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Microencapsulation of casein hydrolysate by complex coacervation with SPI/pectin

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ABSTRACT

The aim of this work was to encapsulate casein hydrolysate by complex coacervation with soybean protein isolate (SPI)/pectin. Three treatments were studied with wall material to core ratio of 1:1, 1:2 and 1:3. The samples were evaluated for morphological characteristics, moisture, hygroscopicity, solubility, hydrophobicity, surface tension, encapsulation efficiency and bitter taste with a trained sensory panel using a paired comparison test. The samples were very stable in cold water. The hydrophobicity decreased inversely with the hydrolysate content in the microcapsule. Encapsulated samples had lower hygroscopicity values than free hydrolysate. The encapsulation efficiency varied from 91.62% to 78.8%. Encapsulated samples had similar surface tension, higher values than free hydrolysate. The results of the sensory panel test considering the encapsulated samples less bitter ($P < 0.05$) than the free hydrolysate, showed that complex coacervation with SPI/pectin as wall material was an efficient method for microencapsulation and attenuation of the bitter taste of the hydrolysate.

1. Introduction

Hyprol is a hydrolyzed enzymatic digest of casein providing a high quality source of peptides, including casein phosphor-peptides. This product is recommended as a soluble peptide source in entheral nutrition, infant food formulations, and protein enrichment of food and beverages (Kerry Bio-Science, 2005). Despite these properties, Hyprol presents an intense bitter taste, which limits its direct addition as food ingredient.

The development of the bitter taste in the hydrolysates is due to the release and/or exposition of hydrophobic amino acids from inside the protein molecules, limiting its utilization as functional ingredient (Lin, Nelles, Cordle, & Thomas, 1997). In addition, some protein hydrolysates are very hygroscopic, hydrophobic, reactive, and allergenic. Consequently, the use of these protein hydrolysates is defying, for both food industries and researchers.

Microencapsulation may be an alternative for reducing these problems. For many years, this technique has been used in the pharmaceutical industry for controlled release and enhanced stability of formulations and flavor masking. Moreover, microencapsulation provides a useful technique to protect products from environmental conditions, to extend shelf-life (Favaro-Trindade, Pinho, & Rocha, 2008; Gouin, 2004) and in nutraceutical delivery systems through the incorporation of bioactive compounds into food systems (Chen, Remondetto, & Subirade, 2006).

Microencapsulation by coacervation is accomplished by phase separation of one or many hydrocolloids from the initial solution and the subsequent deposition of the newly formed coacervate phase around the active ingredient suspended or emulsified in the same reaction media (Gouin, 2004). Microcapsules produced by coacervation are water insoluble, possessing excellent controlled release characteristics and heat resistant properties (Dong et al., 2008).

The most classical system of complex coacervation is that where gelatin is used as the positive polyelectrolyte, and various polyanions such as gum arabic, alginate, pectin, and carboxymethylcellulose are used to counterbalance the positive charges of the protein. In the past years, due to the emergence of new diseases such as the prion diseases, regulations concerning safety and health were reinforced (Chourpa, Ducel, Richard, Dubois, & Boury, 2006). So, the research of new biopolymers which could be compatible with microencapsulation processes such as coacervation seems to be very important.

In this study, complex coacervation was used to microencapsulate casein hydrolysate, aiming to attenuate the bitter taste, to facilitate the incorporation into food products, plus enabling

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controlled release. As this technique is appropriate to encapsulate hydrophobic cores or active materials, which was not the case, an adaptation was made by including a double emulsion step at the beginning of the process. Soy protein isolate (SPI) and pectin were used as wall materials. They are abundant, inexpensive and renewable raw materials.

Pectin is a water soluble anionic polysaccharide generally obtained from the skin and pulp of citric fruits and apples, and also from sunflower seeds and beet (Thakur, Singh, & Handa, 1997).

