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Electrospray mass spectrometric studies of L-carnosine (β-alanyl-L-histidine) complexes with copper(II) or zinc ions in aqueous solution

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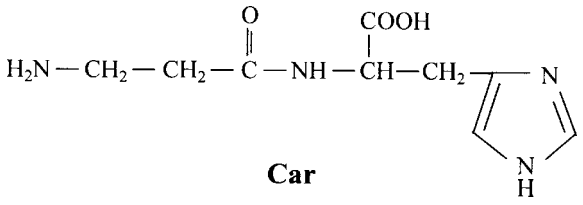
Electrospray ionization mass spectrometry (ESI-MS) was used for the speciation of supramolecular assemblies formed between equimolar amounts of carnosine and copper or zinc ions in dilute aqueous solutions. In the case of pure carnosine and carnosine/copper systems, the effect of pH changes, in the range 2–9, on the complexes surviving in solution was also explored. ESI data, besides supporting previous reported results on the formation of dimeric carnosine/copper and carnosine/zinc complexes, allowed a more complete speciation of the examined systems, bringing to light the existence of bis-complex species and, in the zinc case, the formation of oligomeric species. The data obtained for the systems investigated show that ESI-MS is not only a reliable and fast technique for the analysis of the metal/ligand systems, but also an interesting tool to obtain stoichiometric information on metal complexes formed in very low concentration solutions. Copyright © 2002 John Wiley & Sons, Ltd.

L-Carnosine (β-alanyl-L-histidine), a natural dipeptide discovered by Gulewitsch and Amiradzibi about 100 years ago,[1] is present in the muscle fibres and in various organ tissues of vertebrates.[2,3] Recently, its biological relevance in a variety of functions, apart from the pH-buffering role, as an antiglycating, hydrophilic antioxidant or regulator of specific receptors and enzymes, is attracting great interest.[4,5] Several reports have been published and it seems that, for some carnosine biological actions, the participation of certain heavy metal ions is essential;[6–8] as an example, the formation *in vivo* of a zinc/carnosine complex (a well-known *Helicobacter pylori* inhibitor) appears to have a crucial action in vascular smooth muscle contraction.[9,10] In the case of the carnosine/copper(II) system,[11,12] the formation of a dimer species,[11] stable under physiological conditions, has also been ascertained; it has the same structure as that of the blue crystals obtained by slow evaporation of a concentrated alkaline solution of copper hydroxide and carnosine,[13] whose existence in living organisms is nevertheless believed to be improbable due to the low copper concentration and the presence of competing ligands.[14]

However, as a consequence of their relatively small amounts, the correct speciation of biological supramolecular structures formed under physiological conditions, through the use of traditional tools such as UV/CD, NMR, etc., is often difficult; the attention of many researchers has thus been directed to explore new procedures that can take into account the direct monitoring of complex species.

Recent reports suggest that electrospray ionization mass spectrometry (ESI-MS) could provide a significant contribution.[15] In fact, its high sensitivity, which allows mass spectral acquisition using very small quantities of material (micromoles or less), the potential for high resolution which permits great accuracy in the mass assignments, and the very soft ionization procedure make ESI-MS an excellent method for the direct identification and characterization of labile complexes also at very low concentrations.[16–19] In particular, this technique is assuming an increasingly active role in biological investigations of a wide variety of supramolecular structures such as peptide/transition metal ions, enzyme/inhibitor complexes, etc.;[19–23] several studies have in fact ascertained that protein-metal interactions in aqueous solution can be maintained during the ESI experiments, so permitting the direct speciation of the formed species and the determination of their stoichiometry.[23–25]

In the present work, ESI-MS has successfully been employed in an attempt to obtain a correct speciation of supramolecular assemblies formed between L-carnosine and



