Identification of Oxidation Products and Free Radicals of Tryptophan by Mass Spectrometry

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New oxidation products and free radicals derived from tryptophan (Trp) oxidation under Fenton reaction conditions were identified using mass spectrometry. After the oxidation of tryptophan using hydrogen peroxide and iron (II) system (Fenton reaction), mono- and dihydoxy tryptophans and N-formylkynurenine were identified using electrospray mass spectrometry (ES-MS) and ES-MS/MS. Besides these products, new products resulting from the reaction of tryptophan and oxidized tryptophan and 3-methyl indole derivatives were also identified. The 3-methyl indole derivatives resulted, most probably, from the oxidation process and not from in-source processes. A dimer formed by cross-linking between two Trp radicals (Trp-Trp), similar to the previously described tyrosine dimer was observed, as well as the corresponding monohydroxy-dimer (Trp-Trp-OH). Tandem mass spectrometry was used to identify the structures of these new oxidation products. Free radicals derived from tryptophan oxidation under Fenton reaction were detected using as spin trap the DMPO. The free radical species originated during the oxidation reaction formed stable adducts with the spin trap, and these adducts were identified by ES-MS. New adducts of oxidized tryptophan radicals, namely monohydroxy-tryptophan and dihydroxy-Trp dimer radicals, with one and two DMPO spin trap molecules where identified. Tandem mass spectrometry was used to confirm the proposed structure of the observed adducts. (J Am Soc Mass Spectrom 2003, 14, 406–416) © 2003 American Society for Mass Spectrometry

The oxidation of proteins by free radicals is admitted to play a major role in many oxidative processes within cells. The oxidized forms of proteins accumulate during aging, oxidative stress, and several pathological conditions [1–3]. This has focused attention on physiological and non-physiological mechanisms for the generation of reactive oxygen species (ROS). Hydroxyl radical, in particular, is known to be one of the most reactive ROS in vitro and in vivo systems, capable of reacting with almost all constituents of the cell, such as proteins, lipids, and nucleic acids [4]. The Fenton reaction is one of the main sources of hydroxyl radical in biological systems, with implication in oxidative stress and diseases [4]. The hydroxyl radical, $OH^{\cdot}$, is formed by reduction of hydrogen peroxide with Fe(II) [4]. The other products of this reaction are OH^{-} and Fe(III). This reaction can be catalyzed by other transition metals, such as copper, manganese, cobalt, and vanadium which can be oxidized by hydrogen peroxide, leading to the formation of the $OH^{\cdot}$. The reaction between the hydrogen peroxide and the Fe(II) is the first reaction of a complex process; the reaction of these primary products with solvated molecules lead to the formation of reactive secondary radicals by a series of pathways [4–6].

All amino acid residues are susceptible to oxidation by hydroxyl radical [1, 5]. Kinetic data for the reactions of the OH radicals in aqueous solution have been studied by pulse radiolysis, flash photolysis, and other methods. These results have shown that the hydroxyl radical displays selectivity in its reactions with amino acid residues [7]. However, the relative rates of oxidation of amino acids by the Fenton system, as well as the distribution of the products formed, were found to be significantly different from those reported for oxidation by ionizing radiation [5]. The products formed in the oxidation of amino acids have not been fully characterized [8]. This is probably as a consequence of the fact that hydroxyl radical can react with amino acids at different sites, either in the α carbon or in the side chain of the amino acid, leading to a diversity of products [8]. Recent results published by Goshe et al. [9] proved that the reactivity of any amino acid is not significantly

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altered by being incorporated into a peptide, that the α-hydrogen is not the primary site of reaction of the hydroxyl radical, and that oxidation of side chains occur frequently. Also, it is recognized that aromatic residues are among the preferred targets for ROS attack.

Radicals are formed during oxidation of amino acids, peptides, and proteins by the hydroxyl radical [10]. These species can propagate the oxidative reaction, since they can oxidize other species [11]. The detection and identification of these species is a very difficult task since radicals are very reactive and, in consequence, are very short lived species. Spin trapping agents have been used as free radical interceptors in the study of radicals because they form stable adducts with radicals which are suitable for analysis by electron paramagnetic resonance, EPR [12]. The structural information given by this technique is limited, so there is a need for new techniques that provide more information about the origin and location of radical. Recently, it has been shown that mass spectrometry is a suitable technique to detect the stable adducts formed by radicals and spin adducts of DMPO [13–15], POBN [16, 17], and other [18–20] spin traps. NMR has also been used in determining the sites of reaction of the hydroxyl radical and in identifying the oxidation products [21, 22].

Tryptophan oxidation by hydroxyl radical formed either by Fenton reaction or radiolysis has already been studied [23–25]. However, none of these studies included a complete characterization of the oxidation products. These studies have identified the principal oxidation products of Trp, 2-, 4-, 5-, 6-, 7-hydroxyl derivatives and N-formylkynurenine, and some other less representative species. Several other observed oxidation products haven't been identified by the methods used in the monitoring of these reactions, specially when UV or fluorescence were used to detect and identify the final products of oxidation [23, 25]. To our knowledge, the detection of tryptophan radicals formed by oxidation reactions, other than in proteins using MS, has not been reported. Some studies reported the observation of tryptophan centered radicals using spin traps and ESR [14, 26] or absorbance spectra [27]. The purpose of the present study was to identify oxidation products and radicals formed during oxidation of the tryptophan by hydrogen peroxide, in a Fenton reaction system, in hydrogencarbonate buffer, pH 7.4, similar to physiological conditions. Both pH [28] and hydrogencarbonate [8] seem to be important factors in amino acid oxidation by Fenton reaction. The detection and identification of oxidized species of tryptophan formed under Fenton reaction conditions was done by mass spectrometry using electrospray (ES) as ionization method. The radical species formed in these conditions were detected as DMPO adducts by mass spectrometry. Tandem mass spectrometry was used to confirm the structures of the observed species.

Experimental

Amino acid oxidations were performed by adding the tryptophan solution (5 mM), hydrogen peroxide (25 mM) and $FeCl_2$ (0.1mM) in ammonium hydrogencarbonate buffer (pH 7.4). The systems were left to react at 37° C for different periods of time. Spin trapping experiments were performed by adding the DMPO (5 mM) at the beginning of the oxidation reaction and letting the system react for at least 1 hour at 37° C. The reactions were monitored by ES-MS and the new oxidized species and spin trap radical adducts were analyzed by tandem mass spectrometry.

Electrospray mass spectra and tandem mass spectra were acquired with a Q-TOF 2 (Micromass, Manchester, UK). The instrument resolution was set at 9500 (50% peak valley). The capillary needle voltage was 3 KV and the source temperature was maintained at 150° C. Nitrogen was used as nebulizer gas and argon was used as collision gas. Cone voltage was at 35 V for MS and MS/MS. Collision induced decomposition mass spectra (MS/MS) were acquired by selecting the desired ion with the quadrupole section of the mass spectrometer, and colliding it in the collision cell with argon gas (measured pressure in the penning gauge $\sim 6 \times 10^{-6}$ mBar) using a collision energy of 20–25 eV. The resulting product ions were determined by the TOF analyzer. Data acquisition was carried out with a Micromass MassLynx 3.4 data system.

As solvent system we have used water:methanol: acetic acid 50:50:0.1%. The initial reaction solution was diluted 100-fold with the solvent system prior to the injection in the electrospray source. These sample solutions were introduced into the electrospray source at a flow rate of 5 μl/min. The methanol used was HPLC grade (Riedel-deHaë, Germany).

