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Original article

Physicochemical analysis of casein solubility in water-ethanol solutions

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Abstract – Due to the change in the apparent acid dissociation constant, when ethanol was added to water solutions of an industrially produced spray-dried sodium caseinate, the solution's pH increased proportionally to the reciprocal relative dielectric constant (1/ε). The pH increased by 1 unit when the ethanol volume fraction reached about 0.60. The casein solubility profile at 20 °C as a function of pH (3–8) and the ethanol volume fraction (0–0.75) was quite well represented by the equation describing the ionisation process. The pH inflection point and the slope coefficient were approximately linearly related to 1/ε. The evolution of the electrostatic surface potential (ψ_0) and the zeta potential (ζ) explained the changes in casein solubility as a function of pH and the dielectric constant of the solvent. The increase in ψ_0 by 37 ± 5.2 mV or in ζ by 8.6 ± 1.4 mV raised casein solubility from 10 to 90%.

casein / solubility / ethanol / dielectric constant / electrostatic potential

摘要 – 水－乙醇溶液中酪蛋白溶解性的理化分析。将乙醇加入到由喷雾干燥生产的酪蛋白酸钠水溶液后，由于表观酸离解常数的变化，溶液的 pH 增加与介电常数的倒数(1/ε) 成正比。当加入乙醇的体积分数达到 0.6 时，溶液的 pH 增加了 1 个单位。在 20 °C 时，酪蛋白的溶解性与pH (3–8) 和乙醇体积分数(0–0.75)之间的函数关系可以很好地用电离方程描述。pH拐点和斜率系数与1/ε基本上呈线性关系。表面静电势 (ψ_0)和zeta电势(ζ)的变化进程可以很好地解释酪蛋白的溶解性与 pH 和溶剂的介电常数之间函数变化。当ψ_0增加到 37 ± 5.2 mV 或者 ζ 增加到 08.6 ± 1.4 mV, 酪蛋白的溶解性由 10% 增加达到 90%。

酪蛋白 / 溶解性 / 乙醇 / 介电常数 / 静电势

Résumé – Analyse physicochimique de la solubilité de la caséine dans des solutions d'éthanol-eau. En raison du changement de la constante apparente de la dissociation acide, l'ajout d'éthanol aux solutions aqueuses d'un caséinate de sodium industriel séché par pulvérisation, provoque une augmentation du pH des solutions inversement proportionnelle à la constante diélectrique (1/ε). Le pH augmente d'une unité quand la fraction volumique d'éthanol atteint environ 0.6. Le profil de la solubilité de la caséine à 20 °C, pour des pH de 3 à 8 et pour la fraction volumique d'éthanol entre 0 et 0.75, est assez bien représenté par une équation qui décrit le processus d'ionisation. Le point d'inflexion et la pente sont approximativement linéairement liés à 1/ε. L'évolution du potentiel électrostatique de surface (ψ_0) et du potentiel zeta (ζ) explique les changements de la solubilité de la caséine en fonction du pH et de la constante diélectrique du solvant. L'augmentation du ψ_0 de 37 ± 5.2 mV ou du ζ de 8.6 ± 1.4 mV élève la solubilité de la caséine de 10 à 90 %.

caséine / solubilité / éthanol / constante diélectrique / potentiel électrostatique

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1. INTRODUCTION

Casein, being a mixture of α_{S1}-, α_{S2}-, β- and κ-casein fractions, represents an important and valuable source of ingredients due to its specific nutritional and functional properties [42, 43, 63, 73].

The solubility of main casein fractions in water is a function of the pH, temperature, dielectric constant, ionic strength and ionic environment (calcium and phosphate content) [7, 11, 16, 19, 55, 56, 58, 59].

Since the introduction in the XIXth century of the alcohol test for grading milk, several articles have been published on milk stability in ethanol solutions as a function of both alcohol concentration and Ca, Mg, Na, K, phosphate and citrate ion content, as well as pH, rennet, microbial contamination and genetic variants of casein fractions [8, 14, 28, 31–36, 40, 45, 54, 55, 57, 65, 68, 70, 76, 77].

Alcohol can also be used in the preparation and the purification of individual milk protein fractions [30, 39, 78, 80].

The stability of sodium caseinate in alcohol solutions is an important property for cream liqueur preparations [3–6, 15, 41, 46–48, 50].

Important differences in the secondary structure of milk proteins have been observed in alcohol – water solutions [9, 17, 18].

The aim of this work was to analyse from a physicochemical point of view the combined effects of alcohol concentration and pH on the solubility, the electrostatic surface potential and the electrostatic surface energy of sodium caseinate in water-ethanol mixtures.

2. MATERIALS AND METHODS

Industrially produced, spray-dried sodium caseinate (Armor Proteines, 35466 Saint Brice en Cogles, France) was reconstituted in deionised water at 50 °C using a laboratory mixer, cooled to 20 °C in a water bath and left overnight at room temperature to achieve a complete rehydration. To prevent bacterial growth and limit plasmin activity, 0.1 g·L^{-1} NaN_3 and 0.1 g·L^{-1} soybean trypsin inhibitor were added [11]. The pH of the solutions was adjusted to values in the 2.5–8.0 range with 0.5 mol·L^{-1} HCl or 0.5 mol·L^{-1} NaOH and allowed to equilibrate for 1 h at room temperature. Ethanol was slowly added at 20 °C with continuous mixing, to reach the final ethanol volume fraction of up to 0.75. The solutions were then kept for 1 h at 20 °C and continuously stirred in a water bath. The casein concentration in final solutions was 10 g·L^{-1}. Soluble caseins were separated from aggregates by centrifugation at 2000× *g* for 30 min. Casein content in solutions and supernatants was estimated from nitrogen determinations.

The total solids content of the caseinates was estimated after drying to a constant weight at 102 °C. Total nitrogen (TN), nonprotein N (NPN) and non-casein N (NCN) were measured using Kjeldahl techniques with 6.38 as a nitrogen to protein conversion coefficient [37, 38]. Calcium (Ca), sodium (Na), magnesium (Mg) and potassium (K) concentrations were measured with a Varian SpectrAA 55B atomic absorption spectrometer. The pH of water and ethanol solutions was measured with a Radiometer pHM meter equipped with a combination electrode. Calibration was performed with buffers in aqueous solution. The relative permittivities of aqueous ethanol solutions were calculated according to Åkerlöf [1], Harvey and Prausnitz [27] and Smith et al. [64].

