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Isochoric pressure-temperature behavior of lactalbumin + water reactive mixtures

under subcritical conditions

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abstract

¹*Abbreviations:* AA: amino acids, LA: lactalbumin, P: pressure, PW: Pure water, T: temperature, TSS: total soluble solids, ρ : density, % w/w: weight percentage.

This study examines the pressure-temperature (*P-T*) behavior of reactive mixtures composed of lactalbumin and water under subcritical and isochoric conditions. The impact of density on the *P-T* behavior was investigated for an initial lactalbumin composition of 5%, covering a density range of 0.7597 g/mL to 0.85620 g/mL. Additionally, the effect of varying lactalbumin concentrations, ranging from 3% to 15% (w/w), at a constant initial density of 0.831 g/mL, was also studied. Isochoric lines and density values of the reactive mixtures were compared to those of pure water under identical pressure and temperature conditions in all experiments. Furthermore, the total soluble solids (TSS) contract was analyzed after each experiment, along with the thermal stability of amino acids subjected to prolonged reaction periods and high temperatures.

Keywords: Subcritical water, hydrolysi , is schoric behavior, lactalbumin, high hydrostatic pressure.

1. Introduction

Protein hydrolysates are esed in various industries, including the food industry, as they increase the nutritional value and/or improve the functional properties of the incorporated foods (Abd El-Salam & El-Shibiny, 2017). Additionally, they can be considered nutraceutical products, as they exert potential beneficial effects on the health of consumers (van Loon, 2007). Protein hydrolysates can enhance the functionality of certain food products by modifying their viscosity or improving their emulsifying, foaming, gelling, and flavor properties (He, Franco, & Zhang, 2013). Furthermore, applications of protein hydrolysates from different sources have been reported in the production of biopolymers, adhesives,

surfactants, or as substrates for microbial growth (Mekonnen et al., 2013; Mekonnen, Mussone, El-Thaher, Choi, & Bressler, 2013; Muscio, Cole, McCarty, & Sandu, 1982; Sereewatthanawut et al., 2008).

Protein hydrolysates are commonly obtained through chemical hydrolysis (acid or alkaline catalysis) or enzymatic hydrolysis of the protein (Fountoulakis & Lahm, 1998). An alternative to these conventional methods is the use of subcritical water. In this case, the use of acids, bases, or enzymes is not necessary, as water, due to the operating conditions, acts simultaneously as the reaction medium, reactant, and catalysi (Brunner, 2009). During the hydrolysis process under subcritical conditions (temperatures between 100 °C and 374 °C), water remains in a liquid state due to the high pressure at which the process is carried out (King, 2000). As the temperature increases, the nonic product of water increases due to the higher dissociation of molecules into hydrolysis into (H3O⁺) and hydroxide ions (OH⁻). At temperatures above 200 °C, the dissociation constant of water increases by 3 orders of magnitude, allowing it to act as an acid, base, or "bi-catalyst" (Katritzky, Allin, & Siskin, 1996).

The use of subcritical water has proven to be a viable alternative method for obtaining protein hydrolysates from annual sources (Cheng et al., 2008; Kang, Daimon, Noda, Hu, & Fujie, 2001; Rogalinski, Hermann, & Brunner, 2005; Tavakoli & Yoshida, 2006; Zhu, Zhu, Zhao, & Cheng, 2008), plant-based sources, and marine organisms (Daimon, Kang, Sato, & Fujie, 2001; Quitain, Sato, Daimon, & Fujie, 2001; Sunphorka, Chavasiri, Oshima, & Ngamprasertsith 2012; Watchararuji, Goto, Sasaki, & Shotipruk, 2008). In addition to not requiring chemical or biological catalysts, another advantage of subcritical protein hydrolysis is its high reaction rate due to the elevated temperatures achieved in the process. While acid

hydrolysis typically requires a reaction time of over 4 hours (Fountoulakis & Lahm, 1998; Waitanapat, Nakayama, Beuchat, & Phillips, 1994), subcritical conditions can significantly reduce this time (Ali-Nehari, 2011; Daimon et al., 2001; Rogalinski et al., 2005; Tavakoli & Yoshida, 2006). From an environmental perspective, the use of subcritical or supercritical water is a more favorable alternative to chemical hydrolysis methods, as it does not generate solid waste from both the hydrolysis process and the neutralization of reactants (Lin et al., 2015).