Results and Discussion

Oxidation of tryptophan (Trp) by the hydroxyl radical produced by the Fenton reaction was studied. The reaction was monitored by electrospray MS. In Figure 1, the mass spectra of Trp obtained under oxidative (Figure 1a) and non-oxidative (Figure 1b) conditions are presented. In both spectra, ions of *m/z* 205 and 227, corresponding to the $[M + H]^+$ and to the sodium adduct $[M + Na]^+$ of Trp, respectively, were observed. After oxidation, new ions were observed in the mass spectra indicating that oxidation of tryptophan has occurred and new species have been formed. Comparing the low mass region of spectra (Figure 1a and b), it is possible to observe, in the spectrum of tryptophan after oxidation (Figure 1a*1*), the formation of an ion of *m/z* 221 corresponding to hydroxytryptophan, and an ion of *m/z* 237 corresponding to dihydroxytryptophan and/or the isomeric N-formylkynurenine. These are well known products of oxidation of this amino acid [8, 23, 24]. The hydroxylation of tryptophan occurs in

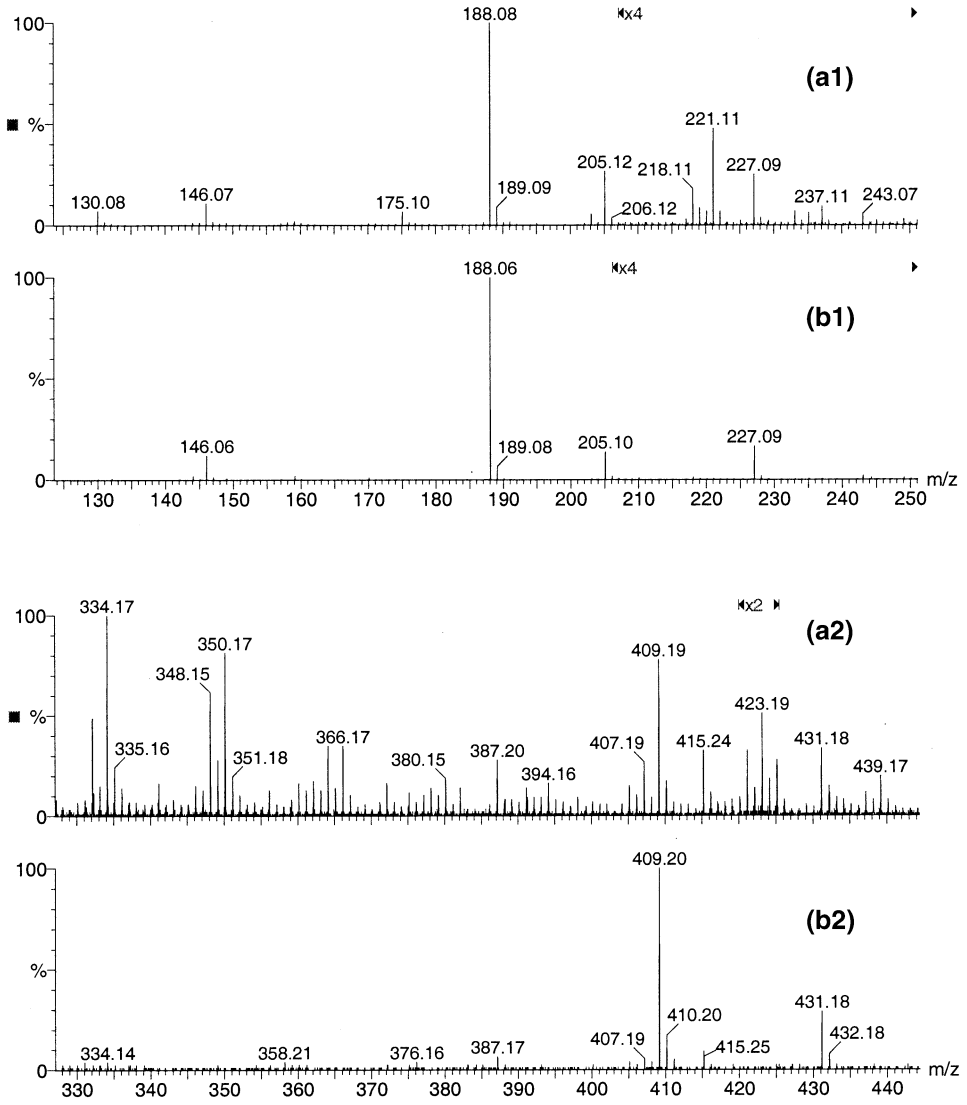
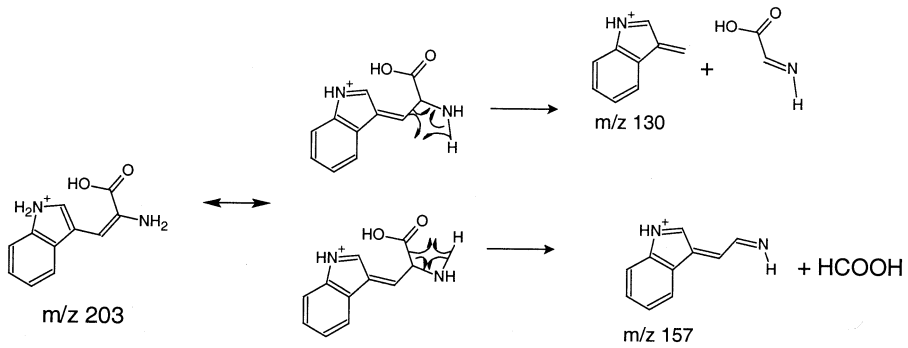


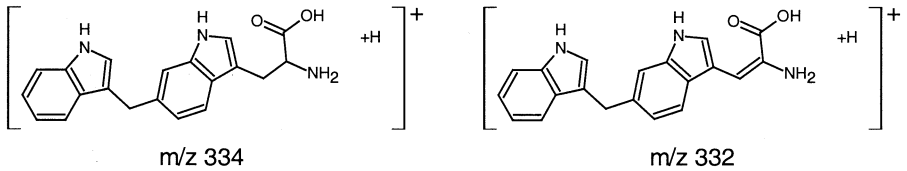
Figure 1. ES mass spectra of tryptophan (**a**) under oxidative Fenton reaction conditions [(**a***1*) low mass region and (**a**2) high mass region] and (**b**) under non oxidative conditions [(**b***1*) low mass region and (**b**2) high mass region].

different positions of the molecule yielding the several possible isomers 2, 4, 5, 6, or 7-hydroxytryptophan [23].

Another oxidation product of tryptophan was observed of *m/z* 203, which probably arises because of the formation of a double bond between carbon α and the adjacent carbon β (Scheme **1**). This structure was proposed based on the MS/MS data of the ion of *m/z* 203, (showing the fragment ions of *m/z* 185, formed because



Scheme 1. Proposed structure and fragmentation pathways for the ion of *m/z* 203.



Scheme 2. Proposed structures for the ions of *m/z* 334 and 332.

of the loss of water and ions of *m/z* 157 and 130), and that the α-β unstauration formed as a result of the oxidative process will be in conjugation with the aromatic system of the indole ring, forming a stable structure. In the interpretation of the fragmentation observed in the MS/MS spectrum it was considered the fragmentation of the ion with the structure proposed in Scheme **1**. The proposed fragmentation patterns are based on the occurrence of rearrangement with hydrogen migration in the precursor ion. In low energy collision induced dissociation, rearrangement of the precursor ion between collisions can occur [29]. Also, product ions may actually represent a more stable isomer of the corresponding substructure in the original molecule [30].

