on direct comparison with corresponding investigations in solution (dotted lines show DEPMPO- $^{13}\text{CH}_3$  spin adduct). Two spin adducts were mainly detected: DEPMPO- $^{13}\text{CH}_3$  and DEPMPO-OH (see Table 2 for *hfccs*). In (A) and (B) DEPMPO-OR/DEPMPO-OOR spin adducts were also detected in tiny proportions but are not showed here for clarification purposes. (C) Radical processes leading to the formation of methyl radicals from Cum-OOH.

Figure 1

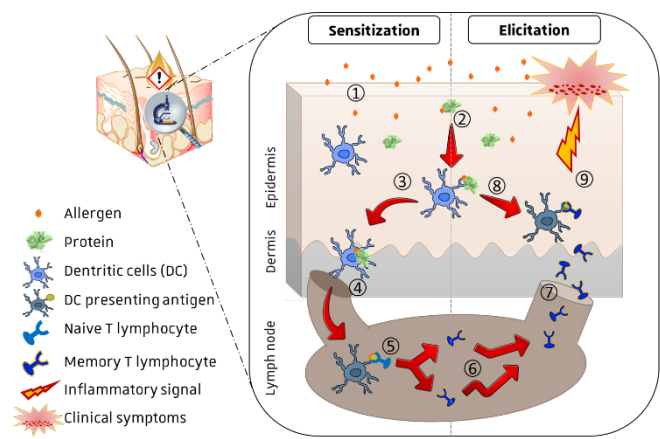


Figure 2

