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Bioaccessibility of the bioactive peptide carnosine during *in vitro* digestion of cured beef meat

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ABSTRACT

A bioactive compound is a food component that may have impact on health. Its bioaccessibility, defined as the fraction released from the food matrix into the gastrointestinal tract during digestion, depends on compound stability, interactions with other food components and supramolecular organization of food.

In this study, the effect of pH on the bioaccessibility of the bioactive dipeptide carnosine was evaluated in two commercial samples of the Italian cured beef meat Bresaola, at two key points of digestion: before the gastric and after the duodenal phases. The digestion process was simulated using an *in vitro* static system, while Capillary Zonal Electrophoresis and ^{1}H Nuclear Magnetic Resonance were used for quantitative analysis.

The gap between the total carnosine content, measured by CZE, and its free diffusible fraction observable by NMR spectroscopy, was 11% and 19% for two independent Bresaola products, where such percentages represent the fraction of carnosine not accessible for intestinal absorption because adsorbed to the food matrix dispersed in the digestion fluid.

Keywords: carnosine, Bresaola, *in vitro* digestion, bioaccessibility, bioactive compounds, ^{1}H-NMR, CZE.

INTRODUCTION

Bioactive compounds are extra nutritional constituents of food that typically occur in small quantities but can have a positive impact on body functions or conditions, and may ultimately influence health.[1] For their potential positive effects, they have been extensively studied in the last decade, isolated from different food sources and chemically and functionally characterised. Most of the research has focused on secondary plant materials such as phytosterols or polyphenols; meat and meat products, despite being recognised as good suppliers of high-grade proteins, vitamins and minerals, are generally not considered as sources of bioactive substances. Yet, research has made progress in the meat segment too, and today many compounds found in meat, i.e. small peptides, carnosine, anserine, taurine, creatinine, and coenzyme Q10 have been recognized as bioactives with a large spectrum of physiological actions.[2-4]

Carnosine is a β-alanine and L-histidine dipeptide, which is distributed widely and abundantly in excitable tissues (particularly muscle and nervous tissues) of several animal species. Although its physiological role has not been completely understood yet, many beneficial actions have been attributed to carnosine, such as being an antioxidant and a free-radical scavenger, an anti-glycating and an ion-chelating agent, and a wound healing promoter.[5] These biological activities may explain the ability of carnosine to exert an antiaging activity at cellular and whole animal levels [6] and to restrain some oxidative-based diseases such as diabetes,[7-9] atherosclerosis,[10] metabolic distress syndrome,[11] and also ischemia–reperfusion damage in different organs.[12-14]

After meat digestion the amount of dietary carnosine becoming available for intestinal absorption depends not only on its original concentration in food (related to animal species and tissue type), but also on its bioaccessibility, i.e. the fraction of the compound that is released from the food matrix in the gastrointestinal tract during digestion, and is available for intestinal absorption.[15] Nutrient bioaccessibility is affected by digestion conditions (pH, enzymes concentration and

activity), compound stability, interactions with other food components, as well as the supramolecular organization of the food.[16] Particularly, the pH of the environment is a critical parameter, being able to modify the net charge of a molecule, its structure and consequently the degree of interaction with the solvent and other food molecules.

In this work, the effect of pH on the solubility and in turn bioaccessibility of carnosine was assessed in Bresaola, a typical beef-based product of Northern Italy (Valtellina), which is produced by curing the intact beef muscle through different processing steps including a careful selection and skilful trimming cuts of meat, followed by a dry salting step, during which wine, spices and sugar (with the aim of encouraging microbial phenomena responsible for much of the aging of the product) are added, then the meat is stuffed into natural or artificial casings and sent to the next stage of drying in special cells for a slow maturation. As this substrate is consumed crude, the evaluation of carnosine content is not expected to be affected by alterations due to the cooking process, which can cause a reduction of carnosine up to 50% in other beef products, depending on the cooking procedure.[17,18] The digestion of Bresaola occurring *in vivo* in the human gastro-intestinal tract was simulated using an *in vitro* static system,[19] and the soluble free compound in the digestion fluid was evaluated by coupling ^{1}H Nuclear Magnetic Resonance spectroscopy (^{1}H-NMR) and Capillary Zone Electrophoresis (CZE). These techniques were operated at different pH values to obtain a global description of the free fraction of the molecule at three key points of digestion, i.e. the end of the oral phase, after 180 and 300 min of duodenal digestion.