Car

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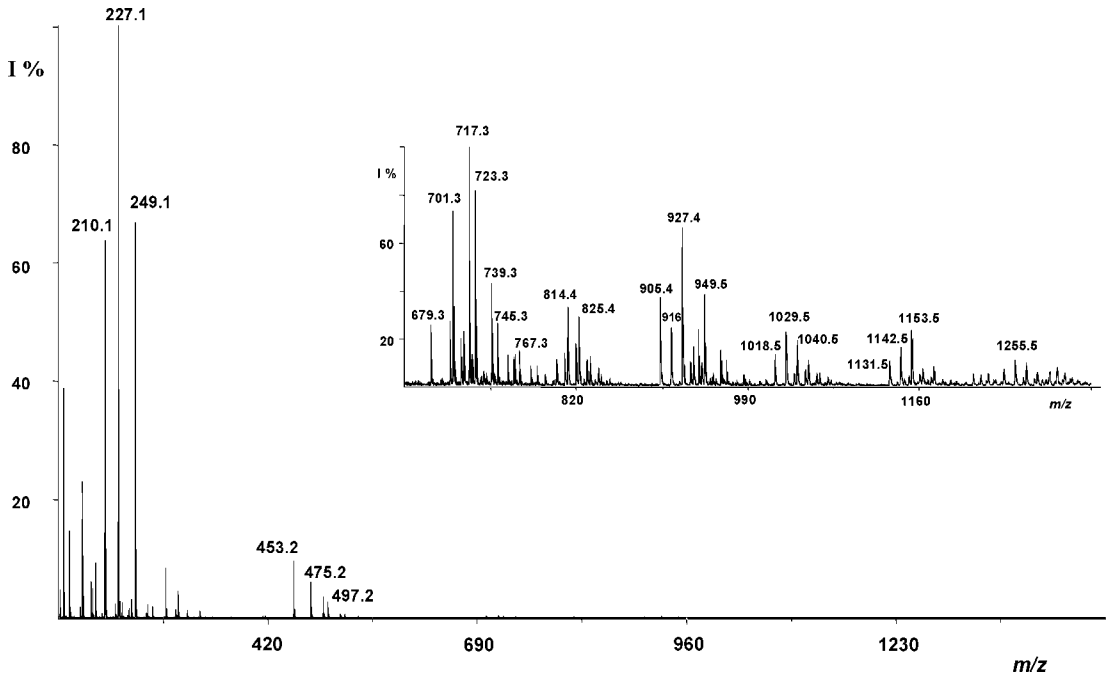

Figure 1. Positive ESI mass spectrum of an aqueous solution of pure carnosine at pH 7.2.

copper or zinc ions in dilute aqueous solution (about 10^{-4} M) in a pH range between 2 and 9.

L-Carnosine (Car) is a polydentate ligand with many potential binding sites, three of which can undergo acid-base reactions in the pH range 1–10. Therefore, the type of complexes formed depends not only on the kind of metal ion, but also on the ligand/metal ratio and on the pH of the solution.[8,11,19] Previous studies on metal(II)/carnosine species formed in aqueous solution, obtained by means of several common techniques (such as UV-visible spectroscopy, potentiometry, ESR, NMR, visible and ultraviolet-circular dichroism),[8,11,19] agree in determining the dimeric complex as the most relevant species formed in the copper case.[25] Similar data were also obtained for the zinc/carnosine system, for which a lower stability of the dimeric complex was demonstrated.[8] However, contrasting data about the presence and relative abundance of other species render further speciation studies necessary.[11,26,27]

EXPERIMENTAL

Materials

L-Carnosine (*β*-alanyl-L-histidine, Car) was purchased from Sigma. ^{63}Cu(II) nitrate solution was standardized by EDTA titration with murexide as indicator. Zn nitrate solution was prepared from ZnO by adding a slight excess of HNO_3.

Solution preparation

All the solutions were freshly prepared using deionized water. The concentrations of carnosine and its complexes were 0.001 mol dm^{-3}. The pH 7.2 of the initial carnosine solution was initially lowered to 2.9 using HCl and subsequently gradually raised up to 9.0 using NaOH solution. The pH changes were measured using a combined Metrohm 125 microelectrode.

ESI mass spectrometric analysis

Positive ion mass spectra were acquired using a Mariner ESI-TOF mass spectrometer (PerSeptive Biosystems) equipped with an API ion source. The ionspray voltage was fixed at 5 kV and the orifice potential (declustering potential) was varied from 50 to 250 V. The aqueous solutions of the samples investigated, mixed with a CH_3OH/H_2O (60:40) mixture (the solute concentration was maintained at about 10^{-4} M in each experiment), were introduced into the source at a flow rate of 7 μL/min. The mass spectra were recorded and processed with the 'BioSpec Data Explorer ver. 3.0.0.1' software (from PerSeptive Biosystems) and, to make the peak assignments in the spectra easier, a nitrate salt of pure ^{63}Cu isotope was used in some experiments.

Because of the isotopic distribution of the elements, molecular species are detected in the mass spectra as clusters of peaks so that, to simplify their assignments, the *m/z* values indicated in the spectra and in the text correspond to the first (lowest-mass) peak of each cluster.