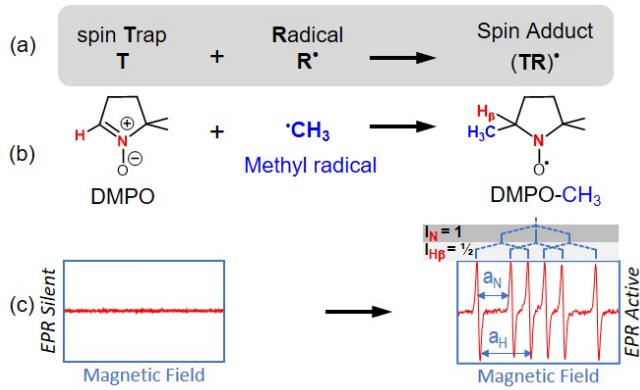


Figure 3

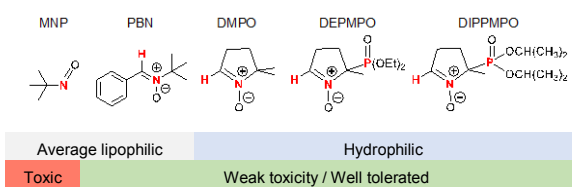


Figure 4

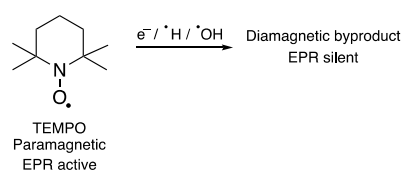


Figure 5

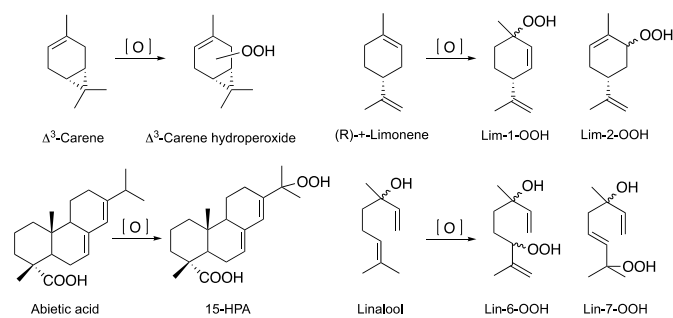


Figure 6

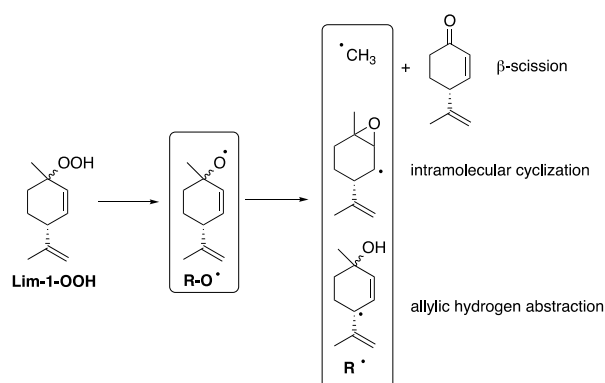


Figure 7

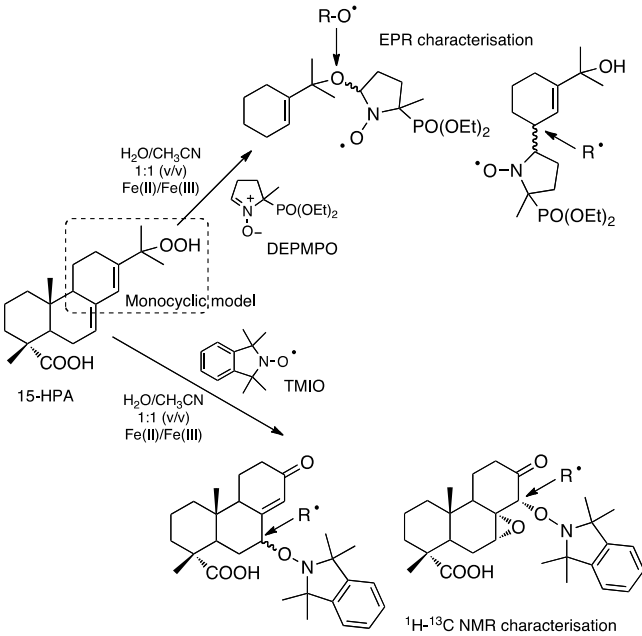


Figure 8

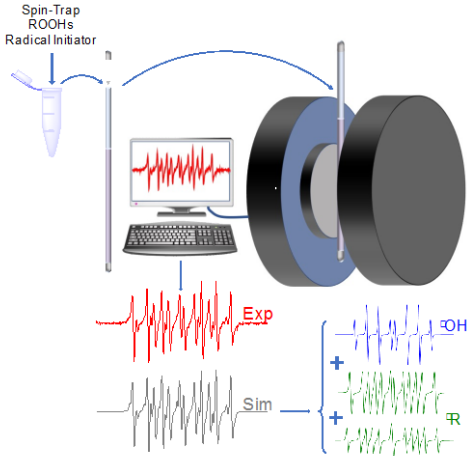


Figure 9

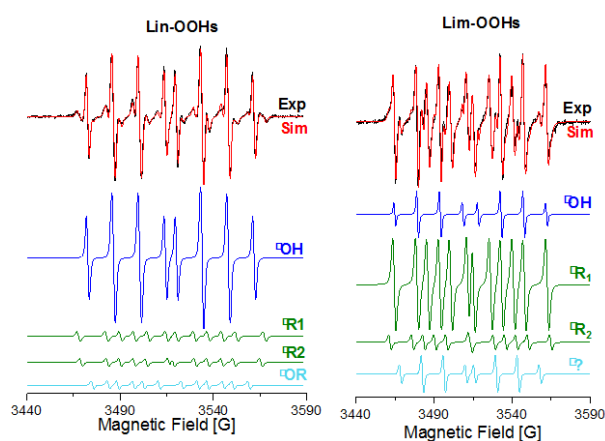


Figure 10

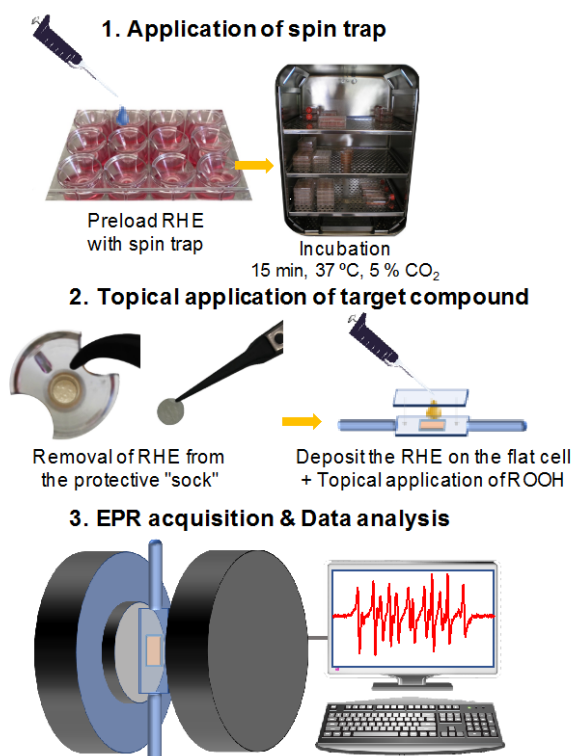




Figure 11

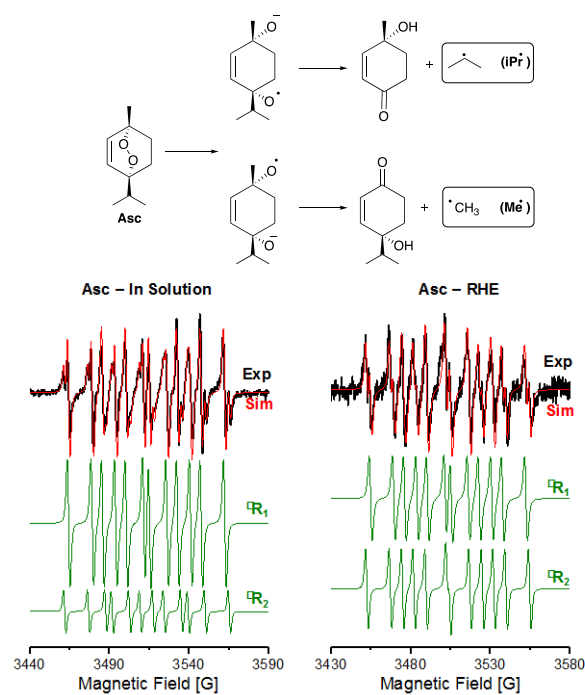


Figure 12