To the best of the Authors knowledge this research work represents the first study estimating the effect of pH on the interactions of carnosine with the digested meat matrix, and therefore on its bioaccessibility. The presence of emulsifiers, metal ions, fats, biopolymers and other chemical species able to bind the bioactive compounds could sequestrate them, thus limiting their free diffusion through the extracting digestion fluid, from food matrix, where bioactive molecules are

embedded, to the gut mucosa where their absorption occurs. The modification of bioaccessibility due to interactions with the food matrix is a key factor that should be considered while evaluating the potential significance of bioactives in human health. Each compound, indeed, is susceptible of chemical exchange between environments, being either freely diffusible in the digestion fluids or being adsorbed on the surfaces of the macromolecular food matrix; this could affect the bioactive bioaccessibility, and therefore effectiveness, when it is delivered in the human diet as part of a complex food matrix and not as a discrete molecule.

MATERIALS AND METHODS

All chemicals and solvents were of the highest analytical grade, and were purchased from Sigma–Aldrich (St. Louis, MO), except where indicated. The two types of commercial Bresaola, named B1 and B2, were bought in a local market.

Cured meat sample preparation for carnosine quantification in raw material.

Bresaola meat samples were prepared for carnosine quantification by grinding them with a common mincer. Five grams of minced meat where then homogenized with 10 mL of distilled water for 4 min at 25000 rpm using a homogenizer. Therefore, the pH was adjusted to 2.5 with 37% HCl and, after centrifugation for 30 min at 11500 rpm at 4 °C, the supernatant was filtered through a 0.45 μm filter, boiled for 10 min, centrifuged for 20 min at 11500 rpm, 4 °C and filtered again using a 0.22 μm filter. For CZE analysis the filtered sample was diluted 1:20 with 100 mM phosphate buffer pH 2.5 (BioRad Laboratories, California). For ^{1}H-NMR spectroscopy 160 μL of deuterium oxide (D_2O), containing 10 mM 3-TrimethylSilyl-Propanoic-2,2,3,3-d4 acid sodium salt (TSP), as chemical shift calibration standard, were added to 1 mL of filtered sample, and the pH was adjusted to the investigated value by adding 10% NaOH or 1 M HCl.

Cured meat sample preparation for carnosine pH dependence evaluation

The pH dependence of carnosine in Bresaola meat was analysed by ^{1}H-NMR in samples prepared as described in the previous section. In these experiments, before the first centrifugation, the pH was adjusted, by adding 10% NaOH or 1 M HCl, to the following values: 2, 2.5, 3.5, 5, 6, 7 and 8. Afterward, the samples were centrifuged at 14000 rpm for 5 min in order to remove precipitates.

Cured meat *in vitro* digestion

The Bresaola samples were digested in triplicate according to Bordoni,[19] following the scheme reported in Figure 1.

In details, human digestion was simulated *in vitro* inside a 100 mL flask, kept at 37 °C in a water bath on a magnetic stirrer equipped with a heating plate.[20] Chemical composition of the digestive fluid, pH and residence periods were adjusted to mimic the physiological conditions. Three samples were collected during digestion: P1, before the gastric phase, when the food enters the stomach and the pH decreases to 2); P2 and P3 after 180 and 300 min from the beginning of the duodenal phase, respectively.

Samples P2 and P3 were acidified to pH 2 with 37% HCl to stop hydrolysis by the pancreatic enzymes. P1, P2 and P3 samples were stored at -80 °C before CZE and ^{1}H-NMR experiments, before any further treatment.

For CZE, 500 µL of samples P1-P3 were boiled for 10 min, immediately cooled and then centrifuged for 30 min at 9,500 X g and 4 °C; the supernatant was filtered through a 0.22 µm before analysis.