After oxidation, other unreported ions were detected in the high-mass region of the ES mass spectrum of *m/z* 334, 332, 350, 348, 364, 366, 407, and 423 (Figure 1a2). The collision induced fragmentation of these ions was studied to obtained information about the composition of these ions and some structures are proposed. These ions have not been previously identified as oxidation products of tryptophan.

The ion of *m/z* 334 is, most probably, an adduct of tryptophan with 3-methyl indole, according to the proposed structures presented in Scheme **2**. The ion of *m/z* 332 is the corresponding oxidized form of this adduct with a double bond between the α-β carbons of the tryptophan molecule, as it is represented in Scheme **2**. This scheme represents the adducts 6-(3-methyl indole) tryptophan, however it is possible that the other isomers where the 3-methylindole is linked to the 2- 3-, 4-, 5-, 7-carbons of the tryptophan, similarly to the different hydroxylation positions in the tryptophan, are also present. This comment should be considered also for the proposed structure of the other new adducts identified and will be addressed in the text.

These proposed structures in Scheme **2** are based on the ES-MS/MS spectra of both ions. The ES-MS/MS spectra of the ion of *m/z* 334 (Figure 2) showed an abundant fragment ion of *m/z* 205, corresponding to the base peak, which indicate that the tryptophan group is present in the structure of this ion. No ions corresponding to mono- or dihydroxytryptophan were observed. Another abundant ion of *m/z* 130 with a relative abundance of 98% was observed. This ion was attributed to the 3-methyleno-indole as is represented in Scheme **3**.

We considered the hypothesis that this ion could be due to the fragmentation of the tryptophan moiety. However, since this ion appeared in the ES-MS/MS spectrum of the $[M + H]^+$ ion of tryptophan with a very low relative abundance of less than 5% (data not shown), this hypothesis, although plausible, seems not to be valid. These data are consistent with the structure of this ion as the 3-methyl indole moiety and that it should be linked to the tryptophan moiety, validating the proposed structure. Another feature that allowed the confirmation of the proposed structure is the concordance between observed mass in the ES-MS spectrum and the calculated exact mass for this ion (334.1555 for the measured mass, −0.2 ppm), since the values obtained with the instrument Q-TOF 2 are characterized by high accuracy. Similar accuracy in the results was obtained for daughter ions.

The ion of *m/z* 332 corresponds to a similar structure of the ion of *m/z* 334, with the tryptophan in its oxidized

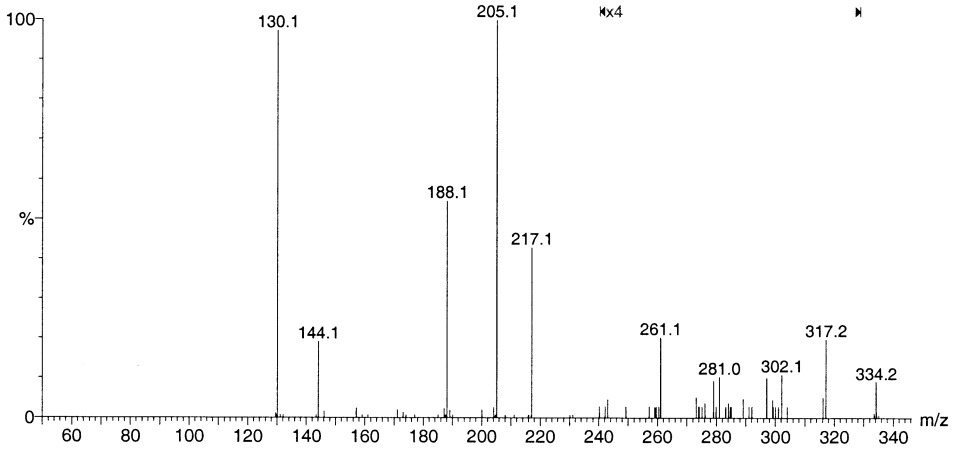
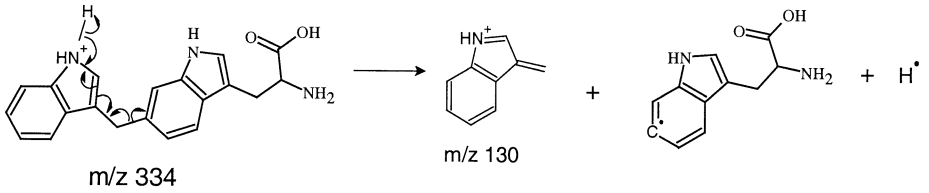


Figure 2. ES-MS/MS spectrum of the ion of *m/z* 334.



Scheme 3. Proposed fragmentation pathway of the ion of *m/z* 334 that originates the fragment ion of *m/z* 130.

form with a double bond between the α and β carbons (Scheme **2**). This structure was proposed considering that in the ES-MS/MS spectrum of the ion of *m/z* 332 (Figure 3) the major fragment ion corresponded to the ion of *m/z* 203. In this spectrum, the ion of *m/z* 130 is also observed, confirming the presence of the 3-methyl-indole group. These adducts have not been previously identified as products of oxidation of tryptophan.

The ions of *m/z* 350 and 366 are 16 and 32 mass units higher than the ion of *m/z* 334 (Scheme **4**). In accordance with the proposed structures for the ions of *m/z* 334, the ions of *m/z* 350 and 366 correspond to the mono- and dihydroxy derivatives of the ion of *m/z* 334. The location of the hydroxyl groups should be discussed since they could be linked either to the tryptophan or the indole moieties, or in both moieties, in the case of the dihydroxy derivative. Analyzing the MS/MS spectrum of the ion of *m/z* 350 (Figure 4), a major ion of *m/z* 221 corresponding to the monohydroxytryptophan (relative abundance of 85%) is observed indicating that the hydroxyl is linked to the tryptophan. The ion of *m/z* 130 was also observed. However, an ion corresponding to the hydroxy-methylene-indole derivative of *m/z* 146 was also observed, although with a significantly smaller relative abundance (<10%). The presence of this ion (*m/z* 146) indicates that hydroxylation of both the tryptophan and methyl-indole moieties contribute to the ion of *m/z* 350. In this ES-MS/MS spectrum (Figure 4) the major ion of *m/z* 175 is attributable to the loss of HCO_2H from the hydroxyl tryptophan (*m/z* 221) (data not shown).

After analysis of the MS/MS spectrum of the dihydroxy derivative, *m/z* 366 (Figure 5), several isomers were suspected to contribute to this ion. The observation of the ion of *m/z* 237 in the MS/MS spectrum confirms that the tryptophan group is doubly oxidized with two hydroxyl groups (Scheme **5**, Structure B). On the other hand, the ions of *m/z* 221 and 146 indicated that the two hydroxyl groups could be linked one to each tryptophan and the methyl-indole moieties of the molecule. The contribution of N-formylkynurenine, an isomer of the dihydroxy tryptophan, should be considered in the proposed structure of these adducts as shown in Schemes **4** and **5** (Structure A). The formation of low abundance ions of *m/z* 322 and 321 in the MS/MS spectrum of the ion of *m/z* 366 can be attributed to the elimination of NHCHO and HNHCHO, respectively, consistent with a minor contribution from this isomer (Scheme **5**, Structure A,) to the adduct of *m/z* 366. The three possible structures for this adduct and their main fragmentation pathways are shown in Scheme **5**.

