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Transferosomes as a delivery system for bioselenized protein hydrolysates as an active ingredient with cosmetic activity

Laura Romero Robles[1], Benjamín Escandón Ródriguez[1], Daniel Alejandro Rodríguez Ramirez[1], Renatta Yanire González Escalera[1], Sayra Serrano-Sandoval[1,2], Marilena Antunes-Ricardo[1,2]
[1]Tecnológico de Monterrey, México; [2]Institute for Obesity Research, México
romero@tec,mx, A00824814@exatec.tec.mx, A00824812@exatec.tec.mx, A00824795@exatec.tec.mx, sayra.serrano@tec.mx, marilena.antunes@tec.mx

Abstract– In higher education, research-based learning could be a valuable addition to the curriculum as a didactic-pedagogical strategy, as it allows students to observe, analyze, reflect, and solve problems. In the context of engineering education, it would be beneficial for students to have the opportunity to apply their learning in a real-world setting during their professional training. It would seem that students in their final semester of nanotechnology engineering have found a way to apply this learning technique in the Integration Project in Chemistry and Nanotechnology. The project developed by the students is about transferomonas with a real application in the dermatological industry. Transferosomes are flexible vesicular carriers capable of deforming up to 10 times their size. They are used to enhance the stability, permeability, and biological activity of the encapsulated active ingredients. In this research, transferosomes were employed to encapsulate bioselenized protein hydrolysates to increase their stability and topical permeability when used in cosmetic formulations to combat signs of aging. The encapsulation efficiency achieved was 70%, while the release assays indicated that 38% of the active ingredient was released after 4 hours of contact with the skin's pH. These results demonstrate the successful optimization of conditions for the formation of transferosomes with bioselenized protein hydrolysates as the active ingredient, as well as their potential use in the cosmetic industry. Future investigations should focus on studying the permeability of transferosomes, considering the dermis as their ultimate destination. The research-based learning technique proved to be an effective approach for solving problems in higher education students.

***Key words: transferosomes,* protein hydrolysates*, antioxidant activity, Higher educatio*n, research-based learning.**

I. Introduction

Research-Based Learning (RBL) is a valuable teaching strategy that can be applied as a learning model in higher education [1].

The objective of this project was to motivate ninth semester students of Engineering in Nanotechnology and Chemical Sciences, under a research-based learning scheme, to develop an applied solution to solve a real problem. The students were encouraged to develop an effective transferomone delivery system that could stabilize a dermal antioxidant complex that could be applied directly to the skin.

Skin conditions affect between 30 and 70% of the global population [2], [3], with aging being the primary condition [4]. This issue arises from an overproduction of reactive oxygen species (ROS) and a reduction in the antioxidant defense mechanism [5], leading to progressive damage and functional decline of cells [6].

To mitigate the effects of aging on the skin, the need to search for natural antioxidants that are safe for the human body has emerged [7]. Among these are protein hydrolysates (PH), which possess antioxidant mechanisms [8][9], including the elimination of free radicals, chelation of metal ions, reduction of peroxide content, or enhancement of antioxidant enzyme activity [10][11,12]. Protein hydrolysates are products derived from the process of protein hydrolysis, in which proteins are broken down into smaller fragments, known as peptides, through the cleavage of peptide bonds in proteins [13].

In recent years, selenization has emerged as a strategy to enhance antioxidant activity [14], as it has been demonstrated to increase it by up to 33% [15], [16]. Selenium fortification enables the generation of selenoproteins through the formation of selenomethionine and selenocysteine, amino acids responsible for the increase in antioxidant activity. These amino acids are produced during the germination of chickpeas with sodium selenite [17]; as studies report chickpeas are a viable option to produce bioselenized protein hydrolysates, not only due to their high availability and low cost but also due to their selective selenium accumulation and selective increase in antioxidant potential [18][19].

To generate protein hydrolysates, the production of peptides during chickpea protein digestion is simulated, making enzymes such as pepsin and pancreatin ideal. Pepsin is a key enzyme generated in the digestion process in the stomach, and pancreatin is released by the pancreas in the small intestine [20].

However, one of the challenges of protein hydrolysates

is their low chemical stability compared to other molecules, whether applied topically or ingested [21]. Therefore, the implementation of a delivery system capable of improving these properties is necessary. Some of the most studied delivery systems include nanocomposites, transdermal proteins, niosomes, and transferosomes. Although niosomes can achieve smaller sizes [22] and can penetrate the skin more easily, transferosomes can achieve higher encapsulation efficiency of active agents, reaching up to 90% in some cases [23], which is why they were chosen as the delivery system to be developed in this investigation.

