„Dunărea de Jos" University of Galaţi

Doctoral School of Fundamental Science and Engineering

DOCTORAL THESIS

Ph.D. student,

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Scientific coordinator,

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Series I.7: FOOD ENGINEERING No. 6

GALAŢI

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ABSTRACT

Functional composites based on the sea buckthorn (*Hippophae rhamnoides*) extract used to produce value-added foods

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Introduction

Food industry shows a high interest in natural bioactive compounds from different sources due to the health benefits provided by these compounds. Numerous studies showed that there is a positive correlation between the consumption of food rich in polyphenols and carotenoids and the low risks of chronic diseases incidence.

In recent years, there was a massive increase in the interests the consumers offered to the functional products with beneficial health properties due mainly to the intensive promotion of a healthy lifestyle. Therefore, food industry seeks to develop new functional foods enriched with bioactive compounds, such as carotenoids and polyphenols.

Hippophae rhamnoides, commonly known as sea buckthorn, is a rich source in bioactive compounds, such as carotenoids (α-carotene, lycopene, lutein, and zeaxanthin), vitamins (C and E), flavonoids (isorhamnetin, quercetin, kaempferol), organic acids, amino acids, micronutrients, and macronutrients. (Yang and Kallio, 2001; Kallio *et al*., 2002) The bioactive compounds composition vary according to the fruits ripening, origin, size, species, and geographic area (Zeb, 2004; Leskinen *et al*., 2010).

The choise for the Ph.D. thesis **"Functional composites based on the sea buckthorn (*Hippophae rhamnoides*) extract used to produce value-added foods"** was based on two main reasons. The first reason was that sea buckthorn loses its property if it is not transported and stored properly. The storage costs are very high and the distribution of these fruits in large quantities to store networks is almost impossible, mainly because the sale of sea buckthorn can last for weeks and in this time there is a great loss in their bioactive compounds content. Consequently consumers will not have the possibility to enjoy the healthy properties the sea buckthorn fruits. Another reason for my choice was the astringent and bitter taste of sea buckthorn fruits, one of the main reasons the customers find difficult to consume these fruits. Therefore, there is a need to find alternatives to improve taste, while retaining health properties.

The Ph.D. thesis entitled **"Functional composites based on the sea buckthorn (*Hippophae rhamnoides*) extract used to produce value-added foods"** aimed to identify and investigate the bioactive compounds of *H. rhamnoides*, but also to exploit and develop them. These fruits are rich in bioactive compounds with numerous beneficial effects, due mainly to their antioxidant properties.

Identification and also the conformational and structural investigation of bioactive compounds from different crops leads to the optimization of the technological processes in order to ensure the functionality of the food products. Therefore, the Ph.D. thesis theme is important and suits perfectly to food industry actual perspectives.

The main scientific objectives of the research conducted during the doctoral studies were:

- Identification and determination of phytochemical content of sea buckthorn extract (*H. rhamnoides*), as well as evaluation of the stability of biologically active compounds, in order to determine the optimal conditions for obtaining and preserving the products rich in these compounds;
  - extraction and characterization of sea buckthorn bioactive compounds by identification and quantification of phytochemical composition of sea buckthorn extract.

- investigations of the thermal stability of the bioactive compounds in sea buckthorn (*H. rhamnoides*) extract at high temperatures, ranging from 50 to 100°C, with different exposure times (0-25 min) and modelling the thermal degradation mechanims of the total carotenoid content, the total polyphenols content, the total flavonoid content, and the antioxidant activity of sea buckthorn extracts on a kinetic basis;

- Evaluation of the binding mechanism of biologically active compounds from sea buckthorn extracts with whey proteins in order to obtain functional composites.
  - Investigation of binding mechanisms between the main whey proteins (β-lactoglobulin and α-lactalbumin) and carotenoids from sea buckthorn extracts, namely based on fluorencence and UV-Vis spectroscopy and molecular modeling;
  - Evaluation of the complexes stability tested under different conditions, such as heat treatment and different pH environments using quenching experiments, UV-vis spectroscopy, and *in silico* techniques.
- Microencapsulation of carotenoid compounds from sea buckthorn extracts in whey proteins isolate in order to obtain functional microcomposites for uses in food products.
  - microencapsulation of lipophilic components (carotenoids from sea buckthorn extract) using whey proteins (whey protein isolate) as shell materials using coancervation and freeze drying as microencapsulation techniques;
  - advanced characterization of the resulted powder.
  - developing of two new value-added food products by addition of whey protein and sea buckthorn-based functional components;
  - establish the manufacturing recipe for the obtaining of the products;
  - comparative analysis of the physicochemical and texture characteristics of the products.

The doctoral thesis is structured in two parts, as follows:

I. DOCUMENTARY STUDY is divided into 3 chapters summarizing various theoretical considerations on the bioactive compounds of *H. rhamnoides* and whey proteins from the literature. Also, the benefits of carotenoids and whey proteins on health are also presented.

II. The EXPERIMENTAL STUDY comprises of 4 chapters where the results of the research studies that were carried out during the doctoral stage are highlighted, as follow:

Chapter 4, entitled **Phytochemical profile of sea buckthorn extract and description of thermal degradation mechanisms of biologically active compounds on kinetic basis**, presents the extraction, separation, identification and quantification of carotenoid compounds (carotenoids, polyphenols and flavonoids) from sea buckthorn (*H. rhamnoides*) extract using spectrophotometric methods, liquid chromatographic techniques (HPLC), and FT-IR. In order to evaluate the thermal stability of these biologically active compounds from the perspectives of using as such in food industry, the thermal degradion mechanisms were described based on kinetic models.

Chapter 5, entitled **Advanced studies of binding mechanisms of carotenoids from sea buckthorn extract and whey proteins**, presents the binding parameters features for the α-lactalbumin-sea buckthorn extract and the β-lactoglobulin-sea buckthorn extract complexes, whereas the evaluation of the structural changes of the complexes induced by thermal treatment and pH were highlighted.

Chapter 6, entitled **Microencapsulation of carotenoids from sea buckthorn extract in whey protein in order to develop functional composites with food industry applications**, describes the microencapsulation of lipophilic components of sea buckthorn extract using whey proteins as shell material, whereas advanced characterization of the resulted powder in terms of encapsulation efficiency, color parameters, total carotenoids content, antioxidant activity, particle structure using FT-IR spectral analysis and confocal microscopy were performed.

Chapter 7, entitled **Development of food technology in order to obtain enriched food products by exploiting the sea buckthorn bioactives and the microencapsulated carotenoid complex potential** describes two technologies for patenting claims of two enriched food products, using the bioactive potential of sea buckthorn extract and the functional properties of the microencapsulated sea buckthorn carotenoids.

Each chapter of the experimental study comprises the following subchapters: Introduction, Objectives, Materials and Methods, Results and Discussion, Partial conclusions and References.

Chapter 8, *General Conclusions*, presents the main conclusions of the thesis experiments.

The doctoral thesis consists of 175 pages, including 75 figures and 25 tables. The documentary study is 25% and the experimental part 75%.

Finally, the original contributions of the Ph.D. thesis and the dissemination of the results are highlighted. Hence, results have been capitalized through the development of 8 scientific articles, published or in the course of publication of which 7 articles in ISI journals (Journal of Food Chemistry, Journal of Macromolecular Science, Part A - Pure and Applied Chemistry, Journal of Molecular Structure, Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, Journal of Food Engineering, Journal of Molecular Structure) and 1 article indexed in international databases (The Annals of the Lower Danube University of Galati, Fascicle VI - Food Technology). The experimental results concluded with the writing of two patents claim, registered at OSIM. The results were also presented at 8 international or national conferences.

Conducting the Ph.D. thesis experiments was possible thanks to the Integrated Center for Research, Expertise, and Technology Transfer infrastructure (BioAliment-Tehnia) (www.bioaliment.ugal.ro), part of the Faculty of Food Science and Engineering, "Dunarea de Jos" University of Galati.

During my doctoral studies, I was enroled in the research team of the project PN-II-RU-TE-2014-4-0115 / 2015-2017 (www.funfood.ugal.ro) entitled "*Functional protein based composites from whey and vegetable extracts for applications in the food industry*", project director Prof. dr. eng. Nicoleta Stănciuc.

The thesis was conducted under the scientific coordination of the steering committee with the following structure:

- **Prof. dr. eng. Elisabeta BOTEZ** - Ph.D. supervisor
- **Prof. dr. eng. Nicoleta STĂNCIUC** - Spectrofluorimetry and chemical kinetics coordinator
- **Prof. dr. eng. Iuliana APRODU** - Molecular modeling and docking studies coordinator
- **Prof. dr. chem. Rodica DINICĂ** – Extraction studies coordinator

4. Phytochemicals profile of sea buckthorn extract and description of thermal degradation mechanisms of biologically active compounds on kinetic basis

4.1. Introduction

The fruits of sea buckthorn (*H. Rhamnoides)* have a high content of carotenoids. Carotenoids exert a number of beneficial actions on the human body, for example: they can reduce the risk of cataracts and prevent some of the eyes' diseases (Michael *et al.*, 2011), the cognitive decline (Plasman *et al.*, 2010), the progression of chronic diseases correlated with obesity (Roberts *et al.*, 2009; Ford *et al.*, 2003), delay the progression of cardiovascular disease and diabetes (Boon, 2010; Palozza *et al.*, 2011; Gouranton *et al.*, 2011).

In order to ensure the functionality of the food products, different experiments that identified and investigated the conformational and structural changes of the bioactive compounds from different plants were carried out. These experiments lead to the optimization of the technological processes in the food industry.

This study focused on extraction, identification, and quantification of the phytochemicals compounds from the sea buckthorn extract. Also, the investigation of the thermal stability of sea buckthorn bioactive compounds (*H. rhamnoides*) in different thermal regimes from the perspective of usage as ingredients in food products or nutraceuticals was also assessed. Quantitative studies have been conducted in order to describe the changes that occur after heat treatment (temperatures ranging from 50 to 100°C, with a different holding time 0-25 min) on the content of the total carotenoids (TCC), the total polyphenol (TPC) and the total flavonoids (TFC) contents, as well as on the antioxidant activity of sea buckthorn extracts.

Fluorescence spectroscopy was used to expand the possibilities of analysis of heat induced changes in the structure of the bioactive compounds, whereas FT-IR spectroscopy was used to obtain more information about the compositional complexity of the extract and to evaluate the thermal behavior of the bioactive compounds.

4.2. Results and discussion

4.2.1. HPLC qualitative and quantitative analysis of sea buckthorn (*H. rhamnoides*) extracts

The HPLC analysis has been carried out by comparing the retention times of the carotenoids samples extracted from the sea buckthorn with those of the carotenoids standard, as well as the data from the literature.

The figure 4.7. displays the HPLC chromatogram of the carotenoids identified and quantified in the sea buckthorn extract. Twelve compounds have been identified, as follows: (1) astaxanthin (peak 1), zeaxanthin (peak 2), zeaxanthin-palmitate (peak 3), γ-carotene (peak 4), cis-β-carotene (peak 5), β-cryptoxanthin (peak 6), lycopene (peak 7), lutein myristate-palmitate (peak 8), lutein di-palmitate (peak 9), β-carotene (peak 10), α-carotene (peak 11), and zeaxanthin-di-palmitate (peak 12). β-Carotene was identified with a content of 15.19 mg/g D.W., followed by astaxanthin with a content of

11.94 mg/g D.W., β-cryptoxanthin with 8.93 mg/g D.W. and lycopene with 2.24 mg/g D.W., while zeaxanthin was found in the largest amount of 81.29 mg/g D.W.

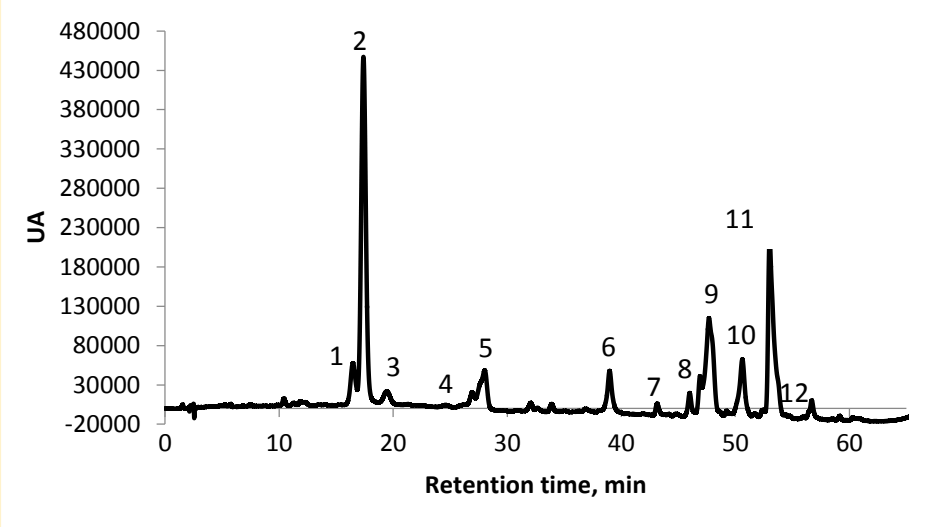


Figure 4.7. HPLC chromatogram for identification and quantification of carotenoids from sea buckthorn extract (Ursache *et al*., 2017)

The results obtained are similar to those reported by Pop *et al.* (2014), who suggested a content of β-carotene between 1.9 and 7.4 mg/100 g D.W., β-cryptoxanthin (1.3-1.6 mg/100 g D.W.), lycopene (1.4-2.3 mg/100 g D.W.) and zeaxanthin (1.8-2.5 mg/100 g D.W.) in six varieties of sea buckthorn from Romania. Andersson *et al.* (2009) suggested some relative percentages regarding the values of different carotenoids in the sea buckthorn fruits: 1% of lutein, 8% of zeaxanthin, 0.3% β-cryptoxanthin, 8% lycopene, 4% γ-carotene, 14% β-carotene, and 10% minor carotenoids.

Table 4.2. displays a comparative analysis of the biologically active compounds from the sea buckthorn extracts with data from the literature.

Table 4.2. The content of bioactive compounds of *H. rhamnoides*

Sea buckthorn bioactive compounds	Ursache *et al.* (2017)	Results reported in the literature	References
Total polyphenols	140.14 ± 6.64 mg AG/g D.W.	28.35±1.31 mg/g	Kumar *et al.* (2011)
		9.64 -107.04 mg AG/g D.W.	Korekar *et al.* (2014)
Total flavonoids	5.04 ± 0.05 mg EC/ g D.W.	345-854 mg/100 mg	Yuzhen *et al.* (1997)
		6.79±0.30 mg rutin/g	Chauhan *et al.* (2012)
		14.14±1.12- 6.40±2.36 mg rutin/g D.W.	Kumar *et al.* (2011)
Antioxidant activity	33.7±0.29%	94.7±3.2 for the ethanolic extract	Papuc *et al.* (2008)
		74.7±2.6 for the water-acetone extract	
***β*-carotene**	38.34 ± 5.71 mg/g D.W.	1.5 – 18.5 mg/100 g	Andersson *et al.* (2009)
		9.4-34.5 mg/100 g	Beveridge *et al.* (1999)
		0.53 – 0.97 mg/g D.W.	Pop *et al.* (2014)

4.2.2. *IR* spectra

In **figure 4.8.** are shown the ATR-FTIR spectra of the sea buckthorn extract after heat treatment (a - 50°C, b - 100°C).

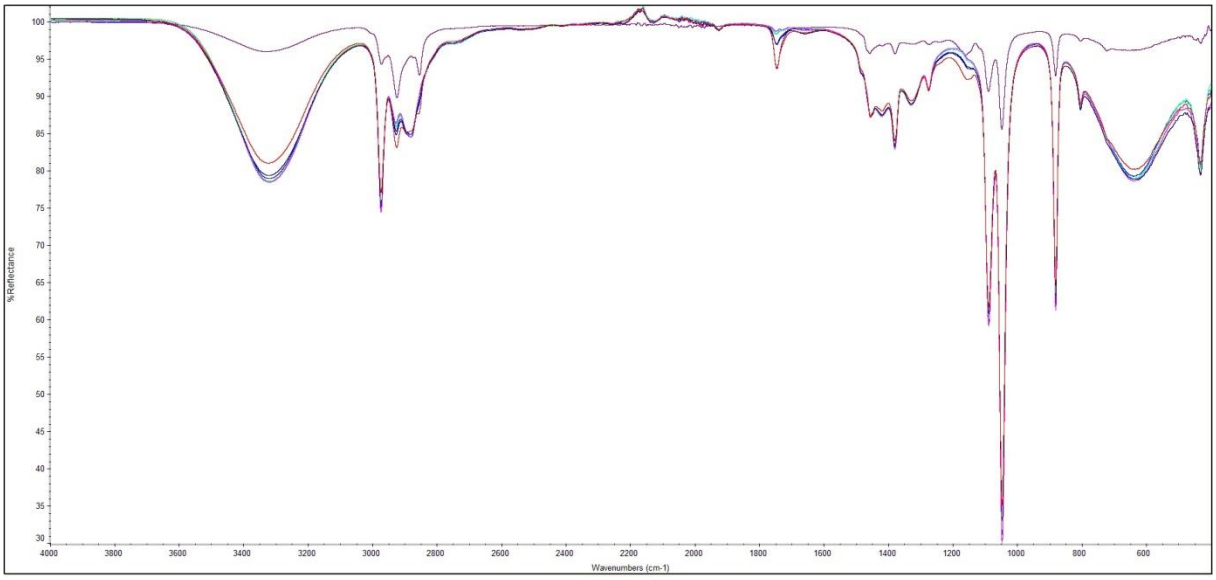


Figure 4.8. ATR-FTIR spectra of the sea buckthorn extract after heat treatment (a - 50°C, b - 100°C) (Ursache *et al.*, 2017)

The IR spectrum of the sea buckthorn extract is complex. The characteristic peaks include ethers and lactones (880 cm^{-1}, 1045 cm^{-1}, 1087 cm^{-1}, 2880 cm^{-1}), the H, O-H and N-H bonds stretch (3320 cm^{-1}), the phenols (1274 cm^{-1}), the C = O of the esters, the dicarboxylic acids and the α-amino acids (1745 cm^{-1}), C-H, and CH_2 stretches and deforms the vibration (1454 cm^{-1}, 2880 cm^{-1}) (Stuart *et al.*, 2009).

