

# Analytical Characterization and Quantification of Histidine Dipeptides, Carnosine and Anserine by Modeling of Potentiometric Titration Data

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In this paper, the relevant acid-base properties of the biologically important dipeptides carnosine and anserine were determined using potentiometric titration. The data obtained were used to calculate the dissociation constants of both carnosine and anserine. Both dipeptides in their fully protonated forms dissociate in three steps with corresponding dissociation constants of  $pK_{a1}= 2.57$ ,  $pK_{a2}= 6.71$  and  $pK_{a3}= 9.57$  for carnosine and  $pK_{a1}= 2.51$ ,  $pK_{a2}= 6.97$  and  $pK_{a3}= 9.51$  for anserine. The distribution diagram of the dissociated species as well as the buffer strength diagrams of carnosine and anserine provide useful information about their behaviors at different pH values. All computations were optimized via mathematical modeling with the *Solver* software.

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**Keywords:** dipeptide, carnosine, anserine, potentiometric titration, modeling, *Solver* optimization

## 1. INTRODUCTION

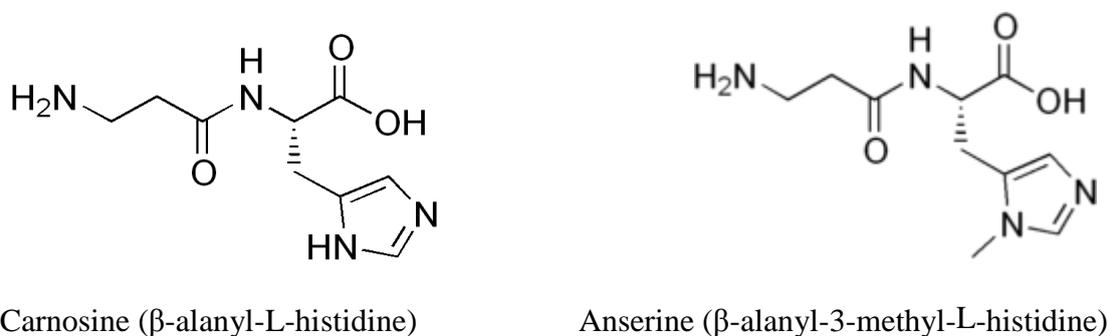
Carnosine and anserine are dipeptides that are contained in the skeletal muscles or brains of vertebrates in high concentrations [1, 2]. These substances have a variety of physiological roles; they may function to reduce muscle fatigue and improve learning ability because of their antioxidative effects [3] and buffering capacities due to the presence of an imidazole group.

L-Carnosine ( $\beta$ -alanyl-L-histidine) is a dipeptide composed of  $\beta$ -alanine and L-histidine and performs multiple biological functions, including pH buffering, membrane-protecting activity, anti-oxidation, anti-glycation, anti-aging, chelation of divalent metal cations and regulation of macrophage function [4-6]. Carnosine is a scavenger of reactive oxygen species that is widely distributed in skeletal

muscles and the central nervous system [7]. Carnosine has a potential role in the treatment of autism [8], ulcers, diabetes and Alzheimer's disease [9, 10].

Anserine ( $\beta$ -alanyl-N-methyl histidine) is an N-methylated analog of carnosine found mainly in fish and birds. Anserine has similar properties to carnosine in many aspects but is mainly found in non-mammalian species.

The chemical structures of carnosine and anserine are shown in Figure 1.



**Figure 1.** Chemical structure of carnosine and anserine.

Since histidine dipeptides were first isolated in 1900, interest in their quantification has been constantly rising. The available colorimetric methods include the diazo procedure [11], which is a method based on diazonium salts of *p*-bromoaniline or *o*-phthalic aldehyde [12] that produce colorful compounds with carnosine. Additionally, fluorimetric methods can be used for determination in biological tissues [13].

The chromatographic methods for the determination of histidine dipeptides in soups, meat products, biological materials, etc. include a) high performance liquid chromatography (HPLC) with UV [14] or fluorescent detection [15-17], b) a new HPLC procedure based on hydrophilic interaction chromatography (HILIC) [18], c) new gradient reversed-phase HPLC methods with pre-column derivatization [19], d) high-performance liquid chromatography/mass spectrometry (HPLC/MS) [20, 21], e) high-performance anion-exchange chromatography (HPAEC) with integrated amperometric pulse detection [22], and f) micellar liquid chromatography (MLC) with a UV detector [23].

