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N^{α}-(1-Deoxy-D-fructos-1-yl)-L-histidine ("D-Fructose-L-histidine"): a Potent Copper Chelator from Tomato Powder

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Dried fruits and vegetables are known for their high content of D-fructose-amino acids, or Amadori compounds, which appear at the initial step of the Maillard reaction and may participate in redox reactions mediated by trace metals. In this study, we investigated complexation between Cu(II) and N^{α}-(1-deoxy-D-fructos-1-yl)-L-histidine (D-fructose-L-histidine, FruHis). The content of FruHis in two types of commercial tomato powders was estimated by GLC-MS, using single ion monitoring of trimethylsilylated FruHis hydroxyoximate, as 40 mg/100 g, whereas the concentration of free histidine in the powder samples was about 53 mg/100 g. The Cu(II)-binding ability of FruHis was studied along with structurally related molecules L-histidine, dipeptide L-carnosine, and N^{α}-(1-deoxy-D-fructos-1-yl)-L-arginine (FruArg) at 25 °C using pH-potentiometric titrations. Analysis of the titration curves showed that formation of Cu(II)−FruHis complex species occurs at pH values as low as 2 and that the complexes were redox stable in the pH range 2−10.5, at least for the time of the experiment. At physiological pH, Cu(II) and FruHis form a dominant coordination species of composition MLH_{-1} (log $\beta = 5.67$), with a presumably deprotonated anomeric hydroxyl group of the fructose portion. The apparent stability constant of 1:1 complexes formed by FruHis and Cu(II) in neutral aqueous solutions is about 10^4 times higher than similar values calculated for L-histidine, L-carnosine, and FruArg. FruHis nearly completely protected hydroxyl radical-mediated fragmentation of polymeric DNA in the presence of the Cu/H_2O_2/ascorbate system, whereas neither of the reference compounds could inhibit the DNA fragmentation as efficiently in similar conditions. These results warrant further investigation of FruHis as a potential food-related antioxidant.

KEYWORDS: Amadori compound; D-fructose-L-histidine; N^{α}-(1-deoxy-D-fructos-1-yl)-L-histidine; d-fructose-L-arginine; N^{α}-(1-deoxy-D-fructos-1-yl)-L-arginine; histidine; carnosine; tomato; antioxidant; copper; chelation; GLC-MS

INTRODUCTION

Free amino acids and monosaccharides are present in foods in significant concentrations and may chemically interact during food processing at elevated temperatures, dehydration, and upon storage. The interaction of amino acids with glucose and other reducing sugars constitutes the first step of the Maillard reaction, one of the mainstays of food chemistry (*1*). Initial condensation of free amino acids with glucose in dried vegetables, fruits, and other food products brings about formation of D-fructose-amino acids, also known as Amadori rearrangement products, which may constitute over 10% of the water-solubles per dry weight (*2*).

The Maillard reaction is often regarded as a detrimental event leading to a loss in nutritional value of foods, a rise of mutagenic agents in foods, glycoxidation, and related physiological problems in diabetes and aging. Nevertheless, the chemical pathways of the Maillard reaction are countless and, in many instances, could as well bring about beneficial effects for human health (*3*). Thus, we previously reported that some Amadori compounds derived from D-lactose possess antitumor properties (*4, 5*), whereas other researchers investigated D-fructose-peptides as prospective immunostimulants (*6*) or analgetics (*7*). There was a number of publications on the antioxidant potential of the Maillard reaction intermediates and products, such as N^{α}-(1-deoxy-D-fructos-1-yl)-L-arginine (*8*), heterocyclic volatiles (*9*), polymeric melanoidins, (*10*) etc. Histidine and histidine-containing peptides, notably L-carnosine, belong to an important class of water-soluble antioxidants in foods (*11*). The accumulating literature data suggest that modification of free histidine with glucose via the Maillard reaction in foods may also result in production of efficient metal chelators and potential antioxidants (*12, 13*). We and others have previously evaluated the ability of some D-fructose-amino acids to chelate Cu(II) and

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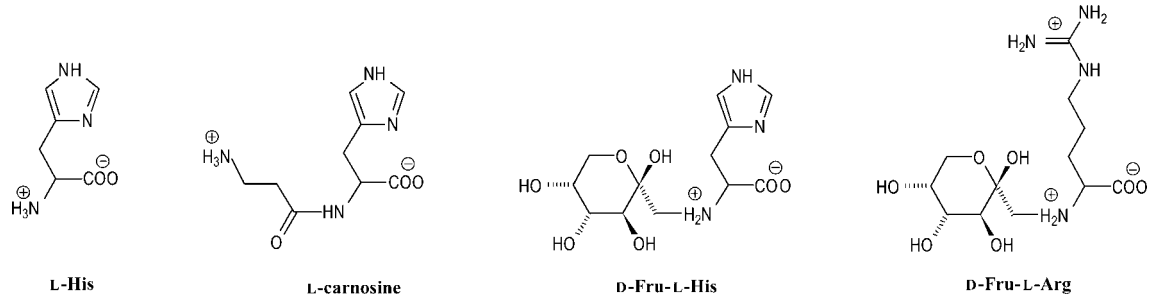
Scheme 1. Structures of major ionic and tautomeric forms of the ligands in equilibria at pH 7



other metal ions (*14–17*). These studies indicated that fructosyl residue in the Amadori compounds may participate in metal binding and thus increase the stability of Cu(II) complexes with D-fructose-α-amino amino acids as compared to the parent-free amino acids. Here we report our initial studies on Cu(II)-binding and antioxidant abilities of an Amadori compound, N^{α}-(1-deoxy-D-fructos-1-yl)-L-histidine (or D-fructose-L-histidine, FruHis), and we compare these to some structurally related antioxidants (**Scheme 1**).

MATERIALS AND METHODS

Samples and Reagents. A panel of D-fructose-amino acid reference standards was from a collection previously prepared in our laboratory using published general methods (*4, 18–20*). Their purity was confirmed by TLC immediately before the derivatization for GLC-MS experiments. The tomato paste samples were from the Morning Star Co. (Los Banos, CA), and the samples of tomato powder (TRANSA, Villanueva de la Serena-Badajos, Spain) were obtained from Henry Broch & Co. (Libertyville, IL). Trimethylsilylimidazole (TSIM) and *N,O*-bis-trimethylsilyl-trifluoroacetamide (BSTFA) were purchased from Pierce (Rockford, IL). Commercial L-histidine hydrochloride and L-carnosine (both from Sigma, St Louis, MO) were of high purity and used without further purification.