In this work, the term "reactive mixtures" is used to define mixtures that, under hightemperature conditions, react due to thermal effects, but at room temperature or slightly above, they do not react without the addition of a catalyst (Cotabarren, Velez, Hegel & Pereda, 2016; Velez, Hegel, Mabe, & Brigne e, 2010a; Velez, Pereda, & Brignole, 2010b). This is the case for water + lactalbumin n. stures analyzed in this study. Due to the fact that these types of mixtures react above e certain temperature without the need for a catalyst, experimental measurements of volumetric properties under high-temperature conditions are challenging to perform without causing compositional changes in the system.

To carry out protein by the system subcritical water in a process developed under liquid and monophasic conditions, or in the case of solid-liquid reactive systems in the absence of a vapor phase, it is important to understand the volumetric properties of the reactive mixtures in the liquid phase. This knowledge will enable the safe design and operation of process equipment. When working with a nearly incompressible fluid in a single phase, studying the relationship between mixture pressure, temperature, and composition under operating conditions is highly relevant (Cotabarren et al., 2016; Velez et al., 2010a). Furthermore, if the process is conducted in a continuous reactor, one of the most important variables to

consider in its design is the residence time of the reactive mixture, which depends on its density. The residence time in a continuous reactor is determined by the ratio of the reactor volume to the volumetric flow rate. The volumetric flow rate, in turn, can be related to the mass flow rate of the reactive mixture through its density value:

$$\tau = (V/\dot{m}) * \rho \qquad \text{(Eq. 1)}$$

Where τ is the residence time, *V* is the reactor volume, *m* is the mass flow rate, and ρ is the mass density of the mixture. By knowing the density of the master mixture under the operating conditions, and assuming that its value remains constant throughout the reactor, the residence time can be calculated by measuring the mass flow rate.

To the best of our knowledge, information regarding d =volumetric properties of mixtures of water + proteins under subcritical reaction conditions is not readily available in the literature. One approach to simplify cal rule ions of residence time in continuous reactors is to assume that the volumetric properties of the reactive mixtures under operating conditions will be approximately equal to those of pure water, which is the major component. However, this assumption can lead to significant errors (Velez et al., 2010a), highlighting the necessity of having experimental data related to the reactive system to make this simplification accurately.

The objective of this study is to experimentally investigate the isochoric pressuretemperature-mass density $(P-T-\rho)$ behavior of water + lactalbumin mixtures with different initial compositions under subcritical hydrolysis reaction conditions in the absence of a vapor phase. The effects of mixture density and composition on the *P-T* behavior will be analyzed using an isochoric method (Velez et al., 2010a; Zhou et al., 2006), which will also provide insights into the stability of free amino acids under elevated temperature, pressure, and extended reaction times.

2. Materials and Methods

2.1. Reagents

All the reagents and solvents used were of analytical grade. Lactalbumin (\geq 92 %), diethyl ethoxymetylenemalonate and AAS18 - Amino Acid Standard were purchased from Sigma-Aldrich (Germany).

2.2. Isochoric experiments

The constant volume cell used for the experiments consisted of a 26.50 ± 0.01 mL stainless steel tube with a nominal diameter of 1/2". A PT-100 cmg erature sensor (platinum resistance thermometer, ± 0.1 °C) was connected to the confide measure the temperature. The pressure was measured using a pressure sensor with an accuracy better than $\pm 0.5\%$ (Dynisco TPT463E-10M-6/18, USA). A schematic alagram of the equipment used for the isochoric measurements is shown in Figure 1.

[Figure 1]

The calculation of the ten perature-induced variation was performed using the following equation (Abdulagatov & Azizov, 2006):

$$\Delta V_T = V_0 * 3\alpha * (T - T_0)$$
 (Eq. 2)

Where V_0 is the initial volume of the cell at a reference conditions, α is the thermal expansion coefficient of the material (α = 1.65 x 10⁻⁵ °C⁻¹), and T_0 is the reference temperature (25°C). On the other hand, the contribution to the cell volume variation due to pressure changes (ΔV_P)

was calculated according to Keyes, (1933) for a cylindrical geometry, with Poisson's coefficient ($\sigma = 0.3$) and Young's modulus (E = 193 GPa) for the material.