The samples showed, in general, a high thermal stability. Heated at 50°C, the peaks were low and displayed a slight increase at 1160 cm^{-1} that can be assigned to the vibration group C-O (Takahashi *et al.*, 2001). A low bandwidth has been recorded in the region of the fingerprints samples heated up to 60-100°C at 1419 cm^{-1}, showing an increase with the increase in temperature. The keto-enol tautomerism could be observed with a significant reduction of the carbonyl peak (1745 cm^{-1}) at 70 and 80°C. This characteristic band also occurs at high-temperature samples (90 and 100°C), while keto formation may be favored by high temperatures (Laurella *et al.*, 2013). Another effect of the heat treatment has been observed in the narrowing and lowering of the 3320 cm^{-1} band, probably due to the rupture of the hydrogen bonds. The oxidation of ascorbic acid at low temperatures could not observe since the spectrum is complex (Munyaka *et al.*, 2010).

4.2.3. Evaluation of the thermal treatment structural changes from the sea buckthorn extracts

Emission spectra for sea buckthorn bioactive compounds were obtained at 250 nm, 340 nm, 410 nm and 448 nm wavelengths with significantly different emission wavelengths. In Figure 4.9. it can be seen that the spectra obtained at these excitation wavelengths are well defined, indicating that several different fluorescent compounds are present in the extract.

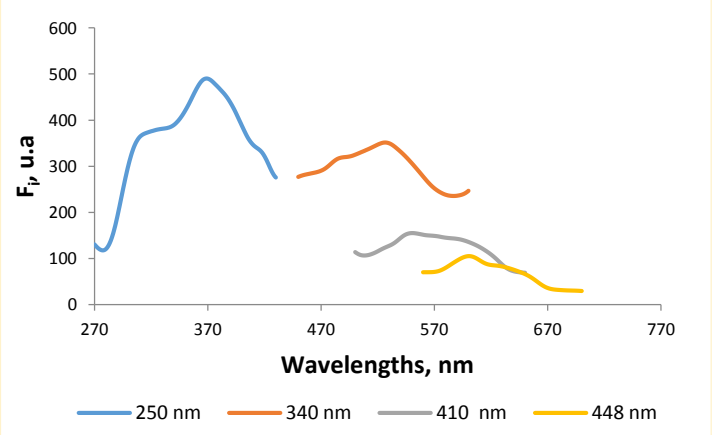


Figure 4.9. The fluorescence spectra of the bioactive compounds in the thermally untreated sea buckthorn extract at different excitation wavelengths (Ursache *et al*., 2017)

According to Zandomeneghi *et al.* (2005) polyphenols absorb at the 260-310 nm wavelength and emit in the range 310-370 nm, while compounds of the vitamin E group absorb at wavelength 365 nm and emit at 525 nm (Kyriakidis *et al.*, 2000). Baran *et al.* (2011) assessed the emission/excitation spectra of quercetin at two excitation wavelengths: 380 nm and 440 nm with a maximum wavelength of approximately 540 nm.

Pawlak *et al.* (2013) reported that the monomeric β-carotene presented the maximum peak intensity at 525 nm, while the form of crystal and aggregates of β-carotene showed maximum emission at 564 nm and 608 nm when excited at 413 nm. Therefore, it can be appreciated, based on the spectral profile that the extract of sea buckthorn is particularly complex in terms of composition, being identified both polyphenols and carotenoids, compounds from the vitamin E group and quercetin.

4.2.4. Thermal degradation studies of bioactive compounds in sea buckthorn extracts

Knowledge on kinetics is essential to predict changes that may occur during thermal processing of food containing natural pigments (Avila *et al.*, 1999). Patras *et al.* (2010) highlighted the importance of understanding the kinetics of the degradation process, the reaction order, the degradation rate constants, and the activation energy. These can have a positive impact in terms of predicting the losses in the quality of the food during thermal treatments. The kinetics studies were conducted for the total carotenoids, polyphenols, flavonoids contents in the sea buckthorn extract, but also for the antioxidant activity, in the temperature range of 60-100°C for different holding time.

4.2.4.1. Total carotenoids content

The fractional conversion kinetic model was used to describe the thermal degradation of the total carotenoid content of the sea buckthorn extract. According to this model, the rate of degradation of the carotenoids compounds increases steadily from $17.83 \pm 2.85 \cdot 10^{-2}$ min^{-1} at 60°C to $24.02 \pm 3.67 \cdot 10^{-2}$ min^{-1} at 100°C (**Table 4.6**).

Table 4.6. Kinetic parameters describing the degradation of total carotenoid content in sea buckthorn extract according to the fractional conversion kinetic model (Ursache *et al.*, 2017)

Temperature, °C	$k \cdot 10^{-2}$ (min^{-1})	R^2
60	17.83 ± 2.85^{a}	0.978
70	18.83 ± 2.78	0.985
80	21.42 ± 3.18	0.976
90	23.47 ± 4.48	0.969
100	24.02 ± 3.67	0.978
E_a (kJ·mol^{-1})	**8.45 ± 0.93**	**0.964**

Fratianni *et al.*, (2010) reported a first order kinetic model for the thermal degradation of the β-carotene content of the microwave-treated orange juice at 60°C with degradation constants (*k*) of $2.30 \cdot 10^{-2}$ min^{-1} and $65.80 \cdot 10^{-2}$ min^{-1} at 75°C. Aparicio-Ruiz *et al.* (2011) demonstrated that the first order kinetic model is appropriate to describe the thermal degradation of β-carotene, β-cryptoxanthin, and lutein in the virgin olive oils, indicating *k* values for β-carotene degradation ranging from 17.16 ± 0.33 min^{-1} to 188.66 ± 5.66 min^{-1} at 60°C and 100°C, respectively.

The thermostability of carotenoids is due to the conjugated double bonds. When a high severity heat treatment is applied, the structures are broken down and molecular reactions occur in which the double bonds are involved. Ty *et al.* (1999) claimed that two types of products were formed following

thermal degradation: a volatile fraction with a low molecular weight which volatilized and a non-volatile fraction, with larger fragments of molecules resulting after the cleavage of the volatile fraction of carotene. Rios *et al.* (2008) have shown that β-carotene under less severe thermal conditions leads to the formation of toluene, xylene, and *m*-2,6-dimethylnaphthalene.

4.2.4.2. The total polyphenolic compounds content

The thermal degradation of the total polyphenols in the sea buckthorn extract followed a fractional conversion kinetic model.

In the **figure 4.18**. the degradation curves of the polyphenolic compounds in the heat-treated sea buckthorn extract at temperatures between 60 and 100°C are presented. At constant temperature up to 5 minutes, a rapid degradation of these compounds can be observed.

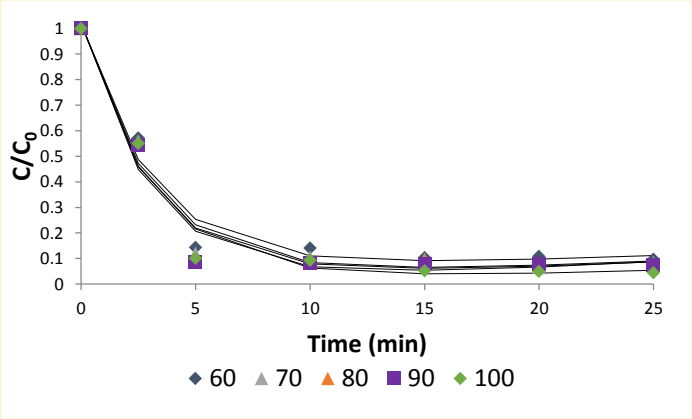


Figure 4.18. Thermal degradation of polyphenolic compounds in sea buckthorn extract according to the fractional conversion kinetic model

According to the fractional conversion kinetic model, *k* ranged from 31.27 ± 3.46·10^{-2} min^{-1} to 34.27 ± 6.19·10^{-2} min^{-1} due to the increase of the temperature from 60 to 100°C (**Table 4.7**).

Table 4.7. Kinetic parameters for the total polyphenol content of sea buckthorn extract according to the fractional conversion kinetic model (Ursache *et al*., 2017)

Temperature, °C	k·10^{-2} (min^{-1})	R^2
60	31.27 ± 3.46	0.989
70	31.40 ± 3.43	0.989
80	31.51 ± 3.17	0.990
90	33.42 ± 4.18	0.986
100	34.27 ± 6.19	0.985
E_a (kJ·mol^{-1})	**2.50 ± 0.66**	**0.827**

In the literature, first-order kinetic models have been reported for the degradation of polyphenolic compounds. Thus, Jaiswal *et al.*, (2012) after applying the blanching treatment of the cabbage obtained *k* values of 37.90 ± 0.00·10^{-2} min^{-1} and 48.40 ± 4.00·10^{-2} min^{-1} for 80°C and 100°C, respectively, and the activation energy reported was 11.54 kJ·mol^{-1}. Turturică *et al.*, (2016) also

suggested a first order kinetic model for the thermal degradation of the polyphenolic compounds in the plum extract, with values for degradation constants of $0.7\pm0.1\cdot10^{-2}$ min^{-1} and of $2.90\pm0.7\cdot10^{-2}$ min^{-1} at 70°C and 110°C, respectively and E_a value of 36.00 ± 8.0 $kJ\cdot mol^{-1}$.

Zorić *et al.* (2014) used also the first order kinetic model to describe the thermal degradation of the quercetin-3-glucoside solution from the cherry pulp by increasing the temperature from 80 to 120°C and obtained degradation constant values ranging from 1.5 up to $2.6\cdot10^{-2}$ min^{-1}.

4.2.4.3. The total flavonoids content

Figure 4.20. displays the degradation curves of the flavonoid compounds in the thermally treated extract at temperatures between 60 and 100°C. In this case, the rapid degradation of these compounds can be observed within the first 5 minutes of constant temperature.

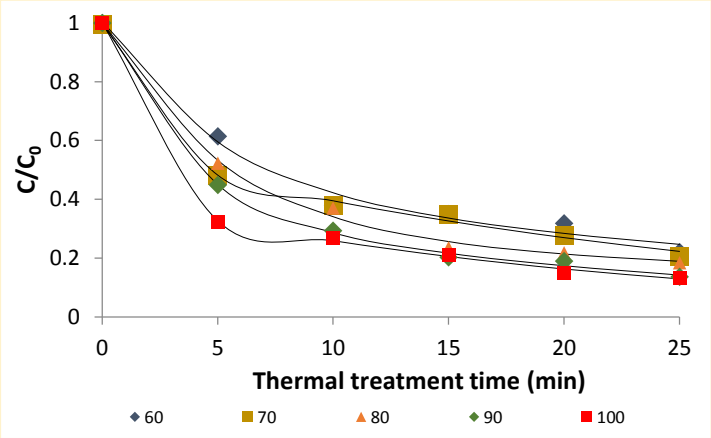


Figure 4.20. Thermal degradation of flavonoids from the sea buckthorn extract described by a fractional kinetic model

According to the fractional conversion kinetic model, the rates of the degradation constants for total flavonoid content increased from $14.13\pm1.89\cdot10^{-2}$ min^{-1} to $38.47\pm13.30\cdot10^{-2}$ min^{-1} for 60°C to 100°C, respectively (**Table 4.8**).

Table 4.8. Kinetic parameters for total flavonoid content in sea buckthorn extracts according to the fractional conversion kinetic model (Ursache *et al.*, 2017)

Temperature, °C	$k\cdot10^{-2}$ (min^{-1})	R^2
60	14.13 ± 1.89	0.988
70	15.47 ± 3.01	0.979
80	18.32 ± 1.56	0.992
90	19.04 ± 1.57	0.993
100	38.47 ± 13.30	0.983
E_a ($kJ\cdot mol^{-1}$)	**22.50 ± 7.26**	**0.761**

Also for the flavonoid content, a first-order kinetic model was reported in the literature, therefore Jaiswal *et al.* (2013) suggested an activation energy for the flavonoid compounds of 9.22 kJ·mol^{-1}, while Turturică *et al.* (2016) reported an activation energy of 18.00 ± 2.0 kJ·mol^{-1}.

4.2.4.4. Antioxidant activity

In order to describe the thermal degradation of the antioxidant activity, the fractional conversion kinetic model was used. The effect of the heat treatment on the antioxidant activity is shown in **Figure 4.22**, where the degradation curves are presented.

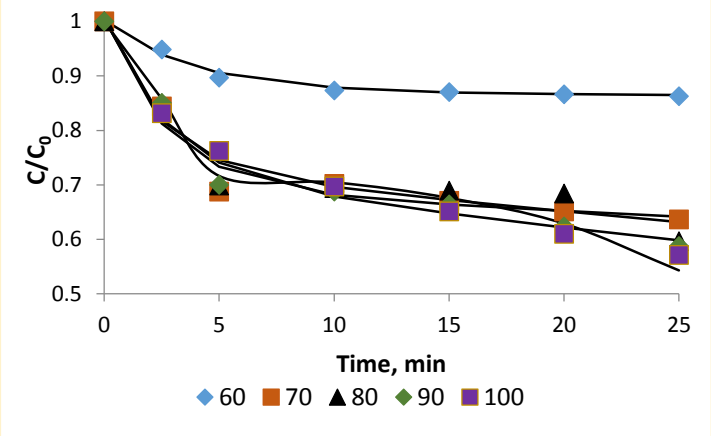


Figure 4.22. Thermal degradation of antioxidant activity in the sea buckthorn extract described by a fractional kinetic model

In the **Figure 4.22**. it can be observed that at 60°C slower degradation takes place compared to higher temperatures which induce a decrease in the antioxidant activity. According to the fractional conversion kinetic model, the rate of degradation of the antioxidant activity increased from 16.30±3.09·10^{-2} min^{-1} at 60 ° C to 30.52±6.40·10^{-2} min^{-1} at 100°C (**Table 4.9**).

Table 4.9. The estimated kinetic parameters and the activation energy of the antioxidant activity in the sea buckthorn extract (Ursache *et al.*, 2017)

Temperature, °C	$k \cdot 10^{-2}$ (min^{-1})	R^2
60	16.30 ± 3.09	0.989
70	23.18 ± 7.26	0.979
80	24.21 ± 9.37	0.932
90	28.53 ± 5.84	0.961
100	30.52 ± 6.40	0.928
E_a (kJ·mol^{-1})	**15.22 ± 2.75**	**0.910**

Given the values of the degradation rate constants, it can be stated that in the case of the total polyphenol content occurs a more rapid degradation, compared to the other compounds.

4.3. Partial conclusions

This study focused on the extraction, the phytochemical characterization and the evaluation of structural changes of bioactive compounds in *H. rhamnoides* extracts, induced by the thermal treatment.

HPLC analysis revealed the presnece of 12 compounds in the sea buckthorn extract, as follow: astaxanthin, zeaxanthin, zeaxanthin-palmitate, γ-carotene, cis β-carotene, β-cryptoxanthin, lycopene, lutein palmitate-myristate, di- carotene, α-carotene, and zeaxanthin di-palmitate. From a quantitative point of view, the extract was characterized by a TCC of 38.34 ± 5.71 mg/g D.W., TPC of 140.14 ± 6.64 mg AG/g D.W., and TFC of 5.04 ± 0.05 mg EC/g D.W., and an antioxidant activity of 33.7±0.29%, corresponding to 2.50±0.02 μM Trolox/g D.W.

The influence of heat treatment was used to quantitatively describe the impact of processing on the content of bioactive compounds in the sea buckthorn extract. The structural complexity of the sea buckthorn extract was highlighted by FT-IR fluorescence and spectroscopy techniques. Four major classes of compounds have been identified, as follows: phenols, flavonoids, tocopherols, and carotenoids. The IR spectrum also highlighted the complexity of the sea buckthorn extract, which involves characteristic peaks for ethers and lactones, H-OH bonds, the stretching of NH bonds, phenols, C=O in esters, dicarboxylic acids and α-amino acids, stretching vibrations and deformation of CH and CH_2. Thermal treatment caused changes in peak emission wavelengths, indicating the sequential character of the phytochemical compounds structural changes, while the ATR of the samples revealed a generally high thermal stability.

The kinetic degradation of the phytochemical content in the thermally treated sea buckthorn extract was modeled using the fractional conversion model. The variation of the degradation constants related to temperature was described by the Arrhenius equation. Calculated values of activation energy for different phytochemicals revealed that polyphenols and carotenoids, respectively, are more stable at heat treatment, while the flavonoids and the antioxidant activity have a low thermal stability.

This study provides important information on the kinetics of phytochemical degradation, which will facilitate the optimization of heat treatment in the food industry regarding food safety and nutritional value.

5. Advanced studies of the binding mechanisms between carotenoid compounds from the sea buckthorn extract and whey proteins

5.1. Evaluation of the binding mechanism between α-lactalbumin and carotenoid compounds in sea buckthorn extract and assessment of complex stability under different environmental conditions

5.1.1. Introduction

The study objectives were to investigate the mechanisms of binding between the main whey proteins (β-LG and α-LA) and the carotenoids in the sea buckthorn extracts, as well as evaluation of the stability of the complexes formed under different pH conditions and thermal treatment from the perspective of the development of composites with high functionality that can be used in foods and/or supplements.