Preparation of N^{α}-(1-Deoxy-D-fructos-1-yl)-L-histidine. Anhydrous D-glucose (36 g, 200 mmoles) was added to 90 mL of glycerol/methanol (1:3), and the mixture was refluxed for 30 min Then, L-histidine (9.3 g, 60 mmoles), sodium pyrosulfite (1.5 g), and acetic acid (7 mL) were added, and the reaction mixture was stirred under reflux for 45 min. The clear, amber-colored solution was cooled and diluted with twice the volume of deionized water. The solution was passed through a column packed with 120 mL of Amberlite IRN-77 in protonated form. After washing the column with 1 L of deionized water, FruHis was eluted with 0.15 M pyridine/0.3 M acetic acid buffer. The combined fractions were decolorized with 4 g of activated carbon and evaporated to a syrup. After dilution with 50 mL of methanol and storage at 4 °C for several days, the solution deposited a crystalline precipitate. This material was collected on a filter, washed with cold water, methanol, and dried overnight over $CaCl_2$. Additional amounts of FruHis were obtained from the filtrates upon evaporation to a small volume. Overall yield of FruHis·H_2O: 7.7 g (38% based on histidine). mp 183–188° (dec.) (lit. for dihydrate (*19*) or anhydrous (*20*) mp 120–130°, dec.); $[\alpha]_D^{25} = -23°$, $c = 1.2$, 0.05 M HCl) (lit. (*19*). −30.5°, $c = 0.3$, water). FAB MS: major, other than solvent (glycerol), peaks are 318 $[M + H]^+$ and 410 $[M + H + \text{glycerol}]^+$. Exact mass of $[M + H]^+$ ion. Calcd for $C_{12}H_{20}N_3O_7$: 318.1301; found: 318.1318. The ^1H and ^{13}C NMR spectra agree with the literature (*19*). The X-ray diffraction data were published elsewhere (*21*). Anal. Calcd for $C_{12}H_{19}N_3O_7 \cdot H_2O$: C, 42.98; H, 6.31; N, 12.53. Found: C, 42.95; H, 6.21; N, 12.43.

Extraction of D-Fructose-Amino Acids from Tomato Products. To 3.0 g of tomato powder or 9.0 g of tomato paste, 20 mL of distilled water was added, which contained 0.1 mg each of xylitol and trehalose as internal standards. After mixing for 30 min, aliquots were centrifuged at 80 000 rpm (Optima TLX Ultracentrifuge, Beckman, Fullerton, CA) for 20 min at 4 °C, and the clear supernatant was subjected to centrifugal ultrafiltration (AmiconUltra-15 Centrifugal Filter Device 5,000 MWCO) at 3,500*g* for 3 h at 4 °C. The resulting filtrate was then freeze-dried for further analysis. Extractions were performed using $N = 10$ samples per batch of tomato powder or paste.

Formation of the Trimethylsilyl Derivatives of Hydroxyoximes of D-Fructose-Amino Acids. To 5.0 mg of the freeze-dried tomato powder extract in a Teflon-capped 2.0 mL autoinjector vial, equipped with a micro stir-bar, 10 μL of distilled water was added. Synthetic FruHis reference was similarly treated with water that contained 0.1 mg of trehalose, as internal standard. After 5 min, and with continued stirring, 100 μL of an oximation cocktail (comprised of 300 mg of hydroxylamine−HCl, 1.0 mL methanol, 2.7 mL pyridine, and 0.47 mL 1-dimethylamino-2-propanol) was added, and the vial was heated at 70 °C for 5 min. Following the reduction of the vial contents to near dryness under a stream of dry nitrogen, 100 μL of pyridine, 100 μL of TSIM, and 300 μL of BSTFA were sequentially added to the capped vial. After heating at 60 °C for 10 min, the vial was then subjected to low-speed centrifugation (300*g*) to pellet any light precipitate that may have formed.

Gas–Liquid Chromatography–Mass Spectrometry (GLC/MS). Aliquots (2 μL, split ratio of 1:25) of the trimethylsilyl derivatives of FruHis hydroxyoximes were analyzed on a Varian CP-3800 gas–liquid chromatograph (GLC), equipped with a CP-8400 autosampler and interfaced with a Varian 1200L triple Quadrupole MS/MS mass spectrometer. Separations were performed on a 30 M × 0.25 mm VF-5 ms Varian (Palo Alto, CA) FactorFOUR GLC capillary column, with a film thickness of 0.25 μm. The GLC was programmed to ramp from 200 to 300 °C at 6 °C per minute with an initial hold at 200 °C for 5 min. With a GLC/MS transfer line temperature of 300 °C and an ion source temperature at 200 °C, mass spectra were recorded at −70 eV with electron ionization in the positive (EI) mode, scanning from 70 to 950 amu with a 0.5 s scan time. Data were processed on Varian MS Workstation software.

Amino Acid Analysis of aqueous extracts from tomato products was performed using a Hitachi L8800 amino acid analyzer. A lithium buffer system and cation exchange column were used for separation of amino acids. Amino acids were derivatized with ninhydrin and were detected spectrophotometrically.

Potentiometric Titration Experiments were performed as follows. All solutions were made up using freshly double-distilled water (Corning NanoPure). A stock electrolyte solution was prepared from crystalline potassium nitrate (Mallinckrodt, AR) to give a 0.2 M concentration. Titrant solutions were prepared by diluting a 50% sodium hydroxide solution (Fisher, certified for content of $Na_2CO_3 < 0.2\%$) or certified 1 M nitric acid (Alfa Aesar) in water under argon and were protected from CO_2. Their concentrations were checked titrimetrically against potassium hydrogen phthalate or TRIS, respectively. All titrations were performed with an automatic titrator, Metrohm 736 GP Titrino, equipped with two titration units delivering the acid and base titrants. The electrodes were a Metrohm SGJ combination glass electrode (No. 6.022.100) and a Phoenix combination copper ion selective electrode. The system was daily calibrated with standard buffers followed by titration of nitric acid, pH 2, in 0.2 M KNO_3 with the alkali until pH 11.3 and back with the acid. The relationship that expresses pH-meter readings as a function of analytical hydrogen ion concentration is shown in eq 1.

$$pH_{read} = C + e \cdot \log[H^+] \quad (1)$$

This procedure typically afforded values for efficiency (e) of ≈ -0.99 and C $\approx$ 0.06 from acidic (pH 2–3) and a pK_W value of 13.82 $\pm$ 0.04 from basic (pH 10.8–11.3) portions of the titration curves. The influence of the liquid junction potential on linearity of the glass electrode function was not substantial within the pH region of these experiments (2–11) and was not taken into account. Normally, at least two series of titration curves were obtained for each ligand concentration (4–8 mM) and metal-to-ligand ratio between 1:1 and 1:3. Titers of the compounds were refined during the calculations of protonation constants by a triangular simplex method. All titrations were performed at 25.0 $\pm$ 0.1 °C. A stream of water saturated purified argon was blanketing the solutions to prevent carbon dioxide influence on the titration data at high pH.