Samples P1-P3 were prepared for 1H-NMR by adding 160 µL of D_2O, containing 10 mM TSP, as chemical shift calibration standard, to 1 mL of each sample. After adjusting the pH to 7, the samples were centrifuged at 14000 rpm for 5 min in order to remove precipitates

Carnosine quantification by CZE

CZE analysis and data processing were performed on a Biofocus 2000 from BioRad (BioRad Laboratories, California). Uncoated fused-silica capillaries with a diameter of 50 µm and effective length of 51 cm, were used for the analysis. The temperature during the analysis was maintained constant at 38 °C and samples were analyzed in a 110 mM phosphate buffer at pH 2.5 (BioRad Laboratories, California). The voltage was kept at 15 KV and the separated components were detected at 200 nm (UV), without derivatization. The quantification was performed in duplicate by area integration.

^{1}H NMR evaluation of carnosine concentration and carnosine pH dependence

All ^{1}H-NMR spectra were recorded at 25 °C on a Bruker US+ Avance III spectrometer operating at 600 MHz, equipped with a BBI-z probe and a B-ACS 60 sampler for automation (Bruker BioSpin, Karlsruhe, Germany). Spectra were collected with a 90° pulse of 14 µsec with a power of 10 W, a relaxation delay of 5 sec and an acquisition time of 2.28 s.

Spectra were registered by means of the first increment of a NOESY sequence, designed to suppress the HOD residual signal, while giving, for each proton, signals proportional to the concentration of the substance they belong.

Statistical analysis

Data normality and homogeneity of the variances were assessed by means of Shapiro-Wilks and Barlett tests respectively. Sample and pH effects on carnosine concentration were investigated

through ANOVA test, followed by *post-hoc* LSD test. Every calculation was performed by means of the correspondent packages implemented in R computational language. A trim value of 0.05 was considered, unless differently stated.

RESULTS AND DISCUSSION

Carnosine quantification in raw Bresaola meat by CZE and ^{1}H-NMR spectroscopy

In undigested raw samples, carnosine content was measured by both CZE and ^{1}H-NMR, using the purified compound as reference standard. Measurements were performed at pH 2.5, which optimizes carnosine resolution through CZE.[21]

By CZE the content of carnosine was estimated at 628 mg/100 g and 671 mg/100 g in raw B1 and B2, respectively.

A typical ^{1}H-NMR spectrum of bresaola extract, with carnosine highlighted, is reported in Figure 2. For the quantification of this molecule the signals at 7.30 and 8.60 ppm, corresponding to imidazole hydrogens Ha and Hb of the histidine residue, were considered, because they are singlets (thus granting the highest signal to noise ratio), and well resolved from signals ascribable to other molecules. In all the registered spectra it was possible to observe a 1:2 ratio between the area of these signals and those from β-alanine appearing in the range 2.65 - 2.80 ppm, corresponding to the two protons Hf and Hg, indicating that the amount of free histidine in all the samples was negligible.

By ^{1}H-NMR the amount of measured carnosine was estimated 623.3 and 664.1 mg/100 g of raw products, in B1 and B2 samples respectively. The very low difference in carnosine concentration detected by ^{1}H-NMR and CZE values (Δ = 0.7% and 1.0% for B1 and B2 samples, respectively) clearly indicates a good agreement between the two techniques, when working at the same acidic pH.

These values are higher than those reported in the literature for beef meat, whose carnosine content ranges from 32.6 mg/100 g in heart to 452 mg/100 g in semitendinosus muscle,[22,23] being generally higher in anaerobic, glycolitic, white muscle than in red, aerobic muscle.[6] It is conceivable that the higher carnosine content is related to the dehydration process that raw meat undergoes during Bresaola production, which increases the protein content from 20.5 to 32 g/100g.[24]

Effect of pH on carnosine quantification in Bresaola meat

Previous investigations have shown that the solubility of low molecular weight molecules during food digestion depends on the physiological pH of the different digestion environments, which can be modulated by the interaction with the food matrix undergoing digestion.[25] This especially holds for carnosine, whose net charge strongly depends on pH for the presence of three ionizable groups, namely the histidine α carbossilic group (pKa = 2.64), the histidine imidazolic group (pKa = 6.83) and the alanine β amminic group (pKa = 9.51).