In the formulas reported in the text and in the table, the substitution of hydrogen atoms of the carnosine molecule with metal atoms is indicated in parentheses; as an example, (Car-2H + Cu) indicates a carnosine molecule in which two H atoms are substituted by a Cu atom; if not otherwise specified, Cu is bivalent. In all cases the cationizing agents are placed outside the parentheses and their charge is indicated.

RESULTS AND DISCUSSION

Before the ESI-MS spectra acquisition, the aqueous solutions of carnosine and carnosine/copper or zinc nitrate were diluted with an H_2O/MeOH (60:40) mixture, fixing the final sample concentration at about 10^{-4} M. Each identified species detected in the spectra has been indicated in the

Table 1. Assignment of the singly and doubly charged ions detected in the ESI mass spectra of carnosine and its mixtures with $^{63}Cu(NO_3)_2$ or $Zn(NO_3)_2$

	m/z values of molecular ions for n=										
Structure	1	2	3	4	5	6	7	8	9	10	11
$(Car)_nH^+$	227.1	453.2	679.3	905.4	1131.5						
$(Car)_nNa^+$	249.1	475.2	701.3	927.4	1153.5						
$(Car)_nK^+$	265.1	491.2	717.3								
$(Car-H+Na)_nNa^+$	271.1	519.2	767.3	1015.4							
$(Car)_nH_2^{2+}$						679.2	792.4	905.4	1018.5	1131.5	1244.6
$(Car)_nH^+Na^+$						690.3	803.4	916.4	1029.5	1142.5	1255.8
$(Car)_nNa_2^{2+}$						701.3	814.4	927.4	1040.5	1153.5	1266.6
$(Car-2H+Cu)_nH^+$			862.3								
$(Car-2H+Cu)_nNa^+$		597.2	884.3								
$[Car(Car-2H+Cu)_n]H^+$	514.2	801.3									
$[Car-H+Cu(I)]H^+$	289.1										
$[Car-H+Cu(I)]Na^+$	311.1										
$(Car-2H+Zn)_nH^+$	289.0	577.0	865.1	1153.1							
$(Car-2H+Zn)_nNa^+$	311.0	599.0	887.0	1175.1							
$[Car(Car-2H+Zn)_n]H^+$	515.1	803.1	1091.2								
$[Car(Car-2H+Zn)_n]Na^+$	537.1	825.1									

following with the *m/z* value of the first (lowest-mass) peak of its isotope cluster of signals. The possible presence of electrostatic or other non-covalent adducts was evaluated by comparing ESI spectra recorded at increasing orifice voltage values from 50 to 250 V (declustering effect).

Carnosine

Literature data indicate that, in aqueous solution at pH 7, carnosine remains in a monomeric form with a 'most probable' molecular conformation (deduced from ^{1}H- and ^{13}C-NMR experiments) in which the β-alanyl moiety is folded toward the imidazole ring.[28]

Figure 1 shows the ESI mass spectrum, recorded with an orifice voltage of 150 V, of pure carnosine in aqueous solution at pH 7.2. The structural assignment of the peaks is reported in Table 1. The more intense signals in the spectrum are two peaks at *m/z* 227.1 (base peak) and 249.1, both corresponding to singly charged carnosine molecules cationized by a proton, $(Car)H^+$, or a sodium ion, $(Car)Na^+$. The peak at *m/z* 210.1 is due to a carnosine fragment formed by loss of NH_3 from $(Car)H^+$, probably during the spray process. This assignment was confirmed by comparison with other carnosine ESI spectra recorded at different orifice potentials: as a consequence of decreasing the potential, a reduction of the intensity of the peak at *m/z* 210.1 is observed, whereas at potential values higher than 150 V it becomes the base peak.

Signals corresponding to carnosine dimeric species, $(Car)_2M^+$, are also well visible in the spectrum at *m/z* 453.2, 475.2 and 491.2 as a function of different cationizing agents M (H, Na or K ions). In addition, on examining the expanded spectral region at higher *m/z* values (inset in Fig. 1), a series of low intensity peaks corresponding to larger structures up to undecamer (detected as singly charged ions up to the hexamer and as doubly charged ions for the remaining species, Table 1) are observed in the spectrum. Peaks corresponding to complexes formed by carnosine (Car) and carnosine sodium salt (Car-$H + Na$) units are also present in the spectra, e.g., at *m/z* 497.2 {[(Car)(Car-$H + Na$)]Na^+ or [(Car-$H + Na$)$_2$]H^+}, *m/z* 723.3 {[$(Car)_2$(Car-$H + Na$)]Na^+ or [(Car)(Car-$H + Na$)$_2$]H^+}, *m/z* 949.4 {[$(Car)_3$(Car-$H + Na$)]Na^+ or [$(Car)_2$(Car-$H + Na$)$_2$]H^+} and, as the doubly charged ion, at *m/z* 825.4 {[$(Car)_6$(Car-$H + Na$)]Na_2^{2+} or [$(Car)_4$(Car-$H + Na$)$_3$]H_2^{2+}}.