A computer program PSEQUAD (*22*) was used to calculate protonation and metal complexation constants in the form of overall formation constants (β_{pqr}) of the equilibrium (eq 2),

$$pCu + qL + rH \rightleftarrows [Cu_pL_qH_r] \quad (2)$$

from which more ordinary acid dissociation constants may be derived (eqs 3−5).

$$pK_{a3} = \log \beta_{011} \quad (3)$$

$$pK_{a2} = \log \beta_{012} - \log \beta_{011} \quad (4)$$

$$pK_{a1} = \log \beta_{013} - \log \beta_{012} \quad (5)$$

The reliability of the data obtained was supported according to IUPAC recommendations (*23*) by the comparison of our glycine and L-histidine acid dissociation constant values with the recommended statistical values (*24*, *25*): glycine pK_{a1} = 2.40 $\pm$ 0.05 (our), 2.37 $\pm$ 0.07 (rec); pK_{a2} = 9.59 $\pm$ 0.01 (our), 9.60 $\pm$ 0.05 (rec); L-histidine pK_{a1} = 1.71 $\pm$ 0.03 (our), 1.72 $\pm$ 0.09 (rec); pK_{a2} = 6.05 $\pm$ 0.01 (our), 6.05 $\pm$ 0.03 (rec); pK_{a3} = 9.09 $\pm$ 0.01 (our), 9.11 $\pm$ 0.02 (rec).

EPR Spectra were obtained with a Bruker EMX spectrometer in the X-band at 120 or 190 K with 100 kHz modulation frequency and 20 G modulation amplitude. The instrument was equipped with a variable-temperature probe accepting samples in 0.6 mm ID × 0.84 mm OD glass capillaries. The sample solutions contained 9 mM Cu(II), 10 mM ligand, and 0.3 M sucrose in double-distilled water; the pH was adjusted to desired values with 1 M NaOH. Normally, 8 scans were averaged for each spectrum.

DNA Oxidative Degradation. To a solution of 50 μg of polymeric DNA (from calf thymus, Sigma) per mL of chelex-treated PBS, pH 7, were added, in order, an antioxidant, $CuSO_4$ to the final 50 μM, H_2O_2 to the final 2 mM, and ascorbic acid to the final 2 mM concentration. The reaction was left to proceed at room temperature for 20 min and was then stopped by the addition of EDTA to bring the solution to the final concentration of 10 mM. Ethidium bromide was added to 20 μg/mL, and the fluorescence of the solutions was measured at 508/590 nm.

RESULTS

Determination of FruHis in tomato paste and tomato powder batches was done by GLC-MS. As shown in **Figure 1A**, the trimethylsilyl derivative of the hydroxyoxime of standard FruHis displayed characteristic syn- and anti- chromatographic peaks that are expected for oxime isomers. As shown in the mass spectral profile (**Figure 1A** insert), nearly identical for both isomers, derivatized FruHis presented with a unique fragment ion at *m*/*z* 747, representing a loss of trimethylsiloxide (TMSO). The imidazolium ion, arising from the loss of the imidazole side-chain (R) of histidine, forms the base ion peak at *m*/*z* 154. Although small molecular ion (M^+) and mass minus 15 (M − 15) fragment ions (i.e., *m*/*z* 836 and 821, respectively) are seen, other significant fragment ions are observed at *m*/*z* 657, 567, and 477, representing sequential losses of trimethylsilanol (TMSOH) from the *m*/*z* 747 ion.

A representative total ion chromatogram (TIC) of derivatized tomato powder extract is shown in **Figure 1B**. Extraction of fragment ion *m*/*z* 747 from this TIC is presented in the selected ion chromatogram shown in **Figure 1C** demonstrating the presence of FruHis with confirmatory *syn*- and *anti*- peaks. Estimated FruHis content in the particular batches of cold break (CB) and hot break (HB) tomato powder was 38.2 $\pm$ 0.2 and 41.4 $\pm$ 0.4 mg per 100 g, respectively. In contrast, FruHis was not detected in any (CB, HB) tomato paste samples. The amino acid analysis of the tomato paste samples gave free histidine content in the range of 77–93 mg/ 100 g dry weight, while in the tomato powder samples this value decreased to 52–54 mg/ 100 g.

The thermodynamic proton- and copper(II)-binding constants of FruHis and its structurally related antioxidant analogs − L-histidine, L-carnosine, and FruArg (**Scheme 1**) − were established pH-potentiometrically. Some of the typical pH-metric titration curves of FruHis and FruArg are demonstrated in **Figure 2**. The titration curves were obtained by first acidifying the ligand or copper/ligand solutions to pH ~2. Then NaOH titrant was added in small increments until pH 11 (ligands only) or pH 10 (copper/ligand mixtures). Immediately after reaching the indicated alkalinity, the solutions were titrated with dilute HNO_3 back to pH 2. The titration curves, expressed as pH vs $C_{titrant}/C_{ligand}$ plots, were overlaid. As shown in **Figure 2**, there is a total similarity between the acid-to-base and the base-to-acid titration curves, which indicates that the fructose-amino acids FruHis and FruArg are stable in the presence of copper(II) within the pH interval from 2 through 10, at least during the titration experiment (30–60 min). It is also evident from the shapes of the titration curves of copper(II)−FruHis, copper(II)−FruArg (**Figure 2**) and copper(II)−L-carnosine (not shown) that more than 3 equiv. of the base/acid titrant per 1 equiv. of ligand are used in the interval of pH 2 through 8. This can be explained either by the hydrolysis of the complexes or by the deprotonation of nonacidic groups, such as amide in peptide L-carnosine or carbohydrate hydroxyl in the D-fructose-amino acids, as a result of their coordination to the copper(II) ion.

