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Detection and characterization of cyclic hydroxylamine adducts by mass spectrometry

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Abstract
Two cyclic hydroxylamines (cHA) bearing pyrrolidine (CPH) and piperidine moieties (TMTH) were evaluated to trap hydroxyl, peptide and phospholipid free radicals using mass spectrometry for their detection. The cHA ionized as $[M+H]^+$ ions, showing higher relative abundances when compared to the DMPO, probably due to higher ionization efficiency. In the presence of hydroxyl radicals, both cHA generated new ions that could be attributed to loss of $^{\bullet}H$ and $^{\bullet}CH_3$, most likely deriving from decomposition reactions of the nitroxide spin adduct. Addition of cHA to Leucine-enkephalin and palmitoyl-lineloyl-glycerophosphatidylcholine free radicals promoted the formation of cHA biomolecule adducts, which were confirmed by MS/MS data. Results suggest that the cHA are not suitable for hydroxyl radical trapping but can be used for trapping biomolecule radicals, having the advantage, over the most used cyclic nitrones, of being water soluble. The biomolecule adducts identified by MS are ESR silent, evidencing the importance of MS detection.

Keywords: *Mass spectrometry, spin trapping free radicals, hydroxyl radical, lipid peroxidation, protein free radicals*

Introduction

Free radicals, which are formed *in vivo* as by-products of biochemical reactions that take place during aerobic metabolism [1], are involved in cell signalling and in oxidation reactions needed to maintain redox homeostasis [2]. Amongst these reactions, free radicals may also lead to structural changes to biomolecules causing deleterious effects [3,4]. Thus, the increase of oxidized biomolecules found with ageing has led researchers to propose that these species may have a significant role in the genesis of age-related diseases such as neurodegenerative diseases [5]. In view of this, the detection of free radicals and consequently the identification of structural changes induced by free radicals and their role within the cell constituents has increased over the last years [6–9]. However, and because free radicals, particularly the hydroxyl radical ($^{\bullet}OH$), are very unstable species with short lives, their detection is particularly difficult. At present, the approaches used in the identification of the hydroxyl radicals are the identification of hydroxy derivatives, such as 2,3- or 2,5-DHBA or 8-OH-dG [6] or through the addition of diamagnetic compounds, also known as spin traps. The stabilization as hydroxyl spin adducts [10,11], which ultimately leads to the formation of more stable compounds with longer lives, allows the accumulation to concentrations that can be detected [10,11]. Detection of spin adducts can then be achieved by Electron Spin Resonance (ESR), which has allowed, so far, the detection of hydroxyl radicals [12–16],

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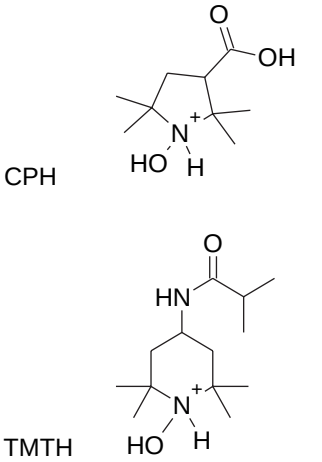
superoxide [12,16–22], alkyl [13,23–25], lipid [25–29] and of amino acids, peptide and protein radicals [30–32] in *in vitro* conditions. These results have been achieved through the use of a wide variety of compounds belonging to the group of cyclic nitrones [15,20,21,25,26,29,33], of imidazole [13,16] and of cyclic hydroxylamines [18,19,34,35], though cyclic nitrones spin traps remain the most popular group among researchers. However, during ESR detection of free radicals researchers often report the decrease of ESR signal intensity attributable to the presence of reducing species that reduce the spin adducts to ESR silent forms (hydroxylamines) [36]. Considering that mass spectrometry (MS) coupled with spin trapping is a technique that provides information about the radical species (spin adducts) present in solution and also of the corresponding ESR silent forms (reduced or oxidized), nonetheless, the application of MS is not yet a popular technique in the identification of radical species. In summary, apart from initial work published in the early 1990s describing the identification of spin adducts by MS, either by fast atom bombardment (FAB) or thermospray (TS) ionization [23,37], only in recent years some works were published that have extended the application from the identification and characterization of oxygen spin adducts [38–42] to the identification and characterization of biomolecule radicals [33,44–48]. Surprisingly, a common feature to all of these works is the use of cyclic nitrones as the spin trap agent. Thus, there is a lack of work devoted to the identification of spin adducts by MS using compounds other than cyclic nitrones.

Enclosed in an ongoing research project aiming at the detection and identification of protein adducts formed in oxidative stress conditions and given the growing effort devoted to the synthesis of new compounds to be used as trapping agents in *in vivo* studies and of new methodologies for the detection of free radicals, two cyclic hydroxylamines, 1-hydroxy-2,2,5,5-tetramethyl-3-carboxy-pyrrolidine (CPH) and 1-hydroxy-4-methoxy-2,2,6,6-tetramethyl-4-carboxy piperidine (TMTH) (Scheme 1) were evaluated for their use in the trapping and detection by mass spectrometry of free radicals, namely of hydroxyl, peptide (leucine enkephalin) and phospholipid (palmitoyl-lineloyl-glycerophosphatidylcholine). The cyclic hydroxylamine, the presence of hydroxyl radicals were detected in the mass spectra and further characterized by tandem mass spectrometry in linear ion trap (LIT) and quadrupole/time-of-flight (Qq-ToF) mass spectrometers, taking advantage of the sequential tandem mass spectrometry provided by the LIT and the accurate mass measurements provided by the QqToF instrument. The results obtained using these compounds on the identification and characterization of biomolecule adducts will also be described.



Scheme 1. Structure of the spin traps CPH and TMTH.

Experimental

Spin traps

The 1-hydroxy-2,2,5,5-tetramethyl-3-carboxy-pyrrolidine (CPH) and the 1-hydroxy-4-methoxy-2,2,6,6-tetramethyl-4-carboxy piperidine (TMTH) were purchased from Alexis Corporation (Lausen, Switzerland), while 5,5-dimethyl-pyrrolidine N-oxide (DMPO) was purchased from Sigma (St, Louis MO, USA). The water used was of MilliQ purity and all the solvents used were HPLC grade. $FeCl_2$ was purchased from Merck (Darmstadt, Germany). Hydrogen peroxide (H_2O_2) used for the peroxidation reactions was also purchased from Merck.