The experimental procedure was as follows: the cell was initially loaded with a known mass of a water + lactalbumin mixture with an accuracy of ± 0.0001 g. This determined the overall mass density (ρ), of the system, which was equal to the mass of the water + lactalbumin mixture divided by the volume of the cell. After loading, the cell was closed and placed in a thermally insulated electric furnace with forced convection and consperature controller. The desired temperature was set, and the heating process start d. Once the required temperature was reached, it was maintained constant until the pressure value stabilized. Once the mentioned conditions were reached, the pressure and comperature values were recorded. Subsequently, the temperature was increased, and the aforementioned procedure was repeated to cover the desired temperature imperature.

The validation of the equipment and x_{1} experimental technique was performed through the *P*-*T*- ρ measurement of two chemical compounds with known properties, namely water and methanol. The results obtained for both reference fluids can be found in Figure A in the Supplementary Material.

In all experiments, the r rocess started at low temperature with the presence of a vapor phase. Due to the high values of ρ used in the experiments, the vapor phase decreased in volume as the temperature increased under isochoric conditions until the entire system collapsed into a single liquid phase. This ensured the presence of a single liquid phase within the cell in all conducted tests, which guaranteed a practically linear behavior of the isochors (Velez et al, 2010b).

After each experiment, the cell was cooled to room temperature, and the liquid product

mixture was collected for further analysis. The studied initial densities were in the range of 0.75 - 0.85 0.7597 - 0.8620 g/mL. The final temperature reached and the duration of each experiment depended on the selected density value, ranging from 230°C to 310°C and lasting from 3.5 to 4 hours. Each of the conducted experiments started with the loading of a fresh mixture of water + lactalbumin according to the corresponding initial density and composition values. All the obtained *P*-*T*- ρ values in this study and the temperature time dependence of the experiments can be found in the tables A a.⁴ B in the Supplementary Material.

The uncertainties associated to density and concentrations values were estimated using the combined expanded uncertainty equation:

$$u^{2}(y) = \sum_{i=1}^{n} \left(\frac{\partial f}{\langle x_{i} \rangle}\right)^{2} u^{2}(x_{i})$$
 (Eq. 3)

2.3. Total soluble solids and amino ack' quantification

Total soluble solids (TSS) were intermined by gravimetric analysis. For this purpose, 5 g of the collected liquid were placed in a previously weighed crucible in an analytical balance (Shimadzu AUW220D, Japan), and placed in an electric oven at 105 °C to constant weight. Then, the crucible with dry matter was weighed and the total solid value was calculated.

Amino acid identification and quantification were performed using a high-performance liquid chromatography (HPLC) system equipped with an UV detector (Perkin Elmer©R 600 Series). Data acquisition and processing were performed using Total Chrom Workstation software (version 6.3). Separation of amino acids was achieved using an Agilent Technologies Zorbax Eclipse Plus C18 column (4.6×150 mm, particle size 5 µm). Prior to

analysis, amino acids were derivatized with diethyl ethoxymethylenemalonate (Alaiz, Navarro, Girón, & Vioque, 1992).

Following the analysis, identification of the sample compounds was achieved using the external standard method, where the obtained chromatogram was compared with AAS18 amino acids standard. For quantification, calibration curves were generated for each amino acid using standard solutions with concentrations ranging from 500 to 25,000 μ g/L. This technique allowed the quantification of 17 out of the 20 protein $\sigma_{\rm E}$ me amino acids (Miranda-Villa, Mufari, Bergesse & Calandri, 2019). The determinations were performed in duplicate.

3. Results and Discussion

3.1. Influence of density on the P-T beh. vic : of the reactive mixtures

To analyze the isochoric P-T behavio: of the reactive mixtures of water + lactalbumin at different ρ values, experiments were conducted using mixtures initially containing 5% w/w lactalbumin. The chosen ρ incial values were 0.75, 0.77, 0.8, 0.82, and 0.85 0.7597, 0.7815, 0.8110, 0.8310, and 0.2522 g/mL. The results of the pressure behavior exerted by the mixtures can be obserted in Figure 2. Each of the isochors for different ρ values of the mixture is compared with the *P*-*T* behavior of pure water (PW) at the same ρ value (Lemmon, Bell, Huber & Mc Linden, n.d.).