The equilibrium speciation models used for thermodynamic calculations in systems containing L-histidine and L-carnosine were taken from the literature (*25, 26*). By convention, amide protons in peptides, such as L-carnosine, are not considered as acidic and are not normally included in calculations of acid–base equilibria for such ligands. Copper(II) ions, however, replace the amide protons upon complex formation with L-carnosine. This reaction leads to formation of species with negative stoichiometric indexes, such as $CuLH_{-1}$, which we have included in the equilibrium models. Similarly, we have assumed that, at pH < 11, acid dissociation of the guanidino group and anomeric hydroxyl group of the fructose in FruArg and FruHis could be neglected, because the acidities for these groups, with respective pK_a values of about 12.3–12.5 (*27, 28*), are too low. In analogy with copper(II)−peptide complexes, coordination species, which were formed by replacement of the carbohydrate hydroxyl protons or the hydrolysis, obtained negative stoichiometric indexes for hydrogen, even though the guanidino group of FruArg remained protonated in such complexes.

A number of spectroscopic and crystallographic studies of the copper(II)−L-carnosine system have established that a major complex species existing in aqueous solution equilibria at low ligand/metal ratio and pH 6−10 is a dimeric complex $[Cu_2L_2H_{-2}]$, with the carboxylate oxygen and the

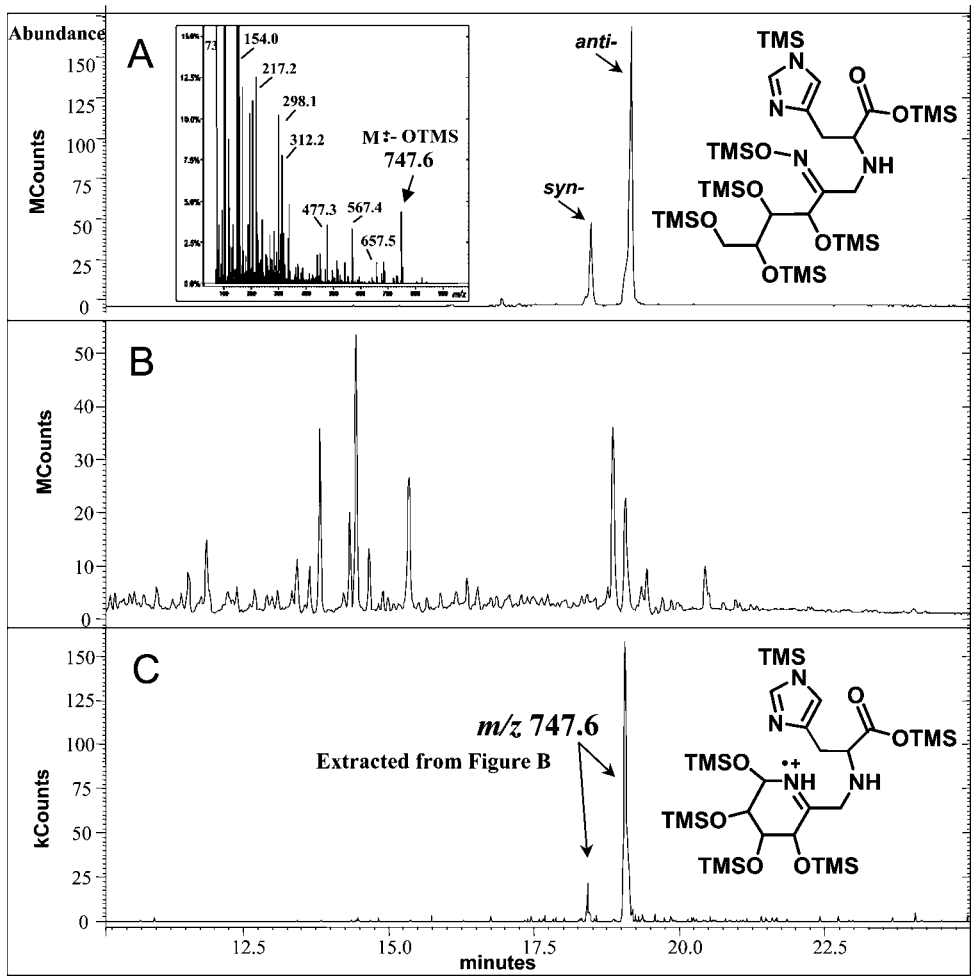


Figure 1. GLC-MS total ion chromatograms (TIC) of the TMS derivatives of the hydroxyoximes of standard FruHis and FruHis in tomato powder. Total ion spectra were scanned from 70 to 950 amu. (**A**) TIC showing the syn and anti ion peaks of the TMS derivatives of the hydroxyoximes of synthetic FruHis with an insert presenting the mass spectral profile of the anti ion peak. With the formation of *m*/*z* 747.6 (M − TMSO), fragment ions at *m*/*z* 657, 567, and 477, respectively, represent sequential losses of trimethylsilanol (−TMSOH). (**B**) Typical TIC of a derivatized tomato powder preparation. (**C**) Selected fragment ion chromatogram at *m*/*z* 747.6 (proposed fragment ion pictured), unique to the TMS derivatives of the hydroxyoximes of FruHis, from the TIC in panel **B**.

amino-, imidazole-, and deprotonated amido nitrogen atoms occupying the coordination square around the copper(II) ion (*26*, *29*–*31*). Because FruHis possesses multiple heteroatom groups, including amino and imidazole nitrogens and carboxylate and hydroxyl oxygen atoms, which may participate in coordination to copper(II), we have considered a possibility that dimeric complexes could be included into the Cu/FruHis speciation model, as well. However, incorporation of the $Cu_2L_2H_{-2}$ species instead of or along with the $CuLH_{-1}$ species into the equilibrium models for copper(II)−FruHis or copper(II)−FruArg systems did not provide the best fit in the calculations of the potentiometric data. The best fit in these calculations was achieved when dimeric complexes were excluded from the model. In addition, we recorded the EPR spectra (X-band) of frozen aqueous solutions containing equimolar concentrations of copper(II) and L-carnosine, FruHis, or FruArg at pH values corresponding to maximal yields of the monodeprotonated complexes (**Figure 3**). The EPR spectrum of the copper(II)−L-carnosine complex shows an expected, for a dimeric $Cu_2L_2H_{-2}$ species, hyperfine pattern of seven peaks in the low field, at $g_{||}$, with apparent intensities of 1:2:3:4:3:2:1, arising from the magnetic coupling of the two copper(II) nuclei having spins of 3/2 each. In contrast, the EPR spectra of copper(II) complexes with FruHis and FruArg in the same field region contain only four 1:1:1:1 signals, as would be expected for a species containing only one copper(II) ion. A broad peak at $g_{\perp}$ (at ∼3400 G) in the copper(II)−L-carnosine EPR spectrum, which has also been attributed (*29*) to the dimeric copper(II), is absent in the spectra of solutions containing copper(II) and either of the D-fructose-L-amino acids. Therefore, no apparent evidence have been obtained for dimeric species in equilibria of copper(II)−FruHis or −FruArg complexes to justify their inclusion into the stability constants calculations.