Transferosomes are flexible and deformable vesicular carriers composed of phospholipids and an edge activator (EA), which is a surfactant that imparts ultra-deformability to the transferosome [24]. This deformability allows them to easily pass through the intracellular pores of the stratum corneum to reach the dermis, and then reform after crossing it [25]. Additionally, these vesicles can store and transport active compounds such as proteins, peptides, and drugs [26][27].

Therefore, the objective of this research was the application of problem-based learning by higher education students of Engineering and Nanotechnology and Chemical Sciences by developing a delivery system based on transferosomes to encapsulate bioselenized protein hydrolysates, obtained by enzymatic hydrolysis applied to bioselenized chickpea flour as previously studied by Guardado-Félix in 2017 [15], [16].

The aim was to increase and improve both the chemical and functional stability of the hydrolysates, as well as to protect and enhance the permeability of the active ingredient for its delivery to the dermal layer of the skin. It is worth mentioning that the system was developed using only reagents commonly accepted within the cosmetic industry.

II. MATERIALS AND METHODS

A. Materials and reagents

For the development of the protein hydrolysates, bioselenized and non-selenized chickpea flour provided by Guardado-Félix was used. The chickpea flour was obtained in the same manner as explained in their 2017 research, were used. Milli-Q water, pepsin, and pancreatin enzymes, 1M solution of sodium hydroxide (NaOH), and hydrochloric acid (HCl) were used. To test the antioxidant activity, 2,2-Diphenyl-1-picrylhydrazyl (DPPH) reagent was obtained from Sigma Aldrich and the bicinchoninic acid (BCA) protein assay kit from Pierce. The absorbance was evaluated using the Biotek Synergy HT multimode microplate reader and Biotek Gen5 software.

For the preparation of the transferosomes, Sigma Aldrich phosphatidylcholine, ethanol, isopropyl alcohol, potassium polysorbate, phosphate-buffered saline (PBS) solution (pH 7.4), an ultrasonic bath, and a rotary evaporator were used.

For the characterization of the transferosomes, the Zetasizer DLS equipment from Malvern Panalytical was used to determine the size, zeta potential, and polydispersity index (PDI). Fourier-transform infrared spectroscopy (FTIR) was performed using the Perkin Elmer Spectrum 400 model.

The scanning electron microscope (SEM) used was the ZEISS EVO MA 25 model with a Bruker secondary detector.

The Quorum Q150 ES equipment was used to coat the samples with gold for SEM analysis.

B. Methodology

1) Phase 1: Preparation and evaluation of protein hydrolysates.

A) Preparation of protein hydrolysates.

Selenized and non-selenized chickpea flour obtained according to the procedure reported by Guardado-Felix et. al [28] was used along with Singh's method [29] with some modifications to obtain protein hydrolysates. The extracted protein was dissolved in Milli-Q water (5% w/v), and combined with pepsin and pancreatin, as well as a sequential reaction of pepsin followed by pancreatin (4% w/w).

The reaction was carried out at 37 °C and at the optimal pH for each enzyme, where they exhibit the highest activity (pepsin: pH 2.0, pancreatin: pH 7.5, sequential reaction: pH 2 and 7.5). In the case of the sequential enzyme reaction, the pH was first adjusted to 2 to add pepsin, and then to 7.5 to add pancreatin.

The pH of the solution was maintained by continuously adding 1M NaOH or HCl. Aliquots were taken at 0, 30, 60, and 120 minutes, replacing the volume with Milli-Q water after adjusting the pH accordingly. The aliquots were then heated in a water bath at 90 °C for 5 minutes and subsequently frozen until their analysis.

The hydrolysate degree was obtained by the pH-stat method [1] using the following equations:

$$(1)\ DH(\%) = \frac{h}{h_{tot}} \times 100 = \frac{BN_b}{\alpha M_p h_{tot}} \times 100$$

$$(2)\ \frac{1}{\alpha} = 10^{pK-pH} + 1$$

Where B represents the volume in ml of acid or base added during the process, N_b the normality of the base, M_p the mass in grams of the hydrolyzed protein, h_{tot} *is* the total of peptide bonds in protein substrate and α the average dissociation degree.

B) Protein determination via BCA assay.

A BCA Pierce assay kit was used to determine the protein concentration in the protein hydrolysates and to normalize the sample concentration for the DPPH antioxidant activity test. A calibration curve was prepared using albumin. For the BCA assay, the AB reagent was prepared by combining reagent A, which includes the bicinchoninic acid (BCA) and sodium and

potassium tartrate, and reagent B, which is copper sulfate.

The original samples were diluted in Milli-Q water at a 1/1000 dilution factor. Then, 25 µL of the dilutions were mixed with 200 µL of the AB reagent. The mixture was incubated at 37°C for 30 minutes, allowed to rest for 5 minutes and its absorbance was measured at 526 nm.

