Detection and Characterization of Hydroxyl Radical Adducts by Mass Spectrometry

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The study of the influence of free radicals in the biological process depends primarily on the capacity to detect these reactive species. In this work we have studied the application of mass spectrometry to the identification of hydroxyl radical species. The detection and identification by collisional activation mass-analyzed ion kinetic energy spectrometry (CA-MIKES) of a spin adduct of DMPO with the hydroxyl radical $[(DMPO + O) + H]^+$ (*m/z* 130) has demonstrated that mass spectrometry can be a powerful tool in the detection and identification of spin adducts of DMPO with hydroxyl radical species. We were also able to detect the capture of secondary free radicals using ethanol by detecting and identifying the corresponding adduct $[(DMPO + ethanol) + H]^+$. Other spin adducts have also been detected and identified. We consider that the use of mass spectrometry is a relevant technique for the detection of free hydroxyl radicals, especially in complex mixtures, since mass spectrometry is able to discriminate these adducts in such situations. Moreover, using this approach, it was possible to identify new spin adducts. (J Am Soc Mass Spectrom 2001, 12, 1214–1219) © 2001 American Society for Mass Spectrometry

Free radical species, particularly the hydroxyl radical, are highly reactive and can react with almost all constituents of the cell. Of special importance appears to be the reaction of free radicals with membrane lipids (to cause non-enzymatic peroxidation) and with DNA (to cause strand breakage) [1]. Several authors have recently developed studies where they have directly implicated oxygen radicals and other derived species as causative agents of aging and of diverse human illnesses such as asthma, cancer, arteriosclerosis, among others [2]. A primary aspect in the study of the influence of the free radicals in the biological processes is the capacity to detect these reactive species. The most common methods for identifying free radicals in biological systems include [3]: (1) Product analysis, (2) inhibition by free radical scavengers, (3) inhibition by superoxide dismutase, and (4) electron spin resonance either directly or by using a spin trap. When free radicals possess long life times, they can be directly detected by using the electronic paramagnetic resonance (EPR). However, for the free radicals with short life times, such as superoxide and hydroxyl radicals, one of the methods more frequently used consists of their detection by EPR using a radical trap. The use of a spin trap involves the addition of the reactive free radical across the double bond of a diamagnetic spin trap (typically a nitroso or a nitrone compound) to form a much more stable free radical, a radical adduct, which can then be examined by EPR [4]. This technique was developed by Iwamura and Inamoto [5, 6] and refined by Janzen and Blackburn [7, 8]. Tomer et al. [9, 10] have shown that determination and identification of spin trapped radical adducts, with a combination of high-performance liquid chromatography (HPLC)-Electrospray (ESI)-Mass Spectrometry (MS) and HPLC-Thermospray (TSP)-MS, is also possible although, because of sensitivity problems, these systems have only been used in in vitro assays. Tomer et al. have also observed in LC/TSP-MS experiments, DMPO adducts identified as quasi-molecular ions $[M + H]^+$ at *m/z* 146 and *m/z* 160 for methanol-and ethanol-derived radical adducts, respectively, and an ion identified has an apparent molecular ion M^+ at *m/z* 130 for the hydroxyl radical adduct in the reaction of DMPO with the hydroxyl radical [11]. Under specific conditions, mass spectrometry has also permitted the correlation of N-tert-butyl-α-phenylnitrone (PBN) signals with GC-MS and has been used to detect hydroxyl and hydroxyethyl free radicals [12].