The methods of investigation used quenching experiments, UV-vis spectroscopy and *in silico* techniques.

5.1.2. Materials

In these experiments, α-LA without Ca^{2+} and β-LG protein solutions, β-carotene, ANS (Sigma-Aldrich Co., St. Louis, MO) were used. White sea buckthorn fruits were purchased, as previously described, from Galati, and were subsequently lyophilized. All reagents used were analytically pure.

5. 2. Results and discussion

5.1.5.1. Study of α-LA capacity to bind carotenoids in model systems

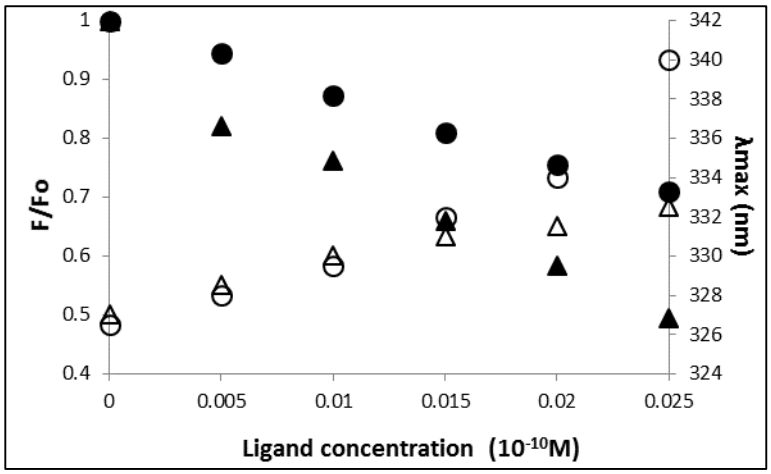
- **The carotenoid binding mechanism of sea buckthorn extract (EC) to α-LA**

The interaction between α-LA and EC was examined by investigating the influence of increasing the EC concentration on the thermally treated protein fluorescence spectra in the temperature range of 25°C and 100°C. Since the total carotenoid content in the extract is expressed as mg β-carotene per gram D.W., similar experiments were carried using a solution of β-carotene as a model system.

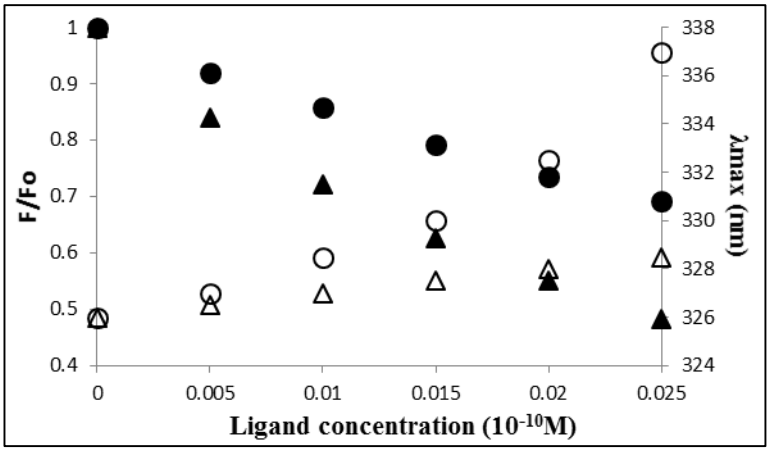
For the evaluation of the exposure of Trp residues, the wavelength value at which the maximum fluorescence intensity (λ_{max}) is recorded is taken into account. If the λ_{max} value is less than 330 nm, Trp residues are blocked in a non-polar medium, and if λ^{max} is greater than 330 nm, Trp residues are exposed to the solvent in a polar medium (Vivian *et al.*, 2001). The λ_{max} values of α-LA in the absence of the ligand recorded a red-shift from 326 nm at 25°C nm to 329 nm at 100°C, indicating that at temperatures above 80°C the protein starts to lose the compact structure of the hydrophobic subdomain where Trp residues are placed. In other words, the red shift is an indicator of changes in the micro medium of Trp residues from α-LA, highlighting the increase in hydrophobicity in their vicinity. The same pattern was observed at the addition of the ligand to the protein solution, indicating hydrophobic interactions between the ligand and α-LA. Changes in the fluorescence of the α-LA Trp residue induced by the hydrophobic ligands can lead to an energy transfer between the excited indole

ring (Trp) and ligands but also changes in polarity of the Trp residue micro medium or both effects (Mensi *et al.*, 2013).

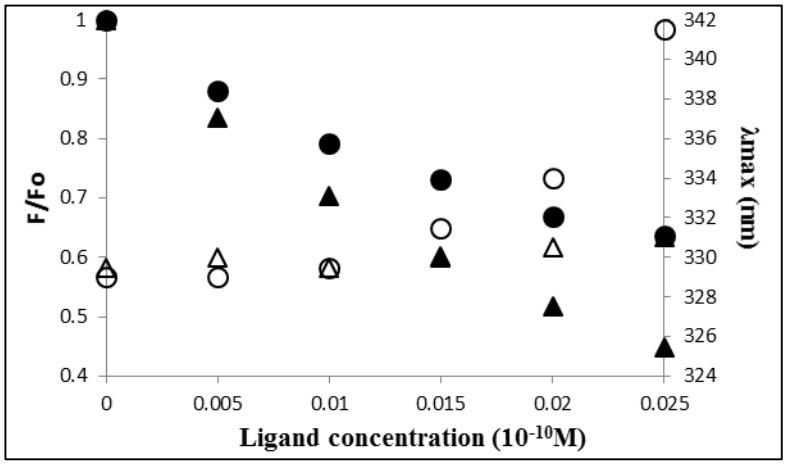
Figure 5.3 shows the ratio between *F* and F_0 of two systems based on α-LA treated at different temperatures (25°C, 60°C, and 90°C). It can be observed that the β-carotene caused a faster quenching of the fluorescence of Trp residues from α-LA compared to EC. The β-carotene at maximum concentration induced a 50.4% decrease in the α-LA native fluorescence, whereas EC only elicited 29% of the initial fluorescence of the protein. The maximum degree of quenching was reached at 100°C, where β-carotene and EC caused the fluorescence of Trp residues to be extinguished by 56% and 47%, respectively.



a)



b)



c)

Figure 5.3. The structural changes of heat treated a-LA at 25˚C (a), 60˚C (b) and 90˚C (c) with EC (circle) and β-carotene (triangle) monitored as F/F_o (● and ▲) and the maximum emission wavelength (○ and ∆)

The addition of 50 µL of EC to the α-LA native solution (**Figure 5.3 a**) lead to a 13-nm red shift, while in the case of β-carotene the addition induced a 6-nm red shift. In the temperature range of 50°C and 90°C (**Figure 5.3 b** and **c**), λ_{max} ranged from 326 nm to 329 nm when β-carotene was added. In the EC quenching experiments, the heat treatment at temperatures between 70°C and 80°C induced variations of λ_{max} from 326 nm to 334 nm, and a more severe thermal treatment led to an 11 nm red-shift.

A similar behavior was reported by Mensi *et al.*, (2013) for the interaction of β-lactoglobulin with different carotenoids, such as β-carotene, β-cryptoxanthin, and α-carotene. The authors found out that, despite its high hydrophobicity, β-carotene has an affinity for β-LG. According to polarity, the carotenoids can be grouped into carotenes and xanthophylls (Victoria-Campos *et al.*, 2013). Since the EC contains both carotenes and xanthophylls, it can be assumed that in the case of EC quenching experiments, there is a competition between the carotenoids compounds in the complex in binding to the protein binding sites. A detailed atomic analysis by Mohammadi and Moeeni (2015) of α-LA at different temperatures indicated the following: Trp^{118} is the most exposed, being located at the α-LA surface, Trp^{26} is blocked in the protein matrix, a hydrophobic region, and Trp^{104} is partially protected by the solvent, while Trp^{60} is almost completely blocked. Therefore, it can be assumed that these Trp residues pass into the more polar environment with a major contribution to the EC binding process.

- **The binding constant and the number of the binding sites**

One of the most important approaches in the elucidation of the mechanisms of the binding of small molecules to proteins is conducting experiments of quenching (Mohammadi *et al.*, 2015), which allows the estimation of the binding parameters.

To elucidate the quenching mechanism of α-LA with EC and β-carotene, the data were analyzed using the Stern-Volmer equation, according to Dumitrascu *et al.*, (2015), and the Stern Volmer constants (K_{SV}) were calculated from the interaction between α-LA-EC and α-LA-β-carotene. The results are presented in **Table 5.1** (**a** and **b**).

Table 5.1. The binding parameters of α-LA with EC (a) and β-carotene (b)

a)

T(˚C)	K_{SV} (10^{-11} L/mol)	R^b	K_b (10^{-11} L/mol)	*n*	R^c
25	1.69±0.02 [a]	0.998	1.60±0.27	1.55±0.13	0.990
50	1.54±0.12	0.997	1.04±0.08	1.13±0.07	0.995
60	1.57±0.02	0.996	1.05±0.19	1.09±0.12	0.998
70	1.91±0.02	0.999	1.02±0.02	1.13±0.05	0.999
80	1.95±0.16	0.999	0.88±0.08	1.02±0.007	0.999
90	1.67±0.07	0.998	0.70±0.08	0.93±0.008	0.998
100	2.29±0.12	0.994	0.99±0.03	0.93±0.02	0.998

b)

T(˚C)	K_{SV} (10^{-6} L/mol)	R^b	K_b (10^{-11}L/mol)	*n*	R^c
25	3.86±0.16[a]	0.997	1.57±0.12	1.13±0.06	0.999
50	4.28±0.07	0.995	1.48±0.03	1.14±0.01	0.999
60	4.25±0.06	0.997	1.43±0.03	1.06±0.01	0.999
70	3.83±0.27	0.996	1.40±0.01	1.10±0.006	0.999
80	4.83±0.15	0.993	1.68±0.06	1.19±0.01	0.999
90	4.6±0.07	0.995	1.55±0.05	1.07±0.002	0.998
100	4.91±0.02	0.994	1.40±0.003	1.13±0.002	0.999

[a] standard deviation
R^b correlation coefficient for the K_{SV} values
R^c correlation coefficient for the K_b values

Regardless of the applied temperature, the K_{SV} displayed lower values when quenching experiments were performed with a β-carotene solution. In the EC quenching experiments, the lowest K_{SV} value was calculated for the temperature of 50°C ($1.54\pm0.12\cdot10^{-11}$ L/mol) and the highest at 100°C ($2.29\pm0.12\cdot10^{-11}$ L/mol), suggesting that the Trp residues are more exposure to EC at higher temperatures. On the other hand, in the case of quenching experiments with β-carotene, K_{SV} values have increased at temperatures between 25°C and 60° C from $3.86\pm0.16\cdot10^{-11}$ L/mol to 4. $25\pm0.06\cdot10^{-11}$ L/mol, while heating at 70°C of α-LA generated a similar degree of Trp exposure as in the case of the 25°C temperature. The maximum K_{SV} value for quenching experiments with β-carotene was reached at 100°C ($4.91\pm0.02\cdot10^{-11}$ L/mol), when quenching experiments with EC were conducted. The increasing of the quenching constants at higher temperatures suggests an altering of the tertiary structure of α-LA, in which Trp residues are more exposed to the quenching agent. Regarding the influence of thermal treatment on molecular flexibility, it can be observed that EC titration led to a more flexible tertiary structure of α-LA compared to experiments where β-carotene was used as a quenching agent.

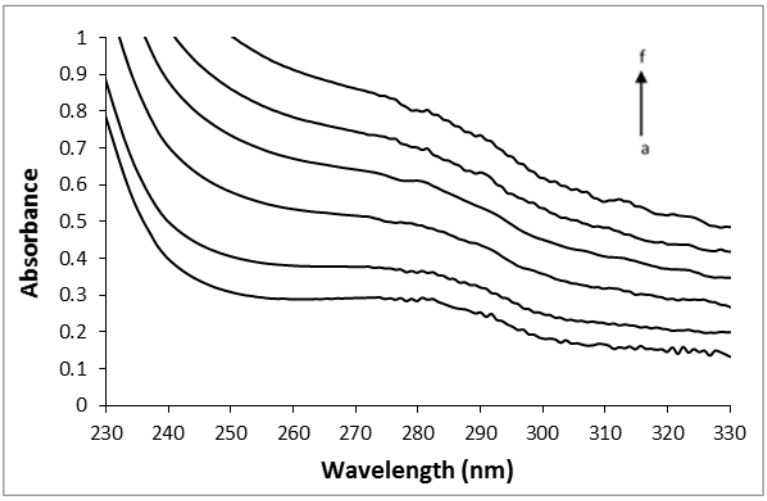
The values for the number of binding sites (*n*) calculated for the α-LA-EC complex (**Table 5.1a**) decreased with increasing temperature, from 1.55±0.13 to 0.93±0.02, indicating that the affinity of α-LA for the EC decreases with increasing the temperature in the thermal treatment of the protein. In the case of β-carotene (Table 5.1b), the *n* values are close together in the entire temperature range,

leading to the hypothesis that, regardless of the thermal treatment applied, the α-LA molecule has at least one binding site with high affinity for β-carotene.

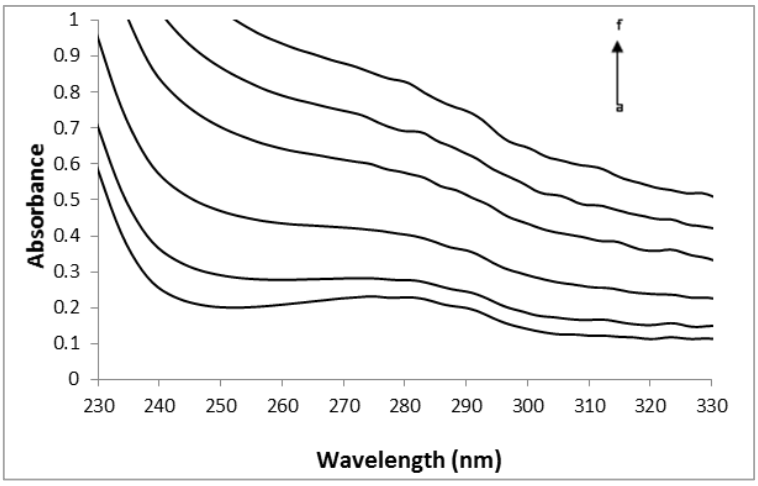
- **Analysis of UV-vis absorption spectra**

UV-vis absorption spectroscopy is one of the most commonly used techniques for studying the conformational protein changes, which can be used successfully in evaluating the formation of complexes with various ligands. The absorption spectrum changes with the modification of the protein structure, which can be observed as a peak or as an increase/decrease in the absorbance intensity (Song *et al.*, 2015). Therefore, information regarding the nature of the interaction between α-LA and the EC, α-LA UV-vis absorption spectra, with and without the EC or the β-carotene, can be obtained.

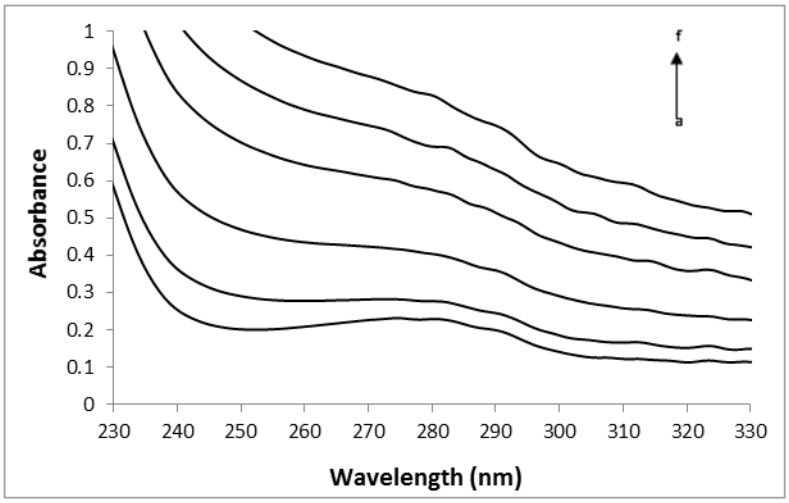
Figure 5.4 shows the UV-vis spectra of thermally treated α-LA in the absence/presence of the EC extract with different concentrations (α-f corresponds to concentrations from 0.005 to 0.02 $\cdot 10^{-10}$ M).



a)



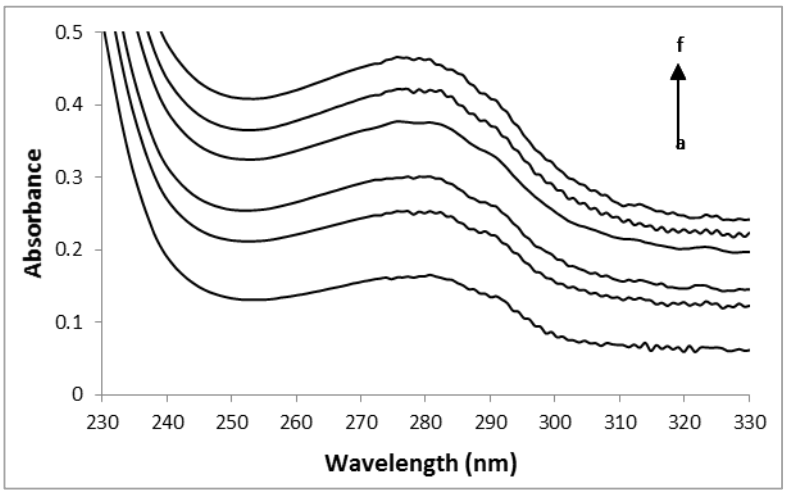
b)