Spin trapping experiments

Both cyclic hydroxylamines are soluble in aqueous solutions according to the manufacturer. Stock solutions of the three spin traps were prepared by solubilization in water in a concentration of 0.25 M. For the radical trapping experiments, 4 μL of $FeCl_2$ (5 mM) and 50 μL of a hydrogen peroxide solution (final concentration of 50 mM) were added in a total volume of 0.5 mL of bicarbonate buffer (5 mM, pH 7.4) and kept in a water bath set at 37°C and after a time of reaction 10 μL of spin trap solution (final concentration 5 mM) were added to the reaction mixture. For the control reaction the cyclic hydroxylamines were solubilized in bicarbonate buffer and the H_2O_2 replaced with water. The reaction mixture was analysed by mass spectrometry at different time periods.

Leucine enkephalin (Leu-enk) trapping experiments, to 50 μL of Leu-Enk stock solution (1 mg/mL) in bicarbonate buffer (5 mM, pH 7.4), were added 50 μL of H_2O_2 (to a final concentration of 50 mM) and 4 μL of $FeCl_2$ (to a final concentration of 5 mM), to a total volume of 0.5 mL left to react in the dark in a water bath at 37°C. For the 1-palmitoyl-2-lineloyl-3-glycero-phosphocholine (PLPC) spin

trapping experiments, the phospholipids were solubilized in bicarbonate buffer (5 mM, pH 7.4), to which were added 50 μL of H_2O_2 (50 mM) and 4 μL of $FeCl_2$ (5 mM), to a final volume of 0.5 mL and left to react in the dark in a water bath at 37°C. The control reaction was performed by replacing the H_2O_2 for 50 μL of H_2O. The cyclic hydroxylamines were added to the reaction mixtures containing the Leu-Enk after 30 min incubation and in the case of the phospholipids this addition was made after 6 h incubation.

Electrospray mass spectrometry

The acquisition of the electrospray mass spectra was performed in the positive mode. Acquisition of the spin traps mass spectra was achieved using a volume of 1 μL of a 5 mM solution of each spin trap and diluted 100 000 fold (50 nM) in methanol with 0.1% formic acid. In the trapping experiments (hydroxyl radical), acquisition of the mass spectra was performed with 1 μL of reaction mixture, prepared as previously described, was diluted 10 000 fold in methanol and injected into the Q-ToF 2 and linear ion trap (LIT) instruments.

In the Q-ToF 2 instrument (Waters, Manchester, UK), the flow rate was 10 μL/min, the needle voltage was set at 3 kV, the cone voltage at 30 V, the ion source set at 80°C and the desolvation temperature at 150°C. Tandem mass spectrometry was also performed on the ions observed in the mass spectra using argon as the collision gas (measured pressure in the penning gauge $\sim 6 \times 10^{-6}$ mBar) and the collision energy used was varied between 18–28 eV according to the ion of interest. Each tandem mass spectra was acquired during the period of 1 min. Data acquisition was carried out on a MassLynx software system (version 4.0).

The LXQ linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA) was operated in positive electrospray mode. Typical ESI conditions were as follows: electrospray voltage was 5.5 kV; capillary temperature was 350°C and the sheath gas flow was 25 units. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a 50 ms and 200 ms maximum ionization time, respectively. Normalized Collision Energy™ (CE) was varied between 15–35 (arbitrary units) for both MS^2 and MS^3 and according to the ion of interest. Data acquisition was carried out on an Xcalibur data system (V2.0).

Results and discussion

The mass spectra of both cyclic hydroxylamines (CPH and TMTH) were obtained in the positive mode using a 5 mM solution with a dilution factor of 1×10^5 fold. The pyrrolidine cyclic hydroxylamine

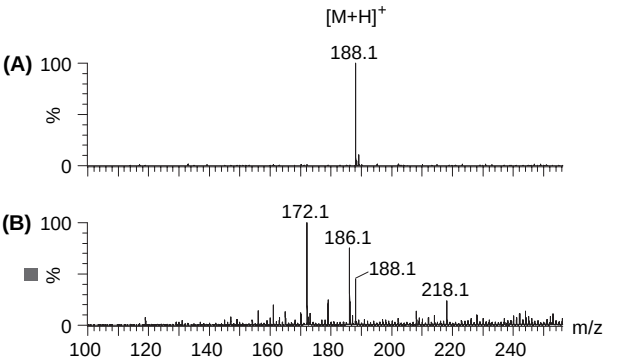


Figure 1. Mass spectra of CPH spin trap obtained in Q-ToF instrument, (A) in the absence and (B) in the presence of H_2O_2 (50 mM).

(CPH) ionized predominantly as protonated molecules ($[M+H]^+$) at m/z 188.1 (Figure 1A). The piperidine cyclic hydroxylamine also ionized predominantly as protonated molecule at m/z 243.2 (Figure 2A). The ions corresponding to the cyclic hydroxylamine sodium adducts ($[M+Na]^+$) were also present, observed at m/z 210.1 (Figure 1A) and m/z 265.2 (Figure 2A), but with low relative abundance (< 5% RA).

The relative abundances observed for the protonated molecules in the mass spectra (Q-Tof instrument) was for CPH of $3.56 \pm 0.66 \times 10^3$ counts ($n = 5$) and for TMTH of $3.30 \pm 0.15 \times 10^3$ counts ($n = 5$), showing, in these acquisition conditions, similar RA. Comparison between the relative abundance of protonated molecule of CPH and TMTH with the relative abundance obtained for the DMPO protonated molecule (m/z 114 - data not shown), for a 5 mM aqueous solution diluted 1×10^5 fold (0.38 ± 0.03 counts ($n = 5$)), suggests that the cyclic hydroxylamines have higher ionization efficiency.