Due to the reactivity of the hydroxyl radical, which results in the formation of new radicals capable of reaction with spin traps, other systems for capturing radicals have been developed. These systems include the capture of secondary free radicals, or the analysis of reaction products involving primary free radicals. In the first case, the use of, for example, ethanol or dimethyl sulfoxide (DMSO) is included, reacting with hydroxyl radicals to form carbon centered radicals which are able

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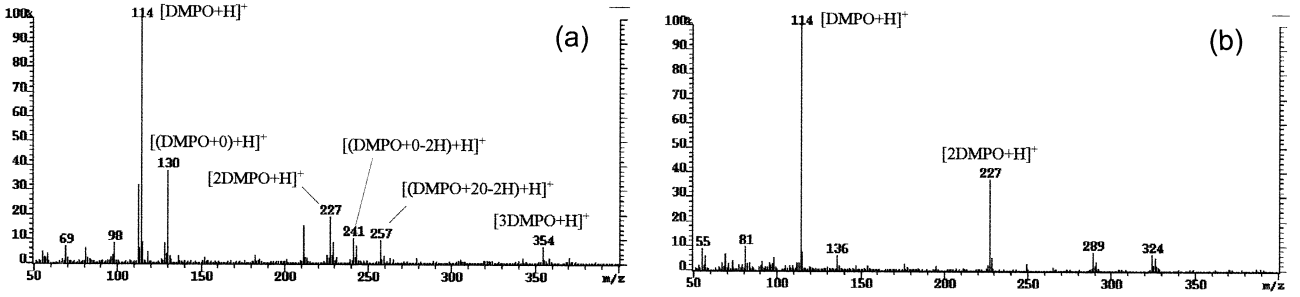


Figure 1. Mass spectrum acquired after incubation of DMPO, $CuCl_2$, and hydrogen peroxide (**a**) and mass spectrum acquired in the same conditions, but in the absence of H_2O_2 (**b**).

to be captured by spin traps [13–15]. In the second case, the detection and quantification of reaction products of DMSO or other similar compounds with the hydroxyl radical can be included [16]. The latter method has been used in the detection of radicals using mass spectrometry [11, 17, 18].

Experimental

Typical spin trapping experiments were performed by adding hydrogen peroxide (H_2O_2) 6 μmol, $CuCl_2$ 0.06 μmol, and then DMPO 9 μmol corresponding to a final concentration of 2, 0.02, and 3 M respectively. Other experiments were performed using lower concentrations up to 0.1 M of DMPO and 0.05 M of H_2O_2. For the experiments where the capture of secondary free radicals was involved, typically ethanol 20 μmol (1 μl) was added corresponding to a final concentration of 5 M. In some experiments, freshly distilled DMPO was used in order to identify possible side reactions due to DMPO impurities. These systems were allowed to react for different periods of time from 10 min to 3 h. Then 1.5 μl of the reactive mixtures were added to a drop of a fast atom-bombardment (FAB) matrix, 3-nitrobenzyl alcohol (NBA). This mixture was immediately analyzed by FAB-MS.

FAB mass spectra and tandem mass spectra were acquired with a VG AutoSpecQ (VG Analytical, Manchester, UK). The instrument is of EBE geometry and is equipped with a caesium gun. The applied accelerating voltage was 8 kV and the caesium ion beam intensity was 3 μA at 20 kV. Decomposition of CAMIKE mass spectra were acquired by selecting the desired ion with the EB section of the mass spectrometer, and colliding the selected ion at 8 kV in the collision cell with sufficient argon gas to reduce the selected ion beam intensity by approximately 50%. The resulting product ions were determined by a scan of the second electric sector. Parent ion spectrum was obtained by a linked B^2/E scan. Data acquisition was carried out with a VG OPUS 3.4C data system and interfaced to the mass spectrometer by a VG SIOS unit (VG Analytical, Manchester, UK).