C) DPPH Radical scavenging assay for antioxidant capacity.

The antioxidant activity was analyzed using the DPPH radical scavenging assay method as described by [30] with minimal modifications. 50 µL of diluted sample in Milli-Q water were mixed with 50 µL of a 5 µM DPPH solution, which was dissolved in 96% ethanol. The samples were placed in a 96-well plate and incubated for 1 hour at 37°C, after which the absorbance was measured at 517 nm. The percentage of DPPH Scavenging was calculated using the following equation:

$$(3)\ DPPHcaptation\ percentage = \frac{A_{blank} - A_{sample}}{A_{blank}} * 100$$

2) Phase 2: Preparation of transferosomes.

A) Solvent selection.

Solubility tests for phosphatidylcholine were performed with different organic solvents to select the most effective. The tests were performed by stirring 100 mg of phosphatidylcholine in 10 ml of solvent. The solvents employed in the tests were isopropyl myristate, absolute ethanol, isopropyl alcohol, and a mixture of isopropyl alcohol and absolute ethanol. The solvents were selected by their dielectric constant, boiling point, and risks that may present when used in cosmetic products.

B) Preparation of transferosomes.

To select the best lipid:surfactant (lip:surf) ratio, three transferosomes with no active ingredient called blanks were prepared. The blanks were prepared with the ratios 50:50, 75:25, and 80:20 following the method of [3]–[5] with some modifications. 100 mg of phosphatidylcholine was dissolved in21 ml of organic solvent and left 24 h in agitation.

Then, potassium polysorbate was added and stirred for 10 min. The organic solvent was removed with a rotary evaporator at 100 rpm, at a temperature of 60 ºC and 175 mbar of pressurefor 30 min or until complete evaporation.

The resultant film was rehydrated with PBS 7.4 and subjected to a rotary evaporator at 60 rpm under ambient conditions for 1 h. The solution was sonicated for 10 min and then refrigerated at 4 ºC. The transferosomes loaded with the bioselenized protein hydrolysates were prepared following the same method used for the blank preparations. This time the protein hydrolysates were added during the rehydration stage at relations of 6, 8, and 10% weight lipid: active (%w lip:act).

3) Phase 3: Characterization of transferosomes.

For size, zeta potential, and PDI determination, DLS equipment was used with the software Zetasizer.

The sample was analyzed directly without previous treatment. The plastic cells were cleaned with Mili-Q water. All samples were tested in triplicate by default.

Before FTIR analysis the samples were lyophilized for two days to remove the water. The FTIR analysis was conducted with a scanning range of 4000 cm^{-1} to 400 cm^{-1} [5] and a resolution of 16 cm^{-1} and 16 scan sweeps.

For SEM analysis the samples were previously coated with gold via sputtering to prevent the transferosomes from being degraded with the electron beam. The analysis was performed with 27 mV in high vacuum mode.

Regarding the release assay, the lyophilized sample was resuspended in 2 mL of Milli-Q water with a pH range of 4.7 - 5.75 to simulate skin acidity conditions. The sample was heated to 37°C and placed under gentle constant agitation. Aliquots of 200 µl were taken at intervals of 0, 15, 30, 60, 120, 240 minutes, and 24 hours, replacing the volume with MilliQ water of skinconditions pH.

The aliquots were frozen until protein analysis was conducted using the same method described in section 1B. The antioxidant activity of the bioselenized protein-loaded transferosomes was analyzed following the same procedure described in section 1C. To calculate the encapsulation efficiency (E.E.) of the transferosomes, the method described by Al Shuwaili [36] was followed with some modifications. The lyophilized sample was resuspended in 2 mL of Milli-Q water and centrifuged at 10,000 rpm at 4°C for 30 minutes to completely break the transferosomes.

The sample was then analyzed for protein determination using the method described in section 1B. Statistical analysis for each result was performed using the Minitab statistical software. One-way analysis of variance (ANOVA) followed by a Tukey posthoc test with a 95% confidence level was conducted.

III. RESULTS AND DISCUSSION

A. PREPARATION OF PROTEIN HYDROLYSATES.

Table I shows the optimal conditions for the preparation of bioselenized protein hydrolysates. The protein hydrolysates were prepared with 3 different enzymes (pepsin, pancreatin pepsin, and pancreatin in sequential order), and the degree of hydrolysis was obtained for each reaction.

TABLE I.
PARAMETERS OF ENZYMATIC HYDROLYSIS OF CHICKPEA FLOUR PROTEIN EXTRACTS AND DEGREE OF HYDROLYSIS FOR 0, 30, 60, AND 120 MIN.