To better elucidate the relationship between carnosine bioaccessibility and pH, the dipeptide concentration in raw B1 and B2 samples was quantified by ^{1}H-NMR in the 2-8 pH range. As reported in Figure 3, in both samples the amount of free carnosine was maximum at pH below 2.5 or above 8, and was characterized by a curvilinear relationship with pH, with a minimum around pH 5.3, corresponding to the isoelectric point of meat proteins. In detail, carnosine concentration in solution appeared significantly different between pH 2 and 7 ($P<0.05$).

The quantification of carnosine alone in water solution did not show any change of concentration depending on pH (data not reported), pointing out that such behavior was peculiarly ascribable to the interaction between carnosine and the food matrix. The lowest recovery around meat proteins isoelectric point suggested in particular the interaction of carnosine with the hydrophobic patches over the surface of meat proteins.

Meat is characterized by its own pH, depending on the quality and manufacturing processes in case of curing, ageing or other technological transformations. The purpose of this study was to describe the effect of the digested meat matrix on the solubility of carnosine, which modulate its accessibility, e.g., the diffusion of the dipeptide towards the gut mucosa where the absorption will occur. The possible implication of this phenomenon on the kinetics of absorption should be taken into account when developing new meat products, by considering that matrix could be modified, thus reacting to digestion in different ways.

Carnosine bioaccessibility during *in vitro* Bresaola digestion

In order to study carnosine bioaccessibility during digestion, its concentration was measured in the soluble fraction of samples collected at different phases of an *in vitro* static digestion process. CZE and ^{1}H-NMR were used in parallel at pH 2.5 and 7 respectively. pH values were adjusted before sample injection (CZE) or transfer to the NMR tube, respectively. As mentioned above, measurement at pH 2.5 optimizes carnosine resolution through CZE;[21] furthermore, it allowed a direct comparison between data obtained on raw samples and digestates. Measurement by ^{1}H-NMR were performed at pH 7 to focus on the carnosine concentration at pH value similar to those found in the intestine, where absorption takes place.

Carnosine concentration in the soluble fractions of digestate obtained at three different time points (P1, P2, and P3), as the result of three repetitions of *in vitro* digestion processes of B1 and B2 products, are reported in Table 1.

Carnosine concentration at P1 corresponds to the amount of compound brought into solution after the oral step, during which the meat is chewed in the presence of saliva. CZE analysis allowed comparing carnosine content at P1 and in raw, undigested samples, evidencing that in both samples

the simple action of chewing and subsequent acidification actually released the most of the dipeptide (96% and 90% in B1 and B2, respectively).

In both samples carnosine concentration measured by ^{1}H-NMR at pH 7 was lower than by CZE, in agreement with the above reported data on the effect of pH on carnosine quantification. Comparison was made between the gastric samples analyzed by CZE at pH 2.5 and the same samples analyzed by NMR after bringing the pH at 7. This change of pH may parallelize the jump occurring when the bolus exit the stomach and enter the duodenum.

As well, carnosine concentration at P2 and P3 (after 180 min and 300 min from the beginning of the duodenal digestion, respectively) was higher by CZE than by ^{1}H-NMR. It is important to consider that the pH of the intestinal environment is close to 7, so ^{1}H-NMR analysis actually mirrors the amount of carnosine available for absorption (i.e. the bioaccessible carnosine), values obtained with CZE being an artefact due to the condition of analysis. ^{1}H-NMR showed that, after 180 min of duodenal digestion, the amount of compound found as the bioaccessible soluble fraction was 89% and 81% of the total extracted from the raw samples B1 and B2, respectively. Prolongation of the *in vitro* duodenal digestion for further 120 min (P3) did not lead to any significant increase in carnosine concentration, indicating that carnosine release from the food matrix is almost completed after 3 h of duodenal digestion.