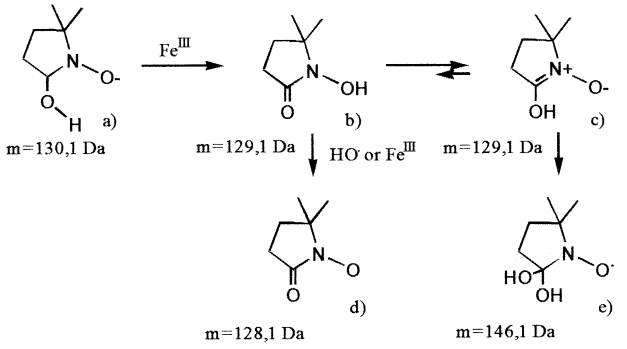
Results and Discussion

In Figure 1a we present a mass spectrum acquired after incubation of 1 μl of DMPO, 1 μl $CuCl_2$ (20 mM), and 1 μl of hydrogen peroxide (30%) and in Figure1b, the mass spectrum acquired in the same conditions, but in the absence of H_2O_2. As it can be observed, new mass peaks appear in the mass spectrum when H_2O_2 was added. These peaks, of *m/z* 130, 211, 229, 241, 243, 257, 259, 354 should be related with the oxidative process that occurs in solution, since they are absent when H_2O_2 is not present. Under this premise, the tentative identification of these peaks is given in Table 1. We have performed these experiments using lower concentrations, up to 0.1 M of DMPO and 0.05 M of H_2O_2, and freshly distilled DMPO and have observed essentially the same spectra, although when lower concentrations were used the relative abundance of the observed species was lower. The main objective in this work was to observe and identify all possible species and for this reason we have used very high concentrations.

The profusion of observed peaks was unexpected since none of these ions has previously been reported. Although it is possible that these ions are formed during the desorption process in FAB because of radical reactions that occur in this process [19], it is likely that its origin is predominantly the result of reactions in liquid phase, since we were unable to detect similar species in the absence of H_2O_2. There is also the possibility that some of these ions are the result of the

Table 1. Identification of observed peaks in the mass spectrum acquired after incubation of DMPO, $CuCl_2$ and hydrogen peroxide

Ion (*m/z*)	Composition
130	$[(DMPO + O) + H]^+$
211	$[(2DMPO - O) + H]^+$
227	$[(2DMPO - 2H) + 3H]^+$
229	$[2DMPO + 3H]^+$
241	$[(2DMPO + O - 2H) + H]^+$
243	$[(2DMPO + O) + H]^+$
257	$[(2DMPO + 2O - 2H) + H]^+$
259	$[(2DMPO + 2O) + H]^+$
354	$[(3DMPO + O) + H]^+$



Scheme 1

formation of adducts and proton bound dimmers of species formed in liquid phase.

The *m/z* of the adducts between DMPO and the hydroxyl radical should have been 131. However the only observed peak was *m/z* 130, which is in agreement with previous results reported by Tomer et al. [11]. Makino et al. [20] have shown that the reaction between the DMPO and Fe (III) in aqueous solution can be complex (Scheme **1**). According to the proposed scheme, the ions detected by mass spectrometry when using the FAB ionization source would be the protonated ion of **b** (hidroxamic acid) or its tautomer **c** (2-hydroxi-DMPO). Another possibility would be the formation of a cyclic structure, during the ionization process, presented in Scheme **3**. We were unable to detect, at least with a significant relative abundance, the protonated ions of structures **d** and **e** (Scheme **1**). On the other hand, the ion of *m/z* 112 was observed with a high relative abundance, approximately 33% of the ion of *m/z* 114. This ion is evidence for the presence of oxidized DMPO specie. It should be noted that the intensity of the peak at *m/z* 112 rapidly increases during the FAB analysis, which suggests that this species is produced, at least partially, in the FAB source.

In order to clarify the identity of these species, MS/MS studies of these ions were performed. The CA-MIKES spectra of the ion of *m/z* 211 did not allow an unequivocal identification of this species.

Fragmentation Pathway of the Ions of m/z 130

The identification of these ions (*m/z* 130) demonstrates the potential of mass spectrometry in the detection of

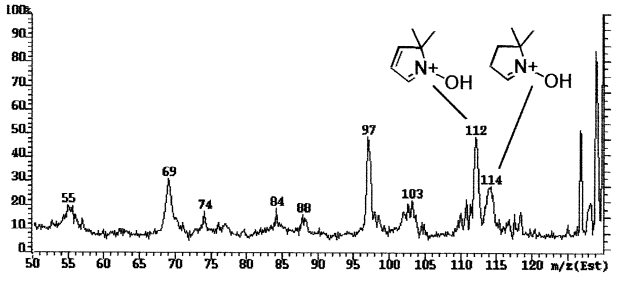
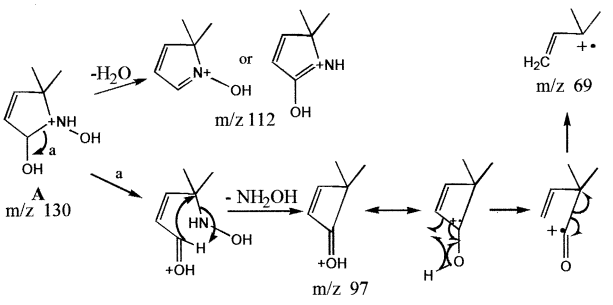