Carnosine and anserine can also be determined in biological materials via capillary electrophoresis with UV detection [24-26], laser-induced fluorescence (LIF) [27], electrospray mass spectrometric detection [28], and microchip electrophoresis with chemiluminescence detection [29]. Electrochemical methods are rarely used for the determination of histidine dipeptides; however, cyclic voltammetry [30, 31] and potentiometry have been reported.

Direct potentiometric pH titrations (aqueous media) represent infrequently used methods for the determination of histidine dipeptide derivatives. However, the carnosine, anserine and balenine buffer capacities have been determined and characterized [32], and a series of carnosine amides with the amido group alkyl substituents have been synthesized and physicochemically and biologically characterized for lipophilicity, dissociation constants and specific complexing to copper (II) ions [33].

Direct potentiometric titration in non-aqueous media can provide useful data for the determination of carnosine and other dipeptide derivatives [34-36], but the use of organic solvents and non-pH scale measurements make these methods unpopular and hard to use. Using potentiometric titrations in aqueous and non-aqueous media provides data about the acid-base characteristics of histidine dipeptides but does not provide data for their quantification.

The objective of this study was to determine the acid-base properties of the histidine dipeptides carnosine and anserine. Throughout this study, potentiometric methods (direct potentiometry and potentiometric titration) were used for the determination of the acid-base properties (dissociation constants, ionization species distribution, buffer strength) of carnosine and anserine.

## 2. EXPERIMENTAL

### 2.1. Reagents and Materials

Carnosine (Sigma-Aldrich, USA) and anserine (Bachem, Switzerland) titrations were performed using standard solutions of NaOH with concentrations of 0.1 and 0.01 M (Kemika, Croatia) as the titrant, and 0.1 and 0.01 M HCl solutions (Kemika, Croatia) were used to adjust the dipeptide solutions to pH 2 and 3. A solution of 0.5 M Na<sub>2</sub>SO<sub>4</sub> (T.T.T., Croatia) was used to adjust the ionic strengths of the solutions. All chemicals were of analytical reagent grade.

### 2.2. Apparatus and Measurements

An 808 Titrand (Metrohm, Switzerland) combined with a Metrohm exchange unit (Metrohm, Switzerland) were used for the titrations. The *Tiamo* software was used for data acquisition and processing (Metrohm, Switzerland). A combined glass electrode (Metrohm, Switzerland) with Ag/AgCl/3 M KCl as the reference electrode (Metrohm, Switzerland) was used for direct potentiometric measurements and for potentiometric titrations. The solutions were magnetically stirred during titrations using an 801 Stirrer (Metrohm, Switzerland).

### 2.3. Procedure

First, 5-mL aliquots of the dipeptide solutions (carnosine or anserine) at concentrations of 0.04 M were diluted with 25 mL of water and titrated potentiometrically with 0.1 M NaOH using the combined glass electrode as the indicator.

The titration parameters were set in dynamic equivalence point titration mode with a 120 s waiting time (before the titration starts), a signal drift of 5 mV/min, and an equilibrium time of 30 s. All of the measurements and titrations were performed at room temperature using a magnetic stirrer and without ionic strength or pH adjustment.

Another titration was performed after adjusting the analyte solution to pH 2 using 0.1 M HCl.