SPI is produced from defatted soy meal by alkali extraction followed by acid precipitation (pH 4.5) (Choa, Park, Batt, & Thomas, 2007). It has a high nutritive value. Indeed, the incorporation of soy by-products in more elaborated formulations represents the perspective of adding value to the product. SPI has already been successfully used to microencapsulate casein hydrolysate by spray drying (Ortiz et al., 2009).

The aim of this research was to study the potential of complex coacervation as encapsulating technique using SPI and pectin as wall materials of a casein hydrolysate and to evaluate the efficiency of the encapsulation process to reduce the bitter taste of this product.

2. Material and methods

2.1. Materials

Soy protein isolate (SPI) (Supro 500L, Solae Company, Brazil) and low-methoxy (LM) pectin (Genu® 8002, CP Kelco, Brazil) were used as encapsulating agents. The core or active material used was casein hydrolysate (Hyprol 8052, Kerry Bio-Science, Brazil).

2.2. Microencapsulation by complex coacervation

Three samples with different core concentrations (50, 100 and 150%) in the total mass of polymers (SPI and pectin) were prepared. The SPI to pectin ratio was fixed in 1:1. The samples were called M1, M2 and M3, respectively.

Aqueous solutions of the polymers (0.25 g/ml) and of the casein hydrolysate (0.25, 0.5 and 0.75 g/ml) were used.

First, the aqueous casein hydrolysate solutions were emulsified in soy oil (Cargill, Brazil) in 1:3 ratios to obtain water in oil emulsions, i.e., the aqueous phase containing the casein hydrolysate was the disperse phase and the oil was the continuous phase. Subsequently, the samples were emulsified with a SPI solution at fixed pH 8, to obtain oil in water emulsion. Both emulsifying steps were realized with an ultraturrax homogenizer (Ika, USA) at 20,000 rpm for 2 min.

The pectin solution was slowly added to these emulsions, aided by a magnetic stirrer. Next, to promote coacervation, the pH was adjusted to 4.4 with 1 M phosphoric acid at approximately 40 °C, under constant magnetic stirring.

The coacervated material was stored at 7 °C overnight to promote decantation. Thereupon the coacervated was slowly freeze in a domestic freezer (−18 °C), and freeze dried (L1500 Lyophilizer, Terroni, Brazil).

All the experiments were performed in duplicate, excepting sensorial evaluation.

2.3. Microcapsules morphology and particle size

The morphology of the microcapsules was observed by optical microscopic (Carl Zeiss, Oberkochen, Germany) aided by the Image Pro Plus 4.0 software for imaging obtaining. Before optical microscopy the samples were colored with Coomassie Blue R250 (Sigma, USA), which is a protein dye-binding.

To calculate the diameter, at least 300 particles were measured for each formulation using the same software mentioned above.

2.4. Solubility

The samples (10 mg/ml solids) were dispersed in distilled water at pH 7.0 and constantly stirred for 90 min and for 24 h at room temperature and then centrifuged at 6000*g* for 5 min at 15 °C.

Protein solubility was determined from supernatants using the Lowry–Folin method (Lowry, Rosebrough, Farr, & Randall, 1951), the protein concentration was calculated from Eq. (1), and absorbance was determined using a Beckman DU 650 spectrophotometer at 750 nm.

$$[\text{protein}] = \frac{\text{Absorbance} - \text{linear coefficient}}{\text{angular coefficient}} \quad (1)$$

The linear and angular coefficients were obtain from the Albumin concentration (mg/ml) vs Absorbance plot.

The Eq. (2) shows the albumin concentration (mg/ml) formula.

$$[\text{Albumin}] = \frac{\text{Absorbance}_{280\text{-UV}}}{0,63} \quad (2)$$

$\text{Absorbance}_{280\text{-UV}}$ was set as the blank absorbance at 280 nm.

The protein solubility was expressed as g of soluble protein per 100 g of total protein. Protein solubility determination was performed during 90 min and 24 h after solubilization of the samples to observe their behavior in both short and extended contact with water.