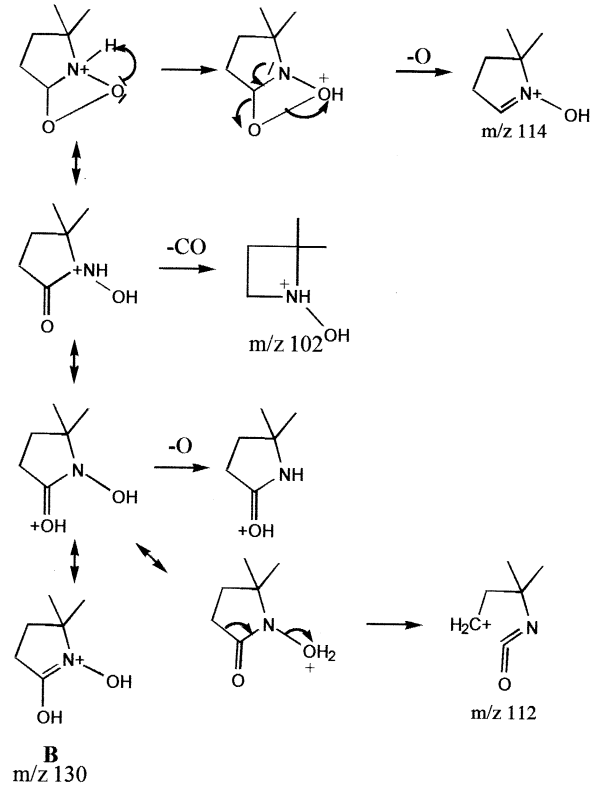
Figure 2. CA-MIKE mass spectrum of the parent ion of *m/z* 130.



Scheme 2

radical species, namely of the hydroxyl radical. The study of the CA-MIKES spectrum of the parent ion of *m/z* 130 suggests that it is formed by two species with the same *m/z*. As it can be seen in Figure 2, the presence of the fragment ion of *m/z* 112 suggests the presence of the oxidized species mentioned above. The mass spectrum also presents an ion at *m/z* 128 that has been assigned as the spin adduct of oxidized DMPO.

In the CA-MIKES spectrum of the ion of *m/z* 130 two series of ions with different abundances can be observed: The more abundant originate from the species that contains the oxidized DMPO (**A**, Scheme **2**), and the lower abundance series originate from the DMPO (**B**, Scheme **3**). This fact is reproduced in all the CA-MIKES studies that we have done and suggests that the DMPO oxidized species has a higher proton affinity. The differences in abundances of the fragment ions may also relate to the relative abundances of the two struc-



Scheme 3

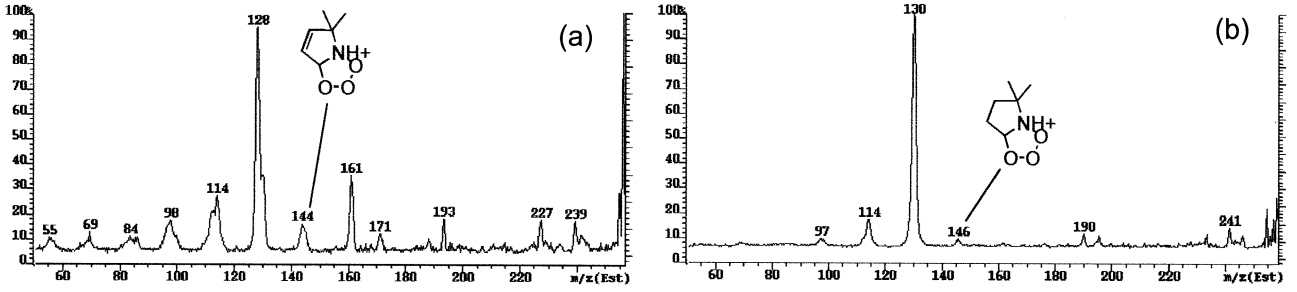


Figure 3. CA-MIKE mass spectrum of the parent ion of *m/z* 257 (**a**) and 259 (**b**).