The fragmentation of the ion of *m/z* 366 leads also to other abundant ions such as the ion of *m/z* 348, formed because of the loss of H_2O, the ion of *m/z* 331 because of the loss of H_2O and OH, and the ion of *m/z* 317 that seems to be due to the loss of O_2 and OH (Scheme **5**). A fragment ion of *m/z* 333 is also observed, formed probably by the loss of OOH. These last two fragmentations suggest that a hydroperoxide, represented in Structure C of Scheme **5**, was also formed during the oxidation process.

The other previously unreported oxidation products observed in Figure 1a are of *m/z* 348 and 364, the α-β unsaturated derivatives of the adducts of methylindole

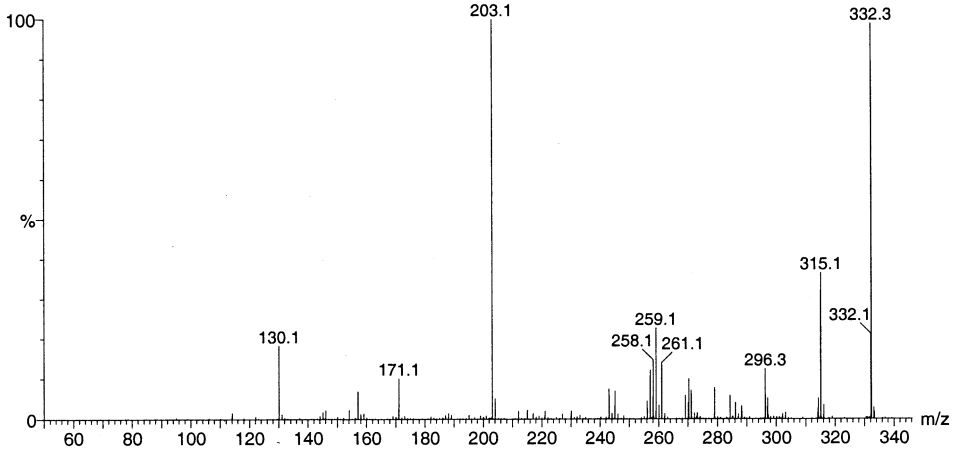
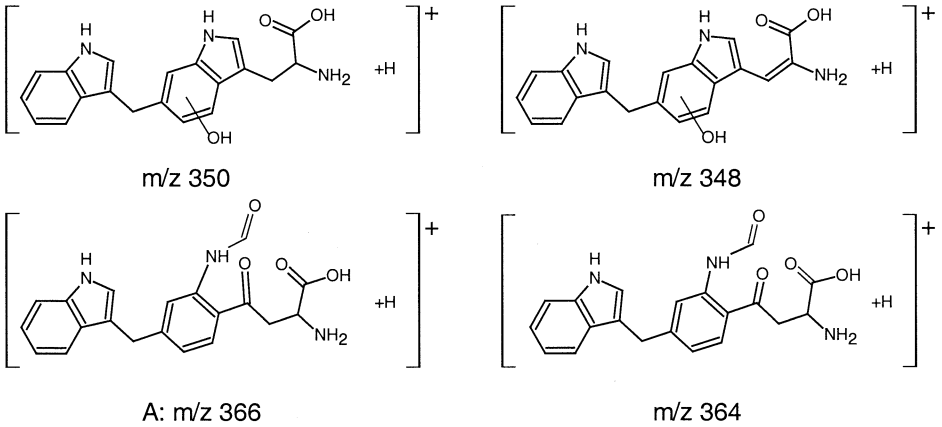


Figure 3. ES-MS/MS spectrum of the ion of *m/z* 332.



Scheme 4. Proposed structures for the ions of *m/z* 350, 348, 366 and 364.

with HO-Trp (*m/z* 350) and methylindole with $(HO)_2$-Trp (*m/z* 366), respectively, (Scheme **4**). In the ES-MS/MS spectrum of the ion of *m/z* 348, the major fragment ions are the ions of *m/z* 203 and the ions of *m/z* 130 and 146, with a relative abundance of approximately 10%. The MS/MS spectrum of the ion of *m/z* 364 shows a fragment ion of *m/z* 203 with a relative abundance of approximately 20% and fragment ions of *m/z* 130 and 146 with a relative abundance of approximately 10%. In this spectrum the major fragment ion is the ion of *m/z* 336, formed by to the loss of CO. This loss is evidence for the presence of a contribution of an isomer of N-formylkynurenine with unsaturation between the α and β carbons, also to the ion of *m/z* 364.

The formation of the adducts of *m/z* 332, 334, 348, 350, 364, and 366 probably occurs in solution by reaction of 3-methylene-indole with tryptophan or hydroxytryptophan. In fact, a new ion of *m/z* 130 (3-methylene-indole) is observed in the ES-MS spectrum of the tryptophan solution after oxidation (Figure 1a).

A bis adduct of tryptophan, Trp-Trp, was observed of *m/z* 407. This specie is probably formed via a radical–radical reaction, by a process similar to the formation of bis-tyrosine, a well known major product of tyrosine oxidation, which is formed by cross-linking two tyrosine radicals [8]. The same process seems to occur with tryptophan, since Mason and coworkers [31] and Minetti and coworkers [26] demonstrated the formation of tryptophan centred radical in proteins under oxidative conditions. Also, formation of a dimer and polymers of tryptophan under oxidative conditions was mentioned by Maskos et al. [23], although the compounds structures were not identified. The formation of the dimer cross-linking Trp-Trp should occur between two Trp radicals, probably at the C3 carbon [26] or C6 carbon [31] of the indole ring, as inferred previously. However, linkage between other positions, such as C2, C4, C5, or C7 carbons of the indole ring should not be excluded. The formation of the dimer was confirmed based on the ES-MS/MS mass spectrum of the ion of *m/z* 407, which shows a fragment ion of *m/z* 203 (see Scheme **1**) as the second most abundant ion with a relative abundance (RA) of approximately 90%. The major ion of the spectrum was the ion of *m/z* 390, formed due to the loss of OH or NH_3.

The hydroxy dimer Trp-Trp-OH, corresponding to the ion of *m/z* 423, was also formed during oxidation by Fenton reaction. The formation of this specie could be due to cross-linking between tryptophan and hydroxytryptophan or due to the hydroxylation of the

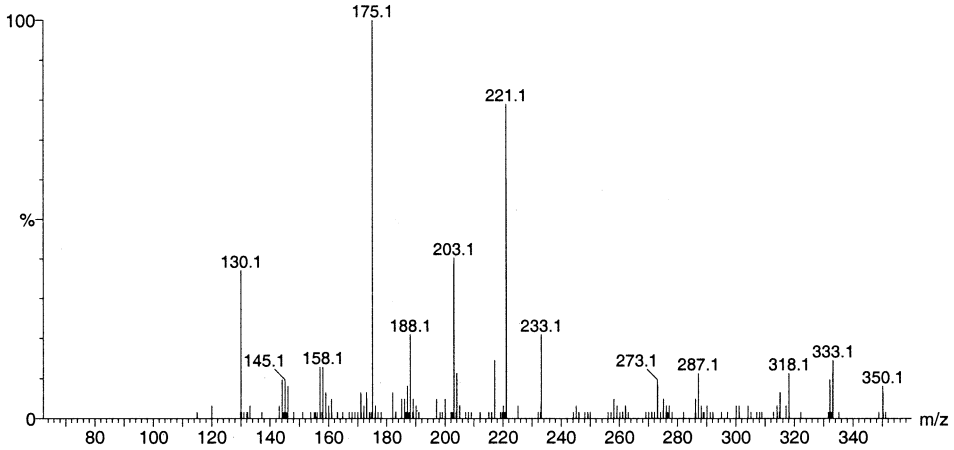


Figure 4. ES-MS/MS spectrum of the ion of *m/z* 350.