The calculated overall protonation and copper complexation constants are listed in **Table 1**. As noted above, the hydroxyl, amide, and guanidino groups are considered as not acidic in these equilibrium models. Therefore, in the cases of L-histidine, L-carnosine, and FruHis, a species of composition L in **Table 1** is assigned to a single negatively charged ligand, with carboxylate as the only negatively charged group. For FruArg, L in **Table 1** stands for a zwitterion with a zero net charge, negatively charged carboxylate, and positively charged guanidine residue. The compositions of copper(II) complex species sets for each ligand were carefully selected based on the above considerations and were confirmed by the best fitting of the experimental data that could be achieved during calculations. In the case of FruArg, because the ligand was available as an acetate salt, the equilibrium model also contained species of

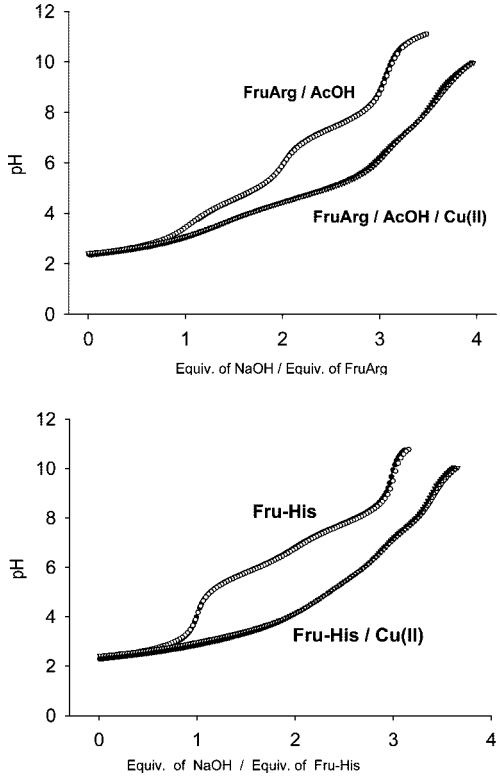


Figure 2. pH-Titration curves of (**A**) copper(II)−FruHis and (**B**) copper(II)−FruArg−acetate systems. $C_{Cu} = 3$ mM; $C_L = 6$ mM. The solutions were titrated first with NaOH, starting at low pH and finishing at pH 10–11 (white circles and triangles), then titrated back with HNO_3 (black circles and triangles). For the illustrative purpose, both titrant volumes were converted into "equivalents of NaOH".

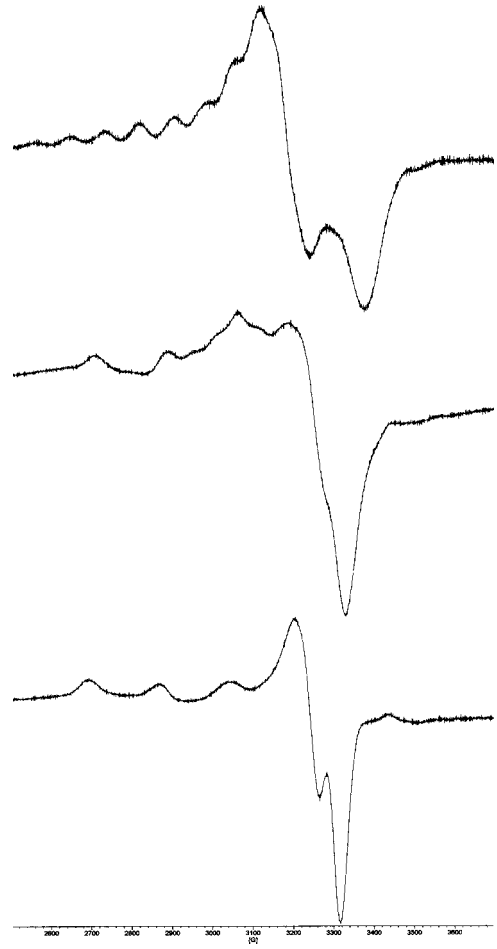


Figure 3. EPR spectra (X-band, $\Delta M = 1$) of frozen solutions containing 9 mM $Cu(NO_3)_2$, 0.3 M sucrose, and 10 mM ligand, from top to bottom: L-carnosine, pH 7.5; FruHis, pH 6.8; and FruArg, pH 6.8.

acetic acid and copper(II)−acetate complexes, with established values of $\log \beta_{011} = 4.54$, $\log \beta_{110} = 2.21$, and $\log \beta_{120} = 3.40$. No ternary complex species were considered in this model.

The concentration distribution of the coordination species formed in the copper(II)−FruHis system at a 2-fold excess of the ligand over the metal is plotted as a function of pH in **Figure 4A**. It is obvious from this diagram that complex species with a metal/ligand stoichiometric ratio of 1:1 strongly dominate, with $[CuLH_{-1}]$ being the most favorable complex in a broad range of pH. In contrast, FruArg forms increasing amounts of 1:2 complexes with copper(II) upon increase in pH, such that $[CuL_2H_{-1}]^+$ becomes a major species at pH > 6.5 (**Figure 4B**). Noticeably, the copper(II) complex formation with FruArg and FruHis occurs in acidic medium; thus, there is over 20−30% of copper(II) bound by these ligands at pH values as low as 2.5, whereas for L-histidine and L-carnosine, such level of metal binding can be achieved only at pH > 3 and 4.5, respectively (data not shown).