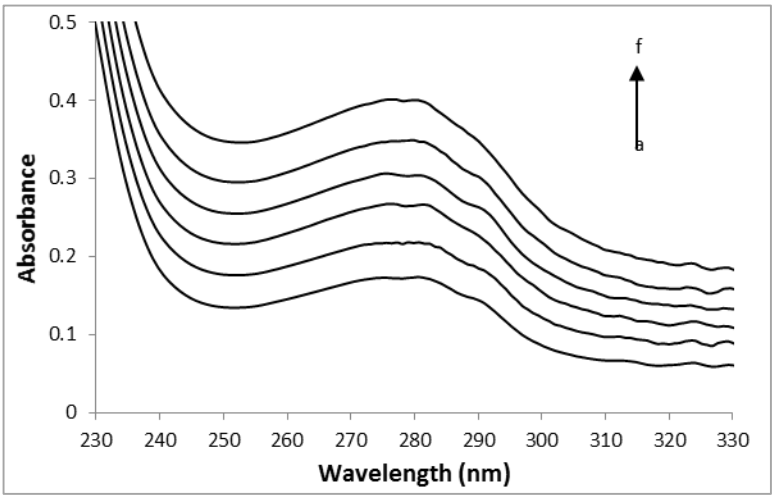
c)

Figure 5.4. The UV-vis absorption spectra of α-LA thermally treated at 25°C (a), 60°C (b) and 90°C (c) in the presence of different EC concentrations (a-f corresponds to concentrations of 0.005 - $0.025 \cdot 10^{-10}$ M)

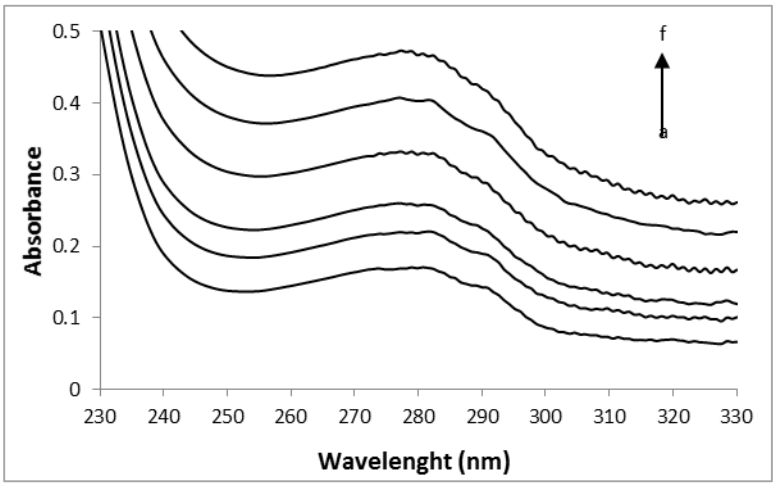
Figure 5.5 displays the UV-vis spectra of the thermally treated α-LA solution in the absence and the presence of different concentrations of β-carotene (a-f corresponds to the concentration of 0.005 to $0.025.10^{-10}$ M).



a)



b)



c)

Figure 5.5. The UV-vis absorption spectra of α-LA thermally treated at 25°C (a), 60°C (b) and 90°C (c) in the presence of different β-carotene concentrations (a-f corresponds to concentrations of 0.005 - 0.025·10^{-10} M)

From the **Figures 5.4** and **5.5**, it can be observed that the native α-LA has a single absorption peak at 280 nm, thus revealing the nature of micro medium chromophores (Trp, Tyr, and Phe). It can also be noticed that the intensity of the absorption spectra resulted from the α-LA interaction with the EC is higher than those obtained with β-carotene. In all cases, it can be remarked that the intensity of the absorption spectra of α-LA increases as the concentration of the added ligand increases.

The increase in the EC concentration led to a decrease in the maximum absorption at approximately 280 nm (**Figure 5.4**). In the temperature range of 25-80°C, no significant changes in the peak characterizing the α-LA-β-carotene complex were observed, and at higher temperatures, a blue-shift from 280 nm to 276 nm (**Figure 5.5**) was recorded. These results reveal that the conformational modifications of the α-LA structure have altered the micromedium around the chromophore, which supports the interaction with ligands. Zhang *et al.*, (2012) studied the binding of bixin and whey proteins. It was established that the addition of bixin resulted in a 7 nm blue-shift in the α-LA absorption spectra, indicating that some Trp residues are blocked in the globular protein.

Barbana *et al.*, (2011) suggested that at temperatures above 80°C, α-LA exhibit a decrease in the accessibility of the quenching agent accompanied by a blue-shift. This highlights the fact that there is an interaction between Trp residues and the apolar phase of the lipid bilayer. Considering that this collision did not change the UV spectrum of the protein, it can be deduced that the absorption spectra obtained after binding indicate the formation of the complex (Zhang *et al.*, 2012).

5.1.5.2. Study of the influence of heat treatment on the stability of complex formed between α-LA and carotenoids in the sea buckthorn extract

- **Phase diagram**

The phase diagram method was used in this study to analyze the unfolding/folding/refolding mechanism of the α-LA-EC complex by identifying the intermediate states resulted from the thermal treatment.

Figure 5.7. presents that the thermal treatment induced significant structural changes in the α-LA-EC complex, the nonlinear correlation emphasizing the sequential character of the structural transformations, which involves the folding and the unfolding of the polypeptide chains with the increase of the temperature.

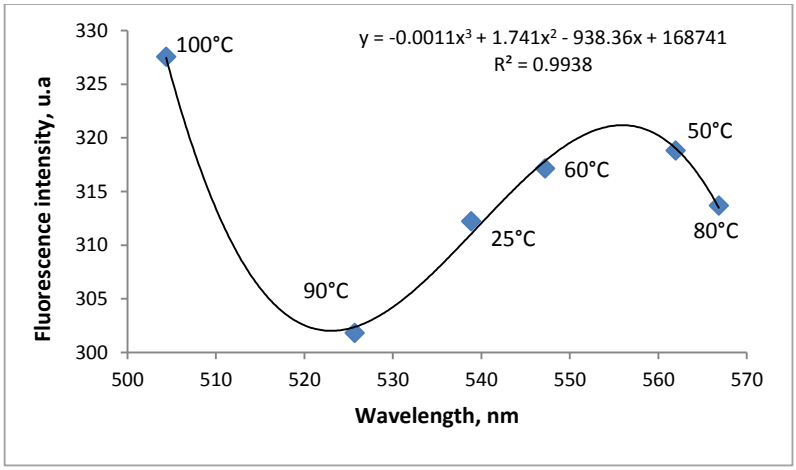


Figure 5.7. *Phase diagram of the α-LA-EC complex resulted from the thermal treatment*

- **Synchronous Spectra**

The synchronous spectra of the α-LA-EC complex were recorded at Δλ = 60 nm and Δλ = 15 nm describing the conformational properties of Trp and Tyr residues respectively (Liang-Liang Shen *et al.*, 2014).

The synchronic spectra obtained at Δλ = 15 nm are shown in **figure 5.9**. It can be noted that the lowest fluorescence intensity was recorded at 100°C, being approximately 10% lower than the one that occurred at 25°C. The thermal treatment did not have a significant influence on the wavelength at which the maximum fluorescence intensity was obtained, indicating that the nature of the environment adjacent to the Tyr residues does not change to thermal treatment.

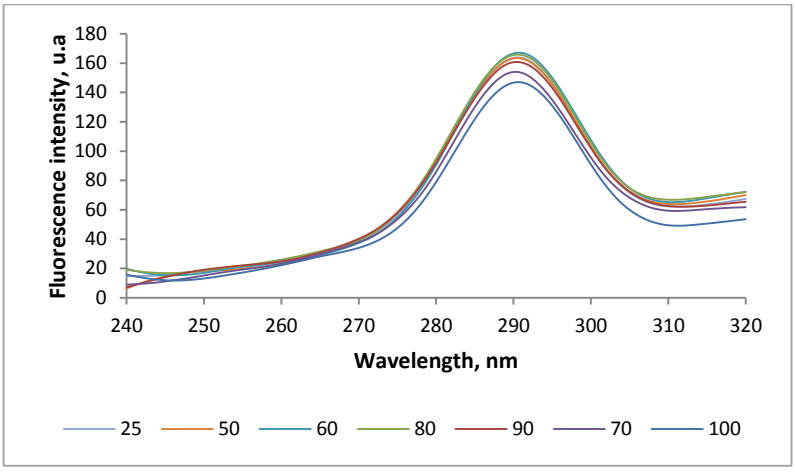


Figure 5.9. Synchronous spectra of the α-LA - EC complex at ∆λ=15 nm

With respect to the spectra obtained at Δλ = 60 nm (**Figure 5.10**.), It can be seen that at temperatures higher than 80°C, higher fluorescence intensity values were obtained (at temperatures higher than 80°C maximum values were recorded). The thermal treatment did not cause significant changes in the wavelength at which the maximum fluorescence intensity was recorded, ranging from 274.5 nm 275 nm at 25°C and 100 C, respectively.

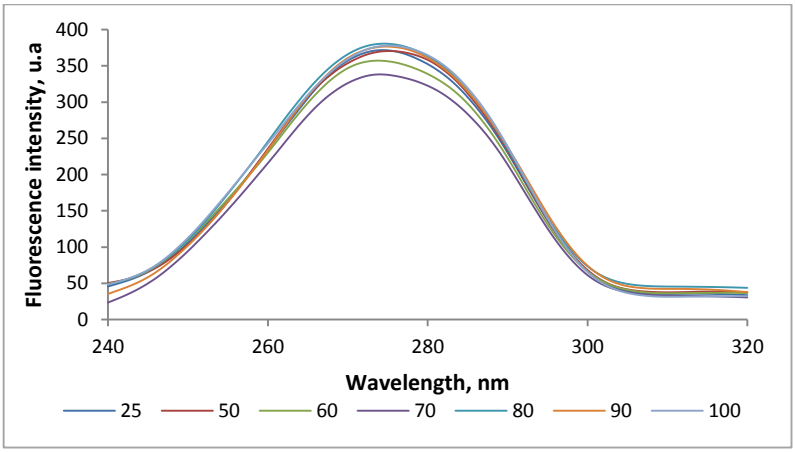


Figure 5.10. Synchronous spectra of the α-LA – EC complex at ∆λ=60 nm

Based on the values obtained for the fluorescence intensity of the synchronous spectra, it can be concluded that the fluorescence of the complex is mainly due to Trp residues and less to those of Tyr. Also, the thermal treatment within the temperature range does not induce significant alterations or spatial reorientation of the hydrophobic residues.

5.1.5.3. Thermodynamic parameters for the α-LA-EC complex

The thermodynamic studies provide the necessary information to understand the molecular forces that lead to complex formation (Bujalowski *et al.*, 2014). In **Table 5.3.** (**a** and **b**), the changes in enthalpy (ΔH) and entropy (ΔS) values, as well as changes in Gibbs free energy (ΔG), are shown.

The graphical representation of the natural logarithm of K_b versus 1/T (K), lead to a linear relationship of the EC-α-LA binding experiments and two linear relationships for the β-carotene experiments (between 25°C and 70°C and between 80°C and 100°C). The ΔG values were negative for the EC binding and positive for the β-carotene binding, indicating that the binding process was spontaneous for the α-LA-EC complex, while in the α-LA-β-carotene complex, binding was achieved gradually. The negative values calculated for ΔH and ΔS suggest that both hydrogen bonds and van der Waals interactions play an essential role in the process of binding both complexes. As can be observed in **Table 5.3a**, the main sources of ΔG values derive from the large contribution of ΔH, suggesting that hydrophobic interaction could play a key role in the EC interaction with α-LA. Moreover, negative values for ΔG and ΔH suggest that the α-LA-EC complex formation process was exothermic.

Table 5.3. Thermodynamic parameters for the thermally treated α-LA associated with EC (a) and β-carotene (b)

a)

T(K)	Δ*H* (J/mol)	Δ*S*(J/mol/K)	Δ*G* (J/mol)	R[a]
298	-4443.83±24.25	-3.94	-3269.71±41.25	0.95
323			-3171.21±13.5	
333			-3131.81±2.16	
343			-3092.41±18.96	
353			-3053.01±29.87	
363			-3013.61±25.38	
373			-2974.21±18.36	

b)

T(K)	Δ*H*(J/mol)	Δ*S*(J/mol/K)	Δ*G* (J/mol)	R[a]
298	-3243.54±169.54	-13.12±2.1	666.22±39.35	0.98
323			994.22±44.27	
333			1125.42±65.24	
343			1256.62±58.3	
353	-9768.95±458.37	-29.43±1.33	619.84±24.82	0.99
363			914.14±54.78	
373			1208.44±49.57	

R[a] correlation coefficient

5.1.5.4. *In silico* investigation on the interaction between α-LA and β-carotene at the level of a single molecule

For the molecular dynamics simulation experiments, the heating of the protein was carried out at 25, 60, and 90°C and the models were balanced so that the potential oscillations of energy were reduced to less than 0.1%.

Increasing the temperature from 25 to 90°C led to an increase in total energy from -364.34±0.72 kJ/mol to -286.21±0.77 kJ/mol. Regarding the initial structure of the crystalline molecule, the thermal treatment up to 90°C resulted in a significant reduction of the α-LA chain and the 3_{10} helix content. Due to thermal destabilization, the Tyr^{18}-Gly^{20} and Ile^{101}-Tyr^{103} sequences, initially involved in the helical structures, lost the hydrogen bond specific to the helical conformation and turned into γ-turn

structures. On the other hand, a new form of type H helix resulted from the β-turn transformation of the Asn^{57}-Trp^{60} chain. Due to the disappearance of the Ala^{40}-Asn^{44} chain, which connects two secondary structures different from the spin in the initial model, the region composed of Ser^{47}-Gly^{51} was transformed at a high temperature in an intrinsically disordered region. In addition, thermally induced molecular rearrangements favored the formation of new secondary structures, such as a G1 antiparallel structure (involving Tyr^{50}, Gln^{54} and Ile^{55}), thus modifying the conformation of the aromatic cluster II and the four beta inverse transformations consisting of four consecutive residues placed at distances of less than 7Å (Ser^{69}-Asn^{71}, Ser^{70}-Ile^{72}, Ile^{75}-Cys^{77} and Cys^{120}-Lys^{122}) (Chrysina *et al.*, 2000; Vanhooren *et al.*, 2006).

Severe thermal treatment applied to the protein resulted in a slight reduction in total surface area compared to the room temperature equilibrium model (**Table 5.4**). These results suggest a partial folding of the α-LA structure at 90°C, accompanied by a 5% reduction in hydrogen bonds stabilizing the protein structure.

The characteristics of the interaction and the affinity between thermally treated α-LA at different temperatures and β-carotene were estimated after docking simulations followed by the complex refinement. No significant differences were found at any simulated temperature in the binding energy between the three complexes. In **Table 5.4**. the interaction details for α-LA-β-carotene models are presented, characterized by the highest total interaction area and the lowest energy interaction values. A detailed analysis of α-LA-β-carotene complexes indicated that the protein did not undergo a major change in the elements of the secondary structure and there were no molecular events responsible for the protein denaturation or destabilization during interaction with the ligand.

Table 5.4. Molecular descriptors of the α-LA-β-carotene complex resulted from the heat treatment

Descriptors	Temperature, °C		
	25	**60**	**90**
Secondary structure descriptors			
Bonds (%)	9.8	8.2	3.3
α-helix (%)	28.7	25.4	29.5
3-10 helix (%)	7.4	8.2	2.5
Helix helix interactions	92±5	88±5	87±5
Energy and thermodynamic descriptors			
Energy bonds α-LA-β-caroten, kJ·mol^{-1}	-167	165.97	127.19
ΔG^f, kJ/mol	-415.05	-431.78	-432.21
ΔG^{int}, kJ/mol	-8.79	-10.04	-20.50
$T\Delta S^{diss}$, kJ/mol	24.69	25.10	24.69
ΔG^{diss}, kJ/mol	-15.9	-15.06	-4.18
Descriptors surface			
Total protein surface, Å^2	6648.6	6499.8	6527.8
Surface interaction of the α-LA-β-carotene complex	340.4	450.2	333.7

Analyzing the contact maps shown in **Figure 5.12**. it is observed that the β-carotene binding sites have been identified in the protein equilibrated at 25°C, 60°C, and 90°C. Due to the temperature-induced conformational changes, the three models do not have common residues. In accordance with the number of amino acids interacting with the ligand, the surface area interacting with the protein increased from 340.4 Å^2 (eight amino acids involved in the interaction: Glu^1, Gln^2, Leu^3, Glu^{11}, Asp^{84}, Leu^{85}, Thr^{86}, Met^{90}) to 450.2 Å^2 (11 amino acids: Phe^{31}, His^{32}, Thr^{33}, Tyr^{36}, Glu^{49}, Ile^{59}, Tyr^{103}, Trp^{104}, Leu^{105}, Ala^{106}, Leu^{101}) at temperature rising from 25 to 60°C and decreases to 333.7 Å^2 (eight amino acids involved: Leu^{15}, Lys^{16}, Val^{21}, Ser^{22}, Leu^{23}, Glu^{25}, Glu^{113}, Lys^{114}) at higher temperatures. It appears that the molecular events recorded in the structure of the protein treated at high temperature affected the properties of the α-LA interface by promoting the expansion of the cavities in which the β-carotene was found to interact.