The ionic strength was calculated according to the relation:

$$I = 0.5\ \Sigma m_i\ z_i^2 \qquad (1)$$

where: (m) denotes the molarity and (z) the valence of the dissolved ions.

The activity coefficient (γ) of the ions was calculated by the equation [74]:

$$\gamma = \exp\left[-42 \times 10^5 \left|z_+ z_-\right| \sqrt{\frac{I_T}{(\varepsilon T)^3}}\right] \qquad (2)$$

where: (z_+) and (z_-) are the valences of the positive and negative ions present in the solution, (ε) is the relative dielectric constant of the solvent and (T) is the absolute temperature.

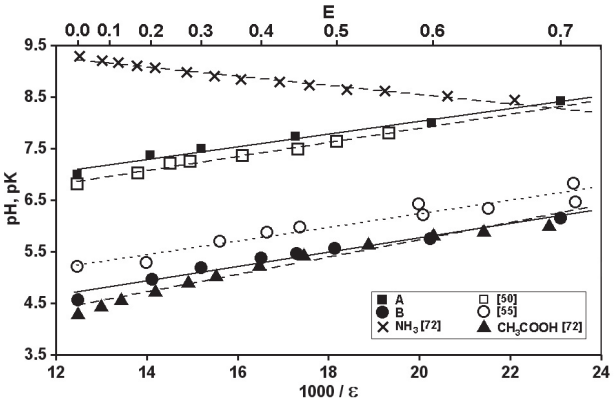

Figure 1. The pH shift of sodium caseinate in water / ethanol solutions as a function of the reciprocal relative dielectric constant (ε): E = ethanol volume fraction. (■) – 10 g·L^{-1} sample (A) – initial pH 7.0; (□) – 30 g·L^{-1} sodium caseinate [50]; (●) – 10 g·L^{-1} sample (B) = sample A with initial pH adjusted to 4.6; (○) – minimum ethanol stability of sodium caseinate [55]; (▲) – apparent pK of acetic acid [72]; (×) – apparent pK of ammonium [72]. Solid, broken and dotted lines represent equation (1) with coefficients A and 1/B given in Table I.

The hypothetical concentration of the ionised acidic (z_-) or basic (z_+) amino acids in mol per mol of protein, as a function of pH, was estimated by applying the relations [74]:

$$z_- = \frac{A_a}{1 + 10^{[pK_a - pH + \log(\gamma)]}} \quad (3)$$

$$z_+ = \frac{A_b}{1 + 10^{[pH - pK_a - \log(\gamma)]}} \quad (4)$$

where: (A_a) and (A_b) are, respectively, the concentrations, in mol per mol of protein, of a given acidic or basic amino acid and pK_a is the intrinsic acid dissociation constant, i.e. the pH level at which the dissociation is exactly 50% for the ionic strength I = 0; (γ) is the activity coefficient.

3. RESULTS AND DISCUSSION

3.1. Composition

The sodium caseinate used in this study contained over 930 g·kg^{-1} dry matter and over 960 g·kg^{-1} casein in total protein. It is consistent with the results of Muir and Dalgleish [48] obtained for industrial sodium caseinates. The sodium content of about 14.6 g·kg^{-1} was in agreement with the results of Towler [69–71]. The potassium and the magnesium contents were 2.5 g·kg^{-1} and 0.3 g·kg^{-1}, respectively. The calcium level (1.5 g·kg^{-1}) was close to that found by Muir and Dalgleish [48] for sodium caseinates from various suppliers.

3.2. pH shift

For ethanol volume fractions up to 0.75, the pH of sodium caseinate solutions rose linearly with the increase in the reciprocal relative dielectric constant (ε) according to the relation (Fig. 1):

$$pH = A + B/\varepsilon + Err \quad (5)$$

where: A is the intercept or the pH level for the hypothetical $\varepsilon = \infty$, and the slope coefficient B indicates the hypothetical pH increase for unitary increase in 1/ε; Err is the standard error term following a normal distribution. More evocative is the reciprocal value of the coefficient B, which indicates the change in 1/ε causing the pH or the apparent pK to change by 1 unit:

$A + B/\varepsilon = A + B/\varepsilon_w + 1$, so $1/\varepsilon = 1/\varepsilon_w + 1/B$

Table I. Intercept (A), slope (B), standard error (Err) and correlation coefficient (R^2) from Equation (5) expressing the relation between the pH or the apparent pK and the reciprocal dielectric constant ($1/\varepsilon$) presented in Figure 1. N = number of experimental points.

Sample	A	B	Err	R^2	N	pH_1 or pK_1	E_1
Sample (A)	5.75 ± 0.27	123 ± 8.1	0.079	0.934	6	8.19	0.63
Sample (B)	3.00 ± 0.17	139 ± 9.8	0.088	0.971	8	5.73	0.57
Pierre [55]	3.56 ± 0.23	132 ± 12.3	0.141	0.934	10	6.23	0.58
CH_3COOH [72]	2.35 ± 0.17	209 ± 6.9	0.113	0.967	12	5.46	0.51
O'Kennedy et al. [50]	5.17 ± 0.11	137 ± 6.9	0.044	0.984	8	7.86	0.58
NH_3 [72]	10.3 ± 0.06	–88.9 ± 3.7	0.039	0.976	15	11.46	0.71

pH_1 or pK_1 shows the pH or pK level for the reciprocal relative dielectric constant $1/\varepsilon = (1/\varepsilon_w + 1/B)$, where $\varepsilon_w = 80.2$ at 20 °C is the relative dielectric constant of water.
E_1 = ethanol volume fraction corresponding to pH_1 or pK_1.

where $\varepsilon_w = 80.2$ at 20 °C is the relative dielectric constant of water.

The values of A and B are given in Table I.

The sodium caseinate samples examined in this study gave very similar tendencies in 1/B to those calculated from the results of Pierre [55] and O'Kennedy et al. [50]. The apparent pK change in the acetic acid [72] was also close to the values obtained for different samples of sodium caseinate. Contrary to carboxyl groups, the apparent pK of amino groups decreases with the increased in the reciprocal relative dielectric constant [72]. The pK reduction rate of the amino groups was about 20% lower than that of the carboxyl groups.

When the reciprocal dielectric constant ($1/\varepsilon$) increased from its initial level ($1/80.2 = 1.25 \times 10^{-2}$) by 1/B from equation (1), the pH or the apparent pK of carboxyl groups increased by one unit to pH_1 or pK_1 (Tab. I). This happened when the ethanol volume fraction reached 0.5 to 0.7 for different samples and groups (E_1 in Tab. I).