The initial evaluation of the antioxidant potential of FruHis as compared to its structural analogs has been performed in a polymeric, double-stranded DNA degradation assay. The copper-catalyzed Fenton reaction was used to generate reactive oxygen species, predominantly hydroxyl radicals, in this assay. Fluorescence of DNA-intercalated dye propidium iodide was used as a measure of the DNA fragmentation level. The effects of FruHis and the related compounds on the protection of the DNA from oxidative fragmentation is summarized in **Figure 5**. D-Fructose, a nonchelator for copper(II) in aqueous solutions, showed no protection. There was a small, but evident inhibition of the DNA degradation by FruArg at the inhibitor concentrations exceeding 100 μM. L-Histidine and L-carnosine exerted similar inhibition activity in this assay, in a concentration-dependent manner. We did not observe, however, a total inhibition of DNA oxidative degradation by these compounds, even at concentrations as high as 1 mM. In contrast, FruHis at 500 μM completely protected DNA from the cleavage, whereas at the lower concentrations, it exerted better protection against the ROS-induced polynucleic acid fragmentation than any of the tested structural analogs within the entire investigated concentration range.

DISCUSSION

That FruHis readily forms in the D-glucose/L-histidine reaction and thus could be present in dried fruits and vegetables, along with other D-fructose-amino acids, was established decades ago (*20*). This report, however, is the first attempt to quantitatively evaluate this Amadori compound in foods, specifically in tomato powder, which is known for a high content of D-fructose-amino acids (*2*). Because the formation and degradation of FruHis do not seem to differ dramatically from those known for the majority of food-related D-fructose-amino acids, one would assume that a content of FruHis in glucose-rich foods will correlate with a relative content of free histidine. Indeed, according to our GLC-MS and amino acid analysis data, free histidine content in the tomato powder is about one-third less than that in the tomato paste samples, which correlates well with the determined content of FruHis in the tomato powder. It may be thus assumed that most of the free histidine loss in the tomato powder is due to its reaction with glucose upon drying.

Table 1. Protonation and Copper(II) Complexation Constants (log β_{pqr}[a]) for FruHis, L-Histidine, L-Carnosine, and FruArg[b]

species	FruHis	L-histidine	L-carnosine	FruArg
HL	7.60 ± 0.01 (7.60)[c]	9.09 ± 0.01 (9.09)	9.36 ± 0.01 (9.36)	7.36 ± 0.01 (7.36)
H_2L	13.28 ± 0.01 (6.68)	15.14 ± 0.01 (6.05)	16.16 ± 0.01 (6.80)	9.03 ± 0.02 (1.67)
H_3L	14.71 ± 0.03 (1.43)	16.85 ± 0.03 (1.71)	18.82 ± 0.01 (2.66)	
CuLH	13.06 ± 0.02	13.92 ± 0.03	13.29 ± 0.02	9.11 ± 0.07
CuL	9.91 ± 0.01	9.8 ± 0.3	8.49 ± 0.01	6.69 ± 0.01
$CuLH_{-1}$	5.67 ± 0.02	1.7 ± 0.2		1.53 ± 0.09
$CuLH_{-2}$			−8.28 ± 0.08	
CuL_2H_2		27.64 ± 0.04		
CuL_2H		23.76 ± 0.01		
CuL_2	15.41 ± 0.03	18.13 ± 0.05		11.88 ± 0.09
CuL_2H_{-1}	8.22 ± 0.03			5.60 ± 0.03
CuL_2H_{-2}				−3.3 ± 0.1
$Cu_2L_2H_{-2}$			8.37 ± 0.05	

[a] $\beta_{pqr} = [Cu_pL_qH_l]/[Cu]^p[L]^q[H]^r$ [b] $I = 0.2$ M KNO_3, $t = 25.0 \pm 0.1$ °C. [c] Acid dissociation constants are given in parentheses.

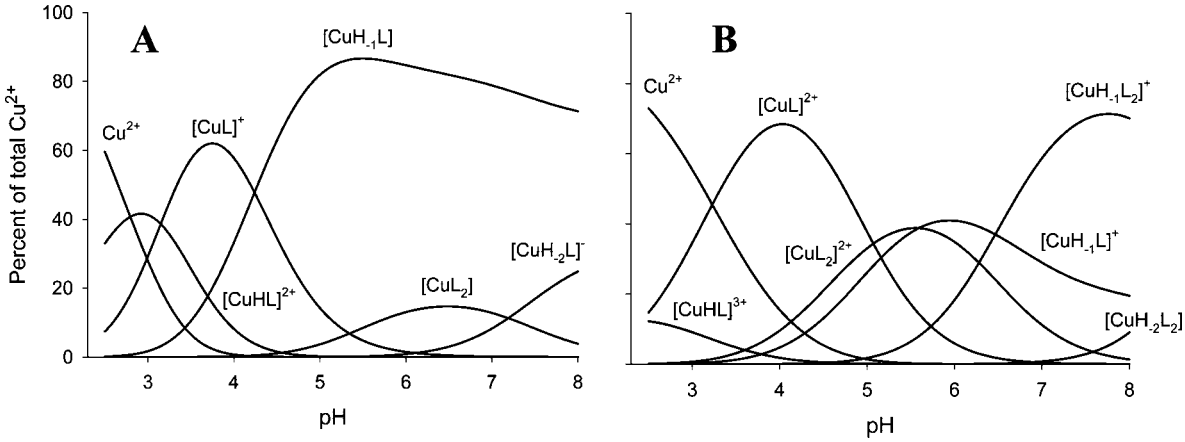


Figure 4. Concentration distribution of the complex species formed in **A**) Cu^{2+} - FruHis system, **B**) Cu^{2+} - FruArg system as a function of pH. $C_{Cu} = 2$ mM; $C_L = 4$ mM.

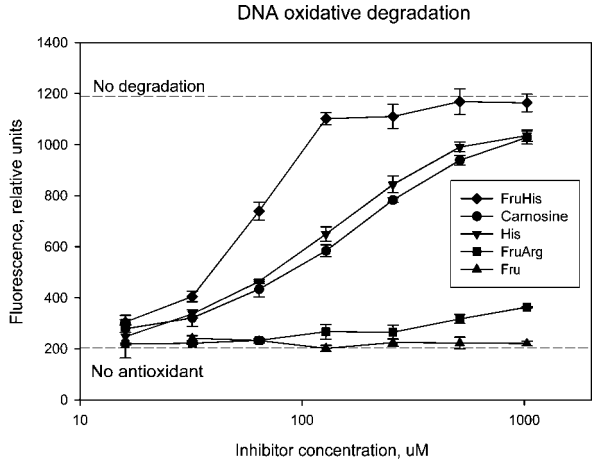


Figure 5. Degradative oxidation of polymeric DNA by Cu^{2+}/H_2O_2/ascorbate in presence of antioxidants. DNA depolymerization is probed by propidium iodide.