Study of cyclic hydroxylamines in the presence of hydroxyl radicals

In the presence of hydroxyl radicals, generated by the Fenton reaction conditions, the mass spectra

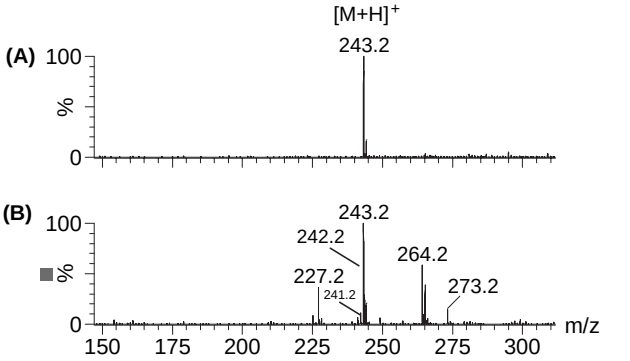


Figure 2. Mass spectra of TMTH spin trap obtained in Q-ToF instrument, (A) in the absence and (B) in the presence of H_2O_2 (50 mM).

obtained for the cyclic hydroxylamine CPH (Figure 1B) and TMTH (Figure 2B) solutions show new ions, when compared to the corresponding mass spectra obtained in the absence of hydroxyl radicals.

For the pyrrolidine cyclic hydroxylamine (CPH), the new ions were observed at m/z 186.1 and m/z 172.1 (Figure 1B), which in the case of the ion at m/z 172.1 can be attributed to the $[M+H]^+$ ion of 1-hydroxy-2,5,5-trimethyl-3-carboxy-4,5-dihydro-1*H*-pyrrole (Scheme 2A) and in the case of m/z 186.1 to the $[M+H]^+$ ion of 1-oxo-2,2,5,5-tetramethyl-4-carboxy-pyrrolidine or its enol form, 1-hydroxy-2,2,5,5-tetramethyl-3-carboxy-3,4-dihydro-1*H*-pyrrole (Scheme 2A). The presence of these ions in solution may be rationalized through the occurrence of secondary reactions of dehydrogenation and demethylation involving the nitroxide spin adduct.

For confirmation of the structure proposed acquisition of the product ion spectra of the ions at m/z 172.1 and 186.1 was performed (Figures 3A and B). The product ion spectrum of ion at m/z 172.1 (Figure 3A) reveals the presence of product ions due to loss of $^{\bullet}OH$ (m/z 155.1), H_2O (m/z 154.1), H_2O and $^{\bullet}OH$ (m/z 137.1), H_2CO_2 (m/z 126.1) and H_2CO_2 and $^{\bullet}OH$ (m/z 109.1) from the precursor ion. The product ion spectrum of m/z 186.1 (Figure 3B) shows the presence of product ions due to loss of H_2O (m/z 168.1), $NO^{\bullet}$ (m/z 166.1), $2H_2O$ (m/z 150.1), H_2CO_2 (m/z 140.1) and combined loss of H_2CO_2 and H_2O (m/z 122.1) or of $NO^{\bullet}$ and CO_2 (m/z 122.1) from the precursor ion. In general, these

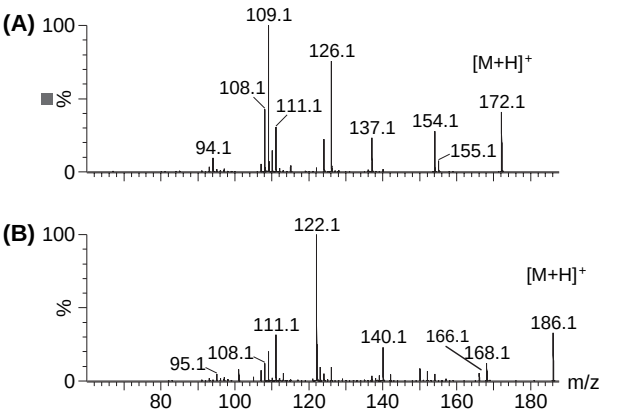
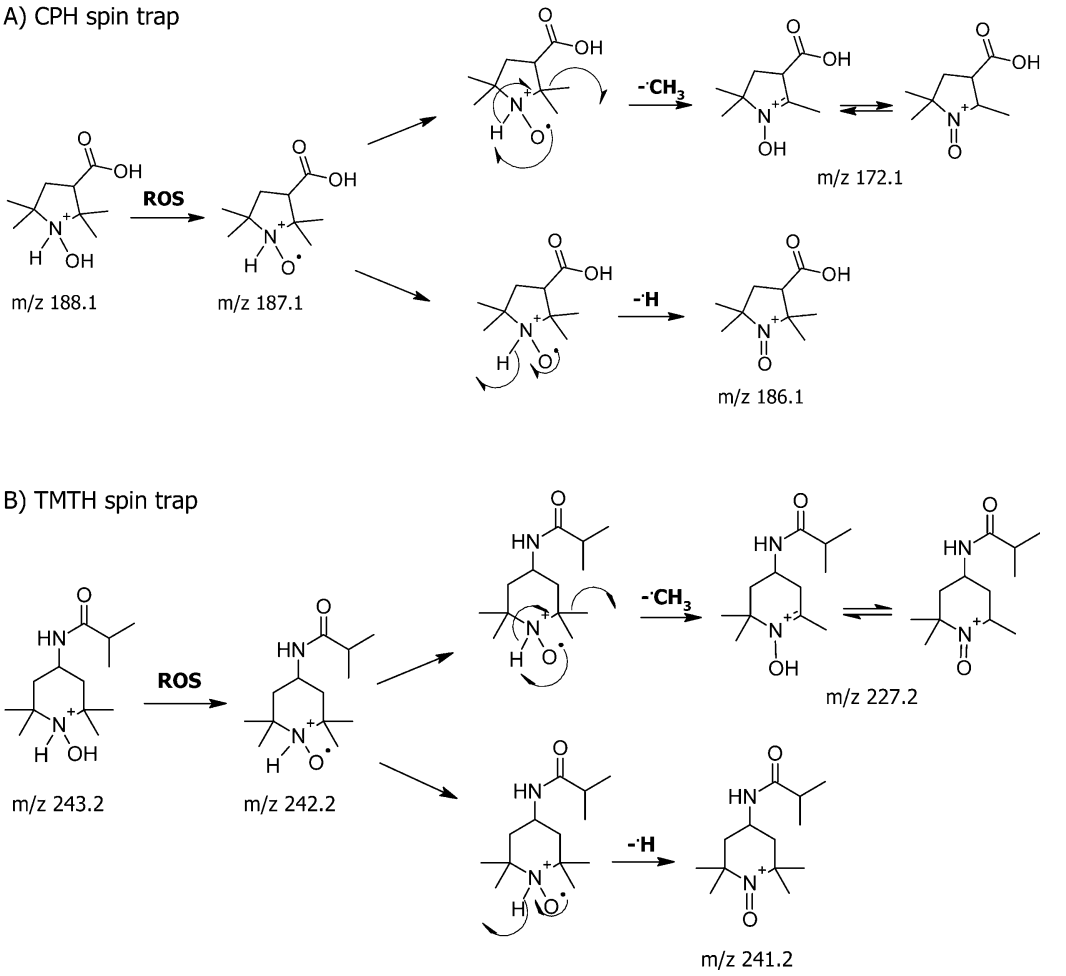