For the entire experimental range studied, a linear isochoric relationship between pressure and temperature was observed in the monophasic region of the mixtures. This linear relationship is typical of pure compounds and has been observed in asymmetric mixtures of different molecular weights, such as methanol + naphthalene, methanol + n-octadecane

(Velez, et al., 2010b), as well as in systems involving gaseous components, polymers, or organic solvents (Menossi, Milanesio, Ciolino, Camy & Zabaloy, 2020). It is important to note that these aforementioned systems are non-reactive under the studied conditions. However, the linearity of the isochoric behavior in monophasic conditions has also been observed in reactive systems, such as those involved in the transesterification of vegetable oils using supercritical methanol for biodiesel production (Velez, et al., 2010a).

In general, as ρ increases, the isochoric lines exhibit steeper sloper as observed in Figure 2. For the value of 0.77 0.7815 g/mL, the slope of the isochor were compared to that of 0.75 0.7597 g/mL. In all cases, the pressure exerted by the mixture was lower than that of pure water under the same ρ and T conditions. This behavior has also been observed in reactive mixtures such as methanol + sunflower viry it a molar ratio of 40:1 for methyl ester production under high pressure and temperature conditions (Velez, et al., 2010a). It was found that the mixtures exerted a lower pressure relative to the major component (methanol) at a given temperature. In the case of 5% lactalbumin mixtures, a maximum pressure difference of 130 134 bar was observed between the isochor of the mixture and that of pure water. This maximum difference occurred at initial ρ of 0.8020 g/mL and T of 283 242.2 °C. For initial ρ of 0.7815 g/mL and T of 277.3 °C, the pressure difference between the mixture and pure water at the same conditions was only 8 24 bar, but as the temperature increased, this difference increased, reaching a maximum of 65 78 bar at 295.5 °C for the same initial ρ value.

[Figure 2]

The residence time in a continuous reactor is determined by the ratio of the reactor volume to the volumetric flow rate. The volumetric flow rate, in turn, can be related to the mass flow rate of the reactive mixture through its density value:

$$\mathbf{r} = (V/\dot{\mathbf{m}}) * \rho \qquad (\text{Eq. 1})$$

Where "" is the residence time, "V" is the reactor volume, "m" is the mass flow rate, and "" is the mass density of the mixture. By knowing the density of the reactive mixture under the operating conditions, and assuming that its value remains constant throughout the reactor, the residence time can be calculated by measuring the mass flow rate.

In the case of water + lactalbumin 5% w/w mixtures, t The data from the isochoric lines in Figure 2 can be used in Eq. 4 to compare the density values of water + lactalbumin mixtures with initial composition of 5% w/w the mixture measured under different *T* and *P* conditions with the density values of pure water under the same conditions. This allows us to estimate the error in calculating the residence time by assuming that the density of the mixture inside the reactor is similar to that of pure water, for which there is a large amount of data available over a wide range of conditions (Lemmon e. al., n.d.).

Figure 3A shows the comparison between the mass density values of 5% lactalbumin mixtures obtained in this study and the corresponding values for pure water under the same P and T conditions. The solid dashed line indicates that the mixture and pure water have the same density value under operating conditions. The dashed dotted lines establish the margins for a difference of $\pm 2.\%$, calculated as follows:

Difference (%) =
$$[(\rho_w - \rho_m)_{P,T} / (\rho_m)_{P,T}] * 100$$
 (Eq. 4)

Where $(\rho_w - \rho_m)_{P,T}$ is the difference between the density of PW and the density of the lactalbumin mixture measured at pressure *P* and temperature *T*, and $(\rho_m)_{P,T}$ is the density of the mixture under the same *P* and *T* conditions.

As can be observed, assuming the density value of the water + lactalbumin 5% w/w reactive mixtures as if it was PW within the studied experimental range would result in an error of

less than 2%. This difference is small compared to the differences in pressure values between the mixtures and PW for the same temperature observed in Figure 2. This is because a change in composition in the reactive mixture will affect its volumetric properties, shifting the isochore on the *P*-*T* diagram and showing significantly different pressure values for a given temperature and density. In the studied experimental range, these isochoric lines have a steep slope. Additionally, as mentioned earlier, the magnitude of this pressure change will be greater as the density value increases since the slope of the isochore increases with density. The mentioned shift of the isochore due to the composition charge will be further analyzed in the following section.