The phenomenon of pH or pK changes is due to the evolution of the electrostatic energy when an ion is transferred from one solvent to another one, both characterised by the relative dielectric constants (ε_1) and (ε_2). According to Max Born's model of the electrostatic work (W in joules J) required to transfer an ion of a radius (r) and the charge (z) is equal to [72]:

$$W = e^2 z^2 (1/\varepsilon_1 - 1/\varepsilon_2) / (8 \pi r \varepsilon_0) \quad (6)$$

where: e = elementary electronic charge = 1.602×10^{-19} C, $\pi = 3.14$, $\varepsilon_0 = 8.854 \times 10^{-12}$ $J^{-1} \cdot C^2 \cdot m^{-1}$ = dielectric permittivity of vacuum.

For casein monomers at pH 7 with a radius of around 4.5 nm [66], the electrostatic work (W) is about 30 $kJ \cdot mol^{-1}$ for the passage from water to 60% ethanol, which is the average alcohol concentration for pH_1, calculated for samples A and B and those of Pierre [55] and O'Kennedy et al. [50].

3.3. Protein solubility

The solubility profile (Fig. 2) below and above the isoelectric point follows the Linderstrøm-Lang equation (7), representing the ionisation process [32–34]:

$$\mathrm{Log}_{10}[(S - S_{min})/(S_{max} - S)] = (pH_{50\%} - pH)/D \quad (7)$$

where: $pH_{50\%}$ is the pH inflexion point or the pH corresponding to 50% casein solubility; D is the slope of the straight line in pH versus $\mathrm{Log}_{10}[(S - S_{min})/(S_{max} - S)]$ coordinates, S_{min} and S_{max} are, respectively, the minimal and maximal casein solubility

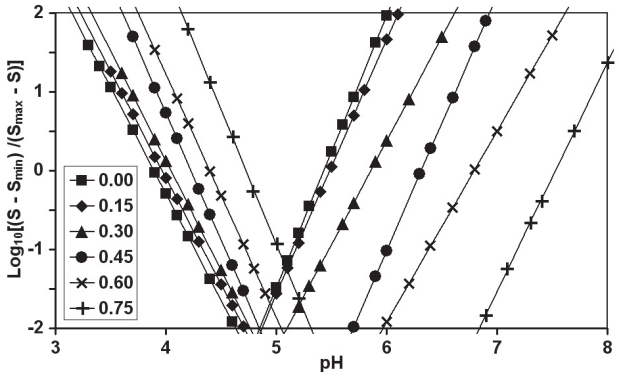

Figure 2. The effect of pH (3–8) and ethanol volume fraction (0–0.75) on the solubility profile of 10 g·L^{-1} sodium caseinate solutions. Solid lines represent Equation (3) with coefficients pH_i and D presented in Figure 3 and Table II.

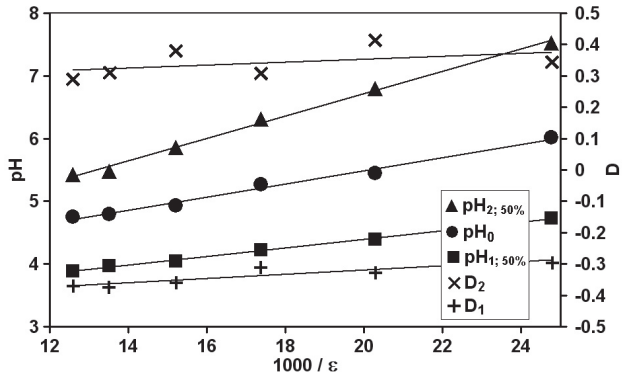

Figure 3. Evolution of pH corresponding to 50% casein solubility ($pH_{1;50\%}$ and $pH_{2;50\%}$) and coefficient D from Equation (3) as a function of the reciprocal relative dielectric constant (1/ε) for the pH range below (pH_1 and D_1) and over (pH_2 and D_2) the minimal solubility point (pH_0). Solid lines represent Equation (1) with coefficients A and B given in Table II.

and S is the solubility at a given pH and ethanol concentration.

Equation (7) can also be presented in the form:

$$S = \frac{S_{min} + S_{max} \times 10^{(pH_{50\%} - pH)/D}}{1 + 10^{(pH_{50\%} - pH)/D}} \quad (8)$$

For the pH range below the minimal solubility point (pH_0), $pH_{1;50\%}$ from equations (7 or 8) increased linearly between 3.7 and 4.6 with the rise in 1/ε (Fig. 3, Tab. II). For pH values over the minimal solubility point, $pH_{2;50\%}$ rose much more (5.2 to 7.4). The lowest level of casein solubility (pH_0) in water occured at pH 4.7 and increased linearly with the rise in 1/ε (Fig. 3). The low solubility zone broadened with the increase in ethanol concentration. A similar tendency for casein solubility in water and in up to 66% ethanol was observed by Zittle and Pepper [79] and by Pierre [55]. The variation in the coefficient D from equation (8) is

Table II. Intercept (A), slope (B), standard error (Err) and correlation coefficient (R^2) from Equation (5) expressing the linear relations between the pH and coefficient (D) from Equation (7) and the reciprocal dielectric constant ($1/\varepsilon$) presented in Figure 3. Number of experimental points N = 6.

	A	B	Err	R^2
$pH_{1;50\%}$	3.03 ± 0.03	68 ± 1.6	0.017	0.998
$pH_{2;50\%}$	3.16 ± 0.10	178 ± 5.6	0.058	0.996
pH_0	3.39 ± 0.10	104 ± 5.7	0.060	0.988
D_1	0.45 ± 0.03	–6.7 ± 1.5	0.015	0.838
D_2	–0.26 ± 0.08	–4.8 ± 4.6	0.048	0.211

relatively small in comparison with the experimental error (Fig. 3). It is positive for the pH range below the minimal solubility point. For the pH range over the minimal solubility point, it is negative and on average does not change with the rise in the ethanol concentration. In absolute terms, there is no statistically significant difference between D_1 and D_2 and their average level is 0.339 ± 0.039.

Around $pH_{50\%}$ points for pH values below and above the minimal solubility point, the casein solubility decreased or increased very rapidly with pH change. Thus, any slight imperfections in the estimation of pH or in the solubility cause quite a significant scattering of the coefficient D between different individual casein solubility curves as a function of pH for a given ethanol concentration (Fig. 3). Nevertheless, for the whole set of data, the regression line between the experimental (S_{EXP}) and model (S_{MOD}) values of casein solubility for N = 150 experimental points gives a standard error of around 3%, which is quite satisfactory:

$S_{MOD} = 0.1(\pm 0.35) + 0.996(\pm 0.005)S_{EXP}$; $R^2 = 0.996$; Err = 2.8; N = 150.