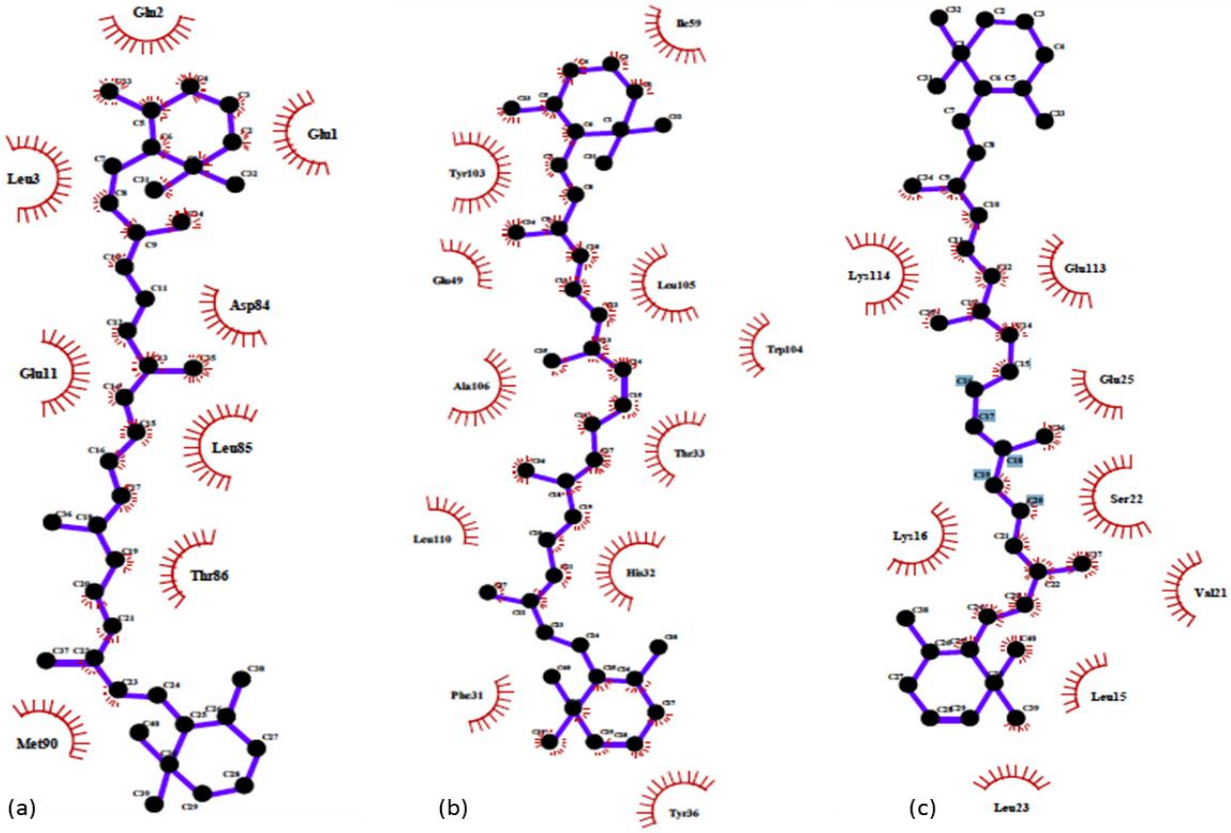


Figure 5.12. The binding sites of the α-LA structure involved in the β-carotene interaction. The protein was preliminarily heated to (a) 25°C, (b) 60°C, and (c) 90°C. The amino acids of the α-LA binding surface that are involved in the hydrophobic contacts with β-carotene molecule are represented by a spoke arc that radiates to the ligand atoms they contact. Figures were extracted using LigPlot +.

In agreement with the observation of Loch *et al.* (2013), which performed thermodynamic studies on the binding of unsaturated fatty acids to β-LG, our results showed that the α-LA-β-carotene complex formation process is both enthalpy and entropic through various events such as ligand-protein interaction, or conformational modifications of the complex components. The increase in temperature did not cause a significant change in entropy in the rigid body ($T\Delta S^{diss}$) at the dissociation of α-LA-β-carotene complex, indicating that the stability of the system involving the denatured protein

was not affected. Compared to the 25°C equilibrated model at temperatures above 60°C, a lower solute energy value for folded protein (ΔG^f) was recorded, indicating that the thermally treated structure is more stable. The negative the free energy solubility values responsible for the formation of the complex (ΔG^{int}) correspond to the hydrophobic interfaces or to the temperature increase of the thermal treatment applied to the protein, indicating that changes occurring in the protein structure following the heat treatment favor the formation of the β-carotene complex. In addition, the increase in ΔG^{diss} from -15.90 to - 4.18 kJ/mol induced by increasing the temperature from 25°C to 90°C (**Table 5.4**) suggests an improvement of the thermodynamic stability of the α-LA-β-carotene complex.

5.2. Evaluation of the binding mechanism between the β-lactoglobulin and the carotenoid compounds in the sea buckthorn extract and assessment of complex stability under different environmental conditions

5.2.1. Introduction

The aim of the study was to deepen the binding mechanisms and effects of the heat treatment on the lyophilized white sea buckthorn extract (hereinafter referred to as β-LG-EC) in accordance with structural modifications of the protein, mainly by the *in situ* fluorescence spectroscopy. Fluorescence spectroscopy methods involved the use of intrinsic and extrinsic intensity fluorescence, the phase diagram, the synchronous spectra, the three-dimensional fluorescence spectroscopy and the quenching experiments. The β-carotene binding sites to β-LG and the effect of complexation on the conformational stability and the β-LG secondary structure were evaluated based on quenching experiments with a pure β-carotene solution, and molecular docking and molecular dynamics simulation.

5.2.3. Materials

β-LG from bovine milk (90% purity, genetic variants A and B), β-carotene, ANS were purchased from Sigma (Sigma-Aldrich Co., St. Louis, MO).

5.2.5. Results and discussion

5.2.5.1. Study of the ability of the β-lactoglobulin to bind carotenoids in model systems

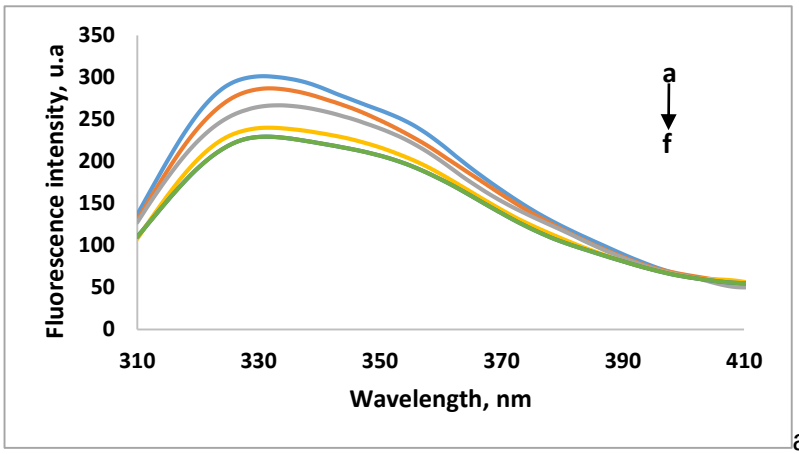
- **The binding mechanism of the sea buckthorn extract carotenoids to the thermally treated β-LG**

The interactions between β-LG and β-carotene were evaluated by investigating the influence of increasing the β-carotene concentration on the β-LG fluorescence intensity spectra which have been thermally treated at temperatures between 25°C and 100°C for 15 min.

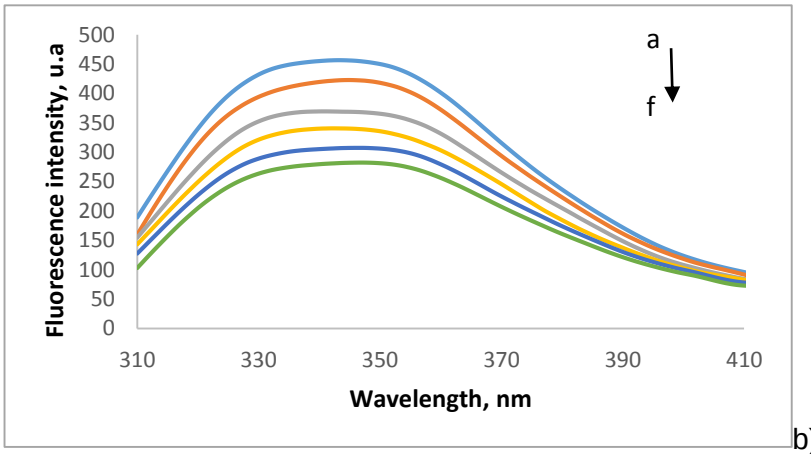
It can be observed that the β-carotene solution extinguished the fluorescence of the Trp residue (**Figure 5.13**). This phenomenon is probably due to the transfer of energy between the excited indole ring (Trp) and ligands or to the polarity changes in the vicinity of the Trp residues (Muresan *et al.*, 2001). According to Mensi *et al.*, (2013), Trp residues are blocked in a nonpolar environment if the maximum fluorescence emission (λ_{max}) is less than 330 nm. If λ_{max} is greater than 330 nm, the Trp residue is located in a polar environment, which in most cases involves exposure to solvents. Thermal treatment at temperatures above 70°C of the β-LG solution resulted in significant red-shifts (from 331

nm at 25°C to 337 nm at 80°C, 343 nm at 90°C to 347 nm at 100°C), suggesting structural changes associated with exposure of the Trp residues.

Increasing the concentration of β-carotene concentration caused the maximum emission wavelength shift from 331 nm to 333 nm at 25°C and 60°C, and at 80°C and 90-100°C a red-shift of 7 nm and 3-4 nm, respectively, was observed, indicating that the addition of the β-carotene caused the loss of the compact structure of the protein, exposing the hydrophobic subdomain where the Trp residues are placed.



a)



b)

Figure 5.13. Fluorescence spectra of the interaction between β-LG and β-carotene, heat-treated at 25°C (a) and 90°C (b). The concentration of β-carotene ranged from 0 to 0.093 μM (a-f).

To elucidate whether the binding mechanism between β-LG and β-carotene is dynamic or static, the K_{SV} values calculated at different temperatures were compared (**Table 5.5**). In the temperature range studied, a high linearity was observed, indicating that the quenching of β-LG fluorescence emission by the β-carotene is static. In the temperature range of 25°C to 90°C, the K_{SV} values increased from $3.48\pm0.55\cdot10^{-10}$ L/mol to $6.83\pm0.24\cdot10^{-10}$ L/mol, and at 100°C, a decrease in the K_{SV} value ($5.70\pm0.24\cdot10^{-10}$ L/mol) was observed.

Table 5.5. The binding parameters between heat treated β-LG and β-carotene at different temperatures

T(˚C)	K_{SV} (10^{-10}L/mol)	R^b	K_b (10^{-8} L/mol)	*n*	R^c
25	3.48±0.55 [a]	0.996	1.11±0.05	1.56±0.04	0.992
50	3.99±0.27	0.989	0.92±0.04	1.31±0.06	0.999
60	4.17±0.19	0.996	0.87±0.02	1.25±0.05	0.991
70	4.36±0.20	0.996	1.07±0.02	1.47±0.03	0.993
80	4.99±0.06	0.993	1.06±0.07	1.34±0.007	0.990
90	6.83±0.03	0.994	0.87±0.05	0.96±0.004	0.998
100	5.70±0.24	0.995	0.78±0.03	1.04±0.02	0.998

[a] standard deviation
R^b correlation coefficient for the K_{SV} values
R^c correlation coefficient for the K_b values

The apparent binding constants (K_b) of the β-LG-β-carotene complex and the number of binding sites (*n*) are shown in **Table 5.5**. The K_b values decreased as a result of the temperature increase from 1.11±0.05·10^{-8} L/mol at 25°C to 0.87±0.02·10^{-8} L/mol at 60°C, suggesting that some changes took place around the binding sites, influencing the binding capacity of the β-LG and the β-carotene. Increasing the temperature up to 70°C induced the increase of the binding constant, but at higher temperatures, the K_b values decreased. The K_b values were consistent with those reported by Mensi *et al.* (2013), which compared variant B (1.23±0.10·10^{-8} L/mol) with variant A (2.07±0.40·10^{-8} L/mol). The values of *n* for the entire temperature range varied from 1.56±0.04 at 25°C to 0.96±0.04 at 90°C, therefore it can be concluded that regardless of the thermal treatment applied, the molecule β-LG has at least one high-affinity binding site for β-carotene.

5.2.5.2. Study of the influence of thermal treatment on the stability of the β-LG-EC complex

- **Synchronous Spectra**

In the **figures, 5.19** and **5.20** are presented the synchronous spectra of the complex at different temperatures at Δλ = 15 nm and Δλ = 60 nm. Changes in the λ_{max} correspond to the polarity change around the chromophore molecules. The addition of the EC caused a blue-shift from 300 nm to 293 nm for Tyr and 280 nm to 276 nm for Trp.

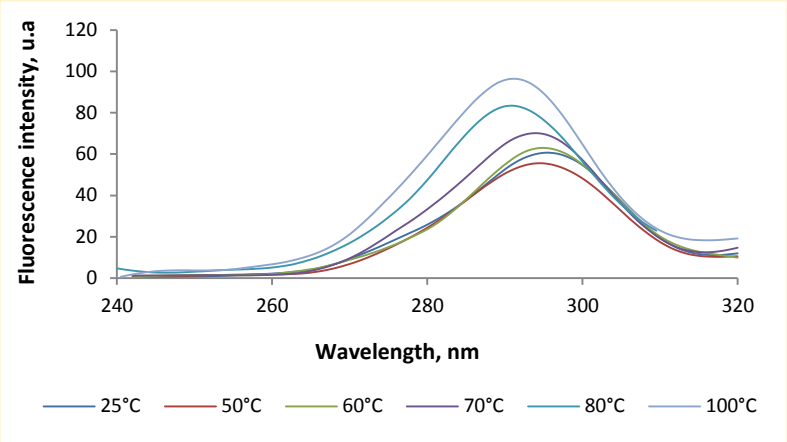


Figure 5.19. Synchronic spectra of the β-LG-EC complex at Δλ=15 nm

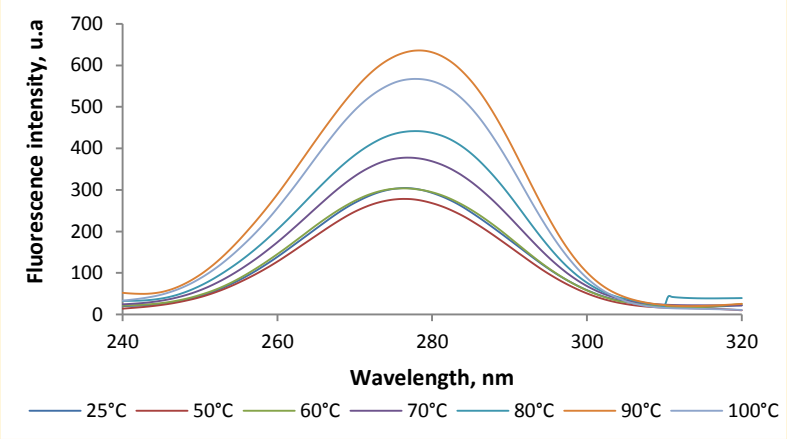


Figure 5.20. Synchronic spectra of the β-LG-EC complex at Δλ=60 nm

As it can be seen from the **Figure 5.19**, the spectrum registered a peak at 293 nm at 25°C, and the thermal treatment at 90-100°C induced a blue-shift of 2-3 nm. In the **Figure 5.20**, a 2-nm red-shift for the Trp residues is highlighted. Therefore, it can be concluded that the thermal treatment induced conformational changes that led to blocking the Tyr residues and exposure of the Trp residues.

A detailed analysis of the models used to simulate the molecular behavior of the β-LG-β-carotene complex revealed that the degree of exposure of Trp residues to the solvent decreases as the temperature increases from 25°C to 90°C. Due to the rearrangement of the side chains at elevated temperatures, it was considered that the Trp^{19} residue was completely blocked in the native state, and it became partially exposed (the solvent accessible surface of 1.23 $Å^2$ at 90°C), while the exposed surface of the Trp^{61} residue decreased from 70.20 $Å^2$ at 25°C to 53.15 $Å^2$ at 90°C. As for Tyr residues, an increase in the exposed surface, 27.11 $Å^2$, 19.94 $Å^2$, and 0.97 $Å^2$ of Tyr^{20}, Tyr^{102} and Tyr^{42} respectively, was observed at 90°C, compared to the native protein. On the other hand, the solvent accessible surface of the Tyr^{99} residue decreased from 64.70 $Å^2$ at 25°C to 28.84 $Å^2$ at 90°C. It is also indicated that polarity has decreased around the Tyr residues and the hydrophobicity increased, while polarity around Trp residues has increased and the hydrophobicity decreased (Hu *et al.*, 2005).

5.2.5.3. *In silico* investigation on the interaction between β-LG and β-carotene at a single molecule level

The atomic events responsible for the thermal behavior of the complex formed between the β-LG and the β-carotene (the representative compound of sea buckthorn extract) were verified after molecular dynamics simulations at 25 and 90°C, temperatures indicated as relevant in the fluorescence studies. A detailed analysis of the molecular models highlighted that only 77% of the native β-LG secondary structure was conserved at 90°C. The heating of the β-LG-β-carotene complex has led to the modification of the hydrogen binding mode, therefore determining the involvement of the amino acid residues in defining different types of secondary structures. Protein treatment at elevated temperatures resulted in the growth of amino acids organized in the strands (from 37.3% to 25°C to 44.3% at 90°C), the most obvious changes being a transformation into β and γ-turn structures. Compared with the initial molecular model, thermal treatment up to 90°C favored the molecular rearrangements that led to the formation of native alpha-helical structures, modifying the 3-10 helical conformation. The increase in the total surface area available to the solvent from 7239.8 to 7976.1 $Å^2$ occurred after the unfolding of the polypeptide chains at high temperatures.

