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Chapter 1. The main structural characteristics of proteins whey

1.1. General aspects

The term of whey proteins is attributed to those proteins that remain in the aqueous phase of milk after precipitation and separation of caseins at pH 4.6 and 20°C. In this class are included: β-lactoglobulin (β-LG), α-lactalbumin (α-LA), serum albumin (SAB), immunoglobulins (Ig) and lactoferrin (LF). β-LG and α- LA are further characterized for structural and conformational features, as are the main whey proteins.

1.2. β-lactoglobulin

β-LG is the major whey protein that it can be found in cow's milk in a concentration of 2.4 g/L (de la Fuente, 2002). Characterizations of protein by composition in amino acid, polypeptide sequence and isoelectric point have revealed the existence of a genetic polymorphism. Ten genetic variants of bovine β-LG have been identified, seven of which being isolated and characterized. All genetic variants of β-LG contain 162 amino acid residues, the difference appearing in one or three positions. Although genetic variants A and B differ only in two positions, the substitution of Gly with Asp in position 4 is especially important because it increases the ability of self-association of variant A. This phenomenon can be explained by the formation of additional salt bridges between the remaining carboxylic group of Asp and any other basic group.

1.2.1. The primary structure of β-LG

The amino acid composition of the reference protein is Asp^{10}, Asn^{5}, Thr^{8}, Ser^{7}, Glu^{16}, Gln^{9}, Pro^{8}, Gly^{4}, Ala^{15}, Cys^{5}, Val^{9}, Met^{4}, Ile^{10}, Leu^{22}, Tyr^{4}, Phe^{4}, Lys^{15}, His^{2}, Trp^{2} and Arg^{3}, with a molecular weight from 18,277 Da. It can be seen that β-LG contains a large number of essential amino acids. However, their bioavailability is low due to the resistance of the protein to proteolysis in the acidic pH range (Reddy et al. 1988).

1.2.2. The secondary structure of β-LG

Monaco et al. (1987) have described the molecule as a spherical shape having a diameter of 2.5 Å, consisting of a short portion of the α-helix and eight antiparallel polypeptide chains with β-sheet folded structure. The eight antiparallel chains are represented by residues 15 to 27 (A), 35-44 (B) 49-56 (C) 65-74 (D), 82-85 (E), 91-97 (F), 102-111 (G), and 116-124 (H). The fragment 131-140 has α-helix structure, whereas fragments 28-31, 45-48, 61-64, 98-101 and 112-115 presents reverse β-sheet structures, while segments 74-82 and 125-130 are rings with structure not well defined. 1.2.3. Conformational flexibility of β-LG At 25°C and pH range of 4.0 to 6.5, bovine β-LG is a dimer. When the pH value changes close to 7.0, the molecule undergoes a number of conformational transitions characterized by increasing of reactivity of the carboxyl groups and SH^{121} group, which under physiological conditions is located within the molecule. Phillips et al. (1994) studied the conformational changes that occur when dimer dissociates at pH 7.5 (so-called Tanford transition). It has been suggested that these structural changes affect the position were β-LG bind the retinol, thus influencing the biological function of the protein.

1.3. α-lactalbumin

α-LA is a small protein, with a molecular mass of 14,174 Da, pHi 4.0-6.0, which is able to bind calcium ions. First, α-LA fulfills an important function in secretory cells breast, being one of the two components of lactose synthase, the enzyme that catalyze the final stage of lactose biosynthesis. Secondly, α-LA has one binding site for $Ca^{2+,}$ being used as a model system for study the effects of calcium ion binding to proteins, peptides, membranes, etc. Thirdly, α-LA can form a series of partially unfolded states, being used as classic molten molecules used to elucidate the mechanisms of protein folding/unfolding.