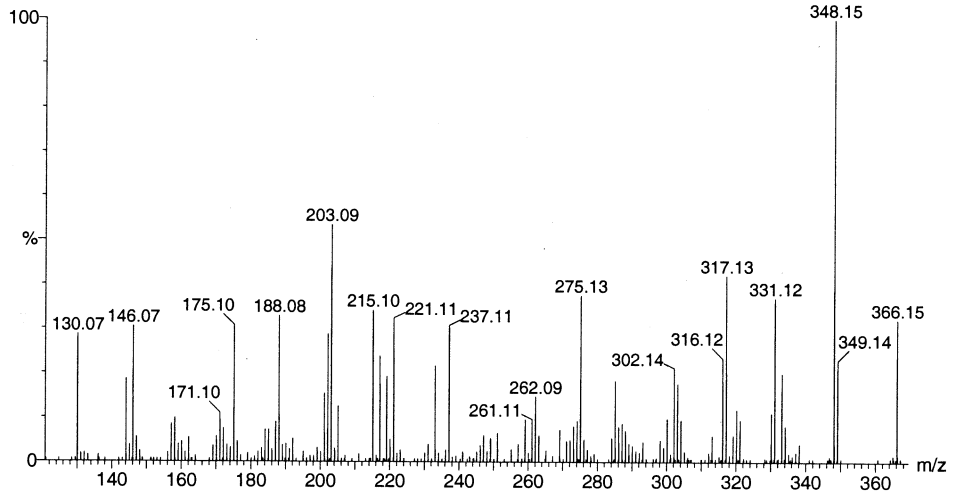
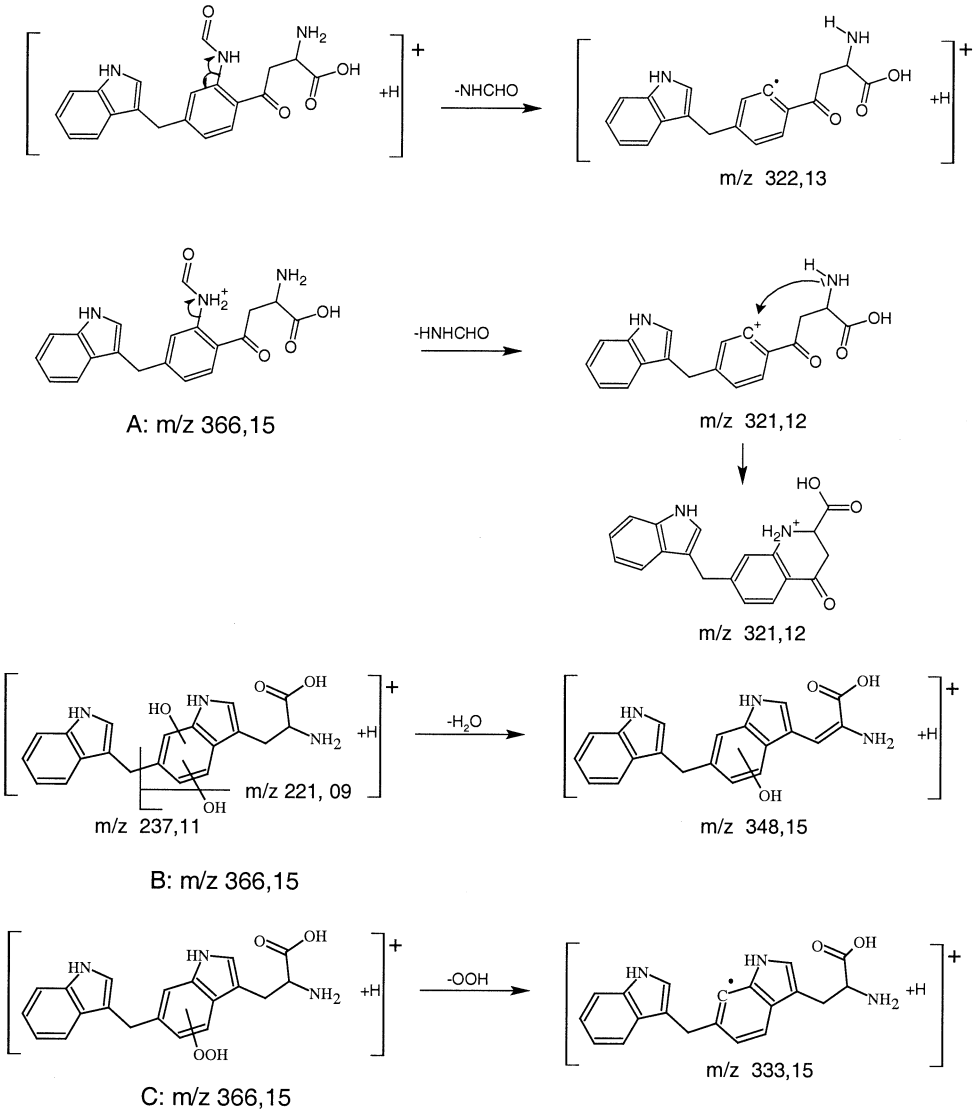


Figure 5. ES-MS/MS spectrum of the ion of *m/z* 366.



Scheme 5. Proposed structures for the ions of *m/z* 366 and correspondent fragmentation pathways.

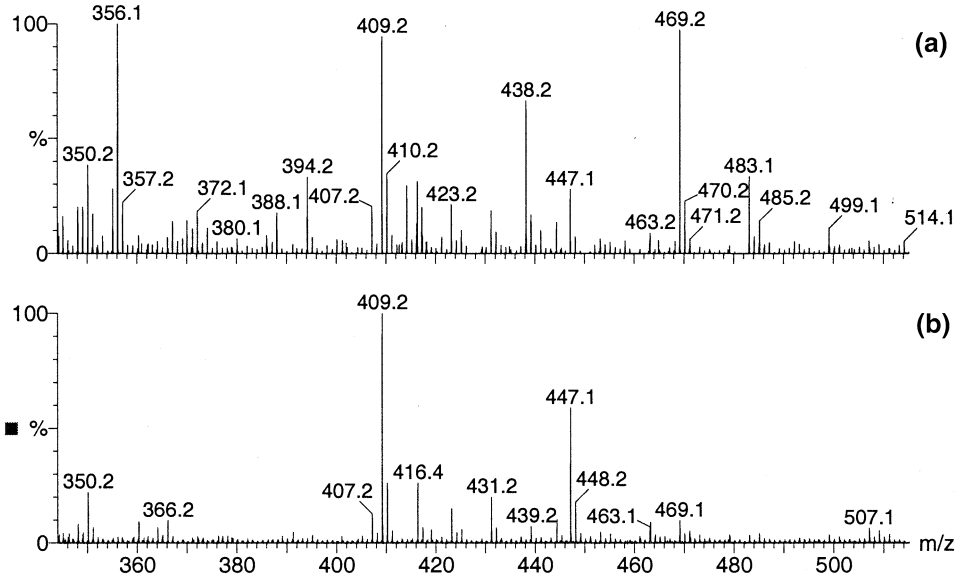
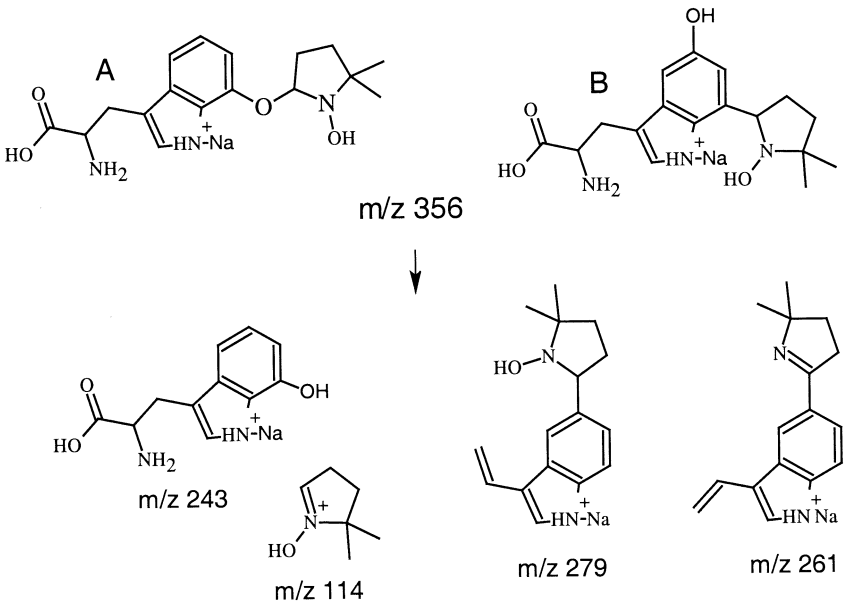