2.5. Moisture

To determine the water content of the microencapsulates, 0.2 g of the encapsulated powder were dried at 100 °C for 24 h inside previously dried and cooled Petri dishes. The water content was calculated by the Eq. (3).

$$\text{Moisture} = \frac{\text{original sample} - \text{dried sample}}{\text{original sample}} \times 100\% \quad (3)$$

2.6. Hygroscopicity

For hygroscopicity determination, samples (about 2 g) of each powder were placed in Petri dishes at 25 °C in an airtight plastic container containing Na_2SO_4 saturated solution (81% RH). After 1 week the samples were weighed and hygroscopicity was expressed as g of water absorbed/100 g of dry solids (Cai & Corke, 2000).

2.7. Encapsulation efficiency (EE)

To determine the encapsulation efficiency, i.e., how much of the hydrolysate used in the formulation was admittedly encapsulated, dispersions were prepared from the M1, M2 and M3 samples, with concentration of 5–10 mg/ml. The dispersions were centrifuged at 3000 rpm, for 5 min; 1 ml samples of the supernatants were used to determined the protein concentration using the Biureto spectrophotometric method (Gornall, Bardawill, & David, 1949), at 540 nm wavelength.

EE of the microcapsules was calculated as follows:

$$\text{EE}(\%) = \frac{\text{Total hydrolysate} - \text{Total free hydrolysate}}{\text{Total hydrolysate}} \times 100 \quad (4)$$

where total free hydrolysate (g) is the protein concentration in the supernatant (protein which was not part of the microcapsules) and

total hydrolysate is the concentration of hydrolysate in the formulations (g).

2.8. Surface hydrophobicity

The surface hydrophobicity was determined according to the method described by Hayakawa and Nakai (1985). The method was standardized adjusting the intensity of the relative fluorescence to 80% of the scale by adding 15 μl of 8-aniline-1-naphthalensulphonate (ANS) 8 mmol/l to 3 ml pure methanol.

Dispersions of samples M1, M2, M3, pure SPI and pure hydrolysate, in water and in buffer, with 10 mg/ml were prepared by magnetic stirring for 1 h and next centrifuged at 8000*g* for 5 min. Next, solutions diluted at 1:10, 1:50, 1:100, 1:150 and 1:200 were prepared from the supernatants.

The fluorescence was measured at excitation wavelength $\lambda_{excitation}$ = 364 nm and emission wavelength $\lambda_{emission}$ = 475 nm (digital fluorimeter, Perkin–Elmer model 2000) (Waltham, USA). The probe amount required for saturation was 25 μl ANS 8 mmol/l for 3 ml of sample. Two series with 3 ml were prepared for each dilution, one as blank and the other with 25 μl ANS added. For each protein concentration, the fluorescence intensity was determined by subtracting the fluorescence intensity of the pure sample from the value of the sample with ANS. The slope of the plot Fluorescence intensity vs Protein concentration (g/100 g) was calculated by linear regression analysis and used as the protein hydrophobicity index (Ho).

2.9. Surface tension

Static and dynamic surface tension measurements were carried out to determine samples adsorption at the air–water interface. Surface pressure isotherm measurements were performed with a drop tensiometer (TVT2 Lauda, Germany) connected to the Lauda-tvt software. This equipment calculates surface tension by measuring the contact angle of the drop using Laplace's Law.

Dispersions of M1, M2, M3, pure SPI and pure hydrolysate, with 10 mg/ml were magnetically stirred for 30 min before measuring the surface tension, both in the dynamic and standard mode (static measurement). The pectin solution was also analyzed in the dynamic mode.

The standard mode enables the reproducibility of speed and other parameters allowing the calculation of tension. In this measurement 0.07 s/μl drop formation time with 5 cycles, was used, i.e., 3 drops per cycle.

The dynamic mode, customarily used for tension measurements, evaluates kinetics. In this mode 10 cycles and 2 drops per cycle were used. The samples (10 mg/ml) were diluted to 1:10.

2.10. Sensory evaluation of mechanically mixed and microcapsules powders

The bitterness attenuation effect of the microencapsulation process was evaluated by sensory comparison of the encapsulated casein hydrolysate with casein hydrolysate mechanically mixed with the wall materials (SPI, pectin and oil).