Enzyme	Temperature (°C)	pH	Degree of hydrolysis (%)			
			0 min	30 min	60 min	120 min
Pepsin	37	2.05	8.30 c	12.77 c	16.46 c	16.51 c
Pancreatin		7.50	22.24 bc	49.24 ab	50.22 ab	50.85 a
Sequential reaction		7.55	4.48 c	11.84 c	13.27 c	14.71 c

Figure 1 shows the percentage of hydrolysis of the chickpea flour with different enzymes at different intervals of 0, 30, 60, and 120 min. The optimal degree of hydrolysis was that of pancreatin at 60 min.

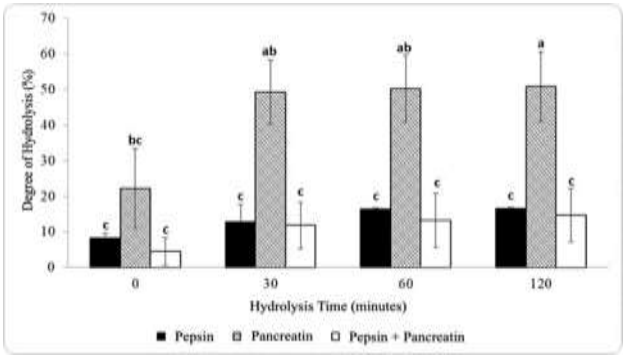

Fig. 1 Degree of hydrolysis of bioselenized protein hydrolysates with different enzymes.

The hydrolysis process is influenced by both the process time and the enzyme used. As the activity time of each enzyme increases, the peptide chain becomes shorter, resulting in a lower molecular weight distribution and an increased amount of free amino acids [37].

Figure 1 shows that pancreatin hydrolysis is superior to pepsin hydrolysis and the sequential reaction of enzymes, stabilizing after 30 minutes. For pepsin and the sequential reaction, the degree of hydrolysis stabilizes after 60 minutes. Pancreatin, being a combination of different proteases, has a greater ability to break down proteins into smaller peptides and amino acids as it acts in different areas ofthe peptide chain compared to pepsin [38].

This difference is because enzymes cleave different peptide bonds within the amino acid chain, resulting in a higher degree of hydrolysis observed with pancreatin. Evaluating the antioxidant capacity of protein hydrolysates allows us to determine how effective they will be against ROSwhen applied to the skin.

To normalize all samples, they were adjusted to the same protein concentration using the BCA assay [39]. The final concentration of protein was 100 µg/mL. The normalized samples were then subjected to the DPPH antioxidant activity test. The results of the BCA assay are shown in Figure 2.

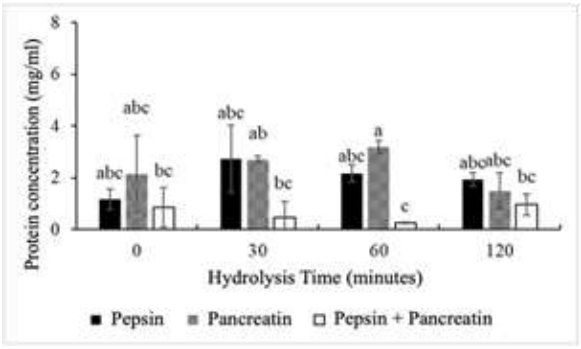

Fig. 2 Determination of protein concentration present in protein hydrolysates performed by BCA assay.

The bioselenized protein hydrolysates exhibited ROS scavenging activity with DPPH at a concentration of 100 µg/mL, as shown in Figure 3.

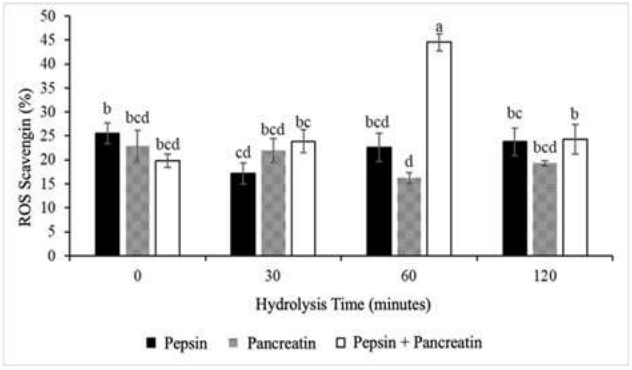

Fig. 3 Antioxidant activity via ROS scavenging with DPPH with different enzymes.

Based on the results obtained by the antioxidant activity, the enzyme that shows better DPPH scavenging is the sequential reaction of pepsin and pancreatin at 60 min.

Due to the different enzymes that work with pancreatin, it shows better antioxidant activity in combination with pepsin. Pancreatin contains different protein enzymes such as trypsin, chymotrypsin, and carboxypeptidase [7], [8].