[Figure 3]

3.2. Influence of the initial composition on the P-T behavior of the reactive mixture.

To analyze the influence of the initial concentration of lactalbumin on the P-T behavior of the mixtures under reaction conditions asochoric measurements were conducted at a constant initial system density value (approximately 0.831 g/mL, see table 1) and initial concentrations of 3%, 5%, 10%, and 15% w/w of lactalbumin in water. The obtained results are shown in Figure 4.

[Figure 4]

It was observed that for low initial concentrations (3% and 5%), the pressure exerted by the mixture at a given *T* was lower than that of PW. For a concentration of 3%, the isochoric line approximated that of PW. For concentrations greater than 10%, it was found that the pressure exerted by the mixture was comparable to or higher than that of PW. This change in trend could be due to the fact that changes in initial concentration generate different final products or in different proportions, which confer different volumetric properties to the mixture

compared to those observed at low lactalbumin concentrations. The hydrolysis products of the protein are amino acids, which, under high temperature conditions, decompose to form a complex mixture of products, including primarily liquid and gaseous products such as carbon monoxide and dioxide, ammonia, hydrogen, methane, alkanes and alkenes, low molecular weight alcohols, amides, aldehydes, and carboxylic acids (Rogalinski et al., 2005; Sato, Quitain, Kang, Daimon & Fujie, 2004). Each of the compounds generated as products will contribute to the volumetric properties of the mixture according to their individual properties, which can be very different. As an example, Figure 5 shows the isochoric lines for a density of 0.75 g/ml corresponding to the pure compounds wate. methanol, and benzene (Lemmon et al., n.d.). It can be observed that the isochoric line of the same ρ-value are within a relatively wide temperature range due to diffe. Set is in the physicochemical properties of these compounds.

[Figure 5]

As mentioned above, the experiment starts at ambient temperatures with a liquid-vapor phase scenario. In the case of the experiments conducted in this study, where it concludes with an incompressible phase, is the temperature increases at nearly constant total volume, the initial liquid phase expands while the vapor phase contracts. Eventually, the vapor phase disappears at a specific temperature, referred to as the transition temperature (Velez et al., 2010b). Above this temperature, the system significantly increases pressure with small temperature changes, and it is in this region where the measurements of the present study were conducted. The mentioned transition temperature, under conditions of constant initial density for the experiments, will be lower or higher depending on both the volumetric properties of the initial liquid phase fed to the cell (properties that depend on its composition) and the changes in

these volumetric properties throughout the course of the experiment due to the chemical reactions generated by the increase in temperature. Additionally, in the monophasic region, the slope of the isochoric lines will also depend on the volumetric properties of the mixture. It should be noted that, throughout the experiment, lactalbumin undergoes hydrolysis processes, generating peptides and free amino acids. Furthermore, at higher temperatures, amino acids will undergo thermal degradation, as mentioned earlier, resulting in a complex mixture of products. The above could explain the observed behavior (shifting of the isochoric lines and slope differences) in experiments for constant initial mass density and different initial compositions of water + lactalbumin mixtures. 1. in a simplified manner, only the presence of proteins, peptides, and amino acids in the mixture is considered, literature provides data on volumetric properties of aqueo. with x tures of these types of compounds and their dependence with composition, ter. per ture and pressure (Häckel et al., 1999; Hakin et al., 1998, 2000; Hedwig et al., 2008; Irbal & Verrall, 1987; Korolev, 2010; Liu et al., 2009; Marriott et al., 2001; Parr et al., 2009 F.oyer & Winter, 2011). However, to comprehensively understand the reasons belind the observed behavior in this study, more detailed investigations into the composition of the system and the physicochemical properties of the components present in the mixture under the analyzed conditions should be conducted.

Figure 6 3B illustrates the comparison between the experimentally measured density values of lactalbumin reactive mixtures of different initial compositions and the density values of PW under the same *P* and *T* conditions. Additionally, the boundaries of a \pm 1% error are plotted by applying the calculation given by Eq. 4. It was observed that for all the studied concentrations, the difference between ρ of reactive mixtures did not exceed \pm 1% compared to ρ of PW. However, as mentioned earlier and can be seen in Figure 4, the differences between the pressure exerted by the mixture and the pressure exerted by PW, for the same ρ

and *T* values, should be taken into account as they could be significant.