tures. We suggest that the series of ions of *m/z* 112, 97, and 69 result from the fragmentation of the spin adduct of oxidized DMPO, as shown in Scheme **2**.

The fragmentation pathways of the spin adduct of the hydroxyl radical with DMPO is less extensive and presents less abundant fragment ions. However, they are important for its identification since the presence of the fragment ion at *m/z* 114, which results from the loss of the N-oxide oxygen from the protonated keto form, demonstrates the presence of DMPO, and the fragment ion of *m/z* 102 demonstrates the presence of a hydroxyl radical (Scheme **3**). Another possibility for the origin of the ion *m/z* 112 is that this species arises partially or primarily from the protonated keto form (**b**, Scheme **1**). Protonation on the N-oxide oxygen lead to water loss through the cleavage of the five membered ring on bond between the keto carbon and the α carbon and formation (Scheme **3**). Further loss of a methyl radical would lead to the *m/z* 97 ion.

Fragmentation Pathway of the Ions of m/z 241 and 243

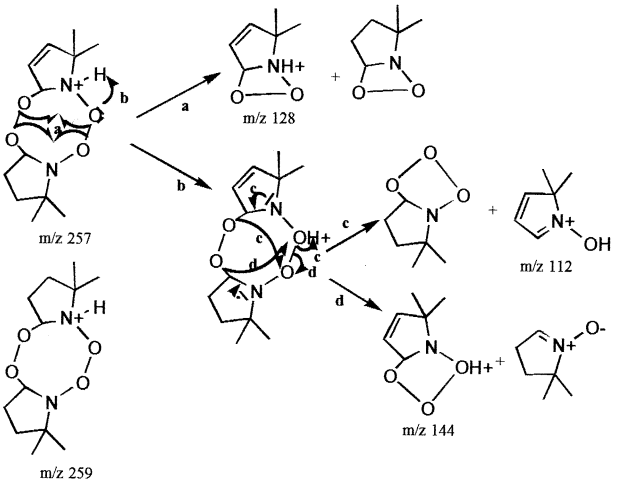
The ion of *m/z* 243 was identified as the result of the formation of spin adducts of a hydroxyl radical with two molecules of DMPO. In the case of the ion of *m/z* 241, one of the DMPO molecules would be oxidized. The study of the fragmentation pathway of both ions did not allow us to exclude the hypothesis of these ions being the result of the formation of proton bound adducts. In fact, the main fragment ions were 130 and 114 for the parent ion of *m/z* 243, and 130, 128, 114, 112, and 98 for the parent ion of *m/z* 241. Although it is possible to explain mechanistically the origin of these ions from the fragmentation pattern of the spin adducts, we cannot exclude the other hypothesis. If these ions are proton bound adducts then the ion of *m/z* 241 would be formed by two different species (*m/z* = 111 + H + 129 and *m/z* = 113 + H + 127).

