MODELLING THE CHARGE ACROSS PH AND ISO-ELECTRIC POINT OF BOVINE COLLAGEN DURING LEATHER MANUFACTURE

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Abstract. Many areas of leather production rely heavily on the manipulation of acidic and basic residues within the primary collagen structure to vary the overall charge of the substrate. For example, it is the basis which enables swelling during liming, deswelling during deliming, penetration of chromium after addition of chrome tanning salts and the fixing of chrome to carboxylate residues during basification. Manipulation of the charge on collagen is readily achieved through the addition of acids or bases into the float which may react with these residues to alter the charge. Often, the increase in anionic charge and reduction in cationic charge with increasing pH are shown to happen concurrently and linearly with the iso-electric point (IEP) given as the point at which the positive and negative charges present on the collagen are equal. However, the pH at which carboxylate/acid groups undergo protonation/deprotonation is significantly lower than that at which an amine/ammonium is protonated/deprotonated, meaning the linear model described above is not a true representation of charge of collagen at varying pH. Here we model the charge of a collagen substrate based off the amino acid profile of bovine skin, considering their relative levels within the collagen and concentrations within a water/collagen matrix, representative for collagen saturated with water. Models are presented for raw and limed bovine hides. This broader approach enables greater understanding of the influence of charge on the collagen substrate compared to IEP on its own, revealing contrasting charge profiles in acidic and alkaline regions of raw collagen, providing greater understanding of their differing behaviour during alkali swelling.

1 Introduction

Collagen is of critical importance in leather due to its unique hierarchical structure based on the triple helix of tropocollagen. However, in the context of leather manufacture the manipulation of charge on the collagen substrate is of equal importance as manipulation of charge is prevalent throughout almost all stages of wet processing. It is primarily responsible for swelling during liming, deswelling during deliming, determination of suitable pickling pH for the appropriate tannage and control of penetration and fixing of tanning, retanning, dyeing and fatliquoring agents. [1]

The charge on collagen originates from the presence of acidic and basic amino acid residues which, depending on the amino acid and pH may be positively charged, negatively charged or neutral. **Fig. 1** shows a peptide composed of the most common amino acids present in collagen that may hold charge. Aspartic acid (Asp) and glutamic acid (Glu) have side chains that have a carboxylic acid group present which, depending on the pH may either be in its neutral protonated form at lower pH or its negative deprotonated carboxylate form at higher pH. Histidine (His), lysine (Lys) and arginine (Arg) each contain side chains that may be in a protonated in positively charged form at lower pH or a deprotonated neutral form at higher pH. Tyrosine (Tyr), an amino acid containing a phenolic ring is also acidic, being in either its neutral phenolic form at low pH or as an anionic phenoxide at high pH. [2]