The effect of pH changes on the composition of the carnosine species observed in the ESI mass spectra is shown in Fig. 2, in which spectra of solutions at pH 2.9, 4.0 and 9.0 are reported. By comparison with the spectrum of Fig. 1, it can be observed that the spectra at pH 9.0 and 4.0 (Figs 2(a) and 2(b)), which are similar to each other, show new relatively abundant peaks at higher *m/z* values. As expected, at pH 9 (Fig. 2(a)), the sodiated species at *m/z* 249.1 becomes the base peak and the other signals present in the spectrum also correspond exclusively to species cationized with sodium ions. Furthermore, new peaks due to oligomeric species, in which a sodium ion also substitutes a carboxyl hydrogen in each carnosine unit, appear at *m/z* 271.1 [(Car-$H + Na$)Na^+], *m/z* 519.2 [(Car-$H + Na$)$_2Na^+$], *m/z* 767.3 [(Car-$H + Na$)$_3Na^+$], *m/z* 1015.4 [(Car-$H + Na$)$_4Na^+$] and at higher *m/z* values (peaks with lower intensity). These peaks, absent in the spectrum of the solution at pH 7.2 (prepared without NaOH addition), appear in all the spectra at pH values higher than 3 (see Fig. 2(b)). In contrast, in the ESI mass spectrum of the carnosine solution at pH 2.9 (Fig. 2(c)), these peaks disappear and the spectrum is essentially composed of peaks due to protonated species at *m/z* 227.1, 453.2 and 679.3 (Table 1), and, with lower intensity, sodiated species at *m/z* 249.1 and 475.2.

To ascertain if the peaks at higher *m/z* values present in the spectra of Figs 1 and 2 are really due to carnosine oligomeric species or to electrostatic adducts, the dependence of the peak intensities on the orifice voltage of the ESI mass spectrometer was monitored (declustering effect). The ESI spectra of the solutions at different pH values, acquired with potential values of 50 and 250 V (spectra omitted for brevity), were then compared with those recorded at 150 V. Apart