3.3. Analysis of final total soluble solids (TSS) and recovered free amino acids.

Figure 5 shows the behavior of the final TSS (as percentage) relative to the initial value of this parameter as a function of the final temperature reached in each experiment (see Table 1). There is a strong dependence of the TSS on this variable, as the percentage of this parameter decreases significantly with increasing temperature. C. \circ possible explanation for this loss is the greater generation of insoluble, volatile \sim gaseous products at higher temperatures due to the thermal degradation of amino acids (Rogalinski et al., 2005), which negatively impacts the final value of TSS.

% LA	Initial density (7/mL)	T max (°C)	% Solids	% AA
5	0.862	242.2	57.01	3.29
5	C 8310	265.6	39.68	3.36
5	9.8110	275.4	35.32	2.8
5	0.7815	295.5	30.85	1.75
5).7597	310.7	27.63	0.66
15	0.8305	253.1	41.87	2.92

255.4

258.1

3.02

3.58

42.75

41.4

Table 1. Final TSS:initial TSS ratio (% 50l² ds), AA% in the final soluble solids and final temperature values (T max) reached in the Exochoric experiments.

%LA is the initial lactalbumin concentration expressed in % w/w.

0.8314

0.8318

10

[Figure 5]

Similarly, Figure 6 shows the effect of temperature on the percentage of the mass of remaining free amino acids (%AA) relative to the final TSS content. It can be observed that the amount of free amino acids follows a similar trend as shown in the final TSS (Figure 7).

[Figure 6]

[Figure 7]

In Figure 7, the effect of temperature on the final content of free amino acids is shown for experiments with an initial lactalbumin concentration of 5% and densities of 0.7597, 0.8110, and 0.8620 g/mL. Only the results for the amino acids that could be quantified appreciably are shown. The final temperature to which the mixture was exposed depended on the density used in each experiment, with lower final temperatures achieved at higher densities (see Table 1). The remaining percentage of each amino acid is calculated relative to the initial mass of each amino acid present in the mixture (see Table C in Supplementary Material). Not all amino acids exhibit the same resistance to high temperatures, and although the thermal degradation of amino acids depends on their individ¹. al substructures, a generalized trend can be observed for the studied amino acids in terms of their recovery values. The best yields were obtained at lower temperatures ($\rho=0.862$ χ/mL , T=242.2 °C), with the exception of lysine, which showed an increase at the highest temperature reached ($\rho=0.7597$ g/mL, T=310.7 °C). This may be because lysine is not released from peptide chains at lower temperatures. Regarding the above, Espinoza, Morawicki, & Hager (2012) found that in the subcritical water hydrolysis process of whey protein isolates, higher concentrations of free amino acids were obtained at 250°C except for lysine and tryptophan, which showed the best yields at a temperature of 300°C.

Although proline and glutamic acid were initially present in a considerable amount in the lactalbumin used, their recovery percentages were practically zero, as well as those of aspartic acid, histidine, arginine, and cysteine. This is consistent with the findings of Abdelmoez, Nakahasi, & Yoshida, (2007) who found that cysteine and glutamic acid are the most labile amino acids, followed by aspartic acid, threonine, and arginine, none of which

withstand temperatures above 230 °C. Despite glycine, alanine, valine, and proline being formed as intermediate products of the degradation of other amino acids (Abdelmoez et al., 2007), the recovery percentages found for these amino acids in this study were very low.

The amino acid with the highest recovery was phenylalanine, which, along with tyrosine, belongs to the group of aromatic amino acids due to the presence of a phenolic group in their structures. It was observed that for these amino acids, there was a decrease in the yields obtained as the temperature of the experiment increased. In the case of tyrosine, degradation was complete above 240 °C. On the other hand, phenylalar ine could still be detected even at temperatures of 310 °C, although a high degree of degradation was observed as the temperature increased, with the remaining percentage decreasing from nearly 80% at 242°C to below 10% at 310 °C.

Regarding the analysis of thermal stability of different amino acids conducted earlier, it should be noted that such stability will depend on the composition of the mixture, as the presence of other amino acids con generate interactions that may positively or negatively affect their reactivity (Abdolm ez et al., 2007). Due to this, it is difficult to establish a general rule for the behavior of different amino acids in an aqueous medium under high-temperature conditions, given the complexity of the mixtures obtained in the protein hydrolysis process from various sources.