The powder samples were submitted to two-sided paired-comparison tests, according to Meilgaard, Civille, and Carr (1999). The sensory evaluation was carried out in laboratory scale individual booths under red light, with 20 trained panelists (n = 20), which were previously selected according to their acuity in perceiving the bitter taste.

A pair of samples (approximately 5 g each) was randomly served to the panelists in white plastic cups coded with three digit random numbers. The panelists were asked to indicate the bitterest sample and were instructed to rinse their mouths with water between samples.

3. Results and discussion

This is the first study to evaluate the possibility of encapsulating casein hydrolysate using SPI and pectin as wall materials by complex coacervation aiming to attenuate the bitter taste of casein hydrolysates.

3.1. Morphology and particle size

Fig. 1 shows that the encapsulation of the casein hydrolysate with SPI and pectin as wall material through the coacervation method was successfully accomplished. The dye used (Coomassie Blue) colored all the protein fractions of the microcapsules evidencing that they were formed different layers.

The microcapsules exhibited round formats. Yeo, Bellas, Firestona, Langer, and Kohane (2005) observed similar formats when encapsulating flavor compounds by complex coacervation.

All the three formulations used in this study generated microcapsules with similar morphologies; therefore the tested concentrations of the casein hydrolysate did not influence morphology.

Fig. 2 clearly shows the presence of oil droplets. There are also protein agglomerates with irregular perimeter unlike the microcapsules; they have intense blue color as the dye colors in all protein material. These agglomerates were present mostly in sample M1.

The microcapsules exhibited assorted sizes, between 16.24 and 24.12 μm (Table 1), values expected for microcapsules obtained by complex coacervation, which according to Favaro-Trindade et al. (2008), may vary from 1 to 500 μm. The increase of the hydrolysate concentration in the formulations did not affect the size of the microcapsules formed, as no significant difference ($P > 0.05$) was observed in particle size among samples.

3.2. Properties

Table 1 lists the values of hygroscopicity, moisture content, solubility (after 90 min and after 24 h), hydrophobicity and encapsulation efficiency of free and encapsulated casein hydrolysate samples.

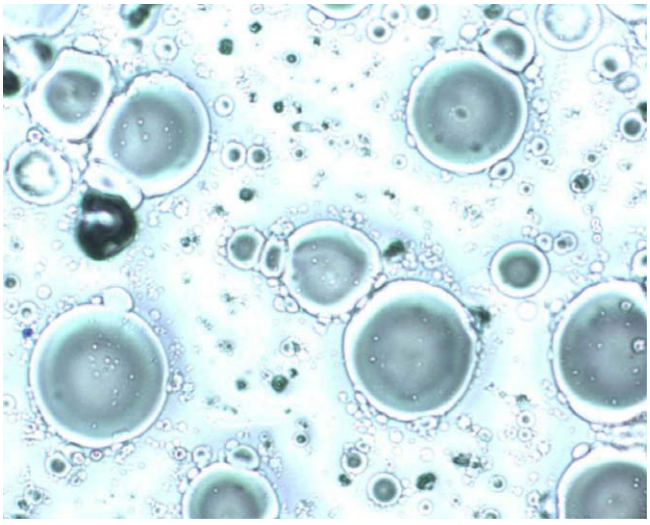

Fig. 1. Optical photograph of microcapsules (sample M1) after treatment with Coomassie Blue (100× magnification).