1.3.1. Primary, secondary and tertiary structures of α-LA

The protein contains 123 amino acid residues with two major genetic variants (A and B). Variant B is present in milk from *Bos taurus*, whereas variant A is present in milk from *Bos indicus* species. Variant A contains Glu in position 10, while in variant B Glu is substituted by Arg (Stănciuc, 2009). The primary structure of α-LA variant B contains: Ala^{3}, Arg^{1}, Asn^{8}, Asp^{13}, Cys^{8}, Gln^{8}, Glu^{7}, Gly^{6}, His^{3}, Ile^{8}, Leu^{13}, Lys^{12}, Met^{1}, Phe^{4}, Pro^{2}, Ser^{7}, Thr^{7}, Trp^{4}, Tyr^{4}, and Val^{6}.

α-LA has a high content of essential amino acids (Trp, Phe, Tyr, Leu, Ile, Thr, Met, Cys, Lys, and Val), representing 63.2% of the protein total amino acids and 51.4% of the essential amino acids from milk proteins. The domain with helical structure contains three α- helix, which are stable at pH variation (fragments 5-11, 23-34 and 86-98), a pH dependent fragment (105- 100) and three portions with small helix structure (18-20, 3 and 115-118). The flexible fragment (105-110) presents a helix configuration at pH ranging from 6.5 to 8.0 (N`Negue et al., 2006). The region with β- structure consists of three antiparallel chains (fragments 41-44, 47-50 and 55-56) and a short chain with helix structure (77-80). These two domains are stabilized by disulfide bond between residues Cys at positions 73 and 91, and to a lesser extent by the disulfide bond Cys^{61} - Cys^{77}. Overall, the structure is stabilized by four disulfide bonds (Cys^{6} - Cys^{120}, Cys^{61} - Cys^{77}, Cys^{73} - Cys^{91} and Cys^{28} - Cys^{111}).

1.3.2. Conformational flexibility of α-LA

The binding of cations increases the stability of the protein conformation. The same effect has binding ions Mg^{2+}, Na^{+} and K^{+}. The binding of Zn^{2+} cause destabilization of the molecule resulting in aggregation of the molecules and increase in susceptibility to proteolysis. In the presence of high concentrations of Zn^{2+}, α-LA partially unfolds and aggregates. In the absence of calcium ions, but in the presence of physiological concentration of Mg^{2+}, Na^{+} and K^{+}, the transition temperature varies in the temperature range of 30-45°C.

Chapter 2. Structural features of carotenoids and polyphenols

2.1. Carotenoids

Chemically, carotenoids are tetraterpenes and their structures are based on 40-carbon polyene chain with conjugated double bonds (3–13) along this chain. Though they seem to be hidden, carotenoids are one of the most wide spread and ubiquitous lipid soluble pigments in nature (e.g. in leaves, fruits, flowers, teguments, etc.) and so far, more than 750 naturally occurring carotenoids have been identified (Britton et al., 2008). Carotenoids are produced by all photosynthetic organisms, by fungi and by non- photosynthetic bacteria and conversely, they are required in the diet of animals as antioxidants or vitamins, but also to produce their tissues pigmentation, as is the case of the feathers of birds or the exoskeleton of crustaceans. Remarkably, carotenoid compounds only differ in the following chemical characteristics, which give rise to the different carotenoids structures: (i) the presence and number of oxygen atoms in the molecule (oxygenated carotenoids are xanthophyll and non-oxygenated are carotenes), (ii) the hydrogenation of the carbon polyene chain, (ii) the cyclization at one/both ends of the molecule, usually with a e-ionone or bionone rings and the (iv) length of the chromophore (Meléndez-Martínez et al., 2007). Carotenoids can be divided into two main groups, depending on the functional groups: xanthophylls, which contain oxygen as functional groups, such as lutein and zeaxanthin; carotene, with a hydrocarbonic chain, containing no functional groups, such as α-carotene, β- carotene and lycopene. Fruits and vegetables are the main sources of carotenoids and play an essential role in the diet through vitamin A activity (Haskell, 2013). In addition to the role of vitamin A, they have antioxidant activity, provides intercellular communication and specific activity of the immune system (Skibsted, 2012; Stephensen, 2013).