Trypsin is known for cleaving peptide bonds at the carboxyl side of lysine and arginine amino acids. Chymotrypsin cleaves bonds at the carboxyl side of amino acids such as phenylalanine, tryptophan, and tyrosine. Carboxypeptidase, on the other hand, completely removes amino acids. Specifically, carboxypeptidase

A separate aromatic or branched amino acids, while carboxypeptidase B separates basic amino acids. Additionally, pepsin cleaves the C-terminal side of amino

acids of methionine, phenylalanine, and leucine. Amino acids containing phenyl branches exhibit enhanced antioxidant activity due to the presence of a hydroxyl group that can readily donate hydrogen atoms to ROS, effectively neutralizing them [42]–[45].

To assess the change in DPPH radical scavenging activity throughout enzymatic hydrolysis between bioselenized and non-selenized protein hydrolysates, the BCA and DPPH radical scavenging tests were repeated using the sequential reaction ofpepsin and pancreatin.

Figure 4 shows the bioselenized and non-selenized protein hydrolysates at a concentration of 200 µg/mL, displaying the percentage of DPPH scavenging.

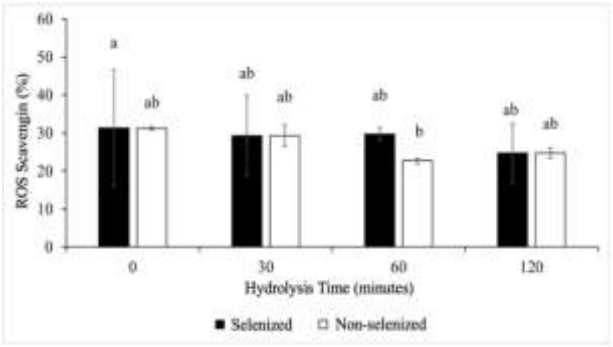

Fig. 4 Antioxidant activity via ROS scavenging with DPPH at differenttimes for bioselenized and non-selenized protein hydrolysates with enzyme combination.

A concentration of 200 µg/mL was chosen because the protein concentration in the samples was outside the calibration curve range. It can be observed that there is a greater difference between the selenium-containing and selenium-free hydrolysates at the 60-minute mark of hydrolysis.

The overall results of the comparison indicate that at a 200 µg/mL concentration of protein hydrolysates, the antioxidant activity of bioselenized protein doesn't increase as expected, however, several studies including the ones from Daniela Guardado and Sayra Serrano, demonstrate that the addition of selenium does increase antioxidant activity [9]–[11].

B. TRANSFEROSOME FORMULATION AND SOLUBILITY TESTS RESULTS.

Table II shows the solvents selected for solubility tests, their dielectric constants, boiling points, and experimental observations when dissolving phosphatidylcholine.

TABLE II
PARAMETERS AND OBSERVATIONS OF ORGANIC SOLVENTS

Solvent	Dielectric constant	Boiling point [°C]	Experimental observations
Diethylamine	3.60	55.50	No tested. Causes skin irritation
Chloroform	4.81	61.20	Completely solubilizes. Causes skin irritation and may cause cancer
Ethyl acetate	6.02	77.00	No tested. Causes skin irritation
Absolute ethanol	24.50	78.37	Partially solubilizes
Isopropyl alcohol	18.60	82.50	Partially solubilizes
Isopropyl myristate	3.25	167.00	Completely solubilizes

According to the observations shown in Table II, isopropyl myristate completely solubilizes phosphatidylcholine. However, its high boiling point makes it difficult to use in the transferosome creation process, as it exceeds the boiling point of the surfactant to be used, which in this case is slightly above 100°C corresponding to Potassium Polysorbate. Due to this, the option was made to use a mixture of isopropyl alcohol and ethanol as a solvent in a 2:1 v/v ratio, as together they fully solubilized the lipid, although this process takes longer toachieve.

Table III shows the results obtained from the Dynamic Light Scattering (DLS) analysis of the 3 transferosome formulations. It shows the sizes, Z-potentials, and Polydispersity Index (PDI) for each of the ratios.

TABLE III
SIZE, ZETA POTENTIAL AND PDI OF THE BLANKS AT DIFFERENT LIPID-SURFACTANT RATIOS OBTAINED VIA DLS.

lip:surf Ratio	Size (nm)	PDI	Z-Potential
50:50	163.75±40.36 b	0.385±0.04 b	-5.368±0.8 a
75:25	134.00±20.20 b	0.385±0.01 b	-5.233±0.54 a
80:20	446.55±4.83 a	0.515±0.05 a	-4.846±0.64 a

According to the results in Table III, there were no statistically significant differences between the 50:50 and 75:25 ratios in terms of size, PDI, and Z-potential. This means that regardless of the ratio chosen, the results obtained should be similar, even though the 75:25 ratio has the lowest standard deviation. However, the decision was

made to use the 75:25 ratio based on some research findings that suggest higher lipid- to-surfactant ratios result in thicker bilayers in the formed transferosomes, providing greater stability to the system [46].