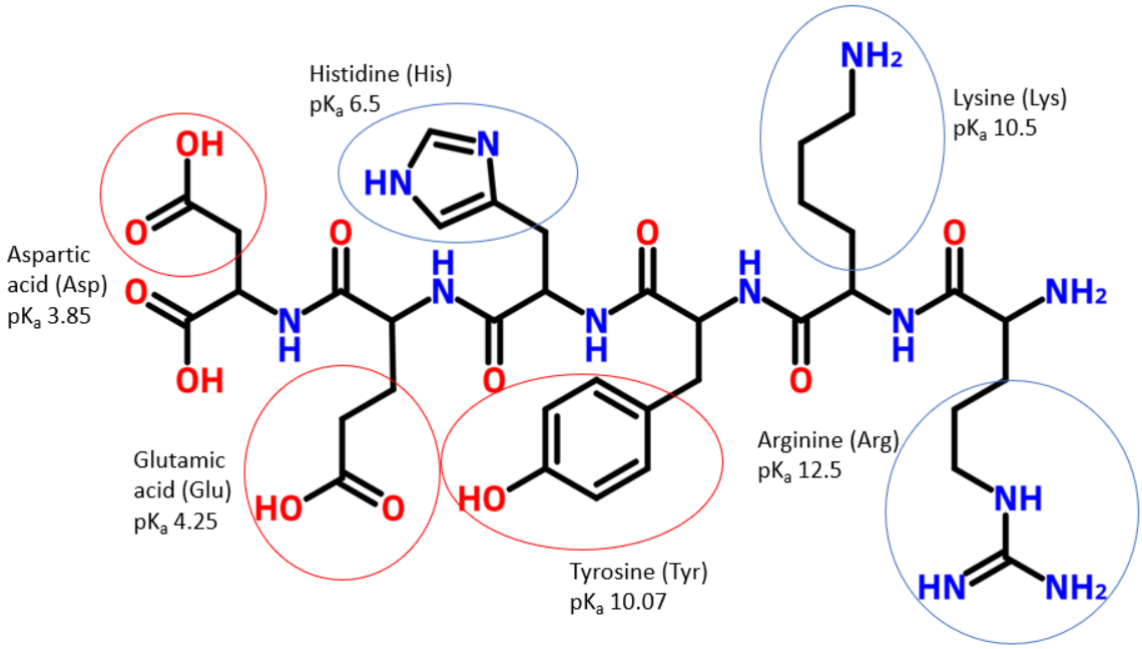

Fig. 1. Common amino acid residues of collagen that can hold charge and respective pK_as of the side chain functional groups. Residues represented in their neutral form.

It is commonly stated that below a pH of 3 all acid groups are protonated and below a pH of 9 all amine groups are protonated. Above a pH of 6 all acid groups are deprotonated and above a pH of 12 all amine groups are deprotonated, **eqns 1 & 2**, however there are notable, important exceptions to this as will be discussed later. [3]

Low pH		High pH	
$R\text{-}CO_2H + H_2O$ (pH < 3)	⇌	$R\text{-}CO_2^- + H_3O^+$ (above pH 6)	**(1)**
$R\text{-}NH_3^+ + H_2O$ (pH < 9)	⇌	$R\text{-}NH_2 + H_3O^+$ (above pH 12)	**(2)**

There is a pH range between pH 6 and 9 where predominantly carboxylate and ammonium groups are present. It is within this region that is generally accepted as "full neutralisation" in leather manufacture. [3] The isoelectric point of collagen can be found within this region at approximately pH 7.4. [4] The IEP is a fixed pH where the sum of positive and negative charges of a substrate, in this case collagen, are equal and thus there is no overall charge. At pHs below the IEP the overall charge will be positive as progressively more carboxylate are protonated, reducing the negative charge component. At pHs above this the charge will be negative as progressively more ammonium groups are deprotonated, reducing the positive charge component. IEP is often discussed in isolation with the implication on charge of collagen at a given pH often only inferred from the IEP value. A more holistic approach is to consider the charge on the collagen substrate across a broad pH range, providing not only information about the pH where there is neutral overall charge (IEP) but also all other pHs as well.

Here we have prepared a model for predicting the overall charge of bovine collagen at differing pHs. Molar concentrations of each individual amino acid residues, the charge of each of the residues at differing pHs, and overall charge on the collagen substrate were calculated. This model was then extended to study changes in charge and IEP during the liming process.

2 Description of model and calculations

2.1 Determination of concentration of acidic and basic amino acid residues per kg hydrated collagen

2.1.1 Molar mass of collagen molecule containing 1000 residues

Bovine skin collagen is predominantly composed of Type I, the composition of which is well understood. The amino acid profile of bovine hides used in the generation of this model is detailed in table 1. [1]

Table 1: Amino acid profile of type I bovine collagen.