Epidemiological studies have shown that a diet rich in carotenoids ensures a lower incidence of cancer, cardiovascular disease, aging and cataract (Meyers et al., 2014; Sharon et al., 2012). Carotenoids deficit results in clinical conjunctivitis and corneal disorders, including dry eye, night blindness, keratomalacia, corneal ulceration, cicatrices and irreversible blindness (Sommer, 2008). Deficiencies in provitamin A lead to visual disability and increased mortality due to immunity innate and low adaptive (Stephensen, 2001). Lycopene shows the highest antioxidant activity.

This feature recommends lycopene in protecting cellular systems against reactive oxygen and nitrogen reactive species, thus preventing cardiovascular disease (Müller et al., 2015).

2.2. Polyphenols

Polyphenolic compounds are of major interest for the food, pharmaceutical and medicine due to their beneficial effects on health, in particular in the treatment and cancer prevention (Chen et al., 2011), cardiovascular disease (Kuriyama et al., 2006; Mursu et al., 2008), anticarcinogenic effects (Jeong et al., 2011; Ogunleye et al., 2009), ulcer (Zakaria et al., 2011), antithrombotic (Han et al., 2012; Tao et al., 2012), anti-inflammatory (Bear et al., 2012; Zimmer et al., 2012), antiallergenic (Chung and Champagne, 2009; Schmitz-Eiberger and Blanke, 2012), anticoagulants (Bijak et al., 2011), immunomodulators (Schütz et al., 2010) and antimicrobial (Silva et al., 2012; Xia et al. 2011), vasodilators and analgesics (Santoz et al., 2010). Polyphenols are naturally occurring compounds found largely in the fruits, vegetables, cereals and beverages. Fruits like grapes, apple, pear, cherries and berries contains up to 200–300 mg polyphenols per 100 grams fresh weight. Polyphenols are secondary metabolites of plants and are generally involved in defense against ultraviolet radiation or aggression by pathogens. In food, polyphenols may contribute to the bitterness, astringency, color, flavor, odor and oxidative stability. More than 8,000 polyphenolic compounds have been identified in various plant species. All plant phenolic compounds arise from a common intermediate, phenylalanine, or a close precursor, shikimic acid. Primarily they occur in conjugated forms, with one or more sugar residues linked to hydroxyl groups, although direct linkages of the sugar (polysaccharide or monosaccharide) to an aromatic carbon also exist. Association with other compounds, like carboxylic and organic acids, amines, lipids and linkage with other phenol is also common. Polyphenols may be classified into different groups as a function of the number of phenol rings that they contain and on the basis of structural elements that bind these rings to one another. The main classes include phenolic acids, flavonoids, stilbenes and lignans (Spencer et al., 2008).

Chapter 3. Investigations on the binding mechanism of compounds from vegetable extracts to β-lactoglobulin and α-lactalbumin and analysis of relevant atomic particularities for assessing stability of complexes

3.1. Introduction

Whey proteins have been extensively studied in terms of binding ability of hydrophobic ligands, such as fatty acids and vitamins, raising a number of advantages, as follows: increase the absorption of fatty acids (Perez et al. 1992), modify the kinetics of enzymatic hydrolysis of the protein (Mandalari et al. 2009), protection of ligands against oxidation or other stress factors and modifying the bioavailability of ligands (Riihimäki-Lampen, 2009). In food products, the binding properties and therefore the biological properties of the complexes can be affected by the structure of proteins and/or the presence of other proteins able to compete for binding sites. For example, the ability of β-LG to bind many hydrophobic compounds, such as retinol, vitamin D, cholesterol, curcumin acids and their derivatives, protoporphyrin IX, aromatic compounds and cations catechin has been demonstrated (Liskov et al. 2011; Le Maux et al. 2012; Puyol et al. 1994; Sneharani et al. 2010; Kanakis et al. 2011; Dufour et al. 1990, 1992; Liu et al. 2011).