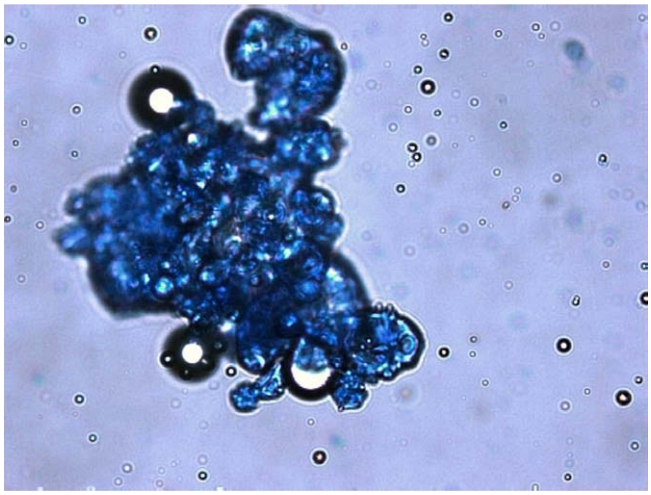

Fig. 2. Optical photograph of protein agglomerates in sample M1 (100× magnification).

The moisture content of the encapsulated samples is within the expected range for powder products and also within the recommended values to grant microbiological stability.

The evaluation of solubility was carried out to observe the behavior of the microcapsules in water; this is to verify if the casein hydrolysate would be released in this medium. As expected for microcapsules produced by coacervation, the solubility in water was low for all the formulations, even after soaking for 24 h, confirming the maintenance of microcapsule integrity. Therefore both the method and the encapsulating agents used permitted to obtain very stable microcapsules in aqueous medium, with favorable controlled release properties.

The solubility increased directly with increasing levels of hydrolysate in the microcapsules. This increasing tendency occurred both in the samples solubilized for 90 min and for 24 h. This result can be associated to the fact that for higher hydrolysate concentration the encapsulation efficiency diminished. Therefore the solubilized matter was certainly composed by the casein hydrolysate and the SPI which did not form microcapsules. Consequently, the solubility results are due to the considerable affinity with water of the casein hydrolysate.

Concerning the free casein hydrolysate, the expected solubility would be 100% (results from an unpublished study done by this group), using the Bradford method (1976). However, this did not happened because the method used in this study (Lowry) is based in the presence of aromatic amino acids, so the mean solubility value was 70%.

The encapsulation efficiency was rather high for all the samples, nevertheless it decreased inversely with casein hydrolysate concentration (Table 1). However these values are approximate, as they surely enclose error due to the methodology used; probably the supernatant contained SPI along with the hydrolysate, but it was not possible to separate these two materials, as they are indistinctively quantified by protein quantification method. Moreover, this methodology assumed that there was not microcapsule solubilization, which might not be true. Therefore, the values obtained for encapsulation efficiency were possibly under-estimated, but they certainly are a useful tool for comparison between samples.

The hygroscopicity of the free casein hydrolysate was almost two times higher than of the samples encapsulated (Table 1); consequently, the encapsulated samples were significantly less hygroscopic than the free casein hydrolysate. There was a slight difference in the hygroscopicity values between the encapsulated samples and this result is related to the encapsulation efficiency, as larger the amount of material out of the microcapsules, greater the hygroscopicity of the sample.

The hydrophobicity results show that this parameter decreased inversely with the casein hydrolysate content in the microcapsule formulation. For higher casein hydrolysate concentration more hydrophobic interactions are established with the SPI, therefore a higher concentration of hydrophobic groups from SPI turn to the core of the capsules causing hydrophobicity reduction; it should be also considered that SPI has high hydrophobicity, near 187, and this property cannot be measured for the casein hydrolysate.

As the surface hydrophobicity determination requires a hydrophobic zone with a "pocket-like" structure to link to the probe, it cannot be accomplished for casein hydrolysate. The low molecular weight of the peptides of the casein hydrolysate does not provide the necessary conformation for the analysis, apart from the high hydrophobicity, which the lateral chains of amino acids may have.

The hydrophobicity results were corroborated by the microscopic observations, discussed above, noticing the presence of dispersed protein agglomerates, mainly in sample M1 were the higher hydrophobicity was observed.

Surface tension is a property of liquids arising from unbalanced molecular cohesive forces at or near the surface, as a result of which the surface tends to contract and has properties resembling those of a stretched elastic membrane. Surface forces, or more generally, interfacial forces, govern such phenomena as the wetting or non-wetting of solids by liquids (Florence & Attwood, 2003).