Amino acid	Residues per 1000	Amino acid	Residues per 1000	Amino acid	Residues per 1000
Glycing (Gly)	330	Proline (Pro)	126	Glutamic acid (Glu)	73 in total
Alanine (Ala)	110	Hydroxyproline (Hyp)	93	Glutamine (Gln)	
Valine (Val)	22	Methionine (Met)	4	Arginine (Arg)	48
Leucine (Leu)	26	Phenylalanine (Phe)	14	Lysine (Lys)	38
Iso-leucine (Ile)	12	Tyrosine (Tyr)	5	Hydroxylysine (Hyl)	6
Serine (Ser)	34	Aspartic acid (Asp)	47 in total	Histidine (His)	4
Threonine (Thr)	18	Asparagine (Asn)			

Initially an overall mass of a collagen molecule containing an arbitrary 1000 amino acid residues was calculated from the above amino acid profile according to **eqn. 3**. below, where the total mass is obtained from the sum of the molecular weights of each amino acid multiplied by the number of their residues. Collagen is a protein formed from the condensation reaction of amino acids, with the elimination of water. To compensate for water elimination the molecular mass of 999 moles of water was then subtracted. This produced a mass of **111 425** g/1000 mol amino acid residues of collagen.

Total mass 1000 amino acid residues =

$$\Sigma \text{ (mwt amino acid * no residues per 1000)} - 999 * \text{mwt } H_2O \quad \textbf{(3)}$$

One limitation of the above calculation is the measurement of acidic amino acids and those containing an amide group but otherwise indistinguishable structures (I.e. Asp/Asn and Glu/Gln) are difficult to measure independently as the amides are converted into acid residues during the protein digestion process necessary for the measurement of concentration. This has limited implication in this case as their respective molecular weights are close. For example, aspartic acid has a mwt of 133.11 whereas asparagine has a mwt of 132.12. At extreme ratios of acid to amide residue this would result in a total mass error of 0.1% and as such is incorporated here as an acceptable error.

2.1.2 Determination of concentration of individual amino acids per 1 kg raw hydrated collagen

Knowing the mass of a theoretical collagen molecule containing 1000 amino acid residues, the concentration of amino acids per unit mass (in this case 100 g) can be calculated as shown in **eqn. 4**.

Conc. individual amino acid per 100 g collagen =

$$(100 \text{ g} / \text{Total 1000 amino acid residues}) * \text{no. residues of individual amino acid} \quad \textbf{(4)}$$

For example, the concentration of glycine per 100 g collagen can be given as follows

Glycine concentration = (100 / 111425) * 330
= 0.296 moles / 100g dry collagen

If hydrated collagen contains 30% collagen and 70% water, the concentration of individual amino acids per kg can be calculated by multiplying the concentration per 100 g dry collagen by 3. The calculated concentrations are detailed in **Table 2**. Concentrations are calculated in mol kg^{-1} hydrated leather.

2.2 Prediction of charge on raw collagen at variable pH

The dissociation behaviour of acids is described by **eqn. 5** where $[H_3O^+]$ can be quantified directly from the pH and the K_a from the pK_a of a given residue of an amino acid. $[A^-]$ and [HA] are the amounts of a functional group, such as a carboxylic acid, that are in deprotonated or protonated forms respectively. Assuming no other additions of chemicals, the sum of $[A^-]$ and [HA] must equal the initial concentration of an acid added $[HA]_i$.

$$K_a = \frac{[H_3O^+][A^-]}{[HA]} = \frac{[H_3O^+]^2}{[HA]} \quad \textbf{(5)}$$

The concentrations of $[A^-]$ and [HA] are linked to the initial concentration of acid added, **eqns 6 & 7.**

$$[HA] = (1\text{-}x)[HA]_i \quad \textbf{(6)}$$

$$[A^-] = x[HA]_i \quad \textbf{(7)}$$

$[A^-]$ and [HA] can be substituted with $x[HA]_i$ and $(1\text{-}x)[HA]_i$ respectively to give **eqn. 8** where x represents the proportion of the acid which is dissociated.

$$K_a = \frac{[H_3O^+]\, x[HA]_i}{(1-x)[HA]_i} \quad \textbf{(8)}$$

Eqn. 8 can then be rearranged to **Eqn. 9** to find an expression for x.

$$x = \frac{1}{\frac{[H_3O^+]}{K_a}+1} = \frac{K_a}{[H_3O^+]+K_a} \quad \textbf{(9)}$$

With a known concentration of acid added the associated and dissociated fractions can then be calculated as in **eqns. 8 & 9** respectively.