Figure 3. Product ion spectra of $[M+H]^+$ ions obtained in Q-ToF instrument of CPH adducts (A) m/z 172.1 and (B) m/z 186.1.



Scheme 2. Proposed degradation pathways of the CPH (A) and TMTH (B) nitroxide spin adducts formed in the presence of ROS through demethylation and dehydrogenation reactions.

losses are in agreement with the fragmentation pattern of the protonated molecules (m/z 188.1) of CPH in low energy decomposition instruments (Q-ToF and LIT instruments, data not shown), which typically show product ions formed by the loss of water, loss of NH_2OH, combined losses of $H_2O + H_2CO_2$, combined losses of $H_2O + NH_2OH$, combined losses of $NH_2OH + CO_2$ and combined losses of $NH_2OH + H_2CO_2$.

In the case of piperidine cyclic hydroxylamine (TMTH), comparison between the mass spectrum obtained in the absence of hydroxyl radicals (Figure 2A) with the mass spectrum obtained in the presence of hydroxyl radicals (Figure 2B), new ions can be observed at m/z 242.2, which may be attributed to the nitroxide spin adduct and also ions with minor relative abundance at m/z 241.2 and 227.2. The new ions at m/z 241.2 and 227.2 are consistent with the occurrence of secondary dehydrogenation and demethylation reactions involving the TMTH nitroxide spin adduct (Scheme 2B). Confirmation of the structures proposed was achieved by tandem mass spectrometry (Figure 4A–C) and particularly by the product ions due to loss of radical species in all ions, namely loss of $^{\bullet}CH_3$ for the ion at m/z 242.2 (Figure 4A), loss of $NO^{\bullet}$ for the ion at m/z 241.2 (Figure 4B) and loss of $^{\bullet}OH$ for the ion at m/z 227.2 (Figure 4C). The loss of radical species was also observed combined with 2-methylpropanamide ($NH_2COCH(CH_3)_2$–87 Da), leading to the product ions at m/z 140.1 (Figure 4A), 124.1 (Figure 4B) and 123.1 (Figure 4C). Particularly, in Figure 4A, the product ion due to combined loss of methyl radical with 2-methyl-propanamide (m/z 140.1) from the precursor ion suggests the involvement of the methyl radical from the piperidine ring leaving one methyl group at the β-position (inset in Figure 4A). The loss of the radicals, namely $NO^{\bullet}$ (Figure 4B) and $^{\bullet}OH$ (Figure 4C) in the product ion spectra of TMTH ions could take place through homolytic cleavage mechanisms, while the loss of $^{\bullet}CH_3$ could involve formation of the nitrone (inset in Figure 4A). Other product ions observed at m/z 168.1 (Figure 4B) and 154.1 (Figure 4C) are due to loss of acetone oxime (73 Da) from the precursor ions and can be rationalized considering the presence of both products (m/z 241.2 and 227.2) as oxo-piperidine derivatives. The losses here described are in agreement with the fragmentation pattern described for hydroxylamine compounds [49] and with the fragmentation pattern of TMTH $[M+H]^+$ ions (m/z 243.2) obtained in Q-ToF and LIT instruments (data not shown) where typically product