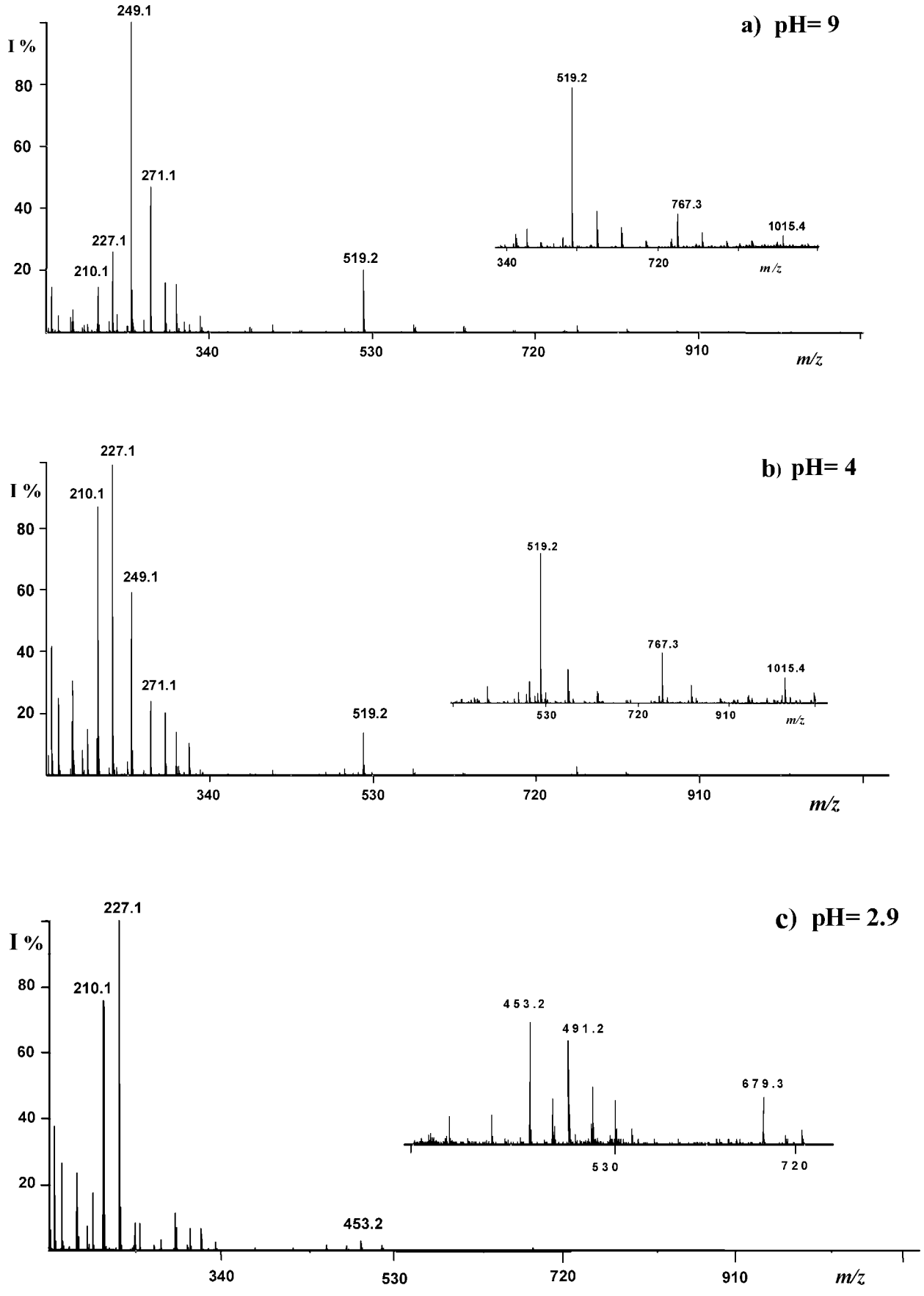

Figure 2. Positive ESI mass spectra of aqueous solutions of pure carnosine at (a) pH 9.0; (b) pH 4; and (c) pH 2.9.