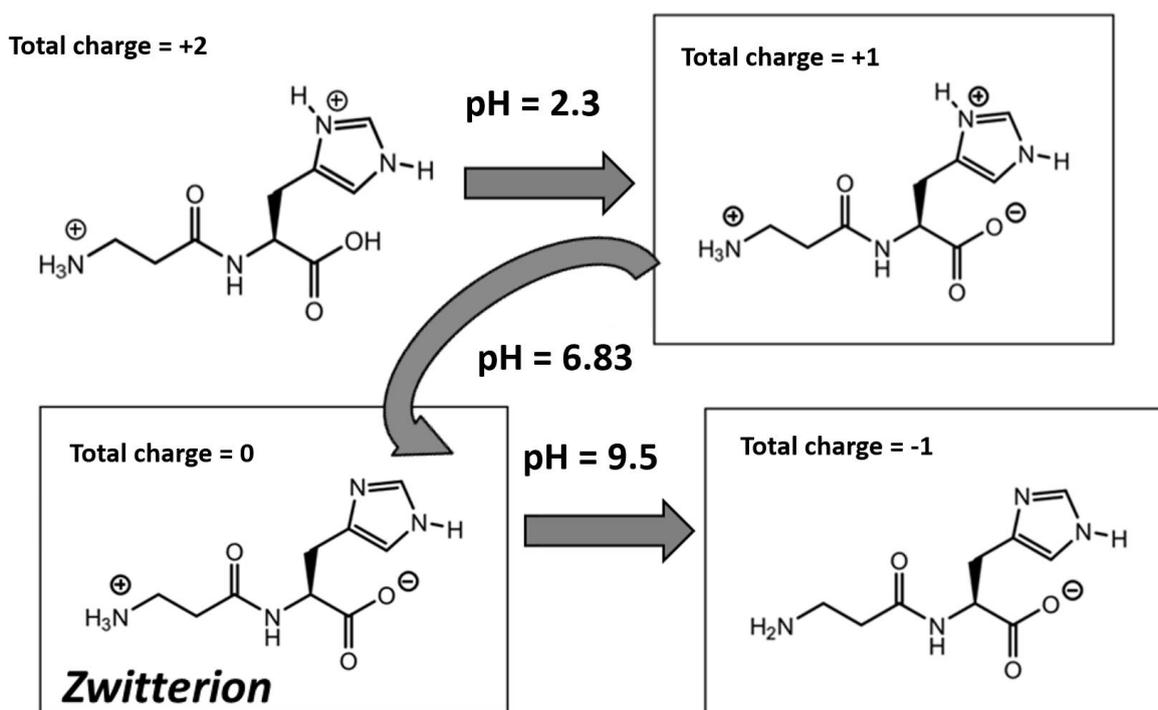
A study of the influence of the ionic strength on the potentiometric titration of the 0.04 M dipeptide solutions was performed by adding sodium sulfate ( $c = 0.1$  M, ionic strength 0.301) to the analyte solution.

#### 2.4 Optimization Using Solver

*Solver* is a tool in Microsoft Excel for Windows that is activated by selecting “*Add ins*” and is used as a spreadsheet optimization modeling system. It is used to solve various linear and nonlinear problems. *Solver* was used to compare an array of data predicted by the model described below using an initial set of parameters over a range of dependent variables with a set of experimental data. Then, the sum of the squared residuals (SSR) between the two arrays was calculated by varying the parameters to minimize the SSR between the theoretical and experimental data sets.

The regression statistics were calculated using the macro SolvStat [37]. The regression statistics included the standard deviations of the parameters, correlation coefficients of the linear function and standard errors of the y estimate,  $SE(y)$ .

### 3. RESULTS AND DISCUSSION



**Figure 2.** Carnosine structure as the cation, *zwitterion* and anion

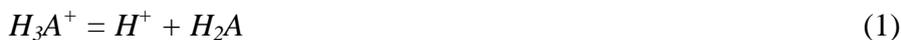
Dipeptides are amphoteric because they contain acidic and basic groups. The pH dictates the dominant ionic form. At low pH values, the groups are protonated to free groups, and the molecule has

an overall positive charge. In contrast, higher pH values result in the gradual loss of the molecule's protons and an overall negative charge of the deprotonated molecule.

There is an intermediate pH (isoelectric point) where the peptide is evenly balanced between the two forms as the dipolar zwitterion with a net charge of zero. The isoelectric point for carnosine is 8.2.

Carnosine and anserine have four ionization states. Figure 2 shows the pH values of the functional group deprotonation in carnosine.