GLC-MS, as we have demonstrated here, is an appealing analytical tool for quantitation of FruHis and other basic Amadori compounds; however, more work is needed to optimize and to validate this method for routine use.

The ability to chelate redox reactive ions of metals such as copper or iron is one of the key properties of many antioxidants. A strong chelator may hinder availability of the catalytically active metal ion to an oxidant or a substrate and/or it may stabilize one of the oxidation states of the chelated metal, thus making the one-electron transition reactions unfavorable. To determine whether FruHis is a "strong" chelator for copper(II) as compared to other structurally related antioxidants, one would need to compare thermodynamic stabilities of copper(II) complexes of FruHis with those obtained for other ligands of interest. This could be readily done by comparison of the complex formation constants in a situation when each of the contesting ligands forms a single metal complex species of the same composition in solution. As it follows from **Table 1** and **Figure 4**, this is not the case for any of the investigated ligands. Every antioxidant studied in this work forms a unique set of coordination species with copper(II) of varied composition and concentration distributions as functions of pH, ligand, and copper(II) concentrations. Therefore, direct use of the overall complex formation constants (β) taken from **Table 1** is not feasible for a comparison of the chelating "strength". From a practical point of view, however, one would like to know which of the chelators provides a better binding of the copper(II) ion in similar conditions (pH, ionic strength, concentrations of metal and ligand, etc.). Here we will apply two approaches: a comparison of the apparent, or conditional, stability constants and a comparison of equilibrium free-metal concentrations.

When a solution contains a ligand at the concentration equal to or lower than that of a metal, it may be assumed that nearly all complex species formed in this solution will have a 1:1 metal/ligand ratio, but may differ in titratable hydrogen content and ability to associate. This assumption excludes a possibility for the existence of complex species with the metal/ligand ratio above unity, but for extensively investigated copper(II) complexation with L-histidine, L-carnosine, or their structural analogs, no stability for such species was credibly established

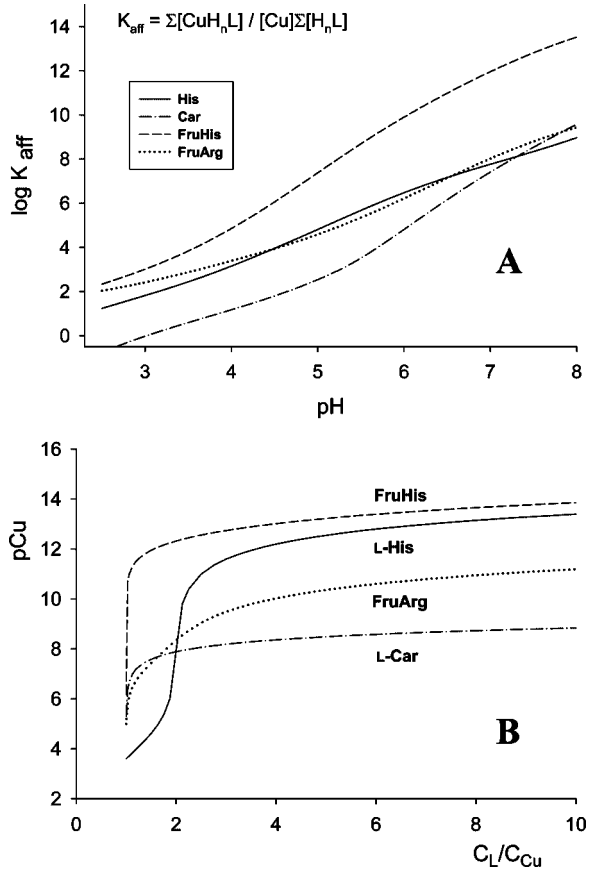


Figure 6. Relative affinities of ligands towards copper(II). (**A**) Apparent stability constants of copper(II) complexes with 1:1 metal/ligand composition, as a function of pH. (**B**) Free $[Cu^{2+}]$ concentration as a function of C_L at pH 7.2 and $C_{Cu} = 2$ mM.

and reported in the literature. Then, at any given pH, we may consider the equilibrium

$$Cu + L \rightleftarrows CuL$$

where L is an unbound ligand, and equlibrium concentration $[L] = \Sigma([L] + [HL] + [H2L] + \ldots)$, Cu is unbound copper(II), and CuL is a sum of all complex species, including dimeric ones, so that the equilibrium concentration is $[CuL] = \Sigma([CuLH] + [CuL] + [CuLH_{-1}] + \ldots)$. Then, the apparent (conditional) stability constant (shown below),

$$K_{aff} = \frac{[CuL]}{[Cu][L]}$$

can be calculated at any given pH using the overall complex formation constants from **Table 1**. The result of such calculations is demonstrated in **Figure 6A** in form of log K_{aff} vs pH graphs. Evidently, the apparent stability of 1:1 copper(II) complexes with FruHis is higher than that calculated for any other three ligands in the entire pH interval. For example, at physiological pH 7.4, values of K_{aff} for copper(II) complexation with L-His, L-carnosine, and FruArg are nearly equal, whereas the K_{aff} for FruHis exceeds the formers by about 4 orders of magnitude.

When the ligand/metal ratio is greater than 1, the above approach cannot be used because 1:1 and 1:2 complexes coexist in solutions containing copper(II) and any of the ligands from **Table 1**, as shown, for example, in **Figure 4**. In this case, the equilibrium free concentration of Cu^{2+} may serve a measure for comparison of relative affinities between copper(II) and the ligands at a given pH. That is, the lower residual free copper ion concentration in presence of a given ligand, as compared to another one, indicates the higher affinity and vice versa. Obviously, this parameter, unlike the apparent stability constants, will depend on both pH and total concentrations of metal and ligand. By fixing pH and total copper(II) concentration and using the overall complex formation constants from **Table 1**, we have calculated the equilibrium residual $[Cu^{2+}]$ as a function of total ligand concentration. The resulting graphs are shown in **Figure 6B** and clearly demonstrate the superior ability of FruHis to bind copper(II), as compared to the rest of the ligands. However, it should be noted that further extrapolation of the pCu vs C_L/C_{Cu} graphs into high values of C_L/C_{Cu} would be speculative as it would ignore the possible formation of complexes with higher stoichiometric ligand/metal ratios that are greater than 2:1 (*30*).