Summarizing, in the present work two different techniques, CZE and NMR spectroscopy, were used in parallel to evaluate the carnosine concentration in two samples of the typical Italian cured meat based product Bresaola. Evaluation was performed on both the raw material and after *in vitro* digestion of samples. Both analysis were performed on raw Bresaola at pH 2.5, and results evidenced a good agreement between the two techniques. In digested samples, CZE analysis was still performed at pH 2.5, which represents the optimum pH value for the analysis, while NMR spectroscopy was performed at a pH value resembling the one in the intestine (pH 7). In this way, it

was possible to evidence the influence exerted by the matrix on the carnosine transfer from the food to the soluble fraction, avoiding interference due to the herein evidenced effect of pH on carnosine quantification.

The nutritional evaluation of a food as source of specific nutrient/s is usually based on the chemical composition of the food itself. This approach does not take into account the digestion process, which regulates the amount of nutrients that can be absorbed, leading to a handy approximation often exceeding the real role of the food in the satisfaction of the human metabolic needs. Nutrients availability depends on the food matrix in which they are embedded, and on food processing, that can interfere with the digestion process. An exhaustive evaluation of the nutritional meaning of a food should take into account, among others, the modifications occurring during the digestion process, since only nutrients that are absorbed have the possibility to modulate the physiological processes occurring in the human body.

Although the present study aimed at investigating the concentration of free carnosine in the gastric and duodenal digestion fluids, the same phenomenon may occur in meat samples before digestion. It is interesting to note that pH 5,3 is typical of meat, and at this pH carnosine has the lowest fraction as free diffusible molecule and this could prevent depletion of such dipeptide during drip loss.

The potentiality of NMR spectroscopy to describe the interactions between a bioactive compound and the food matrix, directly in the digestion fluid and at the physiological pH, provides a tool that may help differentiating meat products on the basis of their matrix effect. Matrix is difficult to be studied during digestion, but its definition may be inferred through the study of its chemical interactions with soluble molecules. The simple quantification of the total amount of each compound extracted from the meat prevent the differentiation of meat products on the supramolecular scale, i.e. on the basis of its matrix. NMR spectroscopy offers the opportunity to

leave the molecules in their physiological environments, including those characterizing the digestion process and for which the food matrix may play an important role for the diffusibility of the bioactive molecules from food to the gut mucosa.

An omic approach is needed to describe the food system and its relationship with the human system. This approach, known as foodomics, has gained momentum in recent years since it allows reaching a deeper level of knowledge about the nutritional value of a food, and of the relationship between food and health.

ABBREVIATIONS

NMR: Nuclear Magnetic Resonance; **CZE**: Capillary Zone Electrophoresis; **NOESY**: Nuclear Overhauser Effect SpectroscopY

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FIGURE CAPTIONS

Figure 1 Flow chart of human *in vitro* digestion. P1, P2 and P3 are the labels of samples collected at the beginning of the gastric phase (P1), and after 180 (P2) and 300 min (P3) from the beginning of the duodenal phase.

Figure 2 ^{1}H-NMR Spectrum of A) bresaola B1 sample at P1 digestion point (pH= 7.0); B) carnosine standard, showing the signals from L-histidine (Ha, Hb, Hc, Hd e He) and β-alanine (Hh, Hi, Hf, Hg) residues.

Figure 3 Carnosine concentration as assessed by ^{1}H-NMR in presence of meat samples B1 and B2 at different pH values. The error bars indicate the standard deviation calculated on 3 replicates.

Table 1: Carnosine Concentration of Bresaola B1 and B2, Measured by CZE and ^{1}H-NMR, at the Beginning of the Gastric Digestion (P1) and After 180 and 300 min from the Beginning of the Duodenal Phase (P2 and P3). The Values of Three Replicates of Digestion and Their Average ± Standard Deviation are Presented, Expressed in mg/100 g of Meat.