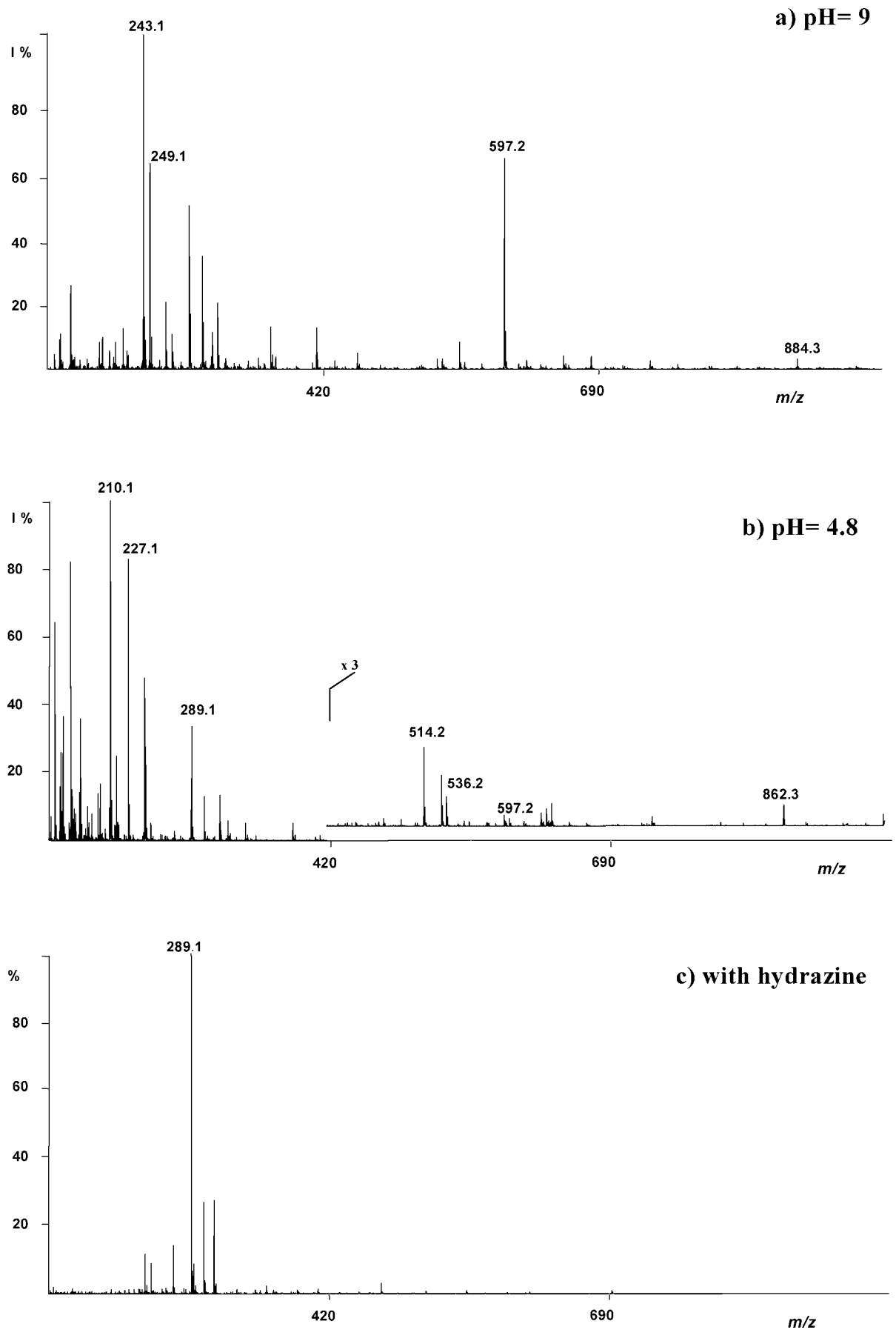

Figure 3. Positive ESI mass spectra of aqueous solutions of stoichiometric amounts of carnosine and $^{63}Cu(NO_3)_2$ at (a) pH 9.0; (b) pH 4.8; and (c) pH 7.2 after addition of 5% (w/w) hydrazine.

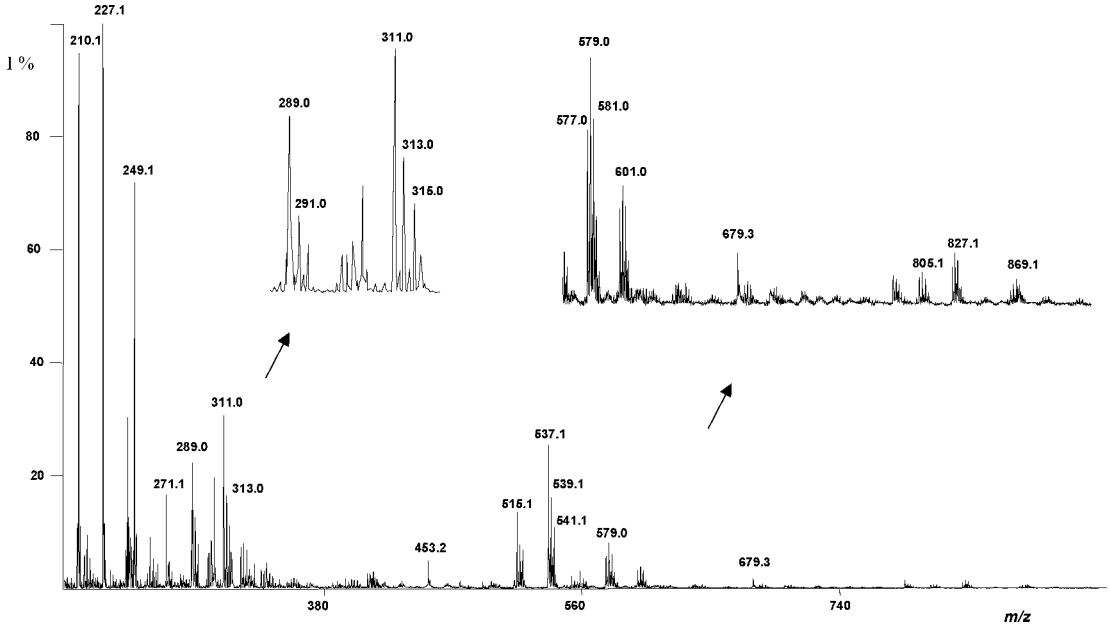

Figure 4. Positive ESI mass spectrum of an aqueous solution of stoichiometric amounts of carnosine and $Zn(NO_3)_2$ at pH 7.2.

from some minor changes in the relative abundances of the different cationized species, the resulting spectra were little affected by the potential variations, thus supporting the hypothesis that the peaks in the spectra are really due to oligomeric carnosine species present in the solution.