α-LA ability to bind oleic acid with the formation of HAMLET/BAMLET (Human/Bovine α- lactalbumin made lethal to tumor cells) is already known for about 15 years (Svensson et al., 2000). This complex has a cytotoxic activity for cancer cell lines, whereas healthy cells are not affected. α-LA can bind other compounds such as resveratrol (Hemar et al., 2011), sodium oleate (Kehoe and Brodkorb, 2014), genistein, kaempferol (Moeen and Mohammadi, 2015) etc.

3.2. Analysis of α-lactalbumin and β-lactoglobulin structure by in silico methods

In order to evaluate the binding mechanism and affinity of biologically active compounds, α-LA and β-LG molecules models were taken from the RCSB Protein Data Bank (www.rcsb.org). Studies at single molecule level were

made using the protein models 1F6S.pdb (one monomer of bovine α-LA it was considered; Chrysina et al., 2000) and 3NPO.pdb (bovine β-LG; Loch et al., 2011) solved by X-ray crystallography. These models showed very good resolution and high stability. Specific structural bioinformatics methods were initially applied for advanced characterization of whey proteins. Based on the physicochemical properties of amino acids the following parameters were analyzed by using ProtParam: molecular weight, isoelectric point, atomic composition, aliphatic and hydrophilicity index (GRAVY), which for a peptide or protein is calculated as the sum of the hydrophobicity index values for all amino acids divided by the number of residues in the sequence. Surface properties of α-LA and β-LG were assessed using the program GETARIA. For an advanced characterization of the models, α-LA and β-LG molecules were first refined by removing all non-protein compounds. Geometry optimization of molecules was then conducted in vacuum; minimization of the potential energy was performed such as to ensure the removal of any possible geometric distortion and repulsive interactions between atoms.

Such optimized molecules were placed in the center of a parallelepidic reaction box, having suitable size for the molecular system, which were subsequently filled with single point charged (SPC) water molecules (explicit solvent). By solvation, it ensures a higher degree of native protein folding due to the involvement of the hydrogen bonds (Mogilner et al., 2002). Solvated molecular systems were then optimized by successively using committed algorithms, Steepest Descent and limited-memory Broyden- Fletcher-Goldfarb-Shanno, as in the case optimization in vacuum. In order to identify specific characteristics of the protein molecules at various temperatures, the systems were heated at 25°C and 80°C using a Berendsen thermostat, followed by the systems equilibration at indicated temperatures. The molecular mechanics and molecular dynamics simulations were performed using the software Gromacs (v. 4.5.5.), which runs with Linux operating environment, using the Gromos96 43a1 or AMBER (Assisted Model Building and Energy Refinement) force fields under parallelization conditions, on a computer equipped with processor Intel(R)Core(TM)2CPU1.86GHz6300. For structural and conformational analysis, specific PDBsum instruments (Laskowski, 2009) and Visual Molecular Dynamics (VMD) were used (Humphrey et al., 1996).

3.3. Studies on the molecules interactions

For interfacing the models (α-LA with β-carotene and β-LG with cyanidin 3-O rutinoside), the PatchDock server was used (Duhovny et al., 2002; Schneidman-Duhovny et al., 2005), that provides the molecular docking on the basis of shape complementarity, generating 100 different complexes, which are then prioritized by scoring function. PatchDock provides rigid interfacing of molecules, resulting models that are then refined using FireDock server (Andrusier et al., 2007). The refinement of ten best models was based on the relative orientation of the specific groups/atoms at the interface between the two molecules in the complex. Then, a new classification of the models was performed, mainly taking into account the interaction energy between molecules, and some others factors such as energetic ones (Andrusier et al., 2007). In order to assess the total number of ligand binding sites in the protein molecule and to evaluate the particularities of interaction between the two molecules within each investigated complex, the ten best results obtained in computational models were considered following docking and refinement methods. The analysis of the resulted complex was done using dedicated software LigPlot+v1.4.5. (Laskowski and Swindells, 2011) and PDBePISA (Krissinel and Henrick, 2007; Krissinel, 2009).