Fig. 3 shows that there were not differences between the dispersions of samples M2 and M3, or between samples M1 and M2. Sample M1 has the higher surface activity which is in conformity with previous analyzes.

As expected the surface tension from the soluble fraction was lower than that of the dispersions, excepting for the casein hydrolysate. This result is associated to the occurrence of insoluble matter in the surface acting as surface tension agent, noticing that without surface tension agents the surface tension decreases.

Table 1
Characteristics of free and encapsulated casein hydrolysate products.

Sample	Properties						
	Moisture (%)	Solubility (90 min)	Solubility (24 h)	Hydrophobicity	Hygroscopicity (g_{H_2O}/100 g_{sample})	Particles size (urn)	Encapsulation efficiency (%)
Free hydrolysate	5.08 ± 0.22^{A}	58.24 ± 4.60^{A}	70.95 ± 18.82^{A}	–	53 ± 7.81^{A}	–	–
M1	4.50 ± 0.33^{AB}	1.79 ± 0.70^{C}	3.71 ± 0.05^{C}	138.2 ± 13.7^{A}	20.08 ± 0.59^{D}	16.24 ± 7.98^{A}	91.62 ± 0.63^{A}
M2	1.96 ± 0.15^{C}	5.32 ± 0.55^{B}	6.48 ± 0.30^{D}	106.46 ± 13.26^{B}	24.38 ± 0.45^{C}	22.55 ± 8.53^{A}	87.92 ± 0.72^{B}
M3	4.40 ± 0.24^{B}	6.18 ± 0.47^{B}	7.68 ± 0.10^{D}	16.93 ± 0.35^{C}	26.0 ± 70.30^{B}	24.12 ± 9.46^{A}	78.80 ± 0.90^{C}

Values represent mean ± standard deviation of duplicate determination.
Different letters in the same line indicate statistically significant difference between the mean values ($P < 0.05$).
M1: 50% casein hydrolysate in total polymer concentration.
M2: 100% casein hydrolysate in total polymer concentration.
M3: 150% casein hydrolysate in total polymer concentration.

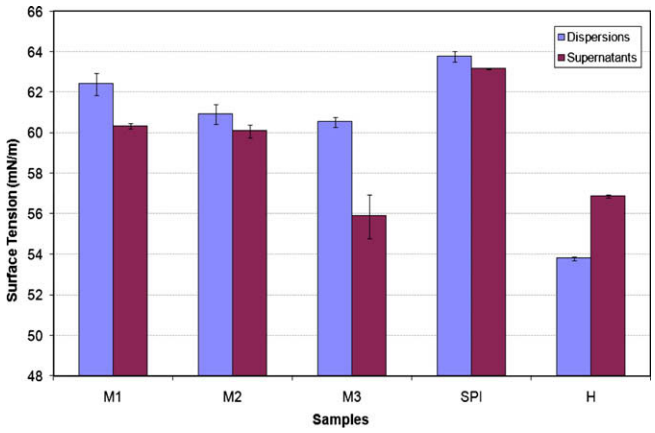

Fig. 3. Surface tension of the dispersions (10 mg/ml) and supernatants, determined by the standard method.

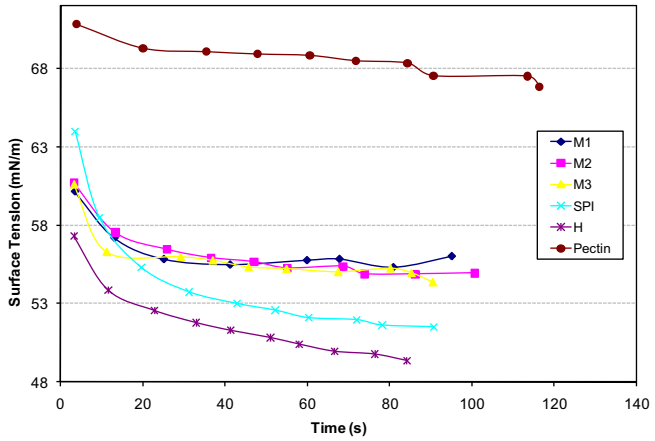

Fig. 4. Surface tension determined by the kinetic method.