The strong intensity of the signal at *m/z* 519.2 in the mass spectra of the solutions at pH values higher than 3 (Figs 2(a) and 2(b)) indicates that, in the presence of sodium ions, the formation of the dimeric Na/carnosine complex [(Car-$H + Na)_2Na^+$] and, to a lesser extent, that of heavier species [(Car-$H + Na)_3$ and (Car-$H + Na)_4$, both cationized by Na^+] is favoured; it can be noticed that these peaks are almost absent in both the spectra of the solutions at pH 7.2 (Fig. 1), to which no sodium compounds have been added, and at pH 2.9 (Fig. 2(c)). In this last case, the pH value is very near to the carboxyl p*K* (about 2.8) so that the carnosine sodium salt cannot be formed. As further evidence, the ESI spectrum (omitted for brevity) of carnosine solution at pH 7.2 after addition of NaCl gave results very similar to those of Figs 2(a) and 2(b).

Copper/carnosine

ESI mass spectra of aqueous solutions of carnosine mixed with an equimolar amount of ^{63}Cu(II) nitrate, at pH values of 9.0 and 4.8, are reported in Figs 3(a) and 3(b). Comparing the spectrum of Fig. 3(a) with that of the pure carnosine solution at the same pH value (Fig. 2(a)), it can be noticed that, apart from some low intensity peaks due to pure carnosine species (at *m/z* 249.1 and 453.2), the spectrum is essentially composed of a very intense peak at *m/z* 597.2 and a peak at *m/z* 884.3. These peaks correspond to the singly charged Cu(II)/carnosine dimer [(Car-$2H + Cu)_2Na^+$] and trimer [(Car-$2H + Cu)_3Na^+$] in which a copper ion substitutes two protons in each carnosine unit. The peak at *m/z* 243.1 is due to an unassigned ion present in all the spectra of carnosine samples containing the copper salt, whereas the peaks at about *m/z* 279, 301, 475, and 701 are background signals (phthalate compounds from tubing).

All the spectra of solutions with pH >5 are very similar to that of Fig. 3(a) and so are omitted here for brevity. In contrast, for the solutions at lower pH values (as an example, that at pH 4.8 is reported in Fig. 3(b)), the peak at *m/z* 597.2 disappears and, besides the intense peaks at *m/z* 210.1 and 227.1 due to pure carnosine species, a peak due to the (Car-$2H + Cu)_3H^+$ ion at *m/z* 862.3, and a peak at *m/z* 514.2 due to [(Car-$H)_2Cu]Na^+$ in which the copper(II) ion is bound at two carnosine ligands by means of their carboxylate groups as well as by means of amine and imidazole nitrogens, appear in the spectra. Furthermore, an intense peak at *m/z* 289.1, present with lower intensity also in the spectra at higher pH values, accompanied by its sodiated ion at *m/z* 311.1, is found in the spectra. This peak corresponds to the [Car-$H + Cu$(I)]H^+ species containing a monovalent copper atom bound to a carboxyl group, probably formed by partial reduction induced (especially at acidic pH values) by the hydrazine found in trace amounts in the commercial carnosine sample.[29] To verify this hypothesis, a further amount of hydrazine (about 5%) was added to the carnosine/Cu(II) nitrate solution before recording the ESI mass spectrum. Comparing the obtained spectrum (Fig. 3(c)) with those of Figs 3(a) and 3(b), a strong increase of the intensity of the peak at *m/z* 289.1 can be observed, whereas almost all the peaks at higher *m/z* values disappear. Moreover, when a natural copper(II) nitrate salt was used, the characteristic pattern of peaks at *m/z* 289.1 and 291.1, this last due to the ^{65}Cu isotope, appears in the spectrum (omitted for brevity).

These results show that the interaction in aqueous solution between carnosine and copper ions determines the formation of several Cu/carnosine species, whose relative abun-

dances change as a function of the pH. When the pH of the solution is higher than 6.7 (Fig. 3(a)), the equilibrium is strongly shifted towards the formation of the dimer complex (Car-2$H + Cu$)$_2$ (peak at *m/z* 597.2) in which, as reported in previous literature data,[11,13,30] each copper(II) ion is bound to the carboxyl group, to the deprotonated amide N-atom and to the amine nitrogen of one carnosine molecule and to the 3-N imidazole atom of the second carnosine molecule.[11,13,30]

At pH values lower than 5.8, in contrast, both the reduced deprotonation of the amide group and the protonation of the 3-N imidazole atom (pK about 6.6)[8] interfere with the structural arrangement between the metal and the carnosine molecules: the formation of the dimeric (Car-2$H + Cu$)$_2$ complex is hindered and the above-mentioned equilibrium is shifted towards the formation of pure carnosine (peak at *m/z* 227), [(Car-H)$_2$Cu] bis-complex species (*m/z* 514.2) and, as a consequence of a partial reduction of the copper atoms, monomeric [Car-$H + Cu$(I)] species (*m/z* 289.1) in which a copper(I) ion is bound to the carboxyl group of a carnosine molecule.