Carnosine and anserine dissociate in aqueous solutions, such as triprotic acids, and the corresponding equilibria in their protonated form,  $H_3A^+$ , are



with the corresponding dissociation constants  $K_{a1}$ ,  $K_{a2}$  and  $K_{a3}$ , and related concentration fraction of the particular species in the mixture  $\alpha_{H_3A^+}, \alpha_{H_2A}, \alpha_{HA^-}, \alpha_{A^{2-}}$ :

$$\alpha_{H_3A^+} = \alpha_3 = \frac{[H_3A^+]}{[H_3A^+] + [H_2A] + [HA^-] + [A^{2-}]} \tag{4}$$

By substituting the concentration of each species from the dissociation constants and after rearrangements, the following expression is obtained:

$$\alpha_{H_3A^+} = \alpha_3 = \frac{[H^+]^3}{[H^+]^3 + [H^+]^2 K_{a1} + [H^+] K_{a1} K_{a2} + K_{a1} K_{a2} K_{a3}} \tag{5}$$

Analogously, the following expressions for the other species are

$$\alpha_{H_2A} = \alpha_2 = \frac{[H^+]^2 K_{a1}}{D} \tag{6}$$

where

$$D = [H^+]^3 + [H^+]^2 K_{a1} + [H^+] K_{a1} K_{a2} + K_{a1} K_{a2} K_{a3} \tag{7}$$

$$\alpha_{HA^-} = \alpha_1 = \frac{[H^+] K_{a1} K_{a2}}{D} \tag{8}$$

and

$$\alpha_{A^{2-}} = \alpha_0 = \frac{K_{a1}K_{a2}K_{a3}}{D} \quad (9).$$

From the mass and charge balance equilibria occurring by titration of the fully protonated triprotic acid (carnosine or anserine) with a strong monoprotic base (NaOH) in aqueous solution, the following species concentrations are obtained:

$$[H_2A] = \frac{C_{H_2A}V_a}{V_a + V_b} \quad (10),$$

$$[HA^-] = \frac{C_{HA^-}V_a}{V_a + V_b} \quad (11),$$

$$[A^{2-}] = \frac{C_{A^{2-}}V_a}{V_a + V_b} \quad (12),$$

where

$[H_2A]$ ,  $[HA^-]$  and  $[A^{2-}]$  are the concentrations of the particular species,

$V_a$  = volume of the protonated dipeptide solution and  $V_b$  = volume of the strong monoprotic base of the concentration  $C_b$ .

By combining of mass and charge balance equations and substituting Eq. 5, 6, 8, and 9 into the resulting expression and performing additional rearrangements, the following equations for the progress of the titration of the fully protonated form of a triprotic acid with a strong monoprotic base are obtained:

$$\frac{V_b}{V_a} = \frac{C_a(\alpha_2 + 2\alpha_1 + 3\alpha_0) - \Delta}{C_b + \Delta} \quad (13),$$

or

$$V_b = \frac{V_a [C_a(\alpha_2 + 2\alpha_1 + 3\alpha_0) - \Delta]}{C_b + \Delta} \quad (14),$$