3.4. α-lactalbumin-β-caroten complex

In order to study the mechanism of β-carotene binding to α-LA, the atomic particularities of all refined docking solutions were analyzed. Four binding sites for β-carotene were observed on the surface of α-LA. The main binding site involves the following amino acids: His^{32}, Thr^{33}, Asn^{44}, Asn^{45}, Asp^{46}, Ser^{47}, Glu^{49}, Asn^{56}, Lys^{58}, Tyr^{103}, Trp^{104} and Leu^{105}. The second binding site involves the following amino acids: Cys^{28}, Phe^{31}, His^{32}, Lys^{108}, Ala^{109}, Leu^{110}, Ser^{112}, Glu^{113}, Lys^{114}, Gln^{117} and Trp^{118}. The third binding site involves the common amino acids Gln^{2}, Lys^{5} , Gln^{117} and Trp^{118} and is characterized by low levels of contact surfaces and high binding energy, indicating a low affinity of molecules. A low affinity was also found for the fourth binding site of β-carotene to α-LA surface, with the participation of the following amino acids: Asn^{74}, Ile^{75}, Ser^{76}, Asp^{78}, Lys^{79}, Asp^{87} and Met^{90}.

3.5. β-lactoglobulin - cyanidin-3-O-rutinosid complex

A similar approach was considered in evaluating the atomic particularities regarding the binding of cianidin-3-O-rutinosid to β-LG and the affinity between these two molecules. Two different binding sites were defined on the surface of β-LG molecule. The primary binding site for cyanidin 3-O rutinozid involves the following amino acids Arg^{10}, Glu^{11}, Lys^{13}, Asp^{14}, Asp^{84} and Leu^{85}, whereas the second one consists of Gln^{43}, Asn^{44}, Asn^{45}, Thr^{48}, Tyr^{50}, Gln^{65}, Asn^{66}, Pro^{67} and His^{68}. A detailed analysis at atomic level indicates that heat treatment lead only to minor conformational changes at protein surface, which includes the sites of interaction with the ligand.

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Chapter 4: Obtaining and characterization of sea buckthorn and sour cherries extracts

4.1. Obtaining and characterization of sea buckthorn and sour cherries extracts

4.1.1. Introduction

The increased interest in the food industry to use natural ingredients from plant sources, led to the extensive studies on plants constituents. The research focused mainly on the study of certain fruits (berries, grapes, cherries, etc.) with high content of bioactive compounds, especially polyphenolic compounds and carotenoids (Kraujalyte et al., 2013). This is highly correlated with the increased demand for safety and nutrional foods, which lead to the diversification of products rich in bioactive compounds. The attention was focused on the plant polyphenolic compounds due to their nutritional and functional benefits; the most studied being the anti-inflammatory and antioxidant capacity (Peng *et al.*, 2015).

4.1.2 Objectives

The main aims of these studies were to determine the total carotenoid content (TCC), total monomeric anthocyanins content (TAC), total polyphenolic content (TPC), total falvonoids content (TFC) and antioxidant activity in the extracts of sea buckthorn (*Hippophae rhamnoides L.)* and sour cherries fruits *(Prunus cerasus L)* by using spectrophotometric methods.

4.1.3. Results and discussion

- **Quantitative analysis of sea buckthorn extract compunds**

The content of bioactive compounds in the sea buckthorn extract is shown in **Table 1**.

Table 1. The content of bioactive compounds in the sea buckthorn extract

Phytochemical	Results	Data from literature	References
TPC	2723±1.29 mg GA/100 g	28.35±1.31 mg/g	Kumar *et al.* (2011)
		9.64 -107.04 mg GA/g d.m.	Korekar *et al.* (2014)
TFC	98±0.01 mg CE/ 100 g	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.m.	Kumar *et al.* (2011)
Antioxidant activity	33.7±0.29%	94.7±3.2%	Papuc *et al.* (2008)
β-carotene	35.4±1.11 mg/100 g	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.m.	Pop *et al.* (2014)

The differences between our results and the data from literature can be explained by the different method of extraction, variety, vintage year, etc.