On the other hand, the 80:20 ratio shows significant differences in terms of size and PDI, as the particle sizes obtained are the largest among the three ratios, exceeding 400 nm.

The PDI value above 0.25 indicates inconsistency in the obtained sizes; however, this result may differ after loading theactive ingredient into the transferosomes.

The final transferosome formulation can be seen in Table IV.

It includes the lipid-to-surfactant ratio, the amount of lipid and surfactant used, the solvent ratio (SolvRt), and the quantityof solvents.

TABL IV
FORMULACIÓN FINAL DEL TRANSFEROSOME

Lip:surf Ratio	Lipid	Surfactant	SolvRt	Solvent 1	Solvent 2
75:25	Phosphatidylcholine	Potassium polysorbate	2:1	Isopropyl alcohol	Ethanol

C. CHARACTERIZATION OF TRANSFEROSOMES VIA DLS, FTIR, AND SEM.

In Table V, the results obtained from the DLS analysis for the transferosomes with different weight ratios of lipid to active ingredient are presented. It shows the size, Z-potential, and PDI.

TABLE V
SIZE, ZETA POTENTIAL AND PDI OF TRANSFEROSOMES WITH DIFFERENTWEIGHT RATIOS OF LIPID TO ACTIVE INGREDIENT OBTAINED FROM DLS.

%w lip:act Ratio		Size (nm)	PDI	Z-Potential (mV)
10%	With selenium	303.17±5.36 c	0.42±13.42 b	-4.03±14.41 a
	Without selenium	190.53±2.86 d	0.37±12.05 b	-5.1±4.23 a
8%	With selenium	482.57±18.12 a	0.67±17.04 a	-3.92±11.94 a
	Without selenium	284.47±0.73 cd	0.42±3.83 b	-4.59±12.71 a
6%	With selenium	375.2±1.67 bc	0.54±6.38 ab	-4.02±20.45 a
	Without selenium	413.00±5.22 ab	0.52±19.48 ab	-3.78±7.10 a

The sizes obtained for bioselenized hydrolysate-loaded transferosomes at 10%, 8%, and 6% w/w lip:act were 303.17±5.36 nm, 482.57±18.12 nm, and 375.2±1.67 nm, respectively. In contrast, the sizes of non-selenized hydrolysate- loaded transferosomes were 190.53±2.86 nm, 284.47±0.73 nm, and 413.00±5.22 nm. The transferosomes loaded with bioselenized hydrolysates were found to be out of the expected range, which is between 50-300 nm [12], [13].

Sizes larger than 600 nm can affect permeability since the intercellular space in the stratum corneum measures between 20 and 50 nm, and a transferosome can only deform up to 10 times its size [14], [15]. The obtained sizes may be due to the short sonication time used [16]. According to the results obtained, the transfersomes loaded with 10% hydrolysate are those that exhibit the smallest size, making this concentration consideredoptimal for their formulation.

On the other hand, the PDI, being in values greater than 0.3, indicates a high heterogeneity in the transferosomes. This means that despite the average size being 303.17 nm, it is possible to find vesicles with much smaller or larger sizes, up to micrometers.

The Z-potential obtained for all samples is far from the expected -30 mV. These values close to -1 mV indicate instability in the system, which can lead to rapid rupture of thetransferosomes.

These results may be due to the sonication time to which the samples were subjected. Although many formulations use a sonication time of 10 minutes, other studies vary the times between 5 and 35 minutes depending on the phospholipid used [46]. Figure 5 presents the FTIR spectrum of bioselenized protein hydrolysates (H1), unloaded transferosomes (B), and transferosomes loaded with the active agent (T1).

The band at 3300 cm^{-1} corresponds to the vibration of the OH group in the hydrophilic part of phosphatidylcholine. Additionally, signals at 1075 cm^{-1} and 1750 cm^{-1} are observed, indicating the stretching vibrations of the C=O double bond and the C-O carbonyl bond, respectively, also present in the ester group of phosphatidylcholines. Furthermore, the signal with two peaks at 2800 cm^{-1} and 3000 cm^{-1} denotes the presence of CH_2 stretching vibrations corresponding to the phospholipid chains of the transferosomes [40] [17].

The hydrolysate spectrum exhibits a signal at 3280 cm^{-1} and 1020 cm^{-1} related to the N-H and N-C groups, respectively, of the peptide bond. The absence of hydrolysate signals and bands in the transferosome spectrum confirms that there is no interaction between the hydrolysate with the transferosomeduring the encapsulation process.