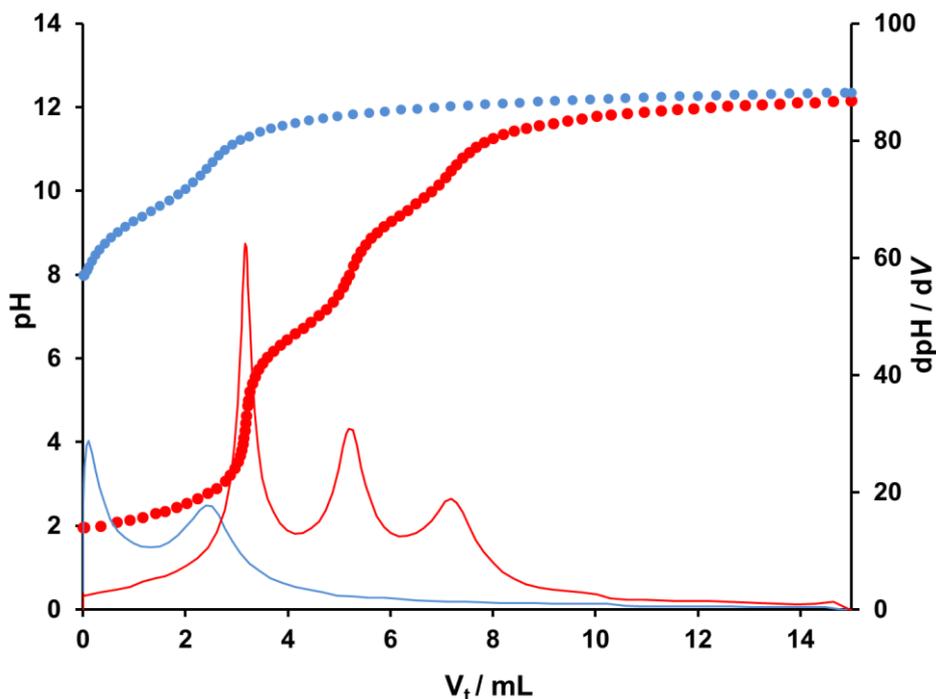
where

$C_a$  = total concentration of all dipeptide species in solution and

$$\Delta = [H^+] - [OH^-] \quad (15).$$

### 3.1. Assay determination

The dipeptide (carnosine and anserine) assay has been shown to exhibit three well-defined potential jumps in the potentiometric titration curve (Figure 3). The equivalence points were located using the first derivative ( $dpH/dV$ ). The results of the determinations are given in Table 1.



**Figure 3.** Potentiometric titration curves of carnosine and its first derivatives obtained by titration of 0.04 M carnosine using 0.1 M NaOH as a titrant (blue: no pH adjustment, red: pH value adjusted with 0.1 M HCl to  $2.0 \pm 0.1$ ).

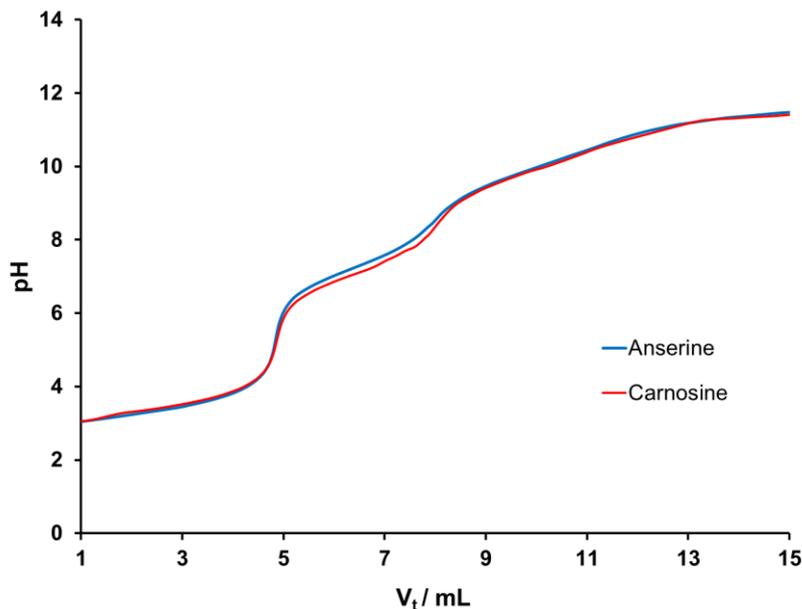
The dipeptides carnosine and anserine are amphoteric substances that contain both acidic carboxylic acid groups ( $-\text{COOH}$ ) and basic amine ( $-\text{NH}_2$ ) groups, and in aqueous solution, they tend to undergo internal proton transfer from the carboxylic acid group to the amino group. As shown in Figure 3, the pH of an aqueous solution of carnosine is ca. 8, and only one inflexion can be observed via titration with NaOH with an equivalence point of ca. 8.5. After acidification of the carnosine solution to  $\text{pH } 2.0 \pm 0.1$ , the stronger acid (HCl) will titrate first and will give an inflexion at its equivalence point. At this equivalence point, a solution of the protonated carnosine form ( $\text{H}_3\text{A}^+$ ) exists, and beyond the equivalence point, a buffer region is established that markedly suppresses the HCl inflexion.

Potentiometric titration curves of both, carnosine and anserine, for the sake of comparison, are shown in Figure 4.

**Table 1.** Results of the carnosine and anserine potentiometric determination assays.

Sample	Mean (%)	Std. dev.	Coeff. Of var. (%)	Confidence limits ( $\pm$ )
Carnosine	99.18	0.64	0.64	0.58
Anserine	99.53	0.54	0.54	0.48

Number of determinations: 5,  $p = 0.95$



**Figure 4.** Potentiometric titration curves of carnosine and anserine obtained by titration of 0.01 M carnosine and anserine using 0.01 M NaOH as the titrant (pH value adjusted with 0.1 M HCl to  $3.0 \pm 0.1$ ).