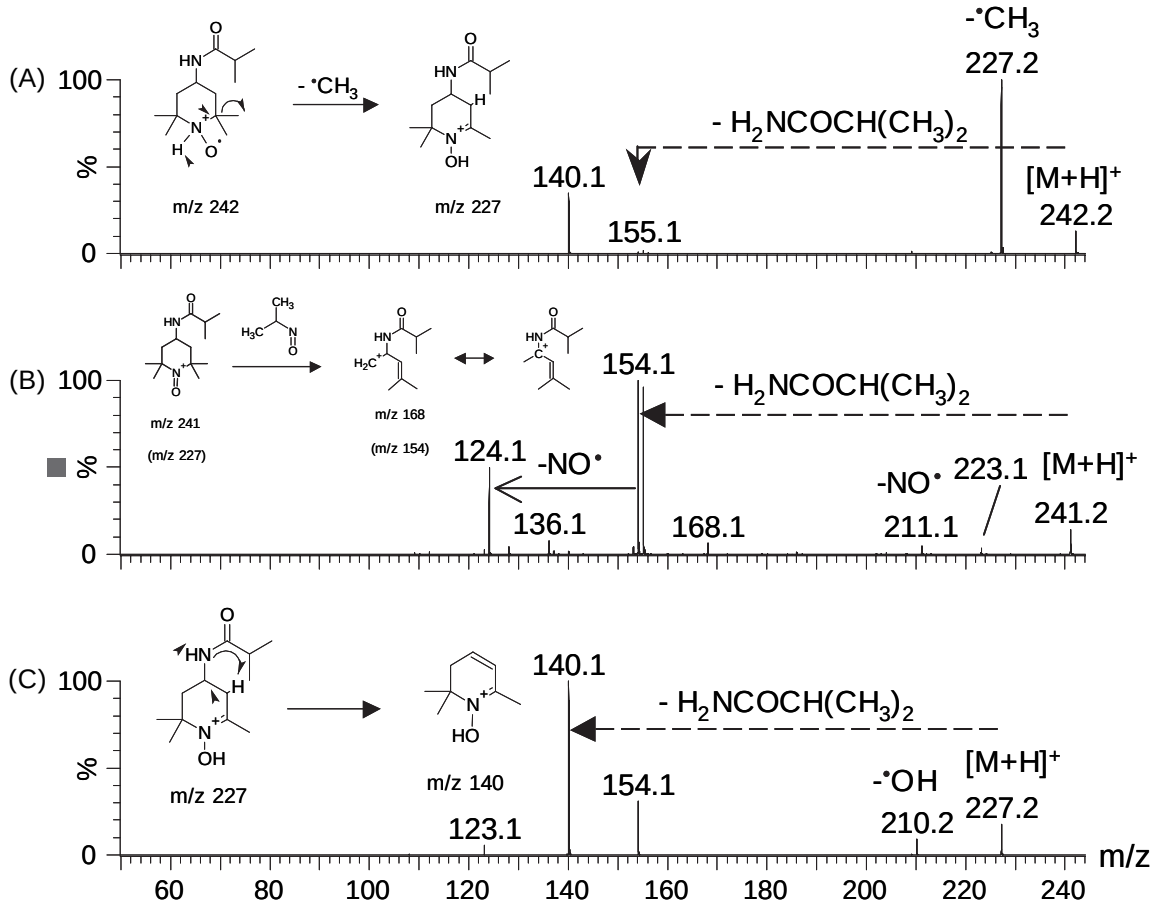


Figure 4. Product ion spectra of $[M+H]^+$ ions of TMTH adducts (A) m/z 242.2, (B) m/z 241.2 and (C) m/z 227.2 (arrows identify loss of 2-methyl-propanamide: 87 Da). Insets in figures are the proposed fragmentation pathways for the ions.

ions due to loss of NH_2OH, 2-methyl-propanamide and combined losses of NH_2OH + i-butene are observed from the precursor ion. The loss of acetone oxime and of 2-methylpropanamide may be rationalized considering an initial ring opening mechanism through cleavage of the α bond, as depicted in the insets of Figure 4, already described for piperidine and pyrrolidine cyclic compounds [49,50] and also in the $[M+H]^+$ product ion spectra of cytidine derivatives formed under ESI conditions [51]. This mechanism triggers the several fragmentation pathways identified, with the charge located at the heteroatom, although the carbocation can also be proposed considering the stability of the tertiary cations [52].

Overall, based on the results here described by mass spectrometry and confirmed by tandem mass spectrometry, new ions observed in the mass spectra for the two cyclic hydroxylamine with pyrrolidine and piperidine moieties that result from the loss of one methyl radical (with m/z 172.1 for CPH and m/z 227.2 for TMTH) and the loss of $^{\bullet}H$ radical (with m/z 186.1 for CPH and m/z 241.2 for TMTH) can be proposed. These products are decomposition products occurring in the cyclic hydroxylamines nitroxide spin adducts originally formed in the presence of hydroxyl radicals (Fenton reaction conditions). Decomposition reactions of spin trapped adducts with rearrangement to other structures are not uncommon in solution [10,11] and are greatly influenced by the stability of the spin trapped adduct, which in turn is dependent on several other factors, such as the intrinsic stability of the spin trap, temperature, concentration of the spin trap, environment in which the spin trap is and the rate at which it dissociates [10,11,21]. In addition, the occurrence of degradation reactions of the nitroxide spin adduct, which is described to take place through bimolecular disproportionation [53], could also have experimental implications, namely the decrease in the spin trap available for trapping. The identification of the decomposition products of the spin traps that we have performed is worthy, as in complex samples these products may difficult the interpretation of the mass spectra due to the formation spin adducts or proton bound adducts.

Remarkably, the detection of the TMTH nitroxide spin adduct (m/z 242.1, Figure 2B) and the absence of the CPH nitroxide spin adduct (m/z 187.1, Figure 1B) in the mass spectra obtained during this study could suggest the higher stability of the piperidine spin adduct when compared to the pyrrolidine spin adduct. In a previous study, researchers described the higher stability of pyrrolidine (CPH) nitroxide adducts in comparison to oxo-piperidine nitroxide adducts (TEMPONE) [18] and attributed this behaviour to the fact that CPH could be less hindered by natural reducing agents than the piperidine compound and, based on this assumption, would therefore be more suitable for free radical trapping in biological systems [18]. At the same time, the authors also determined the rate constant values of cyclic hydroxylamine compounds (CPH, PPH and TEMPONE) and found that the rate constant of the cyclic hydroxylamines adducts [18,34] was hundreds of times higher than the one described in the literature for DMPO [34].

In summary, in the presence of hydroxyl radicals (Fenton reaction) these cyclic hydroxylamine compounds lead to the formation of the nitroxide spin adduct. At the same time, the absence of hydroxyl or peroxyl adducts in the mass spectra of CPH and TMTH cyclic hydroxylamines suggests that these compounds are not suitable for the identification of the hydroxyl radicals in particular, since trapping of the hydroxyl ($^{\bullet}OH$) and superoxide ($O_2^{\bullet}$) radicals all generate the formation of the nitroxide spin adduct of the hydroxylamines. Thus, the identification of the nitroxide spin adduct achieved by ESR data and mass spectrometry data can only be used to provide evidence about the occurrence of free radical reactions in general and not of one free radical in particular, contrary to what is described when using DMPO spin traps through the identification of hydroxyl and peroxyl DMPO adducts [1,38].

Study of cyclic hydroxylamine spin trapping in the presence of biomolecule free radicals

Despite the fact that, in the presence of hydroxyl radicals, no hydroxyl adducts of cyclic hydroxylamines were formed and identified in the mass spectra (Figures 1 and 2), the use of cyclic hydroxylamine for the trapping of biomolecule free radicals was tested, namely peptide (leucine enkephalin – Leu-Enk) and phospholipid (palmitoyl-lineloyl-phosphocholine–PLPC) free radicals.

Leucine-enkephalin was incubated in the presence of hydroxyl radicals to allow the formation of leucine enkephalin free radicals and after a period of time, cyclic hydroxylamines were added to two aliquots.