Figure 6. ES mass spectra of the oxidation of tryptophan by Fenton reaction (**a**) in the presence of DMPO and (**b**) in absence of DMPO.

dimer Trp-Trp. This structure was confirmed from the ES-MS/MS spectrum based on the observation of an ion of *m/z* 203 (RA 30%) and an ion of *m/z* 219 (RA 30%) corresponding to the oxidized hydroxytryptophan.

The formation of radical species during the oxidation reaction was detected by using a spin trap, DMPO. The spin trap was added to the reaction mixture and after a period of time the reaction was monitored by ES-MS. Radicals are very unstable species, but in the presence of spin traps they form a stable adduct with the trap. Previous work has shown that mass spectrometry is a good method to detect the stable adducts of DMPO with hydroxyl radicals [15]. Following the same methodology, new adducts of DMPO and radical species resulting from tryptophan oxidation under Fenton reaction conditions were detected and identified by mass spectrometry. As can be seen in Figure 6 that compares the ES mass spectra after the oxidation of tryptophan under Fenton conditions, Figure 6a in the presence of DMPO and Figure 6b without DMPO, new ions can be observed. ES-MS/MS mass spectra of these ions were obtained in order to define the structure of these adducts of spin.

New ions were observed of *m/z* 356, 383, 385, and 469. The ions of *m/z* 394 and 438 were observed in a control reaction of DMPO, H_2O_2, and Fe(II) in bicarbonate buffer, so they are not spin adducts of DMPO with tryptophan. The ion of *m/z* 356 corresponds to the $[M + Na]^+$ of the spin adduct of hydroxyl-tryptophan with DMPO, and the proposed structure is represented in



Scheme 6. Proposed structure for the spin adduct of *m/z* 356 and correspondingfragment ions.

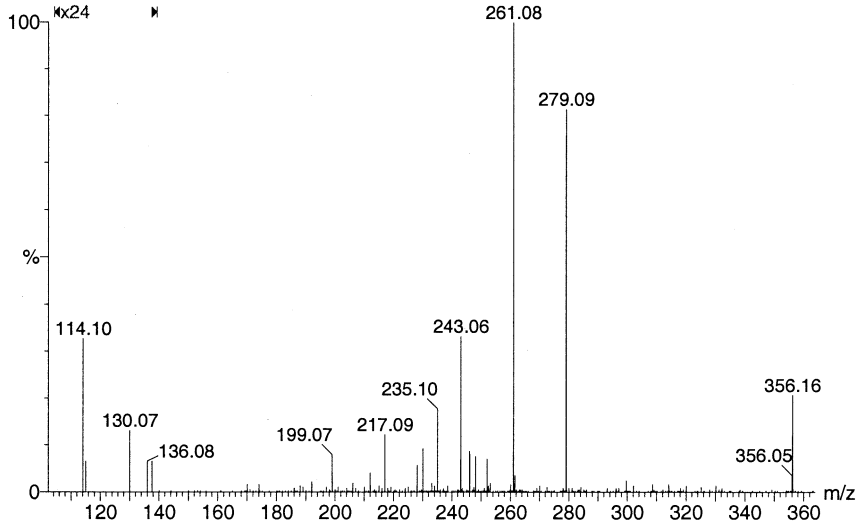


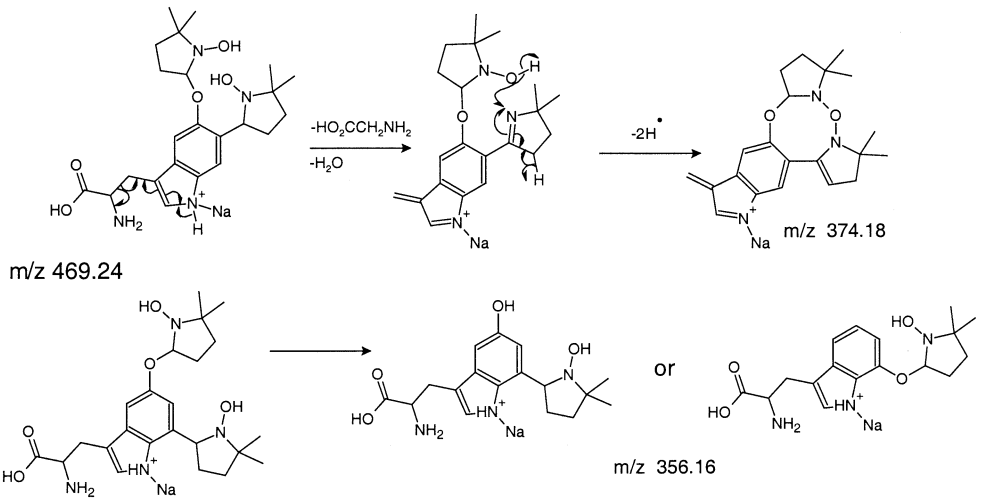
Figure 7. ES-MS/MS spectrum of the spin adduct of *m/z* 356.

Scheme **6**. This structure shows 7-hydroxytryptophan, but the oxygen can be linked to the other possible positions at C2, C4, C5, and C6. This consideration should be extended to the other radical adducts that will be referred to below. The protonated molecule of this spin adduct corresponds to the ion of *m/z* 334, the same *m/z* as the previously discussed oxidized form of tryptophan, so it could not be assigned.

The MS/MS spectrum of the ion of *m/z* 356 (Figure 7 shows an ion of *m/z* 114, attributed to $[DMPO + H]^+$. The ion of *m/z* 130 can be attributed to the protonated molecule of hydroxyl-DMPO [15] that resulted from the cleavage of the spin adduct of HO-Trp with DMPO. Also, an abundant ion of *m/z* 243 formed by the loss of the DMPO (loss of 113 Da) confirms the presence of an DMPO adduct. The ion of *m/z* 243 corresponds to the sodium adduct of hydroxytryptophan. This is consistent with the sodium ion complexed either to the tryptophan moiety or to the DMPO molecule.