### 3.2. The influence of the ionic strength on the shapes of the potentiometric titration curves

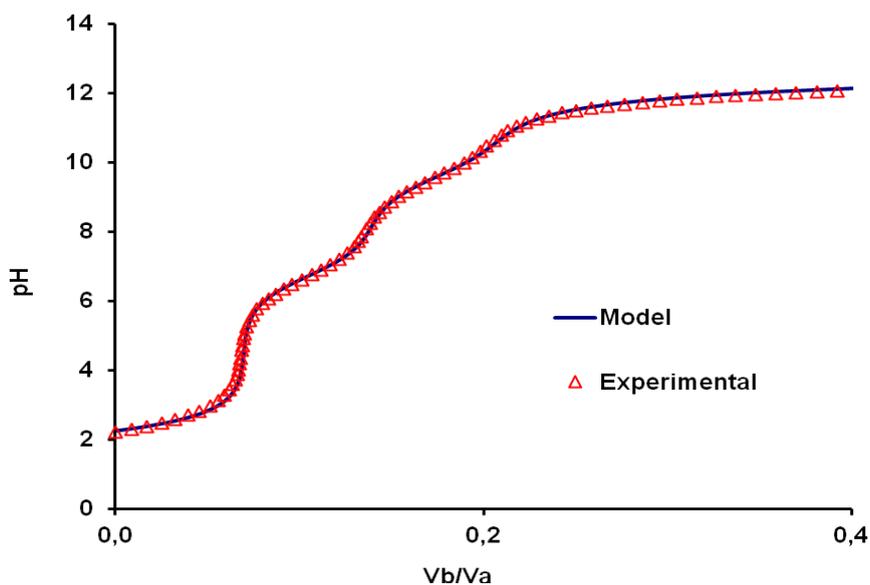
Higher concentrations of inorganic salts are sometimes found in real samples for the potentiometric determination of carnosine and anserine. For those investigations, solutions of  $\text{Na}_2\text{SO}_4$  ( $c = 0.1$  M, ionic strength 0.301) were used. The addition of the  $\text{Na}_2\text{SO}_4$  did not influence the shapes of the potentiometric titration curves.

### 3.3. Determination of the dissociation constants

A model for the determination of the dissociation constant of a fully protonated triprotic acid (carnosine or anserine) with a strong monoprotic base (sodium hydroxide) is based on a mathematical expression for the progress of the titration given above (Eq. 13).

*Solver* (Excel) was used to compare the experimental data with an appropriate theoretical curve. The unknown parameters (the dissociation constants) were optimized. *Solver* uses the entire data set and minimizes the sum of the squares of the differences between the theoretical and the experimental curve using the least-squares criterion to fit a theoretical curve to the experimental data.

The potentiometric titration curve of carnosine, given as titration progress, after the optimization procedure using *Solver* is shown in Figure 5. The data calculated are given in Table 2. For the sake of comparison, the calculated data for anserine, which were obtained using the same procedure, are also shown.



**Figure 5.** Comparison of the experimental and modeled potentiometric titration curves of carnosine with sodium hydroxide.

The determination of the dissociation constants comprises three protonation steps. The remarkable difference between the corresponding  $pK_{a1}$  and  $pK_{a2}$  values (more than 4 pK units) results in clear inflexions in the titration curve. The difference between  $pK_{a2}$  and  $pK_{a3}$  (less than 3 pK units) exhibits a satisfactory inflexion and still reliable determination of the third equivalence point (first derivative curves in Figure 3). The first step corresponds to a medium weak acid ( $pK_{a1} = 2.57$ ), whereas the second one indicates a very weak acid ( $pK_{a2} = 6.71$ ). The third equivalence point is slightly obscured by the competitive ionization of water.