The mass spectrum obtained for the reaction mixture containing Leu-Enk in the presence of hydroxyl radicals and CPH (Figure 5A) exhibits new ions and among these the ion at m/z 741.4 is observed. This ion, based on its m/z value, can be attributed to the CPH Leu-Enk adduct, formed between the Leu-Enk free radical (carbon centred) and the CPH nitroxide spin adduct (oxygen centred) through radical–radical reaction. The ions at m/z 172.1 and 186.1, attributed to decomposition products, are also observed in the mass spectrum, which could lead to a decrease in the spin trap available for Leu-Enk free radical trapping. Through tandem mass spectrometry data obtained (Figure 5A1) it is possible to notice that, upon collision induced

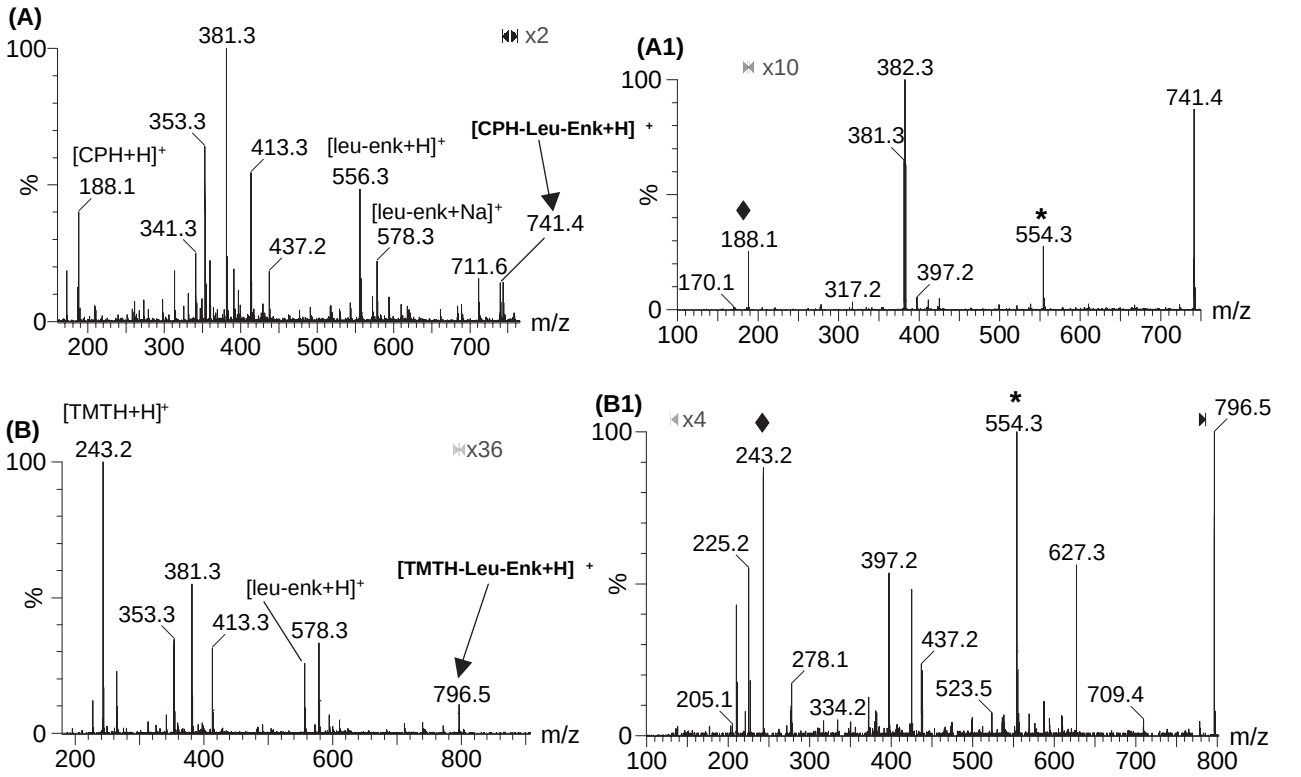


Figure 5. (A) Mass spectrum obtained in Q-ToF instrument acquired after spin-trapping experiments on Leu-Enk radicals using CPH and (A1) product ion spectrum of precursor ion at m/z 741.4; (B) mass spectrum acquired after spin-trapping experiments on Leu-Enk radicals using TMTH and (B1) product ion spectrum of precursor ion at m/z 796.5. (Symbols on the mass spectra (*) assign loss of the cyclic hydroxylamine and (◆) assign cyclic hydroxylamine.)

fragmentation, adducts undergo loss of the cyclic hydroxylamine leading to the peptide product ions at m/z 554.3 (marked with *). It is also noted the product ion attributed to the cyclic hydroxylamine (m/z 188.1, marked with ◆), allowing one to confirm the proposed structure. Location of the CPH may be inferred by the product ions at m/z 464.3 ($b_3^* + OH$) and the product ion at m/z 610.2 (b_4^*) suggesting its presence at the YGGF moiety. This proposal is corroborated by the product ion observed at m/z 317.2 (Fig. 5A1) corresponding to the immonium ion of phenylalanine-CPH. Also, the product ions observed at m/z 397 (a_4) and 425 (b_4) attributed to YGGF allow inferring that CPH is located at the leucine residue. Both locations can be considered although phenylalanine (Phe) and tyrosine (Tyr) are more prone to seize the radical than the glycine (Gly) or leucine (Leu) residues [53]. These results suggest the presence of CPH in different amino acid residues. More detailed information about the presence of positional isomers can be obtained by tandem mass spectrometry coupled with liquid chromatography.