B1	CZE (pH 2.5) mg/100 g		^{1}H NMR (pH 7) mg/100 g		Δ CZE - ^{1}H-NMR
P1	620.3	600.73 ± 19.70	514.7	543.82 ±30.02	10.50%
	601		574.66		
	580.9		542.09		
P2	638.94	654.43 ± 13.85	519.57	557.81 ± 33.93	17.32%
	658.76		584.29		
	665.6		569.58		
P3	608.89	631.78 ± 20.64	524.7	555.80 ± 28.56	13.67%
	637.5		580.85		
	648.96		561.86		

B2	CZE (pH 2.5) mg/100 g		^{1}H NMR (pH 7) mg/100 g		Δ CZE - ^{1}H-NMR
P1	595.32	607.18 ± 17.44	519.3	518.02 ± 4.50	17.21%
	599.02		513.01		
	627.2		521.74		
P2	712.726	659.57 ± 63.92	519.57	542.02 ± 10.52	21.60%
	588.64		584.29		
	677.34		569.58		
P3	681.68	640.74 ± 57.41	524.7	555.02 ± 24.03	15.32%
	575.12		580.85		
	665.42		561.86		

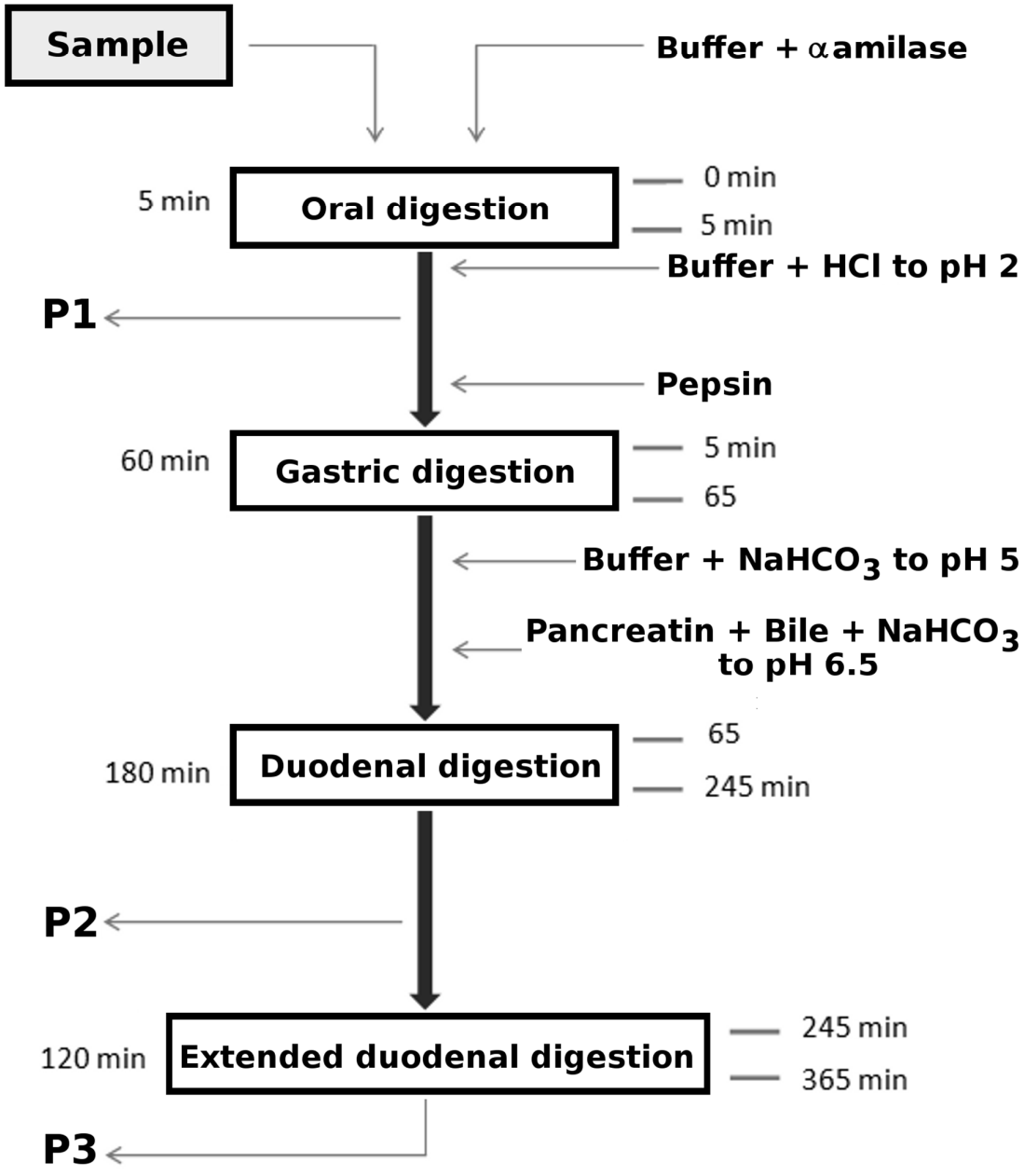
Sample
Buffer + αamilase
5 min
Oral digestion
0 min
5 min
Buffer + HCl to pH 2
P1
Pepsin
60 min
Gastric digestion
5 min
65
Buffer + NaHCO3 to pH 5
Pancreatin + Bile + NaHCO3
to pH 6.5
180 min
Duodenal digestion
65
245 min
P2
120 min
Extended duodenal digestion
245 min
365 min
P3