The casein solubility evolution (Fig. 4), calculated by equation (8) shows two solubility zones over 90%, limited by the isolines joining the points of pH 3.5 for water with pH 4.6 for a 0.8 ethanol volume fraction and pH 5.7 for water with pH 8.3 for a 0.8 ethanol volume fraction. The lower solubility zone (<10%) is limited by the isolines passing from pH 4.2 for water to pH 5.1 at 0.8 ethanol volume fraction and from pH 5.1 at 0% to pH 7.6 for 80% ethanol solutions. Between these relatively flat lower or higher solubility zones, there are two very steep zones of decreasing and

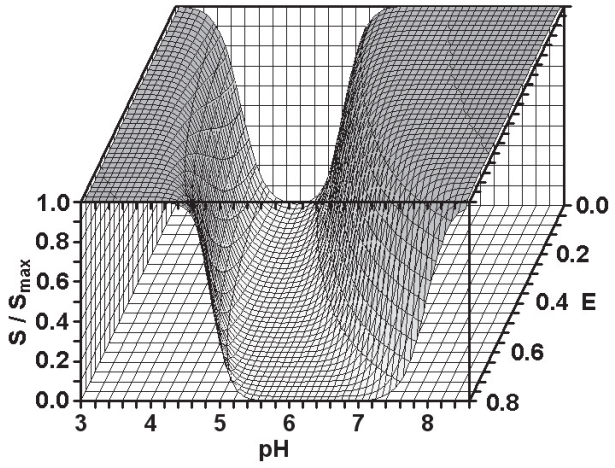

Figure 4. Relative casein solubility (S/S_{max}) as a function of pH and ethanol volume fraction (E), calculated by Equation (8) with $S_{min} = 0$, $S_{max} = 100$ and with average $pH_{50\%}$ and D coefficients calculated by the regression equations from Table II.

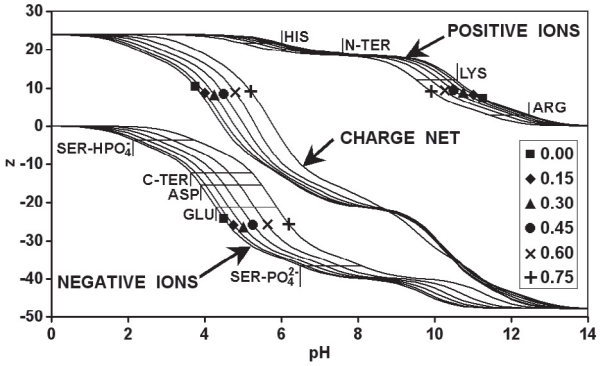

Figure 5. Evolution of the electrical charge (z) of total casein monomers, as a function of pH and ethanol volume fraction (0 to 0.75), calculated by Equations (3) and (4) with $\gamma = 1$ and pK taken from Walstra and Jenness [75]. Horizontal lines with amino acid name abbreviations representing their apparent pK evolution as a function of the ethanol volume fraction (0–0.75).

Table III. Intercept (A), slope (B), standard error (Err) and correlation coefficient (R^2) from Equation (5) representing the isolines of 10% and 90% of casein solubility (Fig. 4) and expressing the linear relations between the pH and the reciprocal dielectric constant ($1/\varepsilon$). Number of experimental points N = 6.

	A	B	Err	R^2
$pH_{1;10\%}$	3.46 ± 0.004	62 ± 0.2	0.003	0.999
$pH_{2;10\%}$	2.71 ± 0.009	166 ± 0.5	0.007	0.999
$pH_{1;90\%}$	2.59 ± 0.006	74.6 ± 0.3	0.005	0.999
$pH_{2;90\%}$	3.41 ± 0.003	182 ± 0.2	0.002	0.999

increasing solubility with rising pH. In pH against ($1/\varepsilon$) coordinates (Fig. 4, Tab. III), the 10% and 90% isolines of casein solubility can be represented by equation (5).

3.4. Ionisation

Protein solubility is in general a function of the intermolecular repulsion and attraction forces that depend mainly on the electrical charge, ionic strength and dielectric constant.

On average, the native casein contains 190 mmol·kg^{-1} of histidine, 560 of lysine, 220 of arginine, 220 of aspartic acid, 740 of glutamic acid and 260 of phosphate groups [75]. In total, casein contains 1.35 mol·kg^{-1} of anionic groups against 0.78 mol·kg^{-1} of cationic groups.

The content of α_{S1}-, α_{S2}-, β- and κ-caseins in total casein is on average equal, respectively, to 40.2, 10.5, 37.4 and 11.9% [66, 67]. On the basis of these proportions and on the known amino acid composition of the main casein fractions, we calculated the average content of the ionic groups in total casein.

The ionic casein groups, dissociated within the 4 to 9 pH range are γ- and δ-carboxyl groups of the aspartic and glutamic acids and the amino group of histidine with pKs of 4.1, 4.6 and 6.4, respectively [55, 74, 75]. Typically for strong acids, the pK of the first H^+ of the serine phosphate (pK 2.12) is only slightly dependent on the dielectric constant changes with ethanol concentration up 80% [72]. For the second H^+ of phosphate esters, the pK 7.21 would increase as for other weak acids. The pK of amino groups decreases by 1 unit with the average increase in the ethanol volume fraction from 0 to 0.73 [72]. So, in 73% (v/v) ethanol the pK of the imidazole group of histidine would be 5.4, that of the ε-amino group of lysine would decrease from 10.6 to 9.6 and of the guanidine group of arginine from 12.5 to

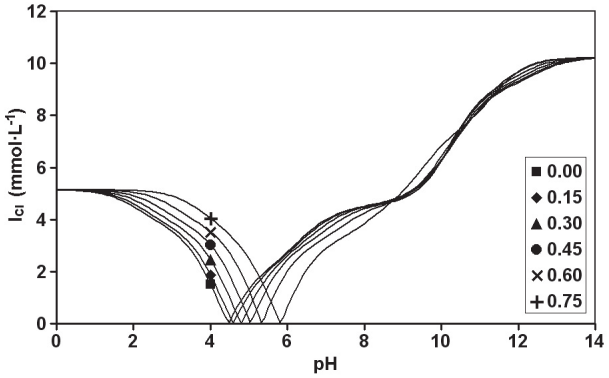

Figure 6. Evolution of the counter ions' ionic strength (I_{CI}) in mol·L^{-1}, as a function of pH and ethanol volume fraction (0 to 0.75), calculated by Equation (1), with m = 0.428 mmol·L^{-1} and the net charge (z) taken from Figure 5.