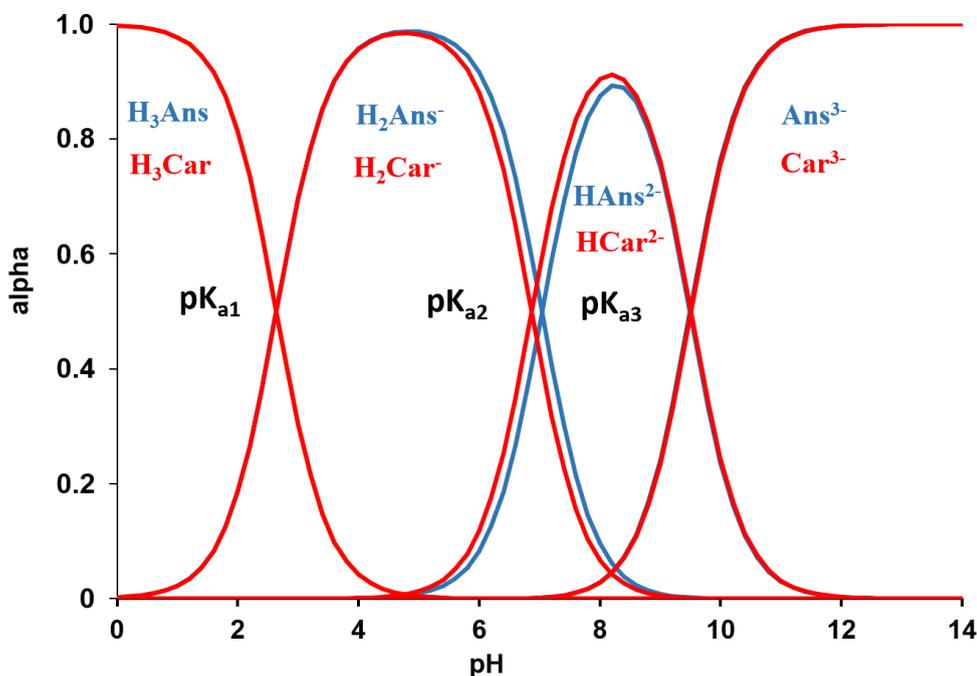
**Table 2.** Dissociation constants of carnosine and anserine obtained using the potentiometric method after the optimization procedure using *Solver*.

Parameter	Carnosine	Anserine
$K_{a1}$	$2.69 \times 10^{-3}$	$2.51 \times 10^{-3}$
$pK_{a1}$	$2.57 \pm 0.12$	$2.60 \pm 0.11$
$K_{a2}$	$1.96 \times 10^{-7}$	$1.07 \times 10^{-7}$
$pK_{a2}$	$6.71 \pm 0.18$	$6.97 \pm 0.14$
$K_{a3}$	$2.68 \times 10^{-10}$	$3.09 \times 10^{-10}$
$pK_{a3}$	$9.57 \pm 0.17$	$9.51 \pm 0.11$
Correl. coef. $R^2$	0.9753	0.9895
SE(y)	0.020	0.012

Table 2 shows that the difference in the dissociation ability of carnosine and anserine is negligible. The pK values obtained after optimization with *Solver* are in good agreement with the data

from the literature [32, 33], where obtained  $pK_a$  values for carnosine were:  $pK_{a1}=2.60$ ,  $pK_{a2}=6.79$  and  $pK_{a3}=9.42$ ; and for anserine:  $pK_{a1}=2.64$ ,  $pK_{a2}=7.04$ , and  $pK_{a3}=9.49$ .

### 3.4. Distribution diagram



**Figure 6.** Distribution diagram of the carnosine  $H_3Car$  (red) and anserine  $H_3Ans$  (blue) species.

A species distribution curve was used to illustrate the variation in the composition of an aqueous solution of a polyprotic weak acid species. Based on the data obtained after the optimization described above, the corresponding distribution diagram of the carnosine species (defined through Eq. 8, 9, 11 and 12) as a function of pH was plotted (Figure 6). As a comparison, the species distribution diagram for anserine is also given.

The distribution diagram of carnosine and anserine shows the distributions of the dissociated species as functions of the pH value, clearly indicating that the concentrations of the dissociated species increase with increasing pH.