At 25°C the β-carotene directly interferes with the following β-LG amino acids: Ile^2, Val^3, Thr^4, Thr^6, Lys^8, Ile^{78} și Glu^{89}, Gln^5, Ala^{80}, Val^{81}, Lys^{91}, Leu^{93}, Ser^{110} și Gln^{115}. The most important contribution to total energy is given by Lys^8 (0.81 kcal/mol), Ile^{78} (0.39 kcal/mol) and Lys^{91} (0.50 kcal/mol). Following the thermal treatment, slight changes in the binding site were observed, but also a change in the total protein surface that increased from 304.7 to 386.1 $Å^2$, contributing to the formation of the β-carotene complex.

When they compared the models equilibrated at 25°C and 90°C, it was observed a significant increase in exposure to the solvent of the Ala^{80} and Val^{81} residues (17.71 $Å^2$ at 25°C to 79.30 $Å^2$ at 90°C) with increasing temperature (**Figure 5.22b**), as well as an increase in the hydrophobic interactions with β-carotene molecule. Due to the molecular rearrangement, the Lys^8 residue is trapped inside the protein molecule and no interactions with the ligand can be observed. On the other hand, although an increase in the degree of exposure due to lateral chain reorientation has been observed, the Glu^{89} and Lys^{91} residues were not implicated in the interaction with β-carotene at 90°C. Migration to the surface molecule of the Leu^{95} residue, which was completely blocked in the native state, favored the expansion of the interaction across the entire exposed surface of 28.98 $Å^2$ with one of the two cyclohexene rings of the β-carotene molecule (**Figure 5.22b**). Changing the ligand binding surface resulted in the masking of the Ala^{142}, Leu^{143}, and Pro^{144} residues when complexed with a β-carotene molecule, thus limiting the potential interaction with other β-LG molecules (Adams *et al.*, 2006).

In agreement with the observation of Loch *et al.* (2013), the β-LG-β-carotene complex formation was determined by enthalpy and entropy changes which consisted of conformational changes of the molecules when bound. The fact that a slight decrease in the complex entropy (TDS^{diss}) (6 kcal/mol at 25°C to 5.4 kcal/mol at 90°C) occurred, indicated that the stability of the complex is not significantly affected by increasing temperature. Additionally, the increase in the free energy (ΔG^{diss}) of the complex from -3.6 to -2.5 kcal/mol revealed that this complex is thermodynamically more stable and the external forces required to dissociate the complex are higher at 90°C. Moreover, the energy of

solvation of the folding of β-LG (ΔG^f) decreased from -127 to -154.4 kcal/mol, indicating that the structure of the thermally treated protein in the complex is more stable.

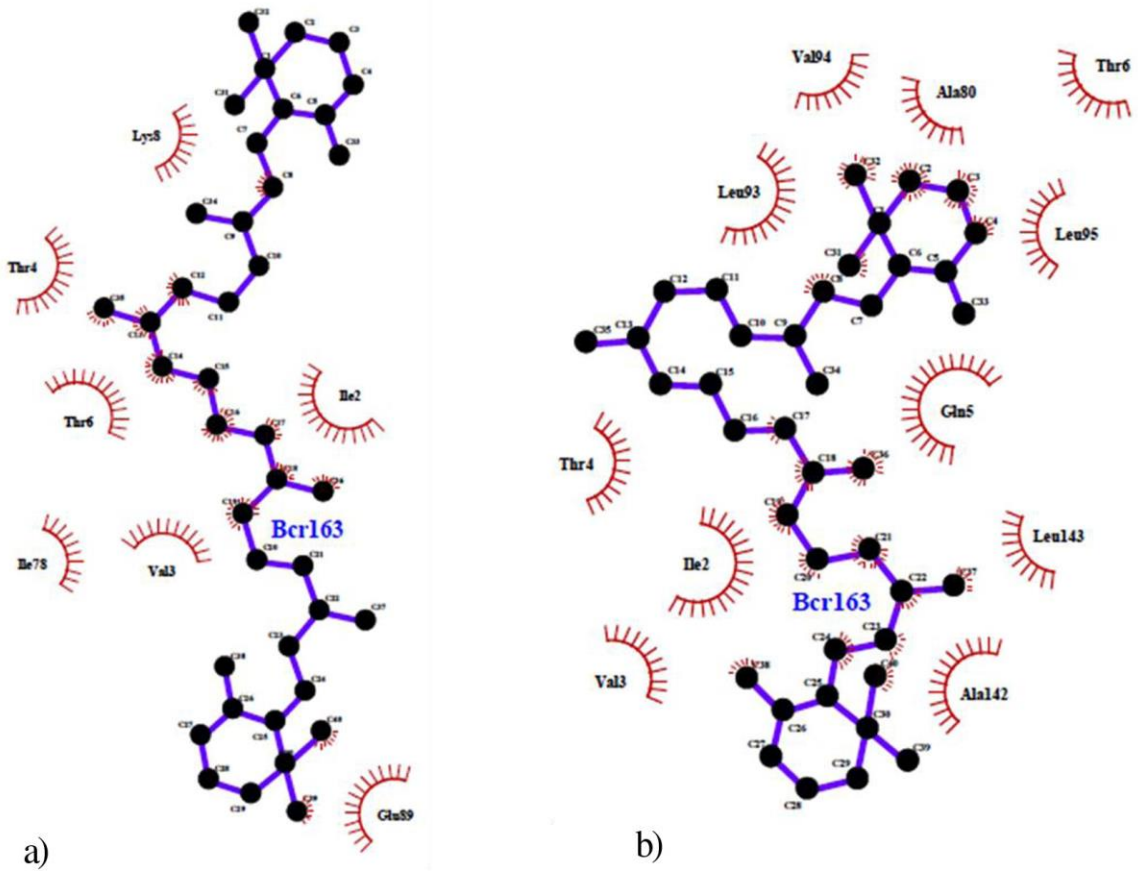


Figure 5.22. The contact surface of β-LG-β-carotene complex equilibrated at 25°C (**a**) and 90°C (**b**)

5.3. Partial conclusions

This study focused on the advanced evaluation of the binding mechanisms and the stability of the complexes formed by the main whey proteins, β-lactoglobulin, and α-lactalbumin, from the perspective of improving the stability of the extracted carotenoid compounds from natural sources such as sea buckthorn, as microencapsulation preliminary stages.

The study implied an advanced assessment based on the fluorescence spectroscopy techniques, UV-vis spectroscopy, and *in silico* techniques.

The first step was to study the binding mechanism between the whey α-lactalbumin protein and the carotenoid compounds in sea buckthorn extract versus the β-carotene by fluorescence spectroscopy, UV-vis spectroscopy and *in silico* techniques.

The thermal treatment of the α-lactalbumin had a minor contribution to the interaction with the carotenoids in the sea buckthorn extract. According to the quenching constants, the carotenoids can extinguish the fluorescence of the α-lactalbumin through a static mechanism.

The UV-vis studies shown that sequential addition of the sea buckthorn extract resulted in an increase in absorbance at 280 nm, suggesting that the interaction between the protein and the carotenoids results in an unfolding of the polypeptide chains.

The thermodynamic parameters evidenced that ΔH has a major contribution to the ΔG, which implies that the binding processes between the molecules are enthalpy driven.

Detailed analysis at a single molecule level after performing the molecular dynamics simulation experiments by thermal treatment of the α-lactalbumin molecule at 90°C displayed a good thermodynamic stability of the β-carotene complex.

Conformational changes induced by the thermal treatment in the α-lactalbumin-sea buckthorn extract complex, in the temperature range 50-100°C, were investigated using fluorescence spectroscopy methods combined with the *in silico* approach.

The intrinsic fluorescence experiments showed that the addition of the sea buckthorn extract resulted in minor conformational changes in the protein structure.

The intrinsic fluorescence data indicated that the highest exposure to Trp residues of α-lactalbumin occurs after the thermal treatment at 60°C and at temperatures above 90°C. Quenching studies revealed that the highest accessibility of intrinsic chromophores to acrylamide and KI were at 80°C and 70°C, respectively.

The extrinsic fluorescence results demonstrated that at 80°C, the ANS is placed in a less hydrophobic environment due to its interactions with the complex formed between the protein and the carotenoids.

The *in silico* experimental results revealed that the complex formation is temperature-independent and white sea buctkthorn carotenoids do not bind to the sites close to the Trp residues of the protein. The thermal treatment favored various molecular events that affect the interaction between the proteins and the ligand and thus the stability of the complex.

In the case of the β-lactoglobulin-sea buckthorn extract, the results suggested a partial unfolding of the protein, especially at temperatures above 80°C. In the studied temperature range, λ_{max} evidenced a red-shifting that indicates an increase in the hydrophilicity in the vicinity of the hydrophobic residues.

Regarding the phase diagram, an *all-or-none* transition was noted, involving the dissociation of the dimers in the temperature range of 25-70°C, followed by unfolding of the protein at higher temperatures.

The carotenoids binding and the thermal treatment resulted in a decrease in the polarity of the adjacent Tyr residue and an increase in the hydrophobicity, while the polarity of the adjacent area to the Trp residue increased while the hydrophobicity decreased.

The flexibility of the protein molecule in the complex was highlighted by the Stern-Volmer quenching constants, which presented higher values at high temperatures, in the case of the quenching experiments with acrylamide, and in the case of those with KI they had lower values.

The molecular dynamics results showed that, regardless of the temperature, the complex between the β-lactoglobulin and the β-carotene is stabilized by the hydrophobic bonds.

The events occurring at a single molecule level at high temperature in the protein molecules resulted in significant alterations in the β-carotene binding site, thus leading to a more thermodynamically stable assembly.

Regarding the study of the effect of the pH variation on the β-lactoglobulin-sea buckthorn extract, it was observed that the Trp residues were blocked within the protein core, a phenomenon evidenced by the fact that the maximum fluorescence intensity was reduced to an excitation wavelength of 292 nm.

The Tyr residues were partially exposed to the solvent because λ_{max} was greater than 330 nm. The ANS fluorescence suggested an exposure of the hydrophobic residues to a non-polar alkali pH. The synchronous spectra revealed the partial obstruction of the Tyr residues in the alkaline solution and the exposure of the Trp residues at pH values greater than 5.2. The acrylamide quenching experiments exhibit a greater molecular flexibility at acidic pH, while in the case of quenching with KI the data displayed a maximum K_{SV} at pH 6.5.

6. Microencapsulation of the carotenoid compounds from the white sea buckthorn extract in whey protein from the perspective of the development of functional composites with applications in the food industry

6.1. Introduction

The aim of this study was to encapsulate the lipophilic components (carotenoids from white sea buckthorn extract) using whey proteins as the shell material. Subsequently, the content of carotenoids in the microparticles, and the encapsulation efficiency were determined. The functionality of the powder was analyzed in terms of the antioxidant activity and color. In order to determine what changes occur in the particle structure, FT-IR spectral analysis and confocal microscopy were also performed.

6.2. Materials

The materials used for microencapsulation of bioactive compounds in the sea buckthorn extract are: a polymeric system consisting of whey protein isolate and acacia gum, extract obtained from 50 g of lyophilized sea buckthorn with a moisture content of 6.87%.

The slightly modified method described by *Jain et al.* (2015) was used for the encapsulation of the sea buckthorn extract in WPI and gum acacia by coacervation. Briefly, 5 g of WPI was dissolved in 100 ml distilled water and maintained at 40°C for 20 min over a mechanical stirrer (IKA RCT Basic, Germany). Subsequently, 10 ml of sea buckthorn extract previously dissolved in blackcurrant oil was added and the mixture was homogenized at 4000 rpm for 30 min to obtain oil-in-water emulsions. Afterwards, 100 ml 1 % (w/v) gum acacia solution was added to the emulsion, and the mixture was allowed to stir for 30 min, while the temperature was maintained at 40°C. In order to promote coacervation, the pH of the solutions was adjusted to 3.75 with 1N HCl solution at approximately 40°C, under constant mechanical stirring at 600 rpm. After that, the reaction mixture was allowed to cool down in ice bath and stored at 4°C overnight to promote decantation. The coacervates were freeze dried (CHRIST Alpha 1-4 LD plus, Germany) at -42°C under a pressure of 0.10 mBar for 48 h. After, the powder was collected and packed in metallized bags, and kept in a freezer at -20°C until further analysis.

6.6. Results and discussion

6.6.1. Phytochemical characterization of the extract

The extract characterization evidenced a total carotenoid content of 57.54±0.28 mg/g D.W., an antioxidant activity of 449.85±0.03 µmol Trolox/g D.W., a pH of 4.0, a dry substance of 80.3±0.54% and a sugar content of 70.07 °Brix.

According to the data obtained in the section 4.6.1, twelve compounds were identified in the sea buckthorn extract as follows: astaxanthin, zeaxanthin, zeaxanthin-palmitate, γ-carotene, cis β-carotene, β-cryptoxanthin, lycopene, lutein palmitate-myristate, lutein di-palmitate, β-carotene, α-carotene and zeaxanthin di-palmitate. The β-carotene displayed a content of 15.19 mg/g D.W., followed by astaxanthin with 11.94 mg/g D.W., β-cryptoxanthin with 8.93 mg/g D.W. and lycopene

with 2.24 mg/g D.W., while zeaxanthin was identified to be in the largest amount, 81.29 mg/g D.W. (Ursache *et al.*, 2017b)

Mihalcea *et al.,* (2017) suggested the following carotenoids and esters in the extracts obtained from the sea buckthorn using CO_2 supercritical fluids extraction: astaxanthin (3.58±0.85 mg/g D.W.), zeaxanthin (4.21±0.78 mg/g D.W.), β-cryptoxanthin (8.11±1.02 mg/g D.W.), α-carotene (4.30±0.96 mg/g D.W.), cryptoxantine palmitate (25.10±2.52 mg/g D.W.), cis β-carotene (35.06±2.36 mg/g D.W.), zeaxanthin di-palmitate (39.21±1.47 mg/g D.W.) and zeaxanthin-palmitate-myristate (31.01±1.21 mg/g D.W.).

6.6.2. Encapsulation efficiency

The microencapsulation efficiency of the carotenoid compounds in the sea buckthorn extract was 56.16±1.24%. Mihalcea *et al.,* (2017) reported an encapsulation efficiency of the extracted carotenoid compounds from sea buckthorn by CO_2 supercritical fluids extraction techniques of 41.34±0.07% for samples encapsulated by the same method and 46.18±0.13% of samples subjected to transglutaminase crosslinking reactions prior to coacervation. Rodríguez-Huezo *et al.*, (2004) used different emulsions for spray drying and obtained an encapsulation efficiency ranging from 25.6% to 87.5% depending on the total solids content and the ratio between the biopolymers and the primary emulsion. An encapsulation efficiency with whey protein and maltodextrin was reported by Esfanjani *et al.,* (2015) of 56.51± 0.31% for picrocrocin, 51.57±0.29% for safranal, and 62.55±0.45% for crocin.

6.6.3. Characterization of the microencapsulated powder

The powder obtained had a total carotenoid content of 2.82±0.17 mg/g D.W. and an antioxidant activity of 548.00±0.23 μmol of Trolox/g D.W.

Mihalcea *et al.,* (2017) suggested values for the antioxidant activity of 473.90±5.01 μmol Trolox/g D.W. for the sample obtained by encapsulating the sea buckthorn extract and 480,00±2,80 μmol Trolox/g D.W. for the pretreated sample with transglutaminase.

6.6.4. Structure and morphology of the microcapsules

Combining the white light with a laser light source, the confocal microscopy is a technique capable of scanning surfaces and capturing optical images at high resolution. The technique essentially scans a point-to-point object using a focused laser beam to allow a 3D reconstruction.

Regarding the native powder, it can be observed that lipophilic pigments such as carotenoids display together with the proteins numerous micro-vesicles that join together in the form of irregular coacervates, with dimensions ranging from 20 to 110 μm (**Figure 6.2.a**). When carotenoids were encapsulated in the whey protein matrix, spheroids of varying sizes between 2 and 30 μm (**Figure 6.1.b**) are formed, probably due to the protein matrix that influences the dimensions of the incorporated oil droplets.

a) b)

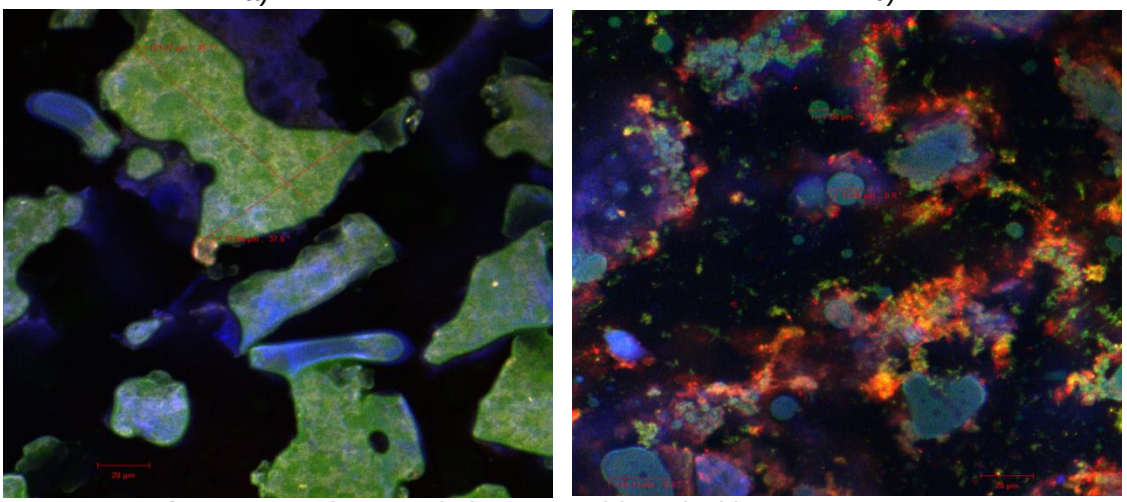


Figure 6.2. Microscopic images achieved with CLSM LSM 710
(a) native powder and (b) fluorochrome stained complex

Some spheroids entwine and generate coacervates that exceed a diameter of 25 μm. Therefore, novel emulsion release systems can be made with whey proteins to control the release profile of lipophilic compounds during digestion with applications in functional foods (Mihalcea *et al.*, 2017).