11.5. In water solution at pH 7 only histidine is at about 80% in its neutral form and at 20% in its protonated form. Lysine and arginine remain almost totally in their protonated forms up to pH 9. In 60% ethanol solutions the carboxyl groups would be 10 times less dissociated than in water. On the other hand, in 73% ethanol the amino groups would be 10 times less protonated, but among them only histidine can play a minor role in electrical charge modification of casein molecules.

For a given pH of casein water solution, when alcohol was added, the pH increased spontaneously, because of the apparent pK changed as an effect of the dielectric constant variation, but the electrical charge of a protein molecule or protein aggregate did not change (Fig. 5). On the other hand, for the same pH level for water and ethanol solutions, the charge differences can be very significant. For pH values between 0 and 3, the amino casein groups are >99.98% protonated. At pH 14 about 99.3% of amino groups are in their neutral form. At pH < 1, over 98.8% of the acidic casein groups are not dissociated. At pH > 11 in water solution and at pH > 12.5 in 75% ethanol solutions, over 99.5% of acidic groups are dissociated. Only at pH < 1 and over 13.5 is there practically only one sort of charge: positive at low pH and negative at high pH levels. At pH < 1, the average electrical charge (z) of casein aggregates is +24 and at pH 14 it is –48. Zero charge, i.e. equal numbers of positive and negative charges in a casein molecule or aggregate, is at pH 4.57 for water solutions and increases to pH 5.79 for 0.75 ethanol volume fractions. Swaisgood [66] found isoionic pH values for water solutions of different casein fractions of between 4.94 and 5.90. For native phosphocaseinate dissolved in water, 50% protein solubility was observed at pH 5.2 [21]. At pH 7.0 the average net charge of total casein would be –18.7 in water solution and –13.6 in 75% ethanol. Because of the opposite effect of alcohol on the pK of amino and acidic groups, for pH > 9 the net charge of casein molecules and aggregates would almost be independent of the ethanol concentration.

3.4.1. Ionic strength

The solution containing 10 g·L^{-1} of sodium caseinate contains 7.09 mmol·L^{-1} Na^+, 0.71 K^+, 0.42 Ca^{2+} and 0.14 Mg^{2+}. The pH of this solution in water is 7.0. The minerals present in the solution represent an ionic strength of 5 mmol·L^{-1}. The contribution of the casein to the ionic strength is as significant as that of the minerals already present. As the solution must be electrically neutral, the counter ions (Cl^- or Na^+) have to be added to neutralise the protonated amino groups and the dissociated phosphoryl, carboxyl and thyrosyl groups. The ionic

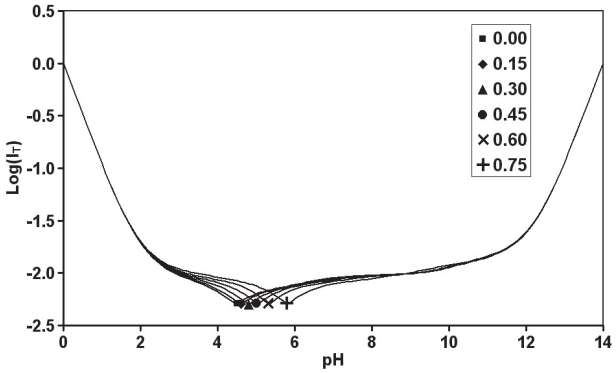

Figure 7. Evolution of the logarithm of the total ionic strength (I_T in mol·L^{-1}), as a function of pH and ethanol volume fraction (0 to 0.75), calculated using Equation (1).

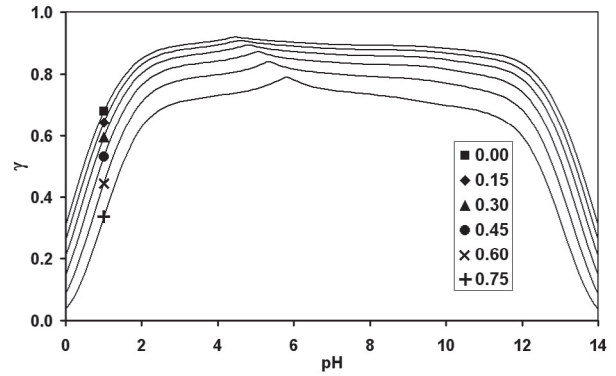

Figure 8. Evolution of the activity coefficient (γ), as a function of pH and ethanol volume fraction (0 to 0.75), calculated by Equation (2) for T = 293 K.

strength, which represents the counter ions, varied between 0 at the isoelectric point and 5.1 mmol·L^{-1} at pH 0 and 10.2 mmol·L^{-1} at pH 14 (Fig. 6). The greatest contribution to the total ionic strength (I_T in Fig. 7) was the ions that were added to the solution to reach very low or very high pH level.

3.4.2. Activity coefficient and electrical charge

The activity coefficient (γ), calculated by Equation (2), varied between 0.04 and 0.92 (Fig. 8). It is at its highest at the isoelectric point and decreases sharply in very low and very high pH regions. The presence of ethanol significantly reduces the activity coefficient.

When taking into account the activity coefficient (γ) from Figure 8 in Equations (3) and (4), instead of $\gamma = 1$, the level of the ionised acidic and basic amino acids became different from that presented in Figure 5. This difference, $\Delta z = z_{\gamma=1} - z_{\gamma}$ (Fig. 9), increased with the rise in the ethanol volume fraction. With the increasing ionic strength and decreasing dielectric constant, the activity coefficient decreased, so less energy is needed to remove a proton from a charged molecule, as the proton senses a smaller electrostatic potential [74]. The observed differences

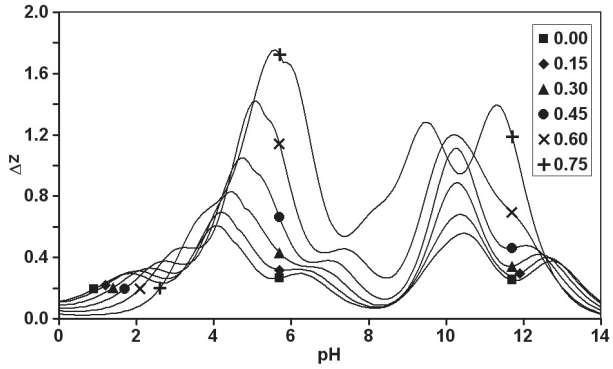

Figure 9. Evolution of the difference ($\Delta z = z_{\gamma=1} - z_{\gamma}$) between the net electrical charge (z) of total casein monomers, calculated by Equations (3) and (4) for the activity coefficient $\gamma = 1$ and for the values of γ taken from Figure 8, as a function of pH and ethanol volume fraction (0 to 0.75).