Quantitative analysis of sou cherries extract compunds

Our study involved the use of several extracts (LE, LA, PE, PA) to observe the difference between extraction with ultrapure water and 70% ethanol, and the ability of the solvent to extract the polyphenolic compounds from different matrices. In **Table 2** are given the TPC, TAC, TFC and antioxidant capacity, expressed as antiradical activity on DPPH (DPPH RSA). LE extract presented the highest content in bioactive compounds compared to the other samples, hence was chosen for subsequent experiments.

Table 2. The phytochemical content in sour cherries extracts

Extract	TAC (mg C3G/ g d.m.)±DS	TFC (mg CE/ g d.m.) ±DS
PA	0,009±0,001	1,012±0,075
PE	0,058±0,001	1,094±0,046
LA	0,055±0,002	1,087±0,077
LE	0,085±0,008	1,981±0,087
Extract	**TPC (mg GAE/ g d.m.)±DS**	**DPPH RSA (%) ±DS**
PA	1,081±0,047	38,90±1,37
PE	1,179±0,070	58,50±1,29
LA	1,363±0,048	65,60±2,98
LE	2,464±0,015	93,80±2,36

The antimicrobial activity of sea buckthorn and sour cherries extracts

The results showed that both extracts showed antifungal activity against the test microorganisms, with an inhibition ratio varying between 3.12 and 29.68% (**Table 3** and **Figure 1**).

Table 3. Antifungal activity of sea buckthorn and sour cherries extracts against *Aspergillus niger* MIUG M5 and *Penicillium expansum* MIUG M11

Microorganism	Diameter of the colony, mm			Inhibition ratio, %	
	Martor	Sea buckthorn	Sour cherries	Sea buckthorn	Sour cherries
Aspergillus niger MIUG M5	29,5	22,5	26,5	23,72	10,16
Penicillium expansum MIUG M11	32,0	22,5	31,0	29,68	3,12

a) b)

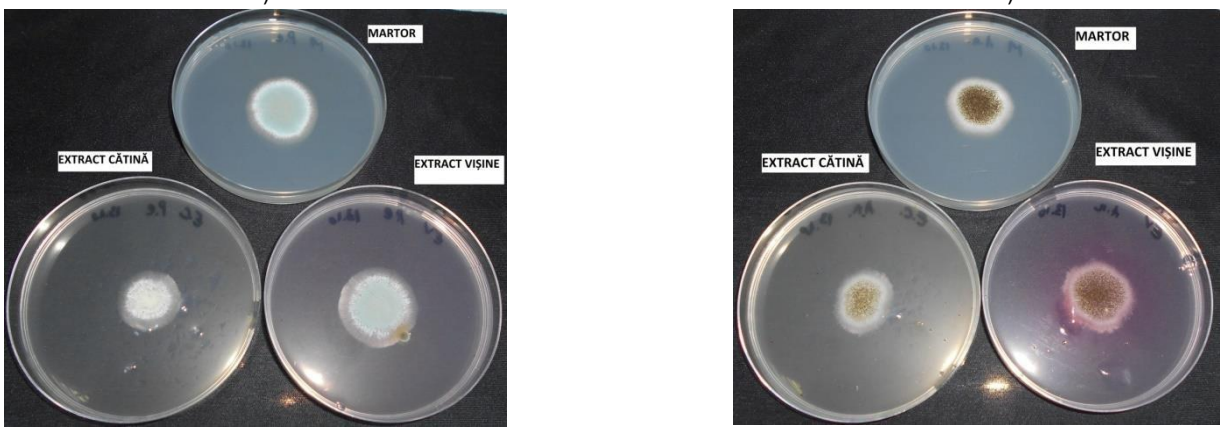


Figure 1. Antifungal activity of sea buckthorn and sour cherries extracts against *Penicillium expansum* MIUG M11 (a) and *Aspergillus niger* MIUG M5 (b)

The maximum value for inhibition ratio was found for sea buckthorn extract against *Penicillium expansum* MIUG M11 (29.68%).