6.6.5. FT-IR analysis

Infrared absorption spectroscopy is a method of analysis used in the food industry to study the structure of different classes of compounds, to determine their purity and to identify the biologically active groups or to form new connections.

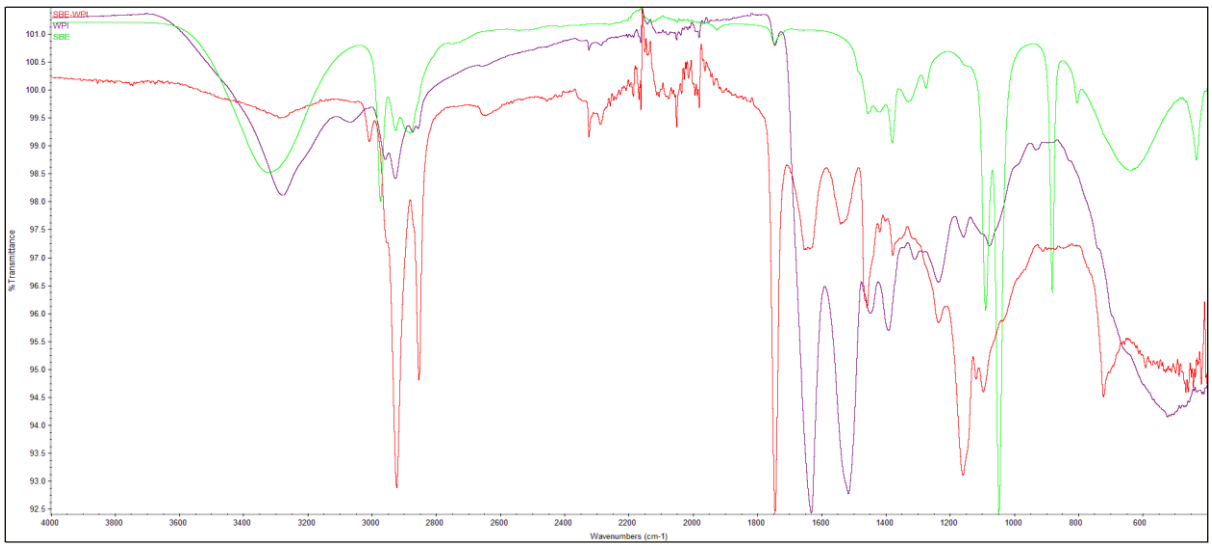


Figure 6.3. The overlapped ATR spectra (4000-400 $cm^{-1)}$ of the EC (red), the whey protein isolate (purple) and the microencapsulated powder (green)

Thus, the spectrum of the whey protein isolate-sea buckthorn extract (WPI-EC) (**Figure 6.3**) revealed the presence of EC specific bands at values of 3000-2800 cm^{-1} (stretch of the C-H bond, a

specific carotenoids bond), 1750 cm^{-1} (stretch of C = O bonds, specific bond for esters groups, dicarboxylic acids etc.) and specific bands for whey protein isolate at 1646, 1539, 1456 and 1377 cm^{-1} (Pretsch *et al.,* 2009). Also, the emergence of a longer band corresponding to the N-H/O-H bonds at 3200 cm^{-1} could suggest the formation of several H-bonds within the obtained coacervates. Another application of FT-IR analysis is the determination of the secondary protein structure (Baltacioglu *et al.,* 2017). Subsequently, in this study, the ATR technique was used to analyze the processes of refolding of the whey protein isolate for the encapsulation process (**Table 6.2**). The results indicate a decrease in the α -helix and β-sheet structures, suggesting a certain degree of aggregation. Since conformational changes are not significant, it can be considered that the process of protein refolding within the WPI-EC complex has been successful.

Table 6.1. displays the secondary structure of the coacervates and the protein isolate (IPZ), calculated from the second derived species, using the method of Goormaghtigh *et al.* (2009).

Table 6.1. WPI and microencapsulated powder secondary structure

Secondary structure	WPI (%)	Microencapsulated powder (%)
α-helix	25.73±1.67	21.42±1.39
β-sheets	32.03±1.12	33.94±2.68
β-turns	27.22±2.15	28.27±0.99
Random coil	15.02±1.25	16.37±1.35

6.7. Partial conclusions

The encapsulated extract was obtained using 50 g of lyophilized sea buckthorn. Before microencapsulation, the extract was phytochemically characterized. Therefore, it had a total carotenoid content of 57.54±0.28 mg/g D.W. and an antioxidant activity of 449.85±0.03 µmol of Trolox/g D.W., a pH of 4.0, a dry substance of 80.3±0.54% and a sugar content of 70.07 °Brix.

The encapsulation of carotenoids from the lyophilized sea buckthorn extract was achieved by coacervation followed by lyophilization. It could be seen that the shell materials used (whey proteins isolate and acacia gum) and the chosen encapsulation method were well suited for making the powder since the efficiency of carotenoid encapsulation was 56.16±1.24 %.

Confocal laser scanning microscopy suggested the presence of carotenoids within the whey protein matrix, and the complexes ranged in size from 2 to 30 µm. However, some spheroids have merged together and generated coacervates that have exceeded the diameter of 25 µm.

The FT-IR specific coacervate spectrum revealed typical bands for carotenoids, esters, dicarboxylic acids and whey proteins, but also the formation of new hydrogen bonds in the complex. Also, the detailed analysis of the protein structure indicated a decrease in the α-helix and β-sheet structures, suggesting an aggregation of the molecules to some extent.

The microencapsulated complex can easily be used in the food industry as an ingredient for the production of value-added food because it does not require large storage space and can be dosed without intermediate steps. It is also expected that the use of microencapsulated powder will improve the rheological and textural properties of the finished product in addition to the beneficial health effects.

7. Development of food technology in order to obtain enriched food products by exploiting the sea buckthorn bioactive compounds and the microencapsulated carotenoid complex potential

7.1. General aspects

The purpose of this study was to develop two technologies for the patenting of two value-added products exploiting the functional potential of sea buckthorn and the microencapsulated sea buckthorn extract, a technology for obtaining a milk-based dessert using whey protein concentrate and sea buckthorn and a technology for obtaining value-added muffins. This study presents the following objectives: establishing the technological recipe for product acquisition, a comparative analysis of physicochemical and texture characteristics of products and a sensory analysis of the added-value muffins.

7.3. Materials and methods

7.3.1. Development of a milk-based dessert using whey proteins isolate and sea buckthorn

The components required to obtain the milk-based dessert are whole milk (3.5% fat), sweet cream (33% fat), sugar, egg yolk, emulsifier, whey protein concentrate and lyophilized sea buckthorn in the proportion of 2 to 6%.

According to the patent application, there are given 3 embodiments of the invention (i.e., 2%, 4% and 6% lyophilized sea buckthorn content). Since the application is being accepted at OSIM, it is not possible to give all the details of obtaining the product.

7.3.2. Enriched muffins formulation

To obtain the muffins, the following raw materials and ingredients were used: coconut butter egg yolk, sugar, 3.5% milk, wheat flour, rice flour, powder, mint essence, baking powder, salt.

The procedure for the obtaining of the added-value muffins has the following steps:

- Continuously mixing coconut butter with salt and white sugar until the sugar is dissolved and a foam is obtained;
- Egg and milk (with a fat content of 3.5%) are added in turn, alternately with the flour, baking powder, and the peppermint essence;
- Finally, the microencapsulated powder (6%, based on the amount of flour) is incorporated in order for the composition to be uniform.

The composition obtained had a soft consistency, facilitating the filling of the special muffins case two-thirds full.

Baking was performed in a convection oven at 185°C for 20 minutes. For comparison, there were also control samples that followed the same technology, but no microencapsulated powder was added.

7.4. Results and discussion

7.4.1. Physicochemical and functional characterization of milk-based dessert containing sea buckthorn and whey protein isolate

According to the invention, the product has the following chemical composition: lipids (12.26-13.46%), proteins (10.96-11.11%), mineral salts (0.65-0.74%) sugar content (13.78-16.58%), and total dry matter (38.85 - 40.65%).

By applying the invention, a milk-based product is obtained that presents a balanced nutritional composition due to the milk protein, concentrate whey protein isolate and egg yolk, milk, and cream fats as well as for sea buckthorn carotenoids.

The total carotenoid content varied with the increase in the percentage of the sea buckthorn in the product. Thus, the highest value (4.15±0.05 mg/100 g D.W.) was recorded in the sample with 6% lyophilized sea buckthorn, while for the 2% sample the lowest value was registered (1.07±0.06 mg/100 g D.W.). It can be affirmed that the sample with 6% addition of lyophilized sea buckthorn brings a high intake of carotenoids, making this product an important source of bioactive compounds with beneficial properties for the human body.

Regarding the antioxidant activity of the dairy product, there were no significant differences between the analyzed samples. This may be explained perhaps by the fact that the emulsion obtained from the egg yolks and the protein concentrate favored the inclusion of the sea buckthorn bioactive compounds.

7.4.2. Textural analysis of the milk-based product containing protein isolate and sea buckthorn

The instrumental analysis of the milk-based products using whey protein concentrate and sea buckthorn aimed to determine the firmness, cohesiveness, and elasticity of the samples in response to their deformation, made by the double compression method (TPA method).

The firmness obtained by instrumental analysis represents the force required to compress the gel for a predefined deformation (in the present case 20 mm) (Trinth and Glasgow, 2012). **Figure 7.5** shows the firmness values for the milk-based product samples based on whey protein concentrate and sea buckthorn. For the control sample, the firmness was 0.085 N. The addition of 2% lyophilized sea buckthorn resulted in a slight decrease in firmness, with a value of 0.0075 N. A more pronounced increases in firmness, by 38% and 176%, were recorded for samples with 4% and 6% sea buckthorn, respectively.

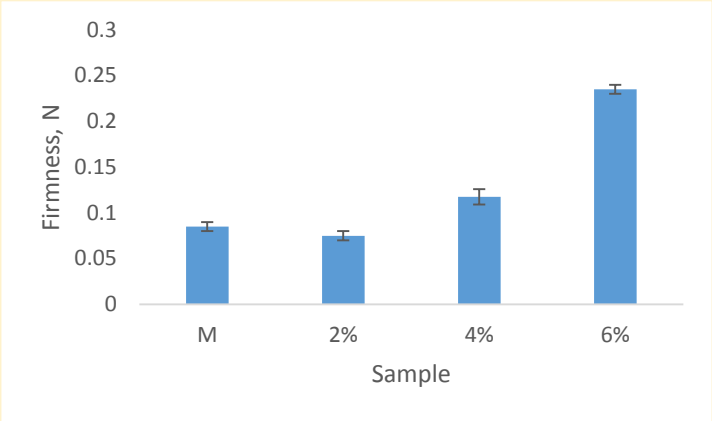


Figure 7.5. Firmness values obtained by the instrumental analysis of the texture

Cohesiveness as a textural parameter, represents a measure of the resistance of the sample during the second compression, relative to the resistance during the second compression. In **Figure 7.6.** there are presented the cohesiveness values of the four samples analyzed.

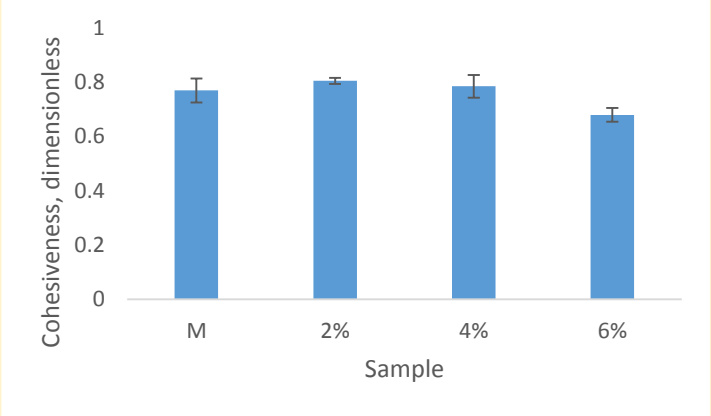


Figure 7.6. *The cohesiveness values using the instrumental analysis of the texture*

For the control sample, the cohesiveness was 0.77. With the addition of 2% lyophilized sea buckthorn, the cohesiveness increased to 0.805, while the increase of the percentage of sea buckthorn added to 4 and 6%, led to a decrease of the cohesiveness to 0.785 and 0.68, respectively. An inversely proportional variation of cohesiveness with firmness can be noticed, behavior also noted by Zheng *et al.,* (2017) for milk charlotte enriched with bamboo shoots fiber.

In **Figure 7.7**. the values of elasticity expressed as the value of the deformation recovered between the two compression cycles are presented.

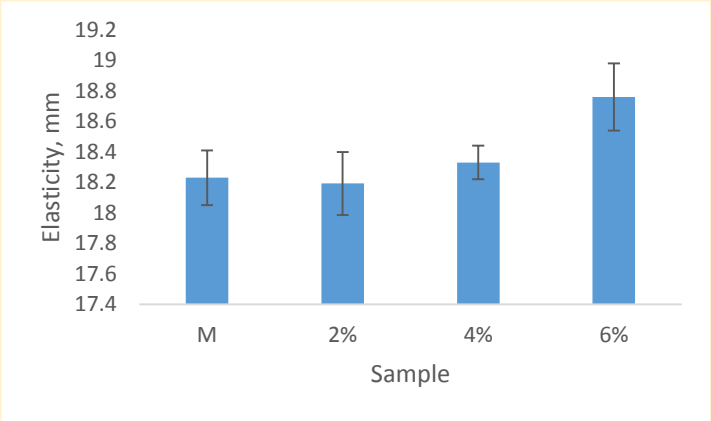


Figure 7.7. *The elasticity values using the instrumental analysis of the texture*

The influence of the addition of lyophilized sea buckthorn on elasticity is very similar to the influence on the firmness. The addition of 2% sea buckthorn led to an insignificant decrease in the elasticity in comparison with the elasticity of the control sample, the most obvious increase in elasticity was recorded for the sample with 6% sea buckthorn added. This behavior is associated with the formation of elastic bonds between the structural elements of the gel when the percentage of sea buckthorn added was increased.

From the analysis of the instrumental texture parameters, it can be concluded that the addition of the lyophilized sea buckthorn, in a percentage over 2%, lead to the improvement of textural properties of the products.

7.4.3. Functional and physicochemical characterization of the added-value muffins

The muffins obtained with microencapsulated powder presented a yellow color, the hue varying according to the percentage of microencapsulated powder added.

The sea buckthorn extract and the microencapsulated powder were characterized prior to use in the preparation of the muffins in terms of moisture content, dry matter, total carotenoids and antioxidant activity (**Table 7.2**).

Table 7.2. Phytochemical characteristics of the sea buckthorn extract and the microencapsulated powder

Ingredient	Dry weight (%)	Carotenoids content (mg/g D.W.)	Antioxidant activity (µmol Trolox/g D.W.)
Sea buckthorn extract	80.30±0.54	57.54±0.28	449.85±0.03
Microencapsulated powder	90.09±0.47	2.82±0.17	548.00±0.23

In order to determine the functionality of the muffins, the total carotenoid content of all samples was determined using the method described by Rodríguez-Huezo *et al.*, (2004). The antioxidant activity and the color parameters were also performed.

The control samples displayed a total carotenoid content of 2.59±0.25 µg/100 g D.W., whereas the value-added muffins had a carotenoid content of 9.10±0.24 µg/100 g D.W.

As for the antioxidant activity, the control sample had a value of 141.03±6.38 µM Trolox / g D.W., while the muffins with microencapsulated powder showed an antioxidant activity of 293.90±2.078 µM Trolox/g D.W. (Ursache *et al.*, 2017)

Figure 7.8. Photos of a cross-section of the muffins

7.4.4. Textural analysis of muffins

The textural parameters analyzed were: the firmness (the force for a given value of the deformation), the cohesiveness (the resistance of the food during the second compression to the resistance during the first compression), the elasticity (the deformation recovered between the two compression cycles) and chewability (energy required to disintegrate food during mastication) (http://texturetechnologies.com/resources/texture-profile-analysis#examples-of-graphs).

In **Table 7.4**. are presented the values of the textural parameters for the muffins with microencapsulated powder and for the muffins that were used as a control sample.

Tabel 7.4. *The values of the texture parameters of the muffins*

Textural parameter, um	Blank	P1	P2
Firmness, N	2.5±0.03^{a}	1.7±0.02^{c}	1.25±0.01^{d}
Cohesiveness, dimensionless	0.33±0.03^{d}	0.51±0.01^{b}	0.65±0.02^{a}
Elasticity, mm	3.53±0.008^{b}	3.84±0.02^{a}	3.93±0.05^{a}
Masticability, mJ	2.82±0.07^{c}	3.17±0.02^{ab}	3.28±0.004^{a}
Moisture lost while baking, g	10.81±2.76^{a}	10.31±0.41^{ab}	12.59±0.81^{b}

*media on the same row that do not share the same letter are statistically significantly different (p <0.001).