were greater around those pH levels close to the pK of the principal ionic amino acids, namely, glutamic and aspartic acids (pK = 4.3 and 3.9) and lysine and arginine (pK = 10.6 and 12.5). Because of the opposing effects of the dielectric constant and the ionic strength on the apparent pK of basic amino acids, quite wide differences in Δz were observed between 0.6 and 0.75 ethanol volume fractions. Close to the isoelectric point ($4 < pH < 6$), the observed differences in Δz were larger than at the potential casein molecule charge within this pH range. However, as values grew further away from the isoelectric point these differences became relatively small, when the overall variation in z between +24 at pH 0 and –48 at pH 14 was taken into account.

3.5. Electrostatic surface and zeta potentials

Casein molecules can be considered as spheres of an average radius r = 4.5 nm [66]. Their average electrostatic surface potential (ψ_0) can be estimated by the relation:

$$\psi_0 = ze / (4 \pi \varepsilon_0 \varepsilon r). \qquad (9)$$

The $\psi_0 = 0$ at the isoelectric point (pH_0), which increases from about 4.5 for water solutions to 5.8 for 0.75 ethanol volume fractions (Fig. 10). For the $pH < pH_0$ range, the ψ_0 is positive and for $pH > pH_0$ it is negative. For casein dissolved in water or in 75% ethanol, the electrostatic surface potential at pH 0 is +95 mV and +191 mV, respectively. At pH 14 the ψ_0 is, respectively, –189 and –376 mV.

The surface potential (ψ_0) did not coincide strictly with the isoline pH_0 from Figure 3. The surface potential, corresponding to the isoline pH_0, increased from 12 mV for water solutions to 30 mV for a 0.75 ethanol volume fraction (Fig. 11, Tab. IV). The absolute values of the electrostatic surface potentials $\psi_{1;10\%}$, $\psi_{2;10\%}$, $\psi_{1;90\%}$, and $\psi_{2;10\%}$, corresponding to 10% and 90% casein solubilities equal for the pH range below ($_1$) and over ($_2$) the minimal pH solubilities, were inversely proportional to the relative dielectric constant (Fig. 11). Within the standard error limits of (± 5 mV), the electrostatic surface potential levels were similar for the corresponding isolines on both sides of the minimal casein solubility or minimal surface potential. The surface potential difference between 90% and 10% casein solubility was on average 37 ± 5.2 mV, independently of the dielectric constant of the solvent. This means that on both sides of the isoelectric point, an increase in the surface potential of about 40 mV augments the casein solubility from 10% to 90%.

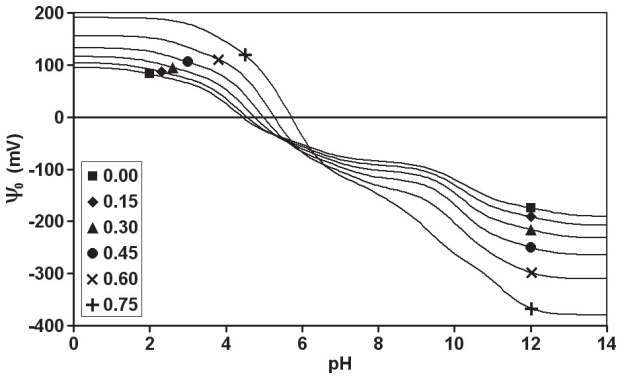

Figure 10. Evolution of the electrostatic surface potential (ψ_0) of sodium caseinate monomers, as a function of pH and ethanol volume fraction (E), calculated by Equation (9) with the charge (z) calculated by Equations (3) and (4) and the activity coefficient (γ) by Equation (2).

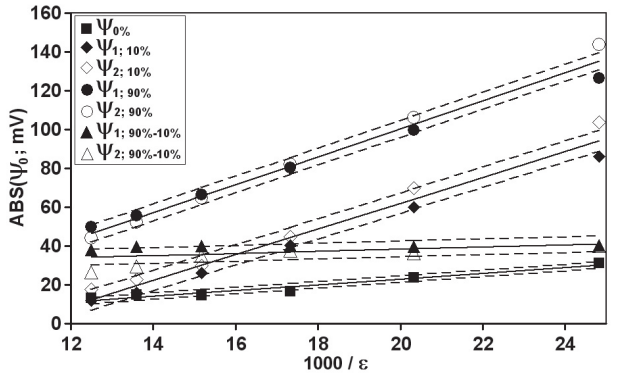

Figure 11. Absolute value of the electrostatic surface potential (ψ_0) of casein monomers as a function of the reciprocal relative dielectric constant ($1/\varepsilon$) for the line pH_0, from Figure 3 ($\psi_{0\%}$) and for the isolines $pH_{1;10\%}$, $pH_{2;10\%}$, $pH_{1;90\%}$ and $pH_{2;90\%}$ from Figure 4, corresponding to casein solubilities of 10% and 90% for the pH range below ($_1$) and over ($_2$) the minimal surface potential (ψ_0). Solid lines represent Equation (5) with coefficients A and B given in Table IV and broken lines show standard deviation limits.

The electrostatic surface potential of protein molecules in solutions is partly screened by the counter ions present in the double layer. The potential at the double layer, being approximately equal to zeta potential (ζ), can be estimated by the relation [29]:

$$\zeta = \psi_0 \frac{r}{r + \lambda_D} \exp(-1) \qquad (10)$$

where: λ_D is the Debye length or the nominal thickness of the electric double layer, being a function of the relative dielectric constant of the solvent (ε), the absolute temperature (T) and the total ionic strength (I_T), according to the equation [74]:

$$\lambda_D = \sqrt{\frac{\varepsilon_0 \varepsilon k_B T}{2 I_T N_A e^2}} \qquad (11)$$

where: $k_B = 1.381 \times 10^{-23}$ J·K^{-1} is the Boltzmann constant and $N_A = 6.022 \times 10^{23}$ is the Avogadro number.