Fragmentation Pathway of the Ions of m/z 257 and 259

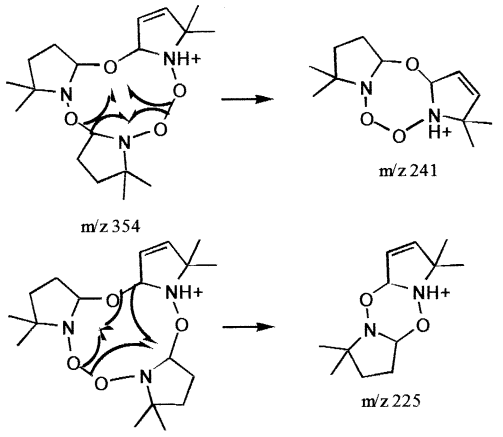
In Figure 3a and b CA-MIKE mass spectra of the ions of *m/z* 257 and 259, respectively, are shown. The ion of *m/z* 257 fragments more extensively and with more abundant fragment ions than the ion of *m/z* 259. We could again question whether these ions are proton bound adducts including the ion of *m/z* 128 and 130, respectively. However, in this case the fragmentation pattern of these ions allows to infer that these species are spin adducts, although it is impossible to exclude the presence of proton bound adducts with that same *m/z*. In Scheme **4** we show the proposed fragmentation pathways for three product ions which we consider essential for the identification of these species: (**a**) the ion of *m/z* 112 that demonstrates the existence of the oxidized DMPO in the species of *m/z* 257; (**b**) the ion of *m/z* 128 that demonstrates to be a spin adduct between the DMPO and the hydroxyl radical; and (**c**) the ion of *m/z* 144 that demonstrates that this species is not a proton bound dimmer. It should be noted that if the charge is located in the non-oxidized DMPO, as can be seen in Figure 3a, the detected ions are those of *m/z* 114, 130, and 146. This fragmentation pathway can also be used for the identification of the product ions of the ions of *m/z* 259 (*m/z* 114, 130, and 146).

Fragmentation Pathway of the Ion of m/z 354

The ion of *m/z* 354 was identified as $[3DMPO + O + H]^+$. The CA-MIKE spectrum presents as product ions those of *m/z* 241, 225, 114, and 112 (spectrum not



Scheme 4



Scheme 5

shown). A mechanistic interpretation for the origin of these ions is presented in the Scheme **5**. The product ions at *m/z* 112 and 114 show the presence in this species of oxidized and non-oxidized DMPO. Although it seems strange not to observe, at least with significant abundance, the ion of *m/z* 356 (in the absence of oxidized species) or *m/z* 352 (when two oxidized species are present) in the mass spectra, it should be noted that the relative abundance of the oxidized species (*m/z* 112) in the CA-MIKE spectrum of the parent ion of *m/z* 354 is 33% of the relative abundance of DMPO (*m/z* 114) and that its percentage in the composition of this species is 33%. These facts added to the absence of the ion of *m/z* 130 allows us to suggest that the ion at *m/z* 354 is not a proton bound adduct.

The Detection of Radicals OH˙ by Using DMPO, Ethanol, and Mass Spectrometry

Due to the very reactive characteristics of the hydroxyl radicals resulting in the formation of new radicals capable of reacting with spin traps, other systems of capture of spins have been developed. These systems include the capture of secondary free radicals or the analysis of reaction products involving primary free radicals. In the first case the use of, for example, ethanol, it is included [13–15]. We also have considered that it would be important to test the use of mass spectrometry in the identification of radicals by using ethanol and DMPO.

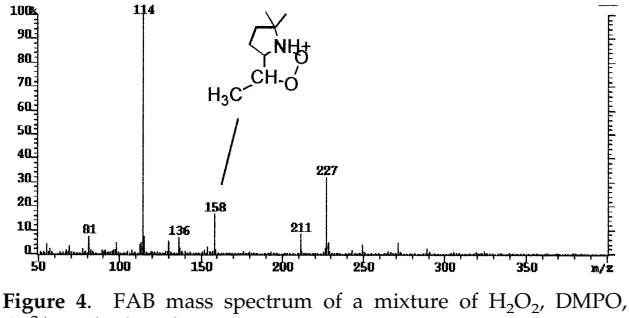


Figure 4. FAB mass spectrum of a mixture of H_2O_2, DMPO, Cu^{2+}, and ethanol.