A potentiometric titration study alone does not provide essential information about a coordination mode in the copper(II) complexes with such novel ligands as FruHis and FruArg. An extensive spectroscopic study would be necessary to draw any credible conclusions about the exact geometry of the complex species. However, we may point out some factors that could lead to the enhanced affinity of FruHis toward copper(II). In the acidic medium, pH < 4, FruHis is likely coordinated to copper ions in the amino acid-like manner (*32*), with the secondary amino group, the carboxylate oxygen (forming a 5-membered chelate ring), and the imidazole nitrogen (forming a 6-membered chelate ring) taking places in the coordination sphere of copper(II). Such mode of coordination would give an advantage for FruHis over other ligands, L-carnosine (no formation of the stable chelate rings), FruArg (no imidazole, bidentate chelation mode only), and L-histidine (higher basicity of both the carboxylate and the amino groups in His as compared to FruHis means higher competition for the chelating atoms from protons). The formation of complex species $CuLH_{-1}$ at a pH as low as 4, and at higher pHs, suggests deprotonation, rather than hydrolysis, of the copper(II)−FruHis complexes. The candidate ligand groups for this process are the fructose anomeric hydroxyl at carbon-2 and the hydroxyl at carbon-3. The rest of the carbohydrate hydroxyl groups are too far from the amino nitrogen to form the chelate rings. The anomeric hydroxyl group is much more acidic ($pK_a \sim 12.3$) as compared to other carbohydrate hydroxyl groups (*27*) and would therefore be expected to deprotonate first upon its coordination to copper(II). Molecular modeling shows that the most abundant tautomer of FruHis, β-pyranose (*19, 21*), can potentially act as a pentadentate ligand for copper(II), thus favoring the formation of 1:1 complexes, with the two nitrogen atoms, water, and the hydroxyl-3 oxygen fitting into the equatorial plane of the coordination sphere and the anomeric hydroxyl oxygen and the carboxylate occupying the axial position, as shown in **Figure 7**.

The multivalent character of FruHis may explain its superior efficiency as copper(II) chelator in its deprotonated complex species as compared to other known copper(II) complexes of D-fructose-amino acids. For comparison, the overall stability of $CuLH_{-1}$ where L = FruHis (log $\beta_{11-1} = 5.67$) is about 14 000 times higher than the stability of $CuLH_{-1}$ with L = FruArg (log $\beta_{11-1} = 1.53$, **Table 1**), 3000–6000 times higher than the stability of the respective copper complex species where L = FruAaa and Aaa = aliphatic α-amino acid (log $\beta_{11-1} = 1.90$ through 2.23, taken from ref. [*15*]), and over three million times higher than that of the respective complex species with L = N^{α}-hippuryl-N^{ϵ}-D-fructose-L-lysine (log $\beta_{11-1} = -0.89$, taken from ref. [*17*]).

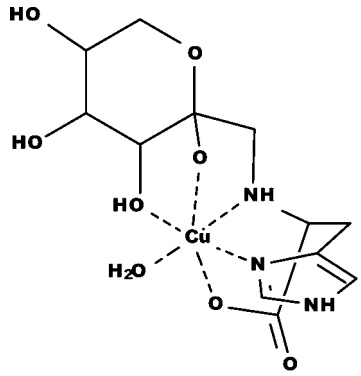


Figure 7. A hypothetical structure of [$CuLH_{-1}$] species in Cu (II)-FruHis complex equilibria.

Ingested D-fructose-amino acids resist digestion and can be partially absorbed into circulation, without being metabolized (*33*). Given that N^{α}-(1-deoxy-D-fructos-1-yl)-L-histidine may be present in measurable amounts in tomato powder and, possibly, in other dried fruits and vegetables, the data presented in this work suggest that FruHis may represent an example of a newly recognized potential dietary antioxidant. The relatively high affinity of FruHis to copper(II) at physiological pH ($\log K_{aff} > 12$, see **Figure 6A**) provides for very low residual free copper in the presence of FruHis (**Figure 6B**), and thus may translate into inactivation of this redox catalyst and protection of biologically important molecules from oxidative degradation in vivo. In tomato products, highly investigated antioxidants, such as lycopene, ascorbic acid, and phenolics, shown to be excellent scavengers of reactive oxygen species (ROS), are poor protectors of the metal-induced DNA degradation (*34*). At comparable concentrations to these antioxidants in tomato powder, FruHis demonstrates the potential to efficiently block metal-dependent oxidative damage of biopolymers but may also exhibit the ROS scavenging capabilities. Possessing these important attributes, FruHis should then be viewed as a prospective nutraceutical agent requiring further investigation.

It has long been recognized that histidine and its dipeptide derivatives carnosine, anserine, and homocarnosine are scavengers and inhibitors of ROS (*35*). The antioxidant capacity of L-carnosine and L-histidine is partly due to the metal-binding properties of these molecules and is also partly attributed to their reducing potential and other electron-donor properties. Specifically, histidine and its derivatives may: (1) inhibit redox reactions leading to production of hydroxyl radical, through chelation of copper and other transition metal ions that catalyze these reactions (*31*); (2) directly interact with singlet oxygen, or other ROS (*36*). The imidazole ring in L-carnosine and other histidine derivatives has been shown to deplete ROS through a mechanism of one-electron oxidation into imidazolone (*37*) or to trap degradative oxidation products through the Michael addition mechanism (*11*). The basicities of the imidazole nitrogens in L-carnosine and FruHis are very close (**Table 1**, pK_a's for the H_2L species are 6.8 and 6.7, respectively), which may reflect the similarity in electron densities on the imidazole rings. It can be assumed then that FruHis could also directly interact with ROS via imidazole oxidation. In addition, we have previously suggested that oxidation of D-fructose-amino acids can occur through a metal-independent one-electron mechanism and that it correlates with the enolization rate of the 1-amino-2-ketose (*38*). A further detailed investigation of the ability of FruHis to directly interact with ROS or other redox metal ions such as iron(II/III) is warranted to further evaluate the antioxidant potential of N^{α}-(1-deoxy-D-fructos-1-yl)-L-histidine in foods.

ABBREVIATIONS USED

BSTFA, *N,O*-bis-trimethylsilyl-trifluoroacetamide; EPR, electron paramagnetic resonance; FruArg, N^{α}-(1-deoxy-D-fructos-1-yl)-L-arginine; FruHis, N^{α}-(1-deoxy-D-fructos-1-yl)-L-histidine; GLC-MS, gas–liquid chromatography–mass spectrometry; PBS, phosphate buffered saline; ROS, reactive oxygen species; TIC, total ion current; TLC, thin layer chromatography; TSIM, trimethylsilylimidazole.

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