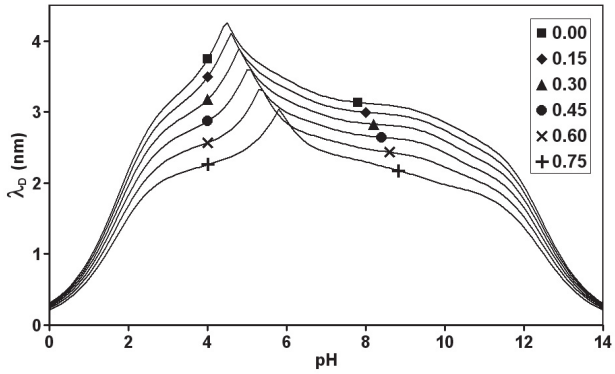

Figure 12. Evolution of the Debye length (λ_D), as a function of pH and of ethanol volume fraction (0 to 0.75), calculated by Equation (11) for T = 293 K.

Table IV. Intercept (A), slope (B), standard error (Err) and correlation coefficient (R^2) from Equation (5) expressing the relations between the absolute values of the electrostatic surface potential (ψ_0 in mV) and the reciprocal dielectric constant ($1/\varepsilon$) for the lines pH_0, $pH_{1;50\%}$ and $pH_{2;50\%}$ from Figure 3, and the isolines $pH_{1;10\%}$, $pH_{2;10\%}$, $pH_{1;90\%}$ and $pH_{2;90\%}$ from Figure 11, corresponding to a casein solubility equal to 10% and 90% for the pH range below ($_1$) and over ($_2$) the minimal solubility point (pH_0). N = number of experimental points.

	A	B	Err	R^2	N
ψ_0	–6.4 ± 2.9	1475 ± 164	1.7	0.953	6
$\psi_{1,2;50\%}$	–54 ± 7.6	7034 ± 429	6.3	0.964	12
$\psi_{1,2;10\%}$	–71 ± 6.5	6645 ± 364	5.3	0.710	12
$\psi_{1,2;90\%}$	–43 ± 5.3	7192 ± 300	4.4	0.983	12
$\psi_{1,2;90\%-10\%}$	29 ± 4.9	547 ± 278	4.1	0.279	12

The Debye length is the distance from the surface of the protein molecule, over which the electrostatic surface potential drops to exp(–1) = 0.368 of its initial level ψ_0. For the experimental conditions applied in this work, the nominal thickness of the electric double layer varied between 0.2 and 0.3 nm at very low or very high pH levels and 3.0 and 4.3 nm near the isoelectric points (Fig. 12). This means that λ_D varied between 5% and 95% of the average radius of casein monomers. For the same pH level, λ_D decreased progressively with the rise in the ethanol volume fraction. For casein micelles in milk, the Debye length is ~1 nm [40, 75].

The casein monomer zeta potential varied between +33 and +67 mV at pH 0 and –65 and –132 mV at pH 14 (Fig. 13). As with the electrostatic surface potential, the absolute value of the zeta potential decreased with drops in the ethanol volume fraction. Within the 4.5 to 6.6 pH range, the effect of the ethanol volume fraction is opposite to that 4.5 > pH > 6.6. For milk pH, the zeta potential decreased from –16 mV for water solution to –26 mV for 75% ethanol solution. The zeta potential of casein micelles in milk and in different buffers varied between –30 and –10 mV [2, 10, 12, 20, 23, 25, 26, 49, 52, 53]. In absolute values, the zeta potential is lower for smaller micelles [51] and also decreases with the addition of calcium, magnesium, copper and iron ions [53], and when the pH is lowered [2, 13]. When the ionic strength is high, the zeta potential may be much

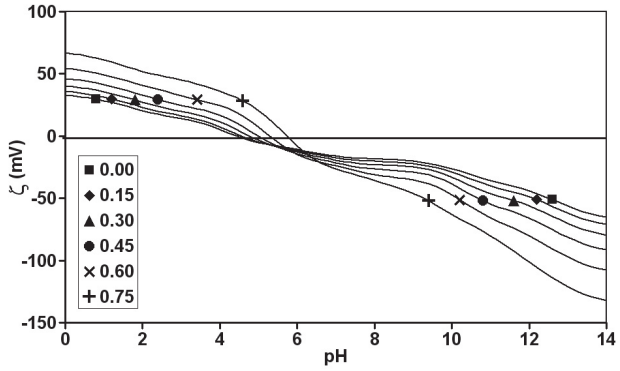

Figure 13. Evolution of the zeta potential (ζ) of casein monomers, as a function of pH and ethanol volume fraction (0 to 0.75), calculated by Equation (10) for T = 293 K.

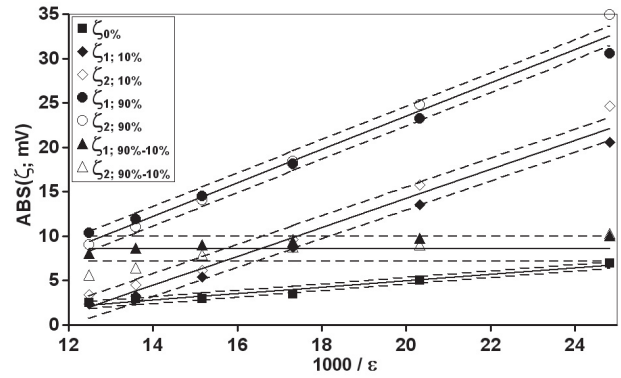

Figure 14. Absolute value of the zeta potential (ζ) of casein monomers as a function of the reciprocal relative dielectric constant (1/ε) for the line pH_0 ($\zeta_{0\%}$), from Figure 3 and the isolines $pH_{1;10\%}$, $pH_{2;10\%}$, $pH_{1;90\%}$ and $pH_{2;90\%}$ from Figure 4, corresponding to casein solubilities of 10% and 90% for the pH range below ($_1$) and over ($_2$) the minimal zeta potential (ζ). Solid lines represent Equation (5) with coefficients A and B given in Table V and broken lines show standard deviation limits.

smaller than the electrostatic surface potential [10, 52, 53].

The zeta potential, corresponding to the isoline pH_0 from Figure 3, increased from +2.5 mV for water solutions to +7.0 mV for ethanol volume fractions equal to 0.75 (Fig. 14, Tab. V). The absolute values of the zeta potentials $\zeta_{1;10\%}$, $\zeta_{2;10\%}$, $\zeta_{1;90\%}$, and $\zeta_{2;10\%}$, corresponding to casein solubilities of 10% and 90% for the pH range below ($_1$) and over ($_2$) the minimal pH solubility, are inversely proportional to the relative dielectric constant. Within the limits of standard error (± 1.5 mV), the zeta potential levels are similar for the corresponding isolines on both sides of the minimal casein solubility or minimal surface potential. The zeta potential difference between 90% and 10% casein solubility is on average 8.6 ± 1.4 mV, independently of the dielectric constant of the solvent. This means that on both sides of the isoelectric point, an increase in the zeta potential of about 9 mV raises the casein solubility from 10% to 90%.