Figure 1

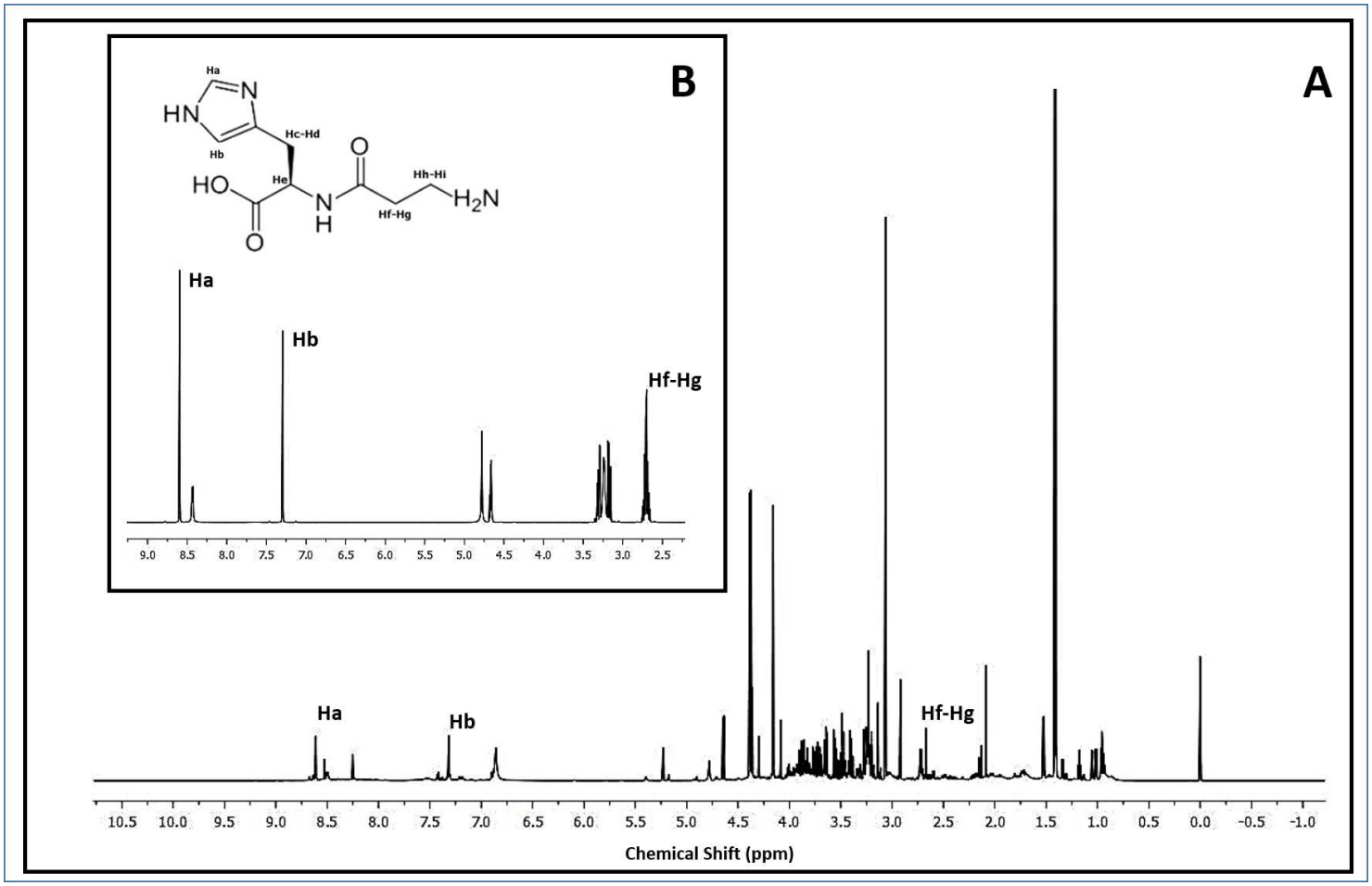
B
A
Ha
Hb
Hc-Hd
He
Hf-Hg
Hh-Hi
HO
HN
N
H
O
H_2N
Ha
Hb
Hf-Hg
9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5
Ha
Hb
Hf-Hg
10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0 -0.5 -1.0
Chemical Shift (ppm)