4. Conclusion

It was possible to study the influence of density and composition of reactive mixtures of lactalbumin and water on the P-T behavior of the system under subcritical and isochoric conditions using a constant volume cell. The P-T relationship was linear in all experiments

under the mentioned conditions, and in general, an increase in density resulted in a steeper isochore. For a fixed initial concentration of 5% w/w lactalbumin and under the same density and temperature conditions, the measured pressure for the mixtures was always lower than that of pure water. However, the behavior of the mixture's pressure compared to pure water varied with changes in the composition of the mixture. It was observed that for concentrations of 3% and 5% w/w lactalbumin, the pressure exerted by the mixture was lower compared to pure water, while the opposite behavior was observed for conce. trations of 10% and 15% w/w. When comparing the density-pressure-temperature v_{1} obtained for the mixtures with those of pure water, it was found that the density of the mixture could be assumed to be equal to that of pure water with an error of less than 2^{12} . However, it was observed that it is necessary to consider that the pressure exerter ov the mixture cannot be assumed to be practically equal to that of pure water f or *e* given temperature and density, as considerable errors could be made in estimating t.'s parameter. Finally, it was possible to quantify the percentage of remaining amino acid. a ter subjecting the mixtures to high temperatures and long reaction times. It was found that phenylalanine had considerably higher recovery than the rest at lower temperatures, although at higher temperatures, appreciable amounts of alanine, leucine, lysin, and threonine were also recovered.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.



Figure 1. Equipment for isoc¹ oric experiments. A: Thermally insulated electric furnace with temperature controller; B: Constant-volume stainless steel cell; C: Temperature sensor; D: Pressure sensor.



Figure 2. Isochoric behavior for reactive mixtures of water + lactalbumin with initial concentration 5% w/w for different initial densities values expressed in g/mL: (•) 0.7597 (**n**) 0.7815 (•) 0.8110 (**A**) 0.8310 (×)0.8620; and eq. indent initial density values for pure water (NIST, Lemmon et al., n.d.): (•) 0.7597 (**D**) 0.7815 (•) 0.8110 (**A**) 0.8310 (+) 0.8620. $u_r(\rho) = \pm 0.04\%$, $u(T) = \pm 0.1^{\circ}$ C, $u_r(P) = \pm 0.5\%$



Mixture density (g/mL)



Figure 3. A: Comparison between the density values c_1^{-1} put 2 water (Lemmon et al., n.d.) and water + lactalbumin 5% w/w reactive mixtures for the same pressure and temperature conditions: (•) 0.7597 (•) 0.7815, (•) 0.8110 (•) 0.0310 (×) 0.8620. The dotted lines correspond to a difference ±2% calculated by eq 12 at n 4. **B:** Comparison between the density values of pure water (Lemmon et al., n.d.) and water + lactalbumin mixtures with initial density value of approximately 0.831 g/ aL and different lactalbumin initial concentrations: (•) 3% (□) 5% (△) 10% (◊) 15%. The dotted lines correspond to a difference ±1% calculated by equation 4. $u_r(\rho \text{ dif}) = \pm 0.1\%$

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Figure 4. Isochoric behavior for reactive mix u_{1} of water + lactalbumin at initial density value of approximately 0.831 g/mL and clifts rent initial lactalbumin composition: (•) 3% (•) 5% (•) 10% (•) 15%, the dashed line consistence of sponds to pure water (NIST, Lemmon et al., n.d.). $u_r(\rho) = \pm 0.04\%$, $u(T) = \pm 0.1^{\circ}$ C, $u_r(P) = \pm 0.5\%$



Figure 5. Final total soluble solids expressed as percentages relative to the initial total soluble solids content.



Figure 6. Recovered amino acids expressed as percentage? relative to the final total soluble solids content.



Figure 7. Recovered amino acids for experiments with an initial lactalbumin concentration of 5% w/w and initial densities of $0.7597 (\blacksquare)$, $0.8110 (\blacksquare)$, and $0.8620 (\blacksquare)$ g/mL, expressed as percentages of final mass of the free amino acids relative to the initial mass of each one.

Highlights

1. Density and composition significantly influence P-T behavior of studied mixtures

2. Linear P-T relationship observed, with steeper isochores at higher density.

3. Mixtures density can be assumed equal to pure water at same T and P conditions

4. Mixtures pressure can significantly differ than that of pure water at same T and ρ

5. Phenylalanine showed high thermal stability below 240°C.

Souther