The addition of powdered microencapsulated decreased the firmness proportional to the percentage of the powder (from 2.5 N for the control sample to 1.25 N for the sample with 12%

powder). This behavior can be correlated with the porosity of the samples, observed visually: the control sample had an uneven porosity, which led to the registration of more resistance during compression. The other textural parameters recorded higher values compared to the control sample. Thus, cohesiveness increased by 97% for the sample with 12% add-on. The better cohesiveness of the samples with the addition of microencapsulated powder confirms the existence of compounds having a role in strengthening the internal linkages between the constituents of the muffin. The same can be noticed by observing the masticability values, which increase up to 16%. Masticability is the energy deposited during mastication to disintegrate the sample. The elasticity of the muffins is enhanced by the addition of powder. This may be associated with a smaller and a more uniform porosity of samples with 8 and 12% microencapsulated powder. As a result of the instrumental analysis of the muffin texture, it can be stated that the sample with 12% sea buckthorn addition had the best values of the textural parameters.

7.4.5. The evaluation of the storage stability of the value-added muffins

In order to assess the stability of value-added muffins during storage, the samples were analyzed for the total carotenoid content, the color parameters, and the antioxidant activity. In this regard, the samples were packaged in plastic foil and stored in the refrigerator for 21 days, the above-mentioned parameters being determined every 7 days.

The **figure 7.9.** presents the variation in the total carotenoid content over the 21 days of storage.

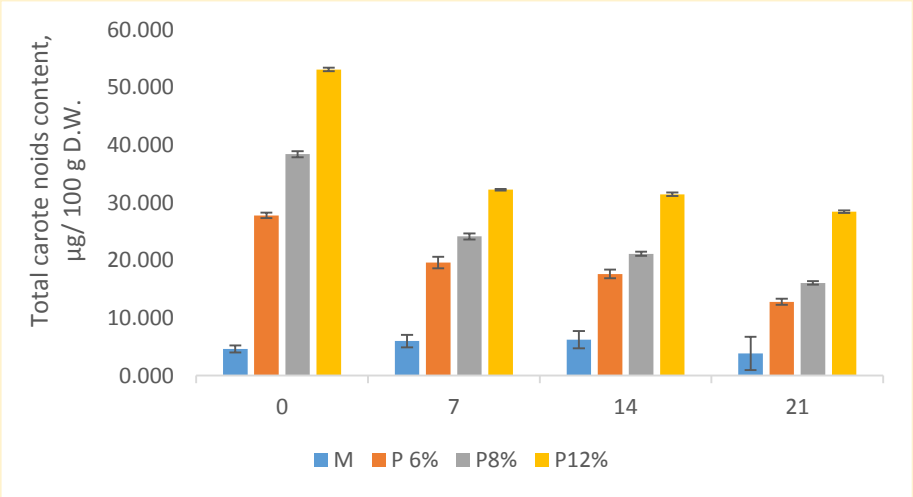


Figure 7.9. Variation of the total carotenoids during storage of the added-value muffins

From the **figure 7.9**. it can be observed that the P1 sample registered a decrease of approx. 38% of the total carotenoid content in the first 7 days of storage and 45% after 14 days. Also, on the 21st day of storage, there was a decrease of approx. 58% of the total carotenoids content, probably caused by degradation reactions. In the case of the P2 sample, there were also decreases in the total carotenoid content during the 21 days of storage. Thus, after 7 days of storage, there was a decrease in the total carotenoid content by approx. 39%, 41% after 14 days and 46% after 21 days. It can be stated that P2 content of total carotenoids decreased less and slower compared to the P1.

Figure 7.10. presents the variation of the antioxidant activity over the 21 days of storage. There may also be noticed a decrease in the antioxidant activity, probably due to variation in the concentration of carotenoids total.

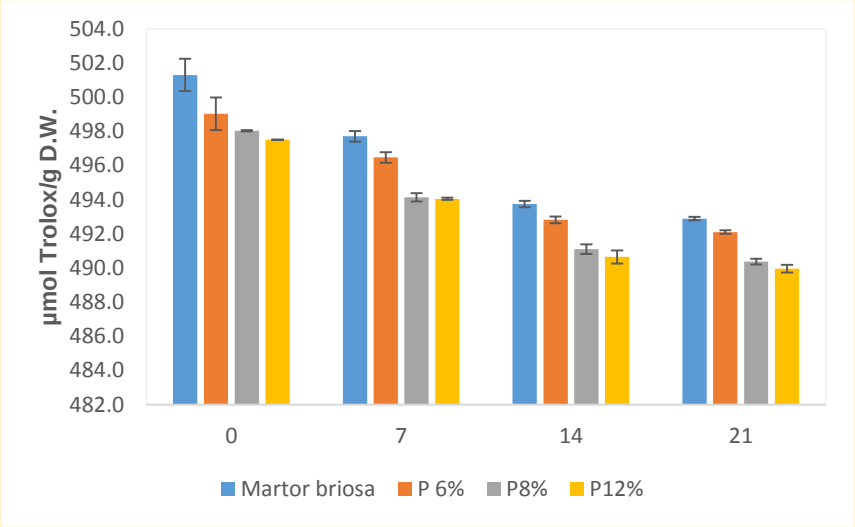


Figure 7.10. Variation of the antioxidant activity during the storage of the value-added muffins

From the **figure 7.10.** it can be observed a decrease of about 36% in the antioxidant activity during the first 7 days of storage and 53% after 14 days, probably due to the carotenoids degradation reactions, largely responsible for the antioxidant activity of the muffins. A slight increase was observed on storage day 21, also without statistical significance.

7.5. Partial conclusions

The milk-based product enriched with sea buckthorn and whey protein concentrate was made in 3 variants: 2%, 4%, and 6%, respectively, lyophilized sea buckthorn. These products have the following chemical composition: lipids ranging from 12.26 to 13.46%, proteins between 10.96 and 11.11%, mineral salts between 0.65 and 0.74%, a sugar content between 13.78 and 16.58%, and the dry substance varied between 38.85 and 40.65%. The content of carotenoids varied according to the percentage of the sea buckthorn added, with values between 1.07±0.06 mg/100 g D.W. and 4.15±0.05 mg/100 g D.W. The antioxidant activity registered a slight variation between the samples, with values of 496.96 µM Trolox/g D.W. for the sample with 2% sea buckthorn and 495.32 µM Trolox/g D.W. for the sample by 6%.

The instrumental analysis of the milk-based products enriched with whey proteins concentrate and sea buckthorn aimed at determining the firmness, cohesiveness, and elasticity of the samples in response to their deformation, carried out by double compression (TPA method). From the analysis of these textural parameters, it can be concluded that the addition of over 2% lyophilized sea buckthorn leads to an improvement of the textural properties of milk-based dessert with sea buckthorn and whey protein concentrate.

The added-value muffins had a satisfactory total carotenoid content and antioxidant activity.

The texture analysis suggested that the addition of the microencapsulated powder determined the increase in firmness correlated with the porosity of the samples. The cohesiveness and elasticity of the muffins with the microencapsulated powder, were lower compared to the control sample, thus this results are correlated with a weaker binding between the constituents, which could cause a fragile texture.

Sensory analysis showed that both samples had a similar taste. The microencapsulated powder sample was preferred by all participants because the added microencapsulated powder improved the appearance of the muffin, bothm texturally and visually.

During the 21 days of storage, it could be noticed that there were no significant changes in the total carotenoid content and the antioxidant activity. Also, in the case of the color parameters (*L*, a*, b**), no significant changes were recorded.

General conclusions

- The research studies were focused on the obtaining of some functional composites based on the sea buckthorn extracts (*Hippophae rhamnoides*) to produce value-added foods.
- Initially, the experiments consisted in the extraction, characterization, and quantification of the phytochemical compounds in the sea buckthorn extract, as well as investigating the stability of the bioactive compounds in the sea buckthorn (*H. rhamnoides*) at different thermal regimes from the perspective of their use as ingredients in food products or nutraceuticals.
- FT-IR techniques and fluorescence spectroscopy have highlighted the structural complexity of the sea buckthorn extract. The thermal treatment, according to the spectrofluorimetric studies, caused changes in the maximum emission wavelengths, indicating the sequential character of the structural changes of the phytochemical compounds, while the ATR of the samples revealed a high thermal stability
- The degradation kinetics studies showed that the polyphenols and the carotenoids, respectively, are stable to thermal treatment, while the flavonoids and the antioxidant activity presents a lower thermal stability.
- The mechanisms of binding of the main whey proteins (α-LA and β-LG) and the carotenoids in the sea buckthorn extract were investigated and the stability of the complexes formed under different pH conditions and thermal treatment were assessed from the perspective of improving the stability of the carotenoids compounds extracted from the sea buckthorn as preliminary microencapsulation steps.
- In the case of the α-lactalbumin experiments, the study was based on the fluorescence spectroscopy techniques, UV-vis spectroscopy and *in silico* techniques. Thus, it could be observed that the thermal treatment did not induce major influences in the interaction between the α-lactalbumin and the carotenoids from sea buckthorn extract. UV-vis studies have shown that the absorbance intensity at 280 nm increased with the sequential addition of the sea buckthorn extract. This highlighted that the interaction between the whey protein and the sea buckthorn extract led to the unfolding of the polypeptide chains. The conclusion from the intrinsic

fluorescence experiments was that the addition of the sea buckthorn extract induced minor conformational changes in the protein structure.

- The *in silico* experimental results revealed that the thermal treatment did not influence the formation of the complex, but the thermal treatment favored different molecular events that affected the interaction between the proteins and the ligand and also the stability of the complex. Therefore, according to the experiments, the carotenoids in the white sea buckthorn extract do not bind in the vicinity of the Trp residues of the protein.
- In the case of the β-lactoglobulin-sea buckthorn extract complex, the results suggested that the thermal treatment caused the partial unfolding of the protein, especially at temperatures above 80°C. Binding of the carotenoids and the thermal treatment led to a decrease in the polarity of the adjacent area of the Tyr residues and an increase in the hydrophobicity, while the polarity of the adjacent area to the Trp residue increased and the hydrophobicity decreased.
- The molecular dynamics results indicated that regardless of the thermal treatment applied, the hydrophobic bonds are the ones that stabilize the complex between the β-lactoglobulin and β-carotene molecules. The changes at an atomic level that occur inside the protein molecules following a more severe thermal treatment indicated the formation of a more thermodynamically stable complex.
- The pH variation in the β-lactoglobulin-sea buckthorn extract complex resulted in the blocking of the Trp residues within the protein molecule, a phenomenon evidenced by a decrease in the maximum fluorescence intensity at the excitation wavelength of 292 nm.
- The encapsulation of carotenoids from the lyophilized sea buckthorn extract was achieved by coacervation followed by lyophilization in order to obtain satisfactory encapsulation efficiency (56.16±1.24%).
- The confocal laser scanning microscopy revealed the presence of carotenoids within the whey protein matrix.
- The specific FT-IR spectrum of the coacervates indicated typical bands for the carotenoids, esters, dicarboxylic acids and whey proteins, but also the formation of new hydrogen bonds in the complex.
- The textured analyzes of the products led to the conclusion that adding the microencapsulated powder to the products improves the rheological and textural properties of the finished product, in addition to beneficial health effects.

Original contributions and prospects for further research

The main objective of the doctoral thesis was to evaluate the stability of the biologically active compounds in sea buckthorn extract (*H. rhamnoides*), the assessment of the structural changes of the extracts induced by the thermal treatment and pH by means of fluorescence spectroscopy (emission spectra) and the kinetics of thermal degradation of biologically active compounds in the sea buckthorn. Therefore, knowing the parameters and the processing conditions (temperature, pH and time) can contribute to achieving a wide range of value-added food products.

The novelty of this study consists in obtaining an ingredient based on the sea buckthorn extract and whey protein isolate that could be used in various foods to increase their nutritional value. The applicative potential of the ingredient (microencapsulated powder) was evidenced by obtaining muffins with or without the addition of microencapsulated powder. It could be observed that the muffins with microencapsulated powder were more appreciated from a sensory point of view compared to those without the addition of the powder. It was demonstrated that the muffins enriched with microencapsulated powder presented a more microbiologically satisfactory stability throughout the shelf life.

Dissemination of the research

A. Articles published in ISI coated journals

Dumitrașcu, L., **Ursache, F.M.**, Aprodu, I., Stănciuc, N. Effect of calcium and magnesium on the structure of β-lactoglobulin bound by carotenoids from sea buckthron berries extract, *submitted to Journal of Molecular Structure.*

Ursache, F.M., Ghinea, I.O., Turturică, M., Aprodu, I., Râpeanu, G., Stănciuc, N. (2017). Phytochemicals content and antioxidant properties of sea buckthorn (*Hippophae rhamnoides L*.) as affected by heat treatment - Quantitative spectroscopic and kinetic approaches, Food Chemistry, 233, 442–449.

Ursache, F.M., Dumitraşcu, L., Aprodu, I., Stănciuc, N. (2017). Screening the thermal stability of carotenoids-α lactalbumin complex by spectroscopic and molecular modeling approach. Journal of Macromolecular Science, Part A - Pure and Applied Chemistry, 54, 316-322.

Dumitrașcu, L., **Ursache, F.M.**, Stănciuc, N., Aprodu, I. (2016). Studies on binding mechanism between carotenoids from sea buckthorn and thermally treated α-lactalbumin. Journal of Molecular Structure, 1125, 721-729, 10.1016/j.molstruc.2016.07.070.

Aprodu I., **Ursache, F.M.**, Turturică, M., Râpeanu, G., Stănciuc, N. (2017). Thermal stability of the complex formed between carotenoids from sea buckthorn (*Hippophae rhamnoides L*.) and bovine β-lactoglobulin. Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy, 173, 562-571.

Ursache, F.M., Andronoiu, D.G., Ghinea, I.O., Barbu, V., Ioniţă, E., Dumitrașcu, L., Botez, E., Râpeanu, G., Stănciuc, N. 2018. Valorizations of carotenoids from sea buckthorn extract by microencapsulation and formulation of value-added food products. Journal of Food Engineering, 219**,** 16-24.

Articles published in BDI coated journals

Ursache, M.F., Botez, E., Râpeanu, G., Stănciuc, N. (2017). pH-induced structural changes of the complex formed between carotenoids from sea buckthorn (Hippophae rhamnoides L.) and bovine β-lactoglobulin. The Annals of the University Dunarea de Jos of Galati, Fascicle VI – Food Technology, 41(2), 62-74.

Abstracts published in ISI coated **journals**

Stănciuc, N., **Ursache, F.M**., Dumitrașcu, L. (2016). Contribution to the binding mechanism of carotenoids from sea buckthorn to the thermally treated α-lactalbumin. Journal of Biotechnology, 1125, 721-729, 10.1016/j.molstruc.2016.07.070

Ursache F.M., Stănciuc N., Nistor O.V., Botez E. (2015). Evaluation of Anthocyanins Thermal Degradation in *Prunus spinosa* on Fluorescence Spectroscopy Basis. Journal of Biotechnology 208:S70, 0.1016/j.jbiotec.2015.06.213.

Papers communicated at international scientific events

Ursache, F.M., Stănciuc, N., Ghinea, I.O., Râpeanu G., Botez, E. (2017). Total carotenoids content degradation from *H. rhamnoides* on a kinetic basis. The 8th International Symposium EuroAliment, Dunarea de Jos University of Galati, 8-9 September, Galati, Romania, poster presentation.

Stănciuc, N., **Ursache, F.M**., Dumitrașcu, L. (2016). Contribution to the binding mechanism of carotenoids from sea buckthorn to the thermally treated α-lactalbumin, European Biotechnology Congress 2016, Riga, Latvia, 05 – 07 May 2016, poster presentation.

Stănciuc, N., **Ursache, F.M.**, Dumitrașcu, L. (2016). Probing the thermal stability of α-lactalbumin-carotenoids complex by fluorescence spectroscopy techniques. 18th IUFoST – World Congress of Food Science and Technology, 21st - 25th August 2016, Dublin, Ireland, poster presentation.

Ursache, F.M, Oancea, A.M., Râpeanu, G., Stănciuc, N. (2016). Thermal degradation of the total carotenoid content and antioxidant activity of sea buckthorn (Hippophae rhamnoides L.) – a kinetic study, 8th Congress Pigments in Food ”Coloured food for health benefits”, 28 June – 1 July, Cluj Napoca, Romania, poster presentation.

Stănciuc, N., **Ursache, F.M**., Aprodu, I., Râpeanu, G. (2016). Exploring the binding affinity between bovine β-lactoglobulin and β-carotene by using fluorescence spectroscopy and molecular modeling techniques. International Conference of Physical Chemistry ROMPHYSCHEM 16, September 21-24, 2016, Galați, Romania, poster presentation.

Stănciuc, N., Dumitraşcu, L., Râpeanu, G., **Ursache, F.M.** (2016). Thermal stability of the complex formed by bovine alpha-lactalbumin and carotenoids from sea buckthorn. International Conference of Physical Chemistry ROMPHYSCHEM 16, September 21-24, 2016, Galați, Romania, poster presentation.

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Ursache, M.F., Stănciuc, N., Râpeanu, G., Aprodu, I., Botez, E. (2016). Investigations of structural changes in carotenoids - β-lactoglobulin complex induced by heat treatment using fluorescence spectroscopy. Ph.D. student conference CSSD-UDJG, Dunărea de Jos University of Galați, Romania, 2nd-3th of June 2016.

Ursache F.M., Stănciuc N., Nistor O.V., Botez E. (2015). Preliminary investigations on the effect of pH on the stability of β-carotene from *Hippophae rhamnoides.* Ph.D. student conference CSSD-UDJG, Dunărea de Jos University of Galați, Romania.

Patents of invention

Ursache, F.M., Botez, E., Stănciuc, N., Andronoiu, D.G., Nistor, O.V, Turturică, M., Râpeanu, G. Desert dairy product based on whey protein concentrate and sea buckthorn and method for making it off. OSIM registration number A/00045/2017.

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