Figure 2

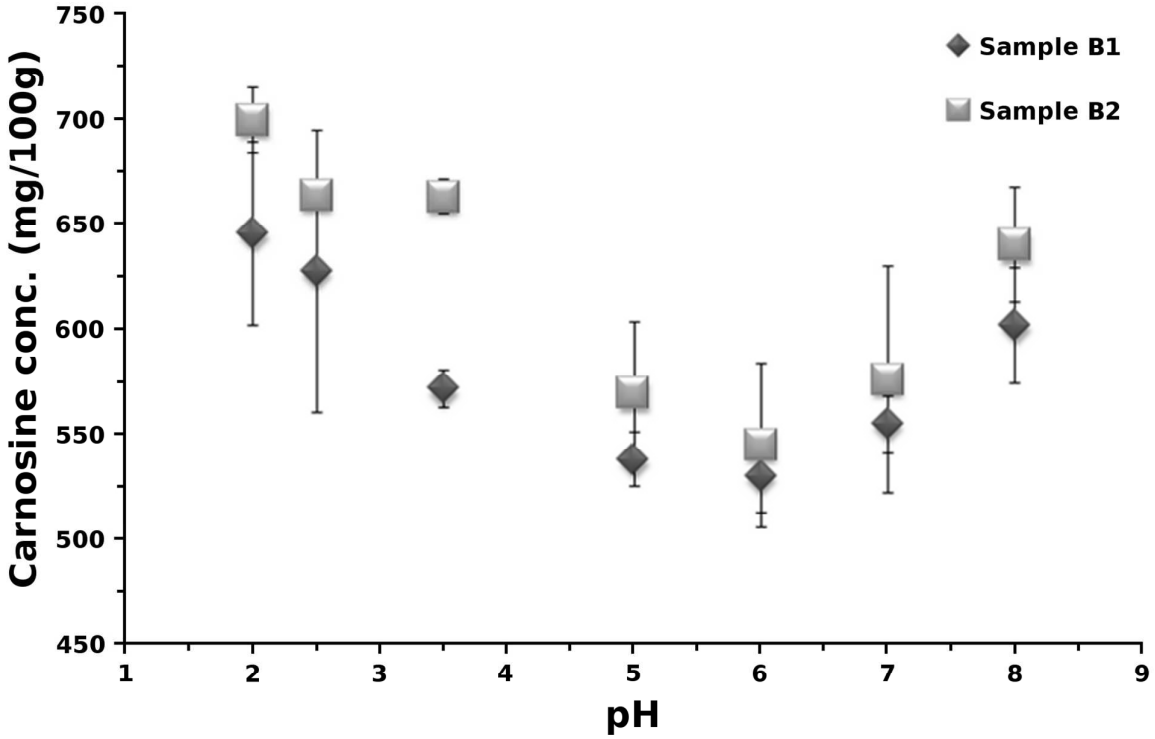
Carnosine conc. (mg/100g)
750
700
650
600
550
500
450
1
2
3
4
5
6
7
8
9
pH
Sample B1
Sample B2

Figure 3

Table of Contents Graphic (TOC)