The mass spectrum obtained for Leu-Enk incubated in the presence of hydroxyl radicals and addition of TMTH (Figure 5B) exhibits new ions and among these the ion at m/z 796.5 was observed. This ion can be attributed to the Leu-Enk TMTH adduct formed between the carbon centred radical of Leu-Enk and the TMTH nitroxide adduct, similarly to what was observed for the CPH cyclic hydroxylamine. Also, decomposition products of TMTH with m/z 227.1 are observed in the mass spectrum. In the product ion spectrum of Leu-Enk-TMTH adduct (Figure 5B1), one can observe the product ions at m/z 554.3 (*) due to loss of the cyclic hydroxylamine from the precursor ion, at m/z 243.2 (marked with ◆) corresponding to the protonated molecule of TMTH, at m/z 709.4 corresponding to the loss of propanamide ($NH_2COCH(CH_3)_3$), already observed in the product ion spectra of nitroxide adduct and degradation products (Figure 4) and others that can be attributed to peptide backbone cleavages. Altogether, these product ions allow corroborating the presence of the proposed adducts. The loss of the spin trap, with formation of the peptide ion is a fragmentation behaviour that is in accordance with the one described in the literature for amino acids [30] and haemoglobin and myoglobin peptides adducts [44,46]. The location of TMTH was inferred at the leucine residue by the product ions observed at m/z 205.1 (internal), 278.1 (b_3), 334.2 (internal), 397.2 (a_4) and 425.2 (b_4), and corroborated by the product ion at m/z 372.3 (Y_1^*). The ion at m/z 627.3 was identified as referring to fragmentation involving the piperidine ring and does not provide information about the TMTH location.

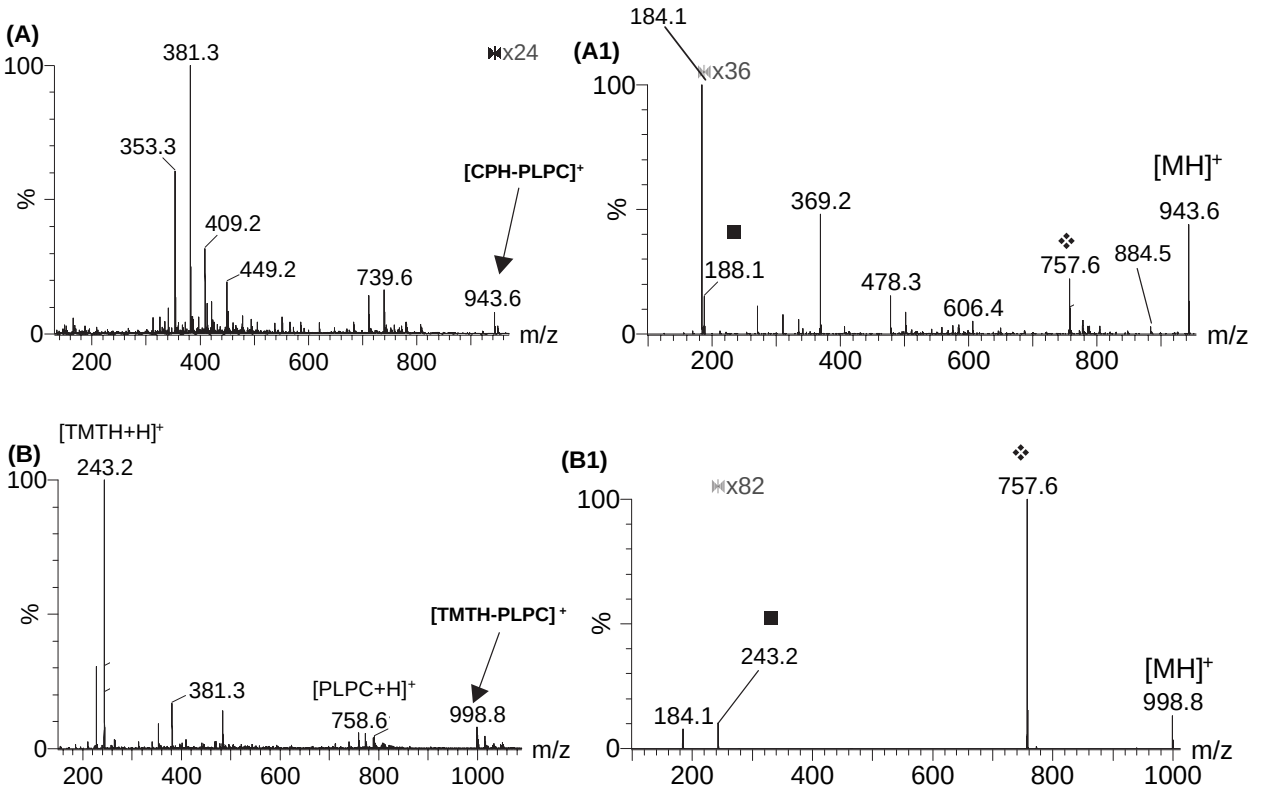


Figure 6. (A) mass spectrum obtained in Q-ToF instrument acquired after spin-trapping experiments on PLPC radicals using CPH and (A1) product ion spectrum of precursor ion at m/z 943.6; (B) mass spectrum acquired after spin-trapping experiments on PLPC radicals using TMTH and (B1) product ion spectrum of precursor ion at m/z 998.8). (Symbols on the mass spectra (❖) assign loss of the cyclic hydroxylamine and (■) assign cyclic hydroxylamine.)

The phospholipid, 1-palmitoyl-2-lineloyl-3-glycerophosphatidylcholine (PLPC), was incubated in the presence of hydroxyl radicals to allow the formation of phospholipid free radicals and, after a period of time, CPH and TMTH cyclic hydroxylamines were added to two aliquots.