Protein molecules in solution are also subjected to van der Waals attractive interactions,

Table V. Intercept (A), slope (B), standard error (Err) and correlation coefficient (R^2) from Equation (5) expressing the relations between the absolute values of the zeta potential (ζ in mV) and the reciprocal dielectric constant ($1/\varepsilon$) for the lines pH_0, $pH_{1;50\%}$ and $pH_{2;50\%}$ from Figure 3, and the isolines $pH_{1;10\%}$, $pH_{2;10\%}$, $pH_{1;90\%}$ and $pH_{2;90\%}$ from Figure 14, corresponding to a casein solubility equal to 10% and 90% for the pH range below ($_1$) and over ($_2$) the minimal solubility point (pH_0). N = number of experimental points.

	A	B	Err	R^2	N
ζ_0	–2.3 ± 0.7	362 ± 37	0.4	0.960	6
$\zeta_{1,2;50\%}$	–16 ± 1.9	1798 ± 105	1.5	0.967	12
$\zeta_{1,2;10\%}$	–18 ± 1.6	1627 ± 88	1.3	0.972	12
$\zeta_{1,2;90\%}$	–14 ± 1.4	1878 ± 76	1.1	0.984	12
$\zeta_{1,2;90\%\text{-}10\%}$	4 ± 1.1	251 ± 62	0.9	0.618	12

which can partly or totally counterbalance the electrostatic repulsion. The Hamaker constant (A_H) has to be known to determine the van der Waals interaction energy. However, the Hamaker constant has been determined for only very few proteins [24, 60]. Probably none of the casein family has been studied so far. For the same protein, depending on the method and the theoretical approach used, the Hamaker constant can vary between 2 and 10 k_BT [24]. For casein micelles Kruif and Tuinier [40] applied $A_H = 1\ k_BT$, assuming that the protein density of the casein micelles is 6 times lower than in globular proteins. Without having more precise evaluation of the Hamaker constant for caseins, it seems premature to estimate correctly the van der Waals attractive energy.

In this work we have presented the electrical charge (z), the electrostatic surface potential (ψ_0) and the zeta potential of hypothetical molecules with a weight average amino acid composition of four basic casein fractions α_{S1}-, α_{S2}-, β- and κ-. The weight average molecular weight of this virtual, total casein would be 23.38 kg·mol^{-1}. This approach seems to work for comparing the solubility of total casein with the electrostatic surface potential or the zeta potential of such a hypothetical total casein molecule.

In reality, the four basic casein fractions in solution coexist and interact independently. Their electrostatic surface potential (ψ_0) as a function of pH is presented in Figure 15. At pH 3 in water solutions, it is α_{S2}-casein that is the most positively charged (+92 mV). At pH 6 to 9 it is α_{S1}-casein that is most negatively charged (–122 mV at pH 9). So-called total casein (t in Fig. 15) evolves somewhere in the middle. In 60% (v/v) ethanol solutions, the amplitude of the electrostatic surface potential increases to +192 mV at pH 3 for α_{S2}-casein and to –204 mV for α_{S1}-casein at pH 9, but the order of fractions does not change significantly. The isoelectric point increases on average by 1 pH unit. The enhancement of the differences of the electrostatic potential between the casein fractions by ethanol could be employed for the preparation of enriched or purified α_{S1}-casein [30, 78].

The evolution of the electrostatic surface potential (ψ_0) of individual casein fractions, or the hypothetical total casein, explains casein solubility quite well as a function of pH and the dielectric constant of the solvent. It has to be stressed, however, that at pH < 1 the molecules have only positive charges and at pH > 13.5 they are almost exclusively negatively charged. Within the pH range between 3 and 9, analysed in this work, the casein molecules have both sorts of charges. For this reason, not only repulsive but also attractive electrostatic interactions exist within the same molecules and between different casein molecules, leading to the formation of casein micelles [19, 22, 61, 62]. The state of casein aggregation in sodium caseinate solutions is not very well known. Dalgleish and Law [11] found particle presence of around 100 nm in water solutions containing 15 mmol·L^{-1} of Ca^{2+} for spray-dried, 50 nm

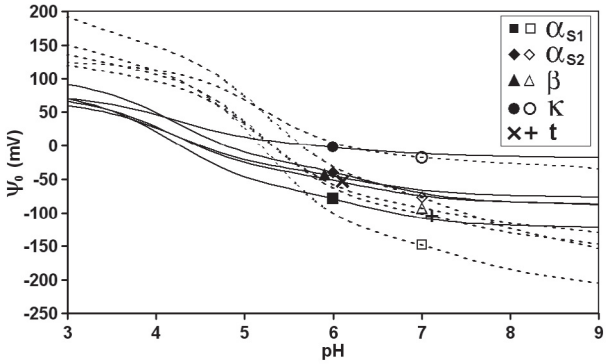

Figure 15. Evolution of the electrostatic surface potential (ψ_0) of principal casein fractions (α_{S1}-, α_{S2}-, β- and κ-) dissolved in water (solid lines) or in 60% (v/v) ethanol (broken lines) as a function of pH. The lines (t) represent total casein.

for roller-dried and 300 nm for laboratory prepared and freeze-dried sodium caseinate samples. For industrial calcium caseinates dissolved in water, Moughal et al. [44] found the particle size distribution to be bimodal, with the radius of the first peak of around 170–200 nm and that of the second peak of around 13–17 μm. In the absence of calcium ions, the aggregates in the sodium caseinate are too small to be detected by laser methods based on the intensity of the scattered light [11, 44, 74].

4. CONCLUSION

Casein solubility is a function of the intermolecular repulsion forces governed by the ionisation process of acidic and amino groups, being a function of pH and of the solvent's dielectric constant.

Equations describing the ionisation phenomenon, the electrostatic surface potential and the electrostatic surface energy quite satisfactorily represent casein solubility in water and in water ethanol mixtures.

The same equations would be applicable for other proteins and other polar solvents such as, for example, alcohols, amides, amines, esters, glycols, ketones, nitriles and sulphoxides.

A similar approach could be applied to studying the solubility of individual casein fractions and especially of α_{S1}-casein in neutral and slightly basic solutions and α_{S2}-casein in acidic conditions.

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