In the case an organic acid, [HA] represents the protonated form $R\text{-}CO_2H$ and $[A^-]$ represents the deprotonated form $R\text{-}CO_2^-$. In the case of an organic base such as an amine [HA] represents the ammonium form $R\text{-}NH_4^+$ and $[A^-]$ represents the amine.

Consequently, the relative fractions of $[A^-]$ and [HA] can be calculated for each individual amino acid from their respective pK_a values and the amino acid concentrations in 1 kg hydrated collagen calculated in **section 2.1.** The overall charge of the collagen can be obtained through subtracting the total sum concentration of anionic residues from the total sum of cationic residues at a given pH **eqn. 10**.

Total charge on collagen =
Σ conc. cationic functional groups – Σ conc. anionic functional groups **(10)**

The isoelectric point is measured as the point at which the overall charge is equal to zero. At this stage the total concentration of acidic and amide residues are known but their relative ratios are not. The IEP of collagen is believed to be at a pH of *ca.* 7.4. The ratio of acid: amide residues for aspartic acid/asparagine and for glutamic acid/glutamine were manipulated to provide concentrations that would result in an overall IEP of 7.4. The calculated concentrations of all amino acid residues contributing to the overall charge of collagen are provided in **Table 2**.

2.3 Modelling influence of liming on collagen charge at variable pH

During the liming process asparagine, glutamine and arginine are known to undergo hydrolysis to aspartic acid, glutamic acid and ornithine respectively. It is predicted that Glu and Arg have a half-life of approximately 18 – 20 hours, similar to that of the duration of the liming process. The concentrations of Glu and Arg during the liming process were calculated through the addition of the initial concentration of Gla and Asp with 20, 40, 50, 60, 80 and 100% of the concentrations of Gln and Arg added to these respectively. The overall charge of collagen at a given pH was then calculated following the same method as described in **section 2.2** from the revised amino acid concentrations. **Table 2** details the calculated concentration of the respective amino acid residues present in limed collagen where 50% of the Gln and Arg have been converted into their acid analogues. There is evidence that Arginine may also undergo hydrolysis during the liming process to produce ornithine, however this process is slow and as such was not incorporated into this calculation.

3 Results and Discussion

3.1 Quantification of amino acid profile of collagen per unit weight

The amino acid residues present in collagen capable of sustaining charge are responsible, along with pH, for the overall charge of collagen. It is necessary to quantify the concentration of these amino acid residues to calculate their contribution to overall charge. The method used to quantify the concentrations is described in section 2 and can be reduced to the following steps:

1. Defining relative amounts of amino acids present in collagen
2. Calculation of the mass of collagen containing 1000 moles of amino acid in the relative quantities defined in **1** above
3. Calculation of the concentration of a given amino acid per unit mass of dry collagen
4. Conversion of concentration of a given amino acid per unit mass of dry collagen to a concentration per hydrated unit mass

As with any model there are unavoidable assumptions that must be made in the absence of specific experimental data which can be used in its place. The amino acid profile for Type 1 Bovine collagen has been extensively studied and as such there is a good understanding of its composition. However, while the combined concentrations of Asp/Asn and Glu/Gln are known the specific concentrations of each can only be estimated. It is understood that the acid residues contribute to the charge of the collagen at pHs where some of the residues are in carboxylate form and the IEP point of collagen is at pH 7.4. Assuming that Asp: Glu and Asn: Gln ratios remain consistent with those overall, described in **Table 1**, then their relative concentrations can be quantified by manipulating their ratios so that the IEP is at pH 7.4. The concentrations of Asp and Glu were calculated as 61.3% of the sum of Asp + Asn or Glu + Gln concentrations, resulting in the values provided in **Table 2**. It is unlikely that there will be an equal ratio of Asp: Asn and Glu: Gln, however, in the absence of other values an assumption such as this must be made. Importantly, the calculated overall concentrations of acid and amide residue amino acids match those calculated for collagen with an IEP of 7.4 and the pK_a values of Asp and Glu are close enough that it is unlikely this assumption will introduce large errors into the model.