Analyzing the ES-MS/MS spectrum of the DMPO adduct of *m/z* 356, we found that it was possible to attribute two possible structures for this adduct of DMPO and the monohydroxy-tryptophan, as it can be seen in Scheme **6**. The ion of *m/z* 130 (the hydroxyl-DMPO) suggests that the DMPO molecule is linked to the hydroxyl oxygen, so the radical is centered in the oxygen (Scheme **6**, Structure A). However, the ions of *m/z* 261 and 279 are only rationalized considering that the radical is centered in a carbon, and that the DMPO is linked to the carbon centered radical (Scheme **6**, Structure B). This is in accordance with the previously observed Trp-Trp dimer in oxidative conditions, similar to the Tyr-Tyr dimer that presupposes the formation of a carbon centered radical.

In the spectrum of the reaction with DMPO present (Figure 6a), all the DMPO adducts of oxidized tryptophan were identified as sodium adducts. It is interesting to note that the spin adducts were observed under ES-MS as sodium adducts rather than protonated molecules. No explanation was found for this observation,



Scheme 7. Proposed structure for the spin adduct of *m/z* 469 and corresponding fragment ions.

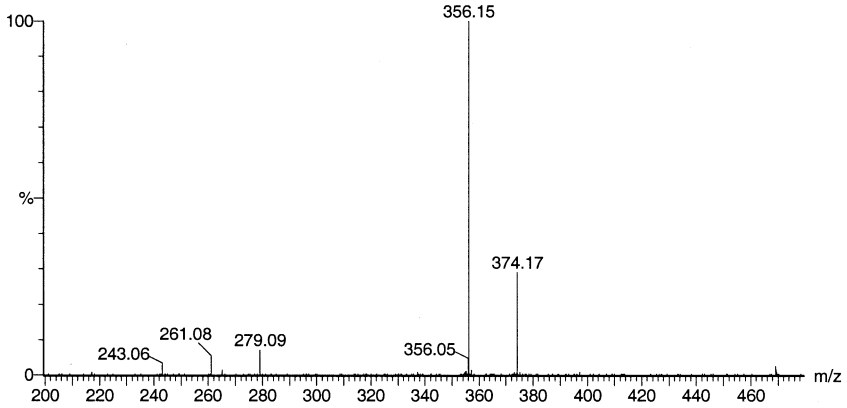


Figure 8. ES-MS/MS spectrum of the spin adduct of *m/z* 469.

although the sodium adducts of both tryptophan and monohydroxytryptophan of *m/z* 227 and 243 respectively, and also the sodium adduct of DMPO of *m/z* 136 (data not shown) were observed in the ES-MS spectra. However these ions were of low abundance relative to the corresponding protonated molecules.

The ion of *m/z* 469 corresponds to a bis adduct of two molecules of DMPO with a hydroxyl-tryptophan as it is shown in Scheme **7**. Although in the ES-MS spectrum of the reaction of Trp without DMPO (Figure 6b) we were able to detect a low abundance ion of *m/z* 469.1, the new ion observed in Figure 6a (after oxidation in presence of DMPO) has an *m/z* of 469.22, which is in accord with the calculated mass for the proposed structure, 469.24 ($C_{23}H_{34}N_4NaO_5^+$). The ES-MS/MS spectrum of this ion (Figure 8) shows as the major fragment ion the ion of *m/z* 356, formed by elimination of one molecule of DMPO. An ion of *m/z* 243 is also observed with relative abundance of approximately 20%, which confirm the presence of the sodium adduct of the HO-Trp.

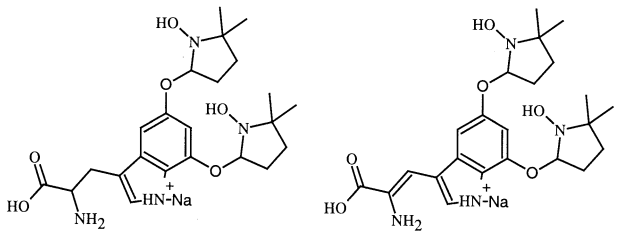
The adduct of *m/z* 469 should have one DMPO molecule linked to a carbon centered radical and the other DMPO molecule linked to an oxygen centred radical, as it is shown in Scheme **7**. Another fragmentation pathway of the adduct ion of *m/z* 469 leads to the formation of the ion of *m/z* 374. This fragment ion is probably formed by combined elimination of H_2O and $HO_2CCH_2NH_2$ followed by elimination of two hydrogen radicals, as proposed in Scheme **7**. The elimination of two hydrogen radicals from the DMPO moieties leads to the formation of an intramolecular seven membered ring, which suggests that DMPO molecules are present in this structure in positions in the vicinity. The intramolecular seven-membered ring is a structure which is similar to previously proposed adducts formed between two and three DMPO with radical hydroxyl [15].

The ions of *m/z* 483 and 485 correspond to the bis adduct of two molecules of DMPO with the dihydroxytryptophan, shown in Scheme **8**. The spin adduct of *m/z* 483 corresponds to the α-β unsaturated form of the bis adduct, of *m/z* 485.

The structures of these ions were corroborated by the ES-MS/MS spectra. In the ES-MS/MS spectrum of the ion of *m/z* 483, we were able to see a major ion of *m/z* 370 (RA 70%) formed due to the loss of 113 that corresponds to the loss of a DMPO molecule. Another fragment ion was observed of *m/z* 257 formed due to the loss of another molecule of DMPO. This ion of *m/z* 257 corresponds to a sodium adduct of α-β unsaturated dihydroxytryptophan. The ES-MS/MS spectrum of the ion of *m/z* 385 showed a major ion of *m/z* 372 (RA 100%) formed due to the loss of 113 Da which corresponds to a molecule of DMPO, and also loss of 129, corresponding to loss of an hydroxyl-DMPO molecule, with formation of the ion of *m/z* 356 (Scheme **6**).

Conclusion

Mass spectrometry proved to be a valuable tool in the identification of new oxidation products of tryptophan formed by Fenton reaction and in the detection of tryptophan oxidation radicals species, using ES as the ionization method. We have identified, using this direct approach, the well known mono- and dihydoxytryptophans and N-formylkynurenine, the Trp-Trp dimer and new oxidized species, new products resulting from the reaction of tryptophan and oxidized tryptophan with 3-methyl indole derivatives. The monohydroxytryptophan dimer (Trp-Trp-OH) was also found to be formed under these oxidative conditions. Using DMPO as a spin trap and analyzing the oxidation products using ES-MS and ES-MS/MS, we were able to identify spin adducts, namely monohydroxyl-tryptophan, with one and two



Scheme 8. Proposed structures for the spin adducts of *m/z* 485 and 483.

spin trap DMPO molecule and dihydroxytryptophan and dihydroxytryptophan α-β unsaturated with two DMPO spin trap molecules. DMPO was found to be linked with oxygen radicals and/or carbon centered radicals.