### 3.5. Buffer strength

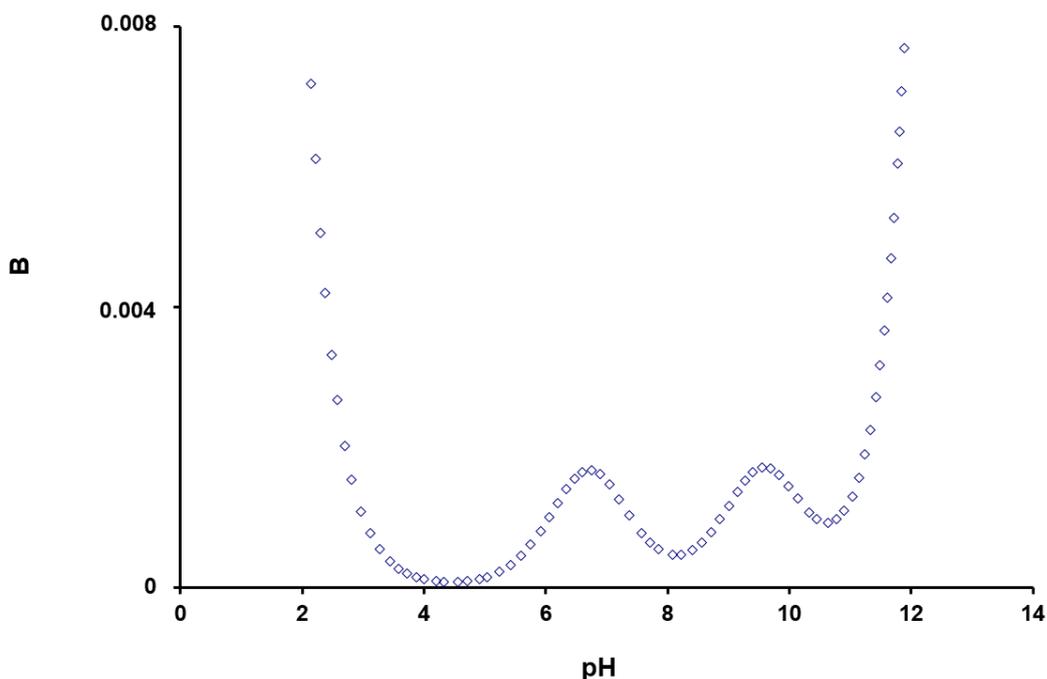
A quantitative expression of the pH-stabilizing buffer action of a triprotic acid, such as carnosine (anserine), is the *buffer strength* B:

$$B = [H^+] + C_a (\alpha_3\alpha_2 + \alpha_2\alpha_1 + \alpha_1\alpha_0 + 4\alpha_3\alpha_1 + 4\alpha_2\alpha_0 + 9\alpha_3\alpha_0) + [OH^-] \tag{16}$$

where  $C_a$  = concentration of acid (M)

$\alpha_0, \alpha_1, \alpha_2, \alpha_3$  = concentration fractions.

The buffer-strength diagram of carnosine and anserine, which was obtained based on the data after the previously described optimization, is shown in Figure 7.



**Figure 7.** Buffer-strength diagram of carnosine and anserine.

The buffer strength diagram of both dipeptides indicates enhanced buffer action at pH values of ca. 6.5 and 9.4, which is important for the buffering of physiological systems.

Direct potentiometry and potentiometric titration in aqueous media are not often used methods for characterization of the histidine dipeptides, like carnosine and anserine. By using the theoretical model, the Solver and the experimental titration data, the histidine dipeptides can be determined and characterized in aqueous media.

#### 4. CONCLUSIONS

Direct potentiometry and potentiometric titration were used for the acid-base characterization of the biologically important histidine dipeptides carnosine and anserine.

The potentiometric titration data were used to estimate some acid-base parameters of carnosine and anserine (i.e., the dissociation constants, buffer capacities, and dissociated species distributions).

A theoretical model for the potentiometric titration curves of both dipeptides was proposed.

*Solver* (Excel) was used to find the values of the variables that minimize the sum of the squares of the differences between the theoretical and experimental curves using the least-squares method to fit the theoretical curve to the experimental data.

The experimental data were compared with the appropriate theoretical curves, and the unknown properties (i.e., the dissociation constants, buffer capacities, and dissociated species distributions) were optimized.

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