The mass spectrum obtained for the reaction mixture containing PLPC in the presence of hydroxyl radicals and CPH (Figure 6A) exhibits new ions and among these the ion at m/z 943.6 is observed. This ion, based on its m/z value, can be attributed to the singly charged ion of the protonated molecule ($[MH]^+$) of PLPC-CPH adduct, formed through radical–radical reactions, and for this reason is ESR silent specie. The identification of PLPC free radical adducts as singly charged ions is somewhat different from the previous works performed with cyclic nitrone spin trap (DMPO), where the PLPC adducts were identified, in the mass spectrum, mainly as doubly charged ions, although the singly charged ions were also observed [45]. Tandem mass spectrometry data performed on the ion observed at m/z 943.6 (Figure 6A) shows the product ions at m/z 184.1 corresponding to the phosphocholine polar head, at m/z 188.1 (marked with ■) corresponding to the cyclic hydroxylamine, at m/z 757.6 (marked with ❖) corresponding to the loss of the CPH hydroxylamine through homolytic cleavage. The loss of the spin trap is a typical behaviour, which is in accordance with the fragmentation pattern described in previous studies for spin trap adducts of lipids [33,43,55] and phospholipids [45,48], allowing confirmation of the proposed structure. Other product ions are observed in the product ion spectra, namely due to loss of trimethylamine ($N(CH_3)_3$ – 59 Da) at m/z 884.5 (Figure 6A), as well as the product ions attributed to the lyso-phosphatidylcholines (m/z 478.3, Figure 6A), both of which are characteristic fragmentation pathways found in the product ion spectra of phospholipids [56] and oxidized phospholipids [57,58]. The predominant product ions at m/z 184.1, 478.3, 502.3, 757.6 and 884.5 do not provide information about the position of CPH, but location of the CPH may be inferred by the product ion at m/z 606.4 that can be rationalized by heterolytic cleavage of the γ-bond (C_7-C_8) considering the cHA to be placed at the C-9 position, which in turn is corroborated by the product ion at m/z 820.5 assigned to homolytic cleavage of C_9-C_{10} carbon bond. Cleavages occurring in the vicinity of the carbon atoms bearing the spin trap seemed to be more prone to carbon-carbon cleavages, either through homolytic or heterolytic mechanisms [44, 47]. Also the product ion at m/z 369.2 (Fig. 6A1) may be rationalized considering the

pyrrolidine at the polar head. In fact, in previous studies performed on the identification of phospholipid DMPO spin adducts [47] and oxidation products [56] have earlier proposed that the polar head appeared to be a site of hydrogen abstraction and further oxidation.

The mass spectrum obtained for the reaction mixture containing PLPC in the presence of hydroxyl radicals and TMTH (Figure 6B) exhibits new ions and among these the ion at m/z 998.8 is observed. This ion, based on its m/z value, can be attributed to the PLPC-TMTH adduct formed through radical–radical reaction between the PLPC free radical and the TMTH nitroxide adduct, similar to what was observed for the peptide cyclic hydroxylamine adducts. The product ion spectrum obtained for this ion (Figure 6B) showed the loss of the cyclic hydroxylamine as the main fragmentation pathway leading to the product ion at m/z 757.6 (marked with ❖), as well as other product ions corresponding to the phosphocholine polar head (m/z 184.1) and the protonated molecule of TMTH (m/z 243.2, marked with ■). The TMTH can be proposed to be located at the C-9 based on the product ion at m/z 606.4 and 621.4 corresponding to heterolytic of the β-bond and homolytic cleavages of the α-bond, respectively.

Interestingly, the peptide and phospholipid adducts of both cyclic hydroxylamines formed in solution through radical–radical reactions, namely between the nitroxide radical and the biomolecule free radicals, behave differently when under collision induced fragmentations, particularly the loss of the cyclic hydroxylamines. The C-O bond cleavage with loss of the cyclic hydroxylamines (CPH and TMTH), in the peptide adducts (Figure 5A and B), took place through heterolytic cleavages leading to the product ion at *m/z* 554.3, most likely as a consequence of charge-driven fragmentation, while in the case of phospholipid adducts (Figure 6A and B) the loss of CPH and TMTH occurred through homolytic cleavages, with formation of the phospholipid free radical (product ions at *m/z* 757.6) due to charge-remote fragmentations. Nevertheless, the observed peptide cleavages are of a- and b-type in accordance with previous published work [59] as well as the loss of fatty acids [56] considering that in the case of the phospholipid adducts the charge is fixed at the N atom of trimethylamine of the phosphocholine polar head and that in the case of peptides adducts the charge could be located at the N atoms of the amide bond or at the N atom of the pyrroline/pyrrolidine ring.

Thus, the results here shown using mass spectrometry reveal the presence of cyclic hydroxylamine products formed from decomposition reactions involving the nitroxide cyclic hydroxylamine adduct and the total absence of hydroxyl adducts of the cyclic hydroxylamines in the mass spectra. The identification of ions resulting from the degradation of the cyclic hydroxylamine nitroxide spin adduct suggest that, at some point, the nitroxide spin adducts exist in solution. The nitroxide spin adducts, formed in the Fenton conditions milieu, will, in the presence of biomolecule free radicals, in turn react with the biomolecule free radicals, also formed in the Fenton conditions milieu, through radical–radical reactions. In fact, the identification of cyclic hydroxylamine Leu-Enk and PLPC adducts in the mass spectra, shows that cyclic hydroxylamines can be used to the detection of biomolecule free radicals by mass spectrometry, although the same cyclic hydroxylamines do not appear to be suitable for the identification of hydroxyl or superoxide free radicals. Curiously, the biomolecule adducts here identified are ESR silent and would not have been observed through ESR detection. Furthermore, the use of MS to the detection has the advantage of allowing further characterization of the peptide and phospholipid adducts identified in the mass spectra and hence allowing confirmation of the ions as cyclic hydroxylamine adducts. In addition, the ion current counts observed for CPH and TMTH peptide and phospholipid adducts (data not shown) exhibit relative abundances similar to the DMPO biomolecule adducts.

Concluding remarks

In this study, mass spectrometry was used in the detection of cyclic hydroxylamine adducts using spin traps containing a pyrrolidine and piperidine moiety. Comparison between the ion intensity of the protonated molecule ($[M+H]^+$) of spin traps in the mass spectrum with $[M+H]^+$ ion of DMPO spin trap showed higher signal intensity, suggesting higher ionization efficiency. The absence of hydroxyl or peroxyl adducts in the mass spectra of cyclic hydroxylamines, obtained under Fenton conditions, shows that these compounds are not suitable for the identification of the hydroxyl radicals. The ions identified in the mass spectra obtained, in the presence of hydroxyl radicals, corresponded to degradation products of the nitroxide spin adducts namely from loss of $^{\bullet}H$ and $^{\bullet}CH_3$ radicals from the nitroxide spin adduct. Nonetheless, the cyclic hydroxylamine spin traps allowed trapping of Leu-Enk (peptide) and PLPC (phospholipid) free radicals, which were confirmed by tandem mass spectrometry. It should be taken into account that these spin traps have the advantage, over the most used cyclic nitrones, of being water soluble and thus more suitable for use in biological environments. Furthermore, the biomolecule adducts identified by mass spectrometry are ESR silent, evidencing the importance of MS detection.

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