Table 2: Concentrations of acidic and basic amino acids in 1 kg hydrated raw and limed collagen

Amino acid	Concentration in hydrated raw collagen (mmol kg^{-1})	Concentration in hydrated limed collagen (mmol kg^{-1})	pK_a
Aspartic acid	77.4	102.0	3.85
Asparagine	49.1	24.6	
Glutamic acid	119.7	159.1	4.25
Glutamine	76.9	38.4	
Arginine	129.2	129.2	12.48
Lysine	102.3	102.3	10.5
Hydroxylysine	16.15	16.1	10.5
Histidine	10.8	10.8	6.2
Tyrosine	37.7	37.7	10.07

The amino acids present in collagen that can be ionised at certain pH ranges are provided in **Table 2**. Both Asp and Glu are the acidic amino acids of highest concentration of 77.4 and 119.7 mmol kg^{-1} respectively, however appreciable amounts of tyrosine (Tyr) are also present at 37.7 mmol l^{-1}. Importantly, Tyr has a significantly different pK_a of 10.07.

The basic amino acids present in highest concentrations are Arg and Lys at concentrations of 129.2 and 102.3 mmol kg^{-1} respectively. In contrast with the acid amino acid residues Asp and Glu, Arg and Lys have substantially different pK_a values from each other of 12.48 and 10.5 respectively, as such they will have significantly different ionisation behaviour. This feature arises due to the differing basic functional group of each of these amino acids (guanidine for Arg and an amine for Lys) and is seldom discussed in the leather literature. Also present are low levels of His, which also has a substantially different pK_a value of 6.2 and hydroxylysine, which has been assumed to have an identical pK_a value to lysine.

3.2 Modelling charge profile on raw, hydrated collagen

The degree of protonation of an acid or amine group on a protein can be described in terms of the concentration of the amino acid residue and a protonation factor, x, as defined in **eqn. 9**. As the value of x is dependent on the pK_a value (K_a) and the pH (H_3O^+), the degree of protonation of the residues of amino acid residues can be easily predicted for the amino acids described in **Table 2**, assuming the pK_a of the residue within the protein is the same as that of the free amino acid.

Fig. 2 (a) plots the value of x across a pH range of 0 to 14 for the amino acids present in collagen capable of being either positively or negatively charged. In each case at low pHs, x has a value of 0 corresponding to a residue that is protonated, as a neutral carboxylic acid for Asp and Glu, neutral phenol for Tyr and protonated cationic basic groups for Arg, Lys and His. At higher pHs, x has a value of 1 for all amino acids corresponding to the residue being fully deprotonated, as an anionic carboxylate for Asp and Glu, anionic phenoxide for Tyr and deprotonated neutral basic groups for Arg, Lys and His. Where x does not equal either 0 or 1, a mixture of the protonated and deprotonated forms will be present, the levels of which are defined by the value of x. The values of these are calculated from **eqns 8** & **9** respectively.

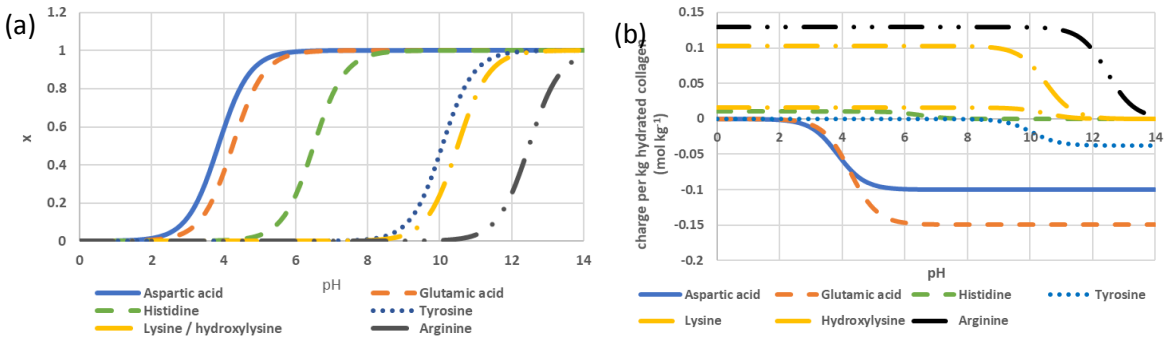

Fig. 2. (a) Theoretical deprotonation ratio of amino acids side chains and (b) calculated molar charge of different amino acid residues per kg of raw hydrated collagen at differing pHs