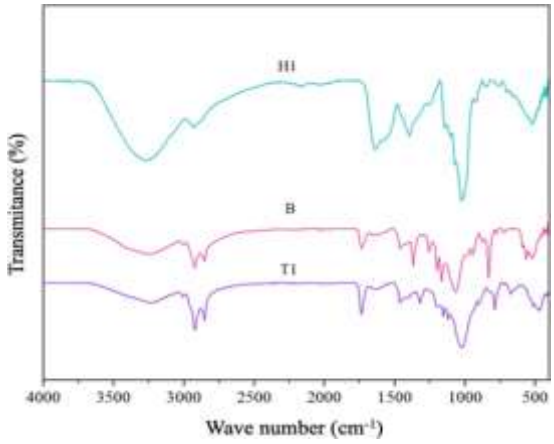

Fig. 5 FTIR Spectrum of H1: bioselenized protein hydrolysates; B: unloadedtransferosomes; and T1: transferosomes loaded with bioselenized protein hydrolysates.

The morphological results obtained through SEM are shown in Figures 6 and 7 for transferosomes loaded with bioselenized and non-selenized protein hydrolysates, respectively.

The prepared transferosomes exhibit a spherical shape, and it is possible to distinguish between the lipid layer and the encapsulated active agent, consistent with the literature [17,18]. Additionally, the images confirm the information obtained by DLS regarding the average size of the loaded transferosomes and their polydispersity index, as vesicles of different sizes canbe observed in the images.

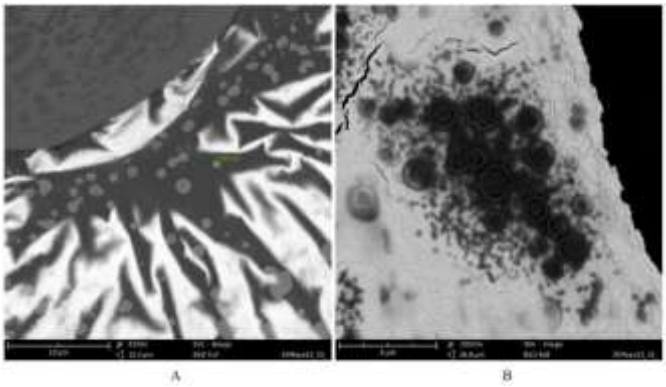

Fig. 6 SEM images of transferosomes loaded with bioselenized protein hydrolysates in suspension. A) Transferosomes loaded with 25 µL of bioselenized protein hydrolysates at a magnification of 8300x. B) Transferosomes loaded with 25 µL of bioselenized protein hydrolysates at amagnification of 10000x.

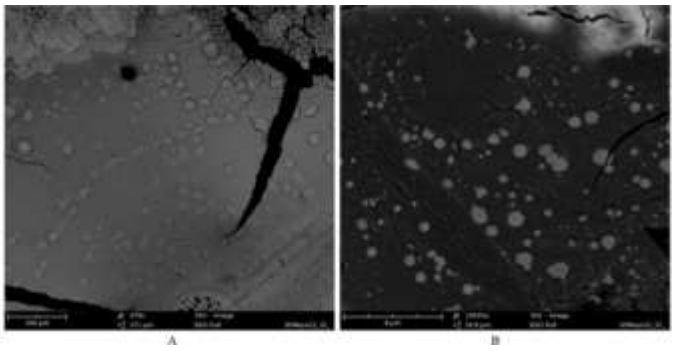

Fig. 7 SEM images of transferosomes loaded with non-bioselenized protein hydrolysates in suspension. A) Transferosomes loaded with 25 µL of non- bioselenized protein hydrolysates at a magnification of 470x. B) Transferosomes loaded with 25 µL of non-bioselenized protein hydrolysatesat a magnification of 10000x.

D. EVALUATION OF DRUG RELEASE AND ENCAPSULATION EFFICIENCY.

Figure 8 shows the results obtained from the release assay. It can be observed that the release of the bioselenized proteolytic hydrolysates starts once the transferosomes come into contact with the acidic medium (pH 4.7-5.75) and continues over time. This rapid release could be due to the low stability of the system, confirmed by its low Z-potential. Within 4 hours, the transferosomes manage to release 38.65% of their encapsulated content, with the fastest release occurring in the first hour, reaching 26.53% at 60 minutes.

Although the shape of the graph may suggest that the release of the active ingredient continues after 4 hours, a longer duration assay would be needed to confirm this. The maximum duration for this assay would be 24 hours, as it is expected to be the maximum time that a person can leave the cosmetic formulation (loaded with transferosomes) applied to their face before washing it off.