Zinc/carnosine

There is agreement in the speciation of the Zn/carnosine system investigated in the pH range below 7.[31–34] Because the possible existence of other species can be limited by solubility restrictions, we have reduced our measurements to the usually critical neutral pH.

The ESI mass spectrum of the aqueous solution at pH 7.2 of an equimolar carnosine/zinc nitrate mixture is reported in Fig. 4. Considerable differences can be noticed on comparing this spectrum with that of Fig. 3(a). The monomeric species region presents essentially intense peaks at *m/z* 210.1, 227.1, 249.1 and 271.1, due to pure carnosine species, whereas two clusters of intense signals (see expanded inset) constituted, respectively, from peaks at *m/z* 289.0, 291.0 and 293.0 and at *m/z* 311.0, 313.0 and 315.0 (whose relative abundances agree with the natural zinc isotopic ratios, Table), correspond to monomeric carnosine/zinc species (Car-2$H + Zn$), detected as H^+ and Na^+ adducts in which a zinc ion, as in the copper case, substitutes two protons of the carnosine molecule.

The region of the spectrum corresponding to the dimeric species of Fig. 4 is also different. In fact, besides a low intensity peak at *m/z* 453.2 due to a proton-bound dimer of carnosine, only low intensity signals due to dimeric (Car-2$H + Zn$)$_2$ species appear in the spectrum as complex clusters at *m/z* 577.0, as the H^+ adduct, and at *m/z* 599.0, as the Na^+ adduct. In contrast, two clusters of intense peaks at *m/z* 515.1 (as H^+ ion) and at *m/z* 537.1 (as Na^+ ion), due to [(Car-H)$_2$$Zn$] species in which the zinc ion is bound to the carboxyl groups of the two carnosine units, appear in the spectrum. Furthermore, other weak signals due to heavier [Car(Car-2$H + Zn$)$_n$] species at *m/z* 803.1 and 1091.2 (as H^+) and 825.1 (as Na^+, see inset in Fig. 4) are also observed in the spectrum. Similar results (omitted for brevity) were also obtained using zinc acetate instead of the nitrate salt.

CONCLUSIONS

Apart from the early investigations by Martin,[31] few data are available on the zinc complexes of carnosine:[32–34] potentiometric studies were limited to pH <7.5 because of precipitation phenomena occurring at higher pH, even for high ligand/metal ion ratios, and thus only the stability constant values of [Zn(Car) H]$^+$ and [Zn(Car)] complexes were reported.[31–34]

ESI-MS allowed us to obtain a more complete speciation and, at the investigated pH, in addition to the (Car-2$H + Zn$) complex, also the bis-complex species [(Car-H)$_2$$Zn$] was determined, whose formation was not ascertained by means of classical potentiometric measurements because of the low stability of zinc complexes. However, the analysis of the precipitate obtained at pH >7.5 showed a ligand/metal ion ratio of 1:1 for the Zn/Car system;[12] the consumption of two base equivalents (with respect to the zinc) was attributed to the metal ion promoted deprotonation of both the terminal amino group and the peptide nitrogen.

Exploring by ESI-MS a concentration range lower than that usually employed in pH-metric titrations (to avoid precipitation phenomena), it was possible to describe the speciation of the Zn/Car system in a more complete manner; as an example, our data indicate the formation also of dimeric and trimeric [Car(Car-2$H + Zn$)$_n$] species (heavier species also appear with lower intensity in the spectrum), supporting previous data[8] about the possibility that zinc can promote oligomeric species formation as other metal ions.

In the copper/carnosine case, several researchers have conducted thermodynamic and spectroscopic investigations in solution to obtain both structural information and a correct speciation,[11,25,27,30,32,35–37] and a general agreement about the existence of species with a ligand to copper(II) ratio of 2 and of a monomeric deprotonated complex was reached. Our ESI-MS data support these previously reported results, throwing light on the existence of the [(Car-H)$_2$$Cu$] complex. As a consequence of the reducing effect of hydrazine (generally present in the carnosine samples), the peak of the monomeric deprotonated species is absent in the ESI spectra whereas the signal due to a monomeric copper(I) complex appears. Furthermore, signals due to a new trimeric species formed in the copper(II)/carnosine system also result.

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