While all the functional groups on the amino acid side chains follow the same trend from x = 0 at low pH to x = 1 at high pH, the range of pH over which these changes occur is specific to each amino acid. For Asp and Glu, the acid containing residues, this occurs within a pH range of *ca.* 3 and 6 as is consistent with the current understanding. Interestingly, the pHs over which the two basic residues of highest concentration, Arg and Lys, are significantly separated. At pH 12 Arg is 24% deprotonated but Lys is 96% deprotonated. These differences between the acid and basic residues at high concentration indicate that the charge response at high and low pH are likely to be different. Additionally, despite containing an acidic proton, Tyr has a similar response to that of Lys, meaning that it will undergo a conversion from neutral to negative charge across a similar pH range as Lys. His undergoes its transition from protonated to deprotonated from pH 5 to pH 8, a pH region that is independent of any other transitions.

Fig. 2(b) shows the calculated charge on collagen contributed by individual amino acid residues at differing pHs as a molar concentration per kg of hydrated collagen. This does not take into consideration deviations arising due to swelling, assuming identical hydration throughout the pH range. The transitions from positive to neutral (for basic residues) and from neutral to negative correspond to the pH ranges observed in **Fig. 2(a)**. The total contribution of each can then be compared. The significant contributors to positive charge are Arg, which is fully cationic below pH 11 and Lys/Hyl which are fully cationic below pH 9. Present at comparatively low concentration His has a small influence on the overall cationic charge. Asp and Gla are the significant contributors to negative charge, present fully as carboxylates at pHs above 6 and neutral below pH 3. Tyr also has a comparatively small contribution of negative charge at pHs above 9.

The total charge on the collagen substrate can be calculated through the sum of the charge of the individual amino acid residues, with the resulting dependence on pH given in **Fig. 3 (a)**. At < pH 3 the collagen structure is close to fully cationic as almost all the amino acid residues are protonated at this point. Within pH 6 and 9 is the “neutralised” region where substantial change of the pH has little influence on the overall charge. In contrast to the acid region, where the protonation of the acidic amino acids occurs within a pH range of 3 – 6, the deprotonation of the basic residues at high pH shows a consistent change from pH 9 through to 14, double the pH range of the acidic region. The origin of this difference arises from the respective pK_a values of the amino acid residues. Asp and Glu have similar pK_as so their values of x at a given pH are close, whereas Arg and Lys have substantially different pK_a values, leading to an extended response across a broader range of pH.