Fig. 4 shows the declining of the surface tension with time, by the kinetic method. It can be noticed that all three samples had similar behavior.

Surface tension dropped for the three samples (M1, M2 and M3), the free casein hydrolysate and SPI during the first 20 s and remained constant afterwards. Surface tension decay occurs due to protein behavior in aqueous solution. First the proteins migrate to the surface and unfold, next they rearrange and form multilayers. In the case of the samples of this study, the initial decay can be accounted for the compounds not involved in the microcapsules (SPI and casein hydrolysate) which initially rearrange at the surface and next stabilized; microcapsules tend to remain stable, without variation. For surface-active proteins, the general adsorption process at the air–water (A/W) interface seems to be assumed a three-step process (Graham & Phillips, 1979; Tripp, Magda, & Andrade, 1995): Step 1. Diffusion of solute molecules from bulk solution to the subsurface region. This period is called "induction period". The induction stage is strongly dependant on the structural properties of the protein. Concretely, the size of the protein, the surface charge and the surface hydrophobicity are key factors for this first step of the adsorption process (Beverung, Radke, & Blanchu, 1999). No induction time was seen for any of the samples tested. This accelerated adsorption can be related to lower electrostatic interactions within protein molecules but also to the absence of protein aggregates in the bulk facilitating the diffusion and the adsorption of protein molecules toward the interface. Step 2. Adsorption of molecules from subsurface to the A/W interface unfolding in the surface layer. In the case of the samples of this study, the initial decay can be accounted for the compounds not involved in the microcapsules (SPI and casein hydrolysate) unfolding in the surface layer and Step 3. Conformational rearrangement at constant surface concentration, i.e., further protein and microcapsules adsorbs beneath the interfacial layer forming multilayers. These do not significantly contribute to the interfacial pressure, which attains a plateau.

The values for pectin happened in the upper part of the plot as their molecules lineup in the surface causing them to have the same behavior of water, i.e., surface tension is almost constant with time with slight surface activity. The pectin surface tension value was similar of that related by Lutz, Aserin, Wicker, and Garti (2009), for apple pectin, that was around 63 mN/m.

According to Dickinson (2003), typically, most of the commercial hydrocolloids, including pectins, are not effective as surfactants (and emulsifiers) regardless of the degree of esterification, and they hardly reduce the surface tension. However, specific pectins and/or modified pectins are more amphiphilic and further reduce the surface tension (Lutz et al., 2009).

3.3. Sensory evaluation of mechanically mixed and microcapsules powders

According to Meilgaard et al. (1999), in a two-sided paired-comparison test with 5% significance level and 20 panelists, the response must contain minimum 15 panelists choosing one of the two samples to find a significant difference between them. Accordingly, encapsulation by complex coacervation using SPI and casein showed to be effective in attenuating the bitterness of the casein hydrolysate. For samples M1 and M2, 18 panelists (90% of panelists) checked the encapsulated ones as tasting less bitter. For sample M3, all panelists checked the encapsulated one as tasting less bitter. It is likely that the coacervation process produced a less bitter product than the free or uncapsulated casein hydrolysate, even for sample M3, were the encapsulation efficiency was lower.

4. Conclusions

The three formulations studied (M1, M2 and M3) formed microcapsules. All formulations reduced the bitter taste, the hygroscopicity and allowed controlled release properties of the casein hydrolysate, however the one used to prepare sample M1 may be considered as the most adequate, as it promotes better hydrolysate encapsulation and lower hygroscopicity.

More than masking the taste of the casein hydrolysate, the microcapsules produced in this study may add nutritional value to the product in which they are incorporated due to inherent properties of the components. The casein hydrolysate has functional properties, additionally pectin is a soluble fiber and soy protein has high nutritional value.

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