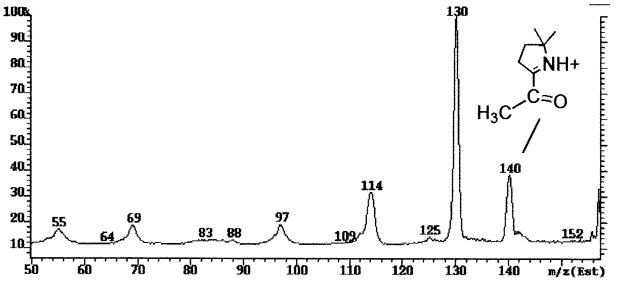
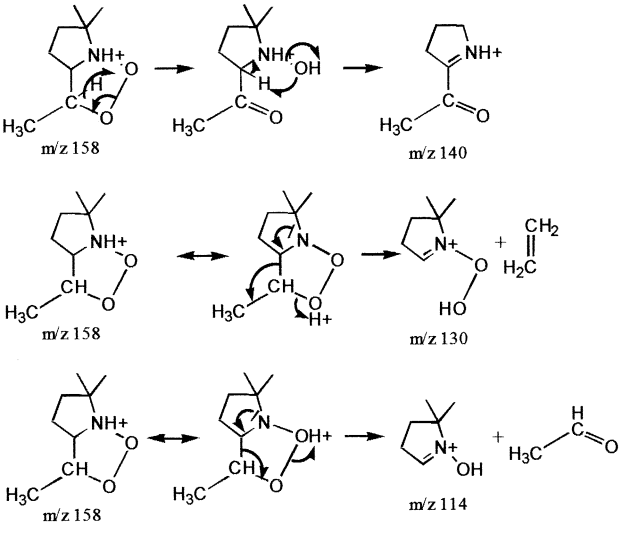


Figure 5. CA-MIKE mass spectrum of the parent ion of *m/z* 158.

In Figure 4 we can see the FAB mass spectrum of a mixture of H_2O_2, DMPO, Cu^{2+}, and ethanol. When compared with the spectrum of a mixture without ethanol, a very significant reduction of the relative abundance of the ions of *m/z* 130 and 271 and the appearance of new peaks at *m/z* 136, 158, 241, 257, and 271 is observed. We have performed CA-MIKES experiments on these ions, and verified that apparently the ions of *m/z* 136, 241, and 257 did not correspond to adducts of DMPO. The CA-MIKE spectra of the ion of *m/z* 158 (Figure 5) and *m/z* 271 show the presence of a product ion at *m/z* 114, which seems to be consistent with both ions being adducts of DMPO. The expected *m/z* for spin adduct between the DMPO and ethanol was 159. Thus, it seems likely that in these adducts a similar cyclization process occurs as the one described for the ion of *m/z* 130.

Fragmentation Pathway of the Ions of m/z 158 and 271

The CA-MIKE spectrum of the ion of *m/z* 158 presents as more significant product ions, the ions of *m/z* 140, 130, and 114 for which we show in Scheme **6** a possible



Scheme 6

mechanistic explanation for their origin. It should be noted that although the proposed structure for the product ion of *m/z* 130 differs from the one presented above, both are equivalent. The CA-MIKE mass spectrum of the ion of *m/z* 271 (data not shown) shows as the main fragment ions those of *m/z* 158, 140, 130, and 114. This ion was thus identified as having the structure $[2DMPO + ethanol + H]^+$. Again we were not able to exclude the hypothesis that this ion is a proton bound dimmer of a DMPO molecule with a molecule of spin adduct, since it was not possible to detect any product ion that clear supported or contradicted this hypothesis.

Conclusion

The method described in this paper has proven to be useful for the detection and identification of DMPO/hydroxyl radical spin adducts. We consider this method to be relevant in detecting free radicals, especially in complex mixtures, since mass spectrometry is able to discriminate these adducts in such cases. Moreover, this method allowed the identification of new spin adducts which had not been previously reported. This study has demonstrated that mass spectrometry allows the identification of hydroxyl radicals in oxidative processes by the capture of secondary free radicals by using ethanol. However, when compared with the method using DMPO alone, the abundance of the protonated molecular ions of the spin adduct with ethanol (*m/z* 158) is significantly less than the abundance of the protonated molecular ions of spin adducts of DMPO (*m/z* 130).

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