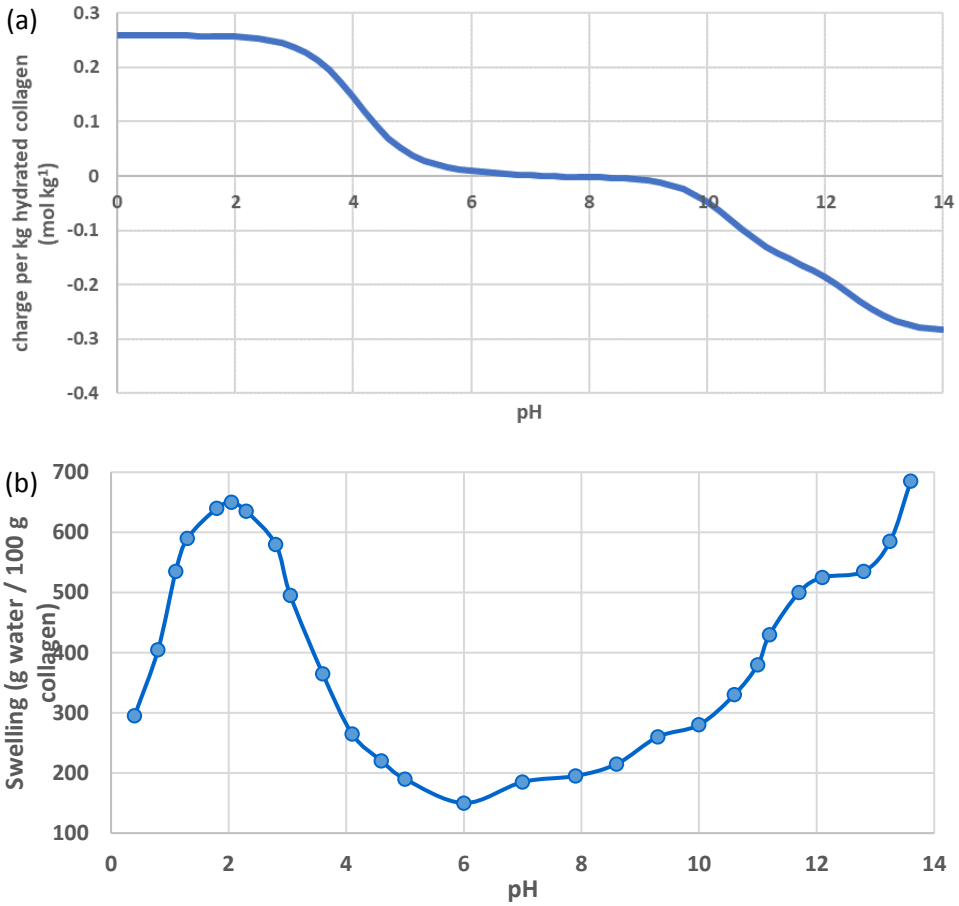

Fig. 3. (a) Total molar charge per kg hydrated collagen at different pHs and **(b)** swelling curve of alkali treated sheepskin reproduced from Bowes, Biochem J. 1950, 46 (1), 1 – 8. [4]

The difference in behaviour at acid and alkaline pHs has important implications for the processes that occur within these regions. Due to its limited solubility, lime has a self-limiting pH of 12.6. In this region 99% of Lys residues are deprotonated, whereas, Arg residues vary considerably within a small pH range: 25% deprotonated at pH 12 and 77% deprotonated at pH 13. Consequently, during liming a small increase in the pH above 12.6 can contribute significantly to the overall charge in this region.

The swelling curve of collagen at variable pH has been reproduced from the original publication by Bowes in **Fig. 3 (b)**. [5] The results plotted on this graph originate from experiments on alkali treated sheepskin so the substrate may be substantially different from the raw bovine collagen modelled here. However, with care some useful comparisons can be made. At progressively lower pH the pelt will swell until a maximum at pH 2. At this pH almost all the carboxylic acid groups are protonated, at pH 2 the total overall charge is 99.2% of the value at pH 0. The swelling is understood to occur due to a combination of electrostatic repulsion from the positively charged sites on the collagen and osmotic swelling. Below pH 2 swelling decreases as, once all acid groups are protonated, additional acid required to reduce the pH acts as an electrolyte, screening charge on the collagen.

At high pH the swelling response does not mirror that observed in acid swelling. There is a gradual increase in the swelling at increased pH, as with acid swelling. However, there is a plateau in the swelling at pH 12 followed by a further rapid increase. This different behaviour has previously been rationalised as a difference in the swelling mechanisms between acidic and alkali conditions, with electrostatic and lyotropic swelling dominant at increased pH. However, this does not consider the comparatively high pK_a of arginine, which leads to the sustained increase in the charge of collagen at high pH. As with swelling under acidic conditions, increase in the charge imbalance on collagen,

breaking salt links, causing electrostatic repulsion of the protonated basic residues leads to progressively larger swelling values up to pH 14. These results suggest that the type of swelling, osmotic or lyotropic, may have less of an implication on swelling behaviour than is currently accepted, with the differing pK_a values of Arg and Lys playing a stronger contribution through their influence on the overall charge, and therefore electrostatic repulsion.