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References

1. Berlett, B. S.; Stadtman, E. R. Protein Oxidation in Aging, Disease, and Oxidative Stress. *J. Biol. Chem.* **1997,** *272,* 20313–20316.
2. Linton, S.; Davies, M. J.; Dean, R. T. Protein Oxidation and Aging. *Exp. Gerontol.* **2001,** *36,* 1503–1518.
3. Arthur J. R.; Duthie G. G. In *Oxygen Radicals and Celular Damage*; Duncan, C. J., Ed.; Press Syndicate of the University of Cambrige: Cambridge, 1991; pp 115-138.
4. Liochev, S. I. The Mechanism of Fenton-like Rreactions and Their Importance for Biological Systems. A Biologist's View. *Metal Ions in Biological Systems, Vol. XXXVI;* In: Sigel, A.; Sigel, H, Eds.; Marcl Dekker, Inc: New York, 1999; pp 1–40.
5. Stadtman, E. R.; Berlett, B. S. Fenton Chemistry. Amino Acid Oxidation. *J. Biol. Chem.* **1991,** *266,* 17201–17211.
6. Maleknia, S. D.; Downard, K. Radical Approaches to Probe Protein Structure, Folding, and Interactions by Mass Spectrometry. *Mass Spectrom. Rev.* **2001,** *20,* 300–401.
7. Buxton, G. V.; Greenstock, C. L.; Helman, W. P.; Ross, A. B. Critical Review of Rate Constants for Reactions of Hydrated Electrons, Hydrogen Atoms, and Hydroxyl Radicals in Aqueous Solutions. *J. Phys. Chem. Ref. Data* **1988,** *270,* 513–886.
8. Stadtman, E. R. Oxidation of Free Amino Acid Residues in Proteins by Radiolysis and by Metal-Catalyzed Reactions. *Annu. Rev. Biochem.* **1993,** *62,* 797–821.
9. Goshe, M. B.; Chen, Y. H.; Anderson, V. E. Identification of the Sites of Hydroxyl Reaction with Peptides by Hydrogen/Deuterium Exchange: Prevalence of Reactions with the Side Chains. *Biochem.* **2000,** *39,* 1761–1770.
10. Dean, R. T.; Fu, S.; Stocker, R.; Davies, M. J. Biochemistry and Pathology of Radical Mediated Protein Oxidation. *Biochem. J.* **1997,** *324,* 1–18.
11. Gieseg, S.; Duggan, S.; Gebick, J. M. Peroxidation of Proteins Before Lipids in U 937 Cells Exposed to Peroxyl Radicals. *Biochem. J.* **2000,** *350,* 215–218.
12. Spin Trapping Free Radicals. In *Free Radicals. Biology and Detection by Spin Trapping*; Rosen, G. M.; Britigam, B. E.; Halpern, H. J.; Pou, S., Eds.; Oxford University Press: New York, 1999; pp 170-186.
13. Iwahashi, H.; Parker, C. E. Mason, R. P.; Tomer, K. B. Radical Identification by Liquid Chromatography/Thermospray Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **1990,** *4,* 352–354.
14. Gunther, M. R.; Tschirret-Guth, R. A.; Witkowska, H. E.; Fann, Y. C.; Barr, D. P. Ortiz; de Montellano, P. R.; Mason, R. P. Site-Specific Spin Trapping of Tyrosine Radicals in Oxidation of Metmyoglobin by Hydrogen Peroxide. *Biochem. J.* **1998,** *330,* 1293–1299.
15. Domingues, P.; Domingues, M. R. M.; Amado, F. M. L.; Ferrer-Correia, A. J. V. Detection and Characterization of Hydroxyl Radical Adducts by Mass Spectrometry. *J. Am. Soc. Mass Spectrom.* **2001,** *12,* 1214–1219.
16. Parker, C. E.; Iwahashi, H.; Tomer, K. B. Spin-Trappped Radicls: Determination by LC-TSP-MS and LC-ESI-MS. *J. Am. Soc. Mass Spectrom.* **1991,** *2,* 413–418.
17. Iwahashi, H.; Albro, P. W.; Grown, S. R.; Tomer, K. B.; Mason, R. P. Isolation and Identification of a-(4-Pyridyl-1-Oxide)-N-*tert*-Butylnitrone Radical Adducts Formed by the Decomposition of the Hydroperoxides of Linoleic Acid, Linolenic Acid, and Arachidonic Acid by Soybean Lipoxygenase. *Arch. Biochem. Biophys.* **1991,** *1,* 172–180.
18. Iwahashi, H.; Parker, C. E.; Tomer, K. B. Combined Liquid Chromatography/Electron Paramagnetic Resonance Spectrometry/Electrospray Ionization Mass Spectrometry for Radical Identification. *Anal. Chem.* **1992,** *64,* 2244–2252.
19. Barr, D. P.; Gunther, M. R.; Deterding, L. J.; Tomer, K. B.; Mason, R. P. ESR Spin Trapping of a Protein-Derived Tyrosyl Radical from the Reaction of Cytochrome C with Hydrogen Peroxide. *J. Biol. Chem.* **1996,** *271,* 15498–15503.
20. Deterding, L. J.; Barr, D. P.; Mason, R. P.; Tomer, K. B. Characterization of Cytochrome χ Free Radical Reactions with Peptides by Mass Spectrometry. *J. Biol. Chem.* **1998,** *273,* 12863–12869.
21. Fu, S.-L.; Dean, R. T. Structural Characterization of the Products of Hydroxyl-Radical Damage to Leucine and Their Detection on Proteins. *Biochem. J.* **1997,** *324,* 41–48.
22. Nukuna, B. N.; Goshe, M. B.; Anderson, V. E. Sites of Hydroxyl Radical Reaction with Amino Acids Identified by ^{2}H NMR Detection of Induced ^{1}H/^{2}H Exchange. *J. Am. Chem. Soc.* **2001,** *123,* 1208–1214.
23. Maskos, Z.; Rush, J. D.; Koppenol, W. H. The Hydroxylation of Tryptophan. *Arch. Biochem. Biophys.* **1992,** *296,* 514–520.
24. Finley, E. L. Dillon, J; Crouch, R. K.; Schey, K. L. Identification of Tryptophan Oxidation Products in Bovine α-Crystallin. *Protein Sci.* **1998,** *7,* 2391–2397.
25. Simat, T. J.; Steinhart, H. Oxidation of Free Tryptophan and Tryptophan Residues in Peptides and Proteins. *J. Agri. Food Chem.* **1998,** *46,* 490–498.
26. Pietrsforte, D. Minetti, M; One-Electron Oxidation Pathway of Peroxynitrite Decomposition in Human Blood Plasma: Evidence for the Formation of Tryptophan Centered Radicals. *Biochem. J.* **1997,** *321,* 743–50.
27. Bloding, W.; Smith, A. T.; Winterhalter, K.; Piontek, K. Evidence from Spin Trapping for a Transient Radical on Tryptophan Residue 171 of Lignin Peroxidase. *Arch. Biochem. Biophys.* **1999,** *370,* 86–92.
28. Strli, M.; Kolar, J.; Pihlar, B. The Effect of Metal Ion, pH, and Temperature on the Yield of Oxidizing Species in a Fenton-Like System Determined by Aromatic Hydroxylation. *Acta Chim. Slov.* **1999,** *46,* 555–566.
29. McLuckey, S. A. Principles of Collisional Activation in Analytical Mass Spectrometry. *J. Am. Mass Spectrom.* **1992,** *3,* 599–614.
30. McLafferty, F. W.; Turecek, F. *Interpretation of Mass Spectra.* University Science Books: Mill Valey , 1993; pp 144–146.
31. Gunther, M. R.; Kelman, D. J.; Corbett, J. T.; Mason, R. P. Self Peroxidation of Metmyoglobin Results in Formation of an Oxygen-Reactive Tryptophan-Centered Radical. *J. Biol. Chem.* **1995,** *270,* 16075–16081.