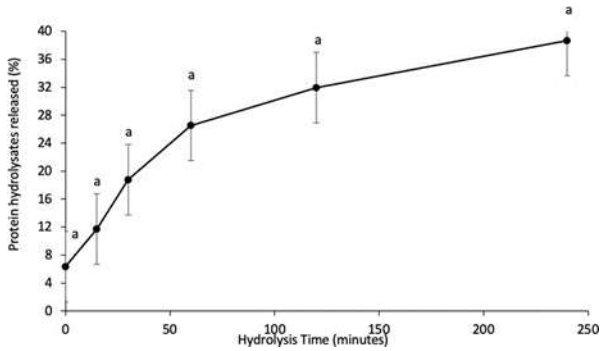

Fig. 8 Release percentage of bioselenized protein hydrolysates encapsulated intransferosomes via BCA assay.

The results obtained for the encapsulation efficiency (E.E.) are shown in Figure 9, where it is noticeable that after the rupture of the transferosomes, there was a significant increase in the protein concentration in the medium. The fact

that the transferosomes before the rupture process show a protein concentration indicates that they were not properly encapsulated or eliminated during any process. The quantified protein concentration after the rupture of the selenized transferosomes indicates an encapsulation efficiency of approximately 70%, while the selenide-free transferosomes indicate an E.E. of 77.5%. Both results are within the expected range as various studies report entrapment percentages between 70 and 79%, varying depending on the encapsulated active ingredient [52].

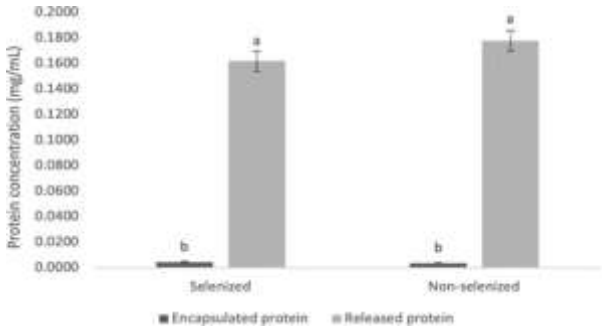

Fig. 9 Concentration of proteins after transferosomes disruption. Data ispresented as means ± standard deviation of three replicates.

IV. Conclusions

Research-based learning encompasses a range of instructional strategies and techniques. This type of learning is particularly suitable for higher education, as it facilitates the development of both cognitive and soft skills in students. The transpheromones developed by 9th semester students demonstrated to be a successful means for the stabilization of complex antioxidant systems based on bioselenized proteins.

Bioselenized protein hydrolysates have potential applications against aging. However, their main challenge is to improve their stability, requiring a delivery system for transdermal applications. In this research, it was possible to determine the optimal conditions for bioselenized protein hydrolysates preparation, as well as the synthesis of transferosomes loaded with bioselenized protein hydrolysates using the film formation method. Potassium Polysorbate and isopropyl alcohol:ethanol was used as a surfactant and organic solvent, respectively. The results of the degree of hydrolysis using pepsin, pancreatin, and a sequential pepsin-pancreatin reaction, along with a DPPH assay, indicate that the optimal degree of hydrolysis is achieved with pancreatin at 60 minutes, while the sequential reaction at 60 minutes exhibits the best antioxidant activity.

Regarding the preparation of transferosomes, the isopropyl alcohol is the solvent that best solubilizes phosphatidylcholine and meets the parameters of dielectric constant and boiling point. Subsequently, a 75:25 lip:surf ratio yielded the best results regarding transferosome physical parameters (size, PDI, and Z-potential). Therefore, a 2:1 solution of isopropyl alcohol: ethanol and a 75:25 lip:surf ratio was chosen for transferosomepreparation.

The sizes obtained for loaded transferosomes were 303.17±5.36 nm, 482.57±18.12 nm, and 375.2±1.67 nm for bioselenized hydrolysates at 10%, 8%, and 6% w/w lip:act, respectively, and 190.53±2.86 nm, 284.47±0.73 nm, and 413.00±5.22 nm for non-selenized ones. Some sizes falling outside the expected range were attributed to the short sonication times. Similarly, the results of PDI and Z-potential exhibited heterogeneity and low stability because of the short sonication times and choice of organic solvent.

Both FTIR analysis and SEM confirmed the proper encapsulation of the hydrolysates. Additionally, the spherical morphology of the transferosomes was verified through SEM. Finally, the encapsulation efficiency was 70% for selenized transferosomes, and the release percentage was 38.65% at 4 hours.

To improve and optimize the physical properties of transferosomes, changes in the formulation focusing on the organic solvent and increased sonication times are necessary.

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