4.2. Carotenoids and anthocyanins identification and quantification

Qualitative and quantitative analysis of the main compunds from sea buckthorn extract by HPLC

Twelfe compounds have been identified, as follows: astaxanthin (peak 1), zeaxanthin (peak 2), zeaxantin-palmitat (peak 3), γ-carotene (peak 4), cis-β-caroten (peak 5), β-cryptoxanthin (peak 6), lycopene (peak 7), lutein palmitat- myristate (peak 8), lutein di-palmitate (peak 9), β-carotene (peak 10), α-carotene (peak 11), and zeaxanthin dipalmitate (peak 12). β-caroten showed a content of 15.19 mg/g d.m., followed by astaxanthin with 11.94 mg/g d.m., β-cryptoxanthin with 8.93 mg/g DW and lycopene with 2.24 mg/g d.m., whereas zeaxanthin was found in the amount of 81.29 mg/g d.m.

Our results are similar to those reported by Pop *et al.* (2014), who suggested a β-carotene content between 1.9 and 7.4 mg/100 g d.m., β-cryptoxanthin (1.3-1.6 mg/100 g d.m.), lycopene (1.4-2.3 mg/100g d.m.) and zeaxanthin (1.8-2.5 mg/100 g d.m.) in the six Romanian varieties of sea buckthorn.

Qualitative and quantitative analysis of the main compunds from sour cherries extract by HPLC

Four anthocyanins were identified in sour cherries extract, as follow: cyanidin-3-rutinosid, cyanidin 3-glucoside, peonidin-3-glucoside and an unidentified cyanidin derivative. Mitic *et al.* (2012) also identified four anthocyanins: cyanidin 3-glucoside-, cyanidin-3-glucosyl rutinosid, cyanidin-3-rutinosid and an unidentified cyanidin derivative. These authors stated that the variety *Oblačinska* presented the highest amount of cyanidin-3- glucozilrutinosid (62.09 mg/100 g fresh sample), while varieties *Cigančica* and *Marela* have a content of 38.42 and 24.73 m /100g fresh sample, respectively.

4.3. Extracts purification and characterization of functionality

4.3.1. Introduction

Although the benefits of biologically active compounds from sea buckthorn and sour cherries are well studied, few studies address the thermal degradation kinetics of these compounds at different temperature-time combinations. Therefore, to describe the functionality of biologically active compounds from sea buckthorn and sour cherries to heat treatment, changes in TCC (sea buckthorn), TPC, TFC, TAC and the antioxidant activity of both fruits were investigated in the temperature range 50-100˚C for 0-25 min (sea buckthorn) and 100-160°C for 0-120 minutes (sour cherries). In

addition, the fluorescence properties of the extracts were analyzed after heat treatment by excitation at 340 nm, 410 nm and 448 nm (sea buckthorn), and 270 nm and 340 nm (sour cherries).

4.3.2. Objectives

The main objectives of this study were:

- ✓ assessment of structural changes induced by thermal treatment by using fluorescence spectroscopy (emission spectra);
- ✓ kinetics of thermal degradation of biologically active compounds from sea buckthorn and sour cherries extracts.

4.3.3. Results and discussion

✓ **Assessment of structural changes induced by thermal treatment by using fluorescence spectroscopy**

The emission spectra of sea buckthorn bioactive compounds have been obtained at the excitation wavelengths of 250 nm, 340 nm, 410 nm and 448 nm. From **Figure 2** it can be observed that well defined spectra were obtained, indicating the presence of a multitudine of fluorescent compounds in the extract.