3.3 Modelling charge profile on limed collagen

The process of alkali treatment (liming) is understood to influence the IEP and charge profile of collagen. [4] Partial deamidation occurs, converting some of the Asn and Gln residues to their carboxylic acid analogues. The increasing the concentrations of Asp and Glu, provide a consequent shift in the IEP to values of 5 or lower. [5] This effect has been modelled here, plotted in **Fig. 4** where the charge across pH has been calculated for systems where 20, 40, 60, 80 and 100% of the Asn and Gln residues have been converted into their acid analogues. There is a consequent increase in the negative charge present from the formation of carboxylate from pH 3 and above. Because of the increased carboxylate concentration, the overall charge of the deamidated samples in the region of full neutralisation, pH 6 to 9, is progressively more negative. As a result, the IEP is shifted to progressively lower values from pH 7.4 for raw collagen to 4.7 where there is 50% deamidation and 4.4 where there is 100% deamidation. Notably, the trend in IEP with degree of deamidation is not linear, with large changes in the IEP initially trending to much smaller changes at large % deamidation, again suggesting that IEP alone is not sufficient to predict the behaviour of proteins at variable pH.

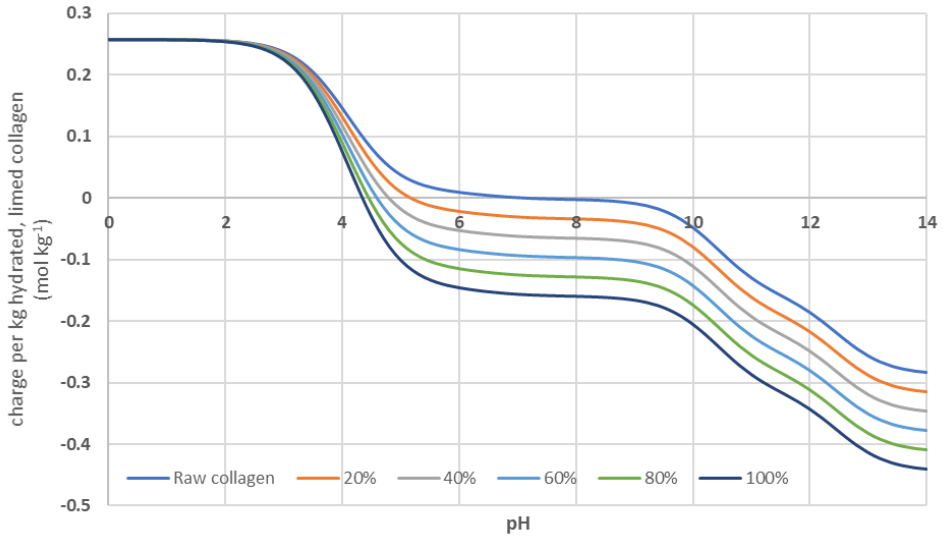

Fig. 4. Molar charge per kg of limed collagen where 20, 40, 60, 80 and 100% of asparagine and glutamine are hydrolysed to their acid analogues

There are suggestions that alkaline hydrolysis of arginine is an important process during alkali treatment. [1] However, measurements by Luck *et. al.* state that, while hydrolysis may occur, there was no conversion at pH 12 after heating at 37 °C for 24 hrs. Elevated temperatures and higher pHs were required to obtain significant hydrolysis. [6] Consequently, hydrolysis of Arg has not been studied here.

4 Conclusions

The importance of the substrate IEP on leather processing is considerable. However, quoting the IEP on its own removes a large quantity of valuable information regarding the collagen charge. For example, it provides little information about the total overall charge at extremes of pH or the rate at which the charge increases with changes in pH. A more holistic approach is to consider the charge on collagen across pHs, providing greater detail regarding it's behaviour. For example, the influence of IEP on the charge of collagen at a given pH is non-linear, showing wide variations depending on the amino acid makeup. Additionally, discussions of IEP of raw collagen do not provide evidence for the difference in the charge behaviour at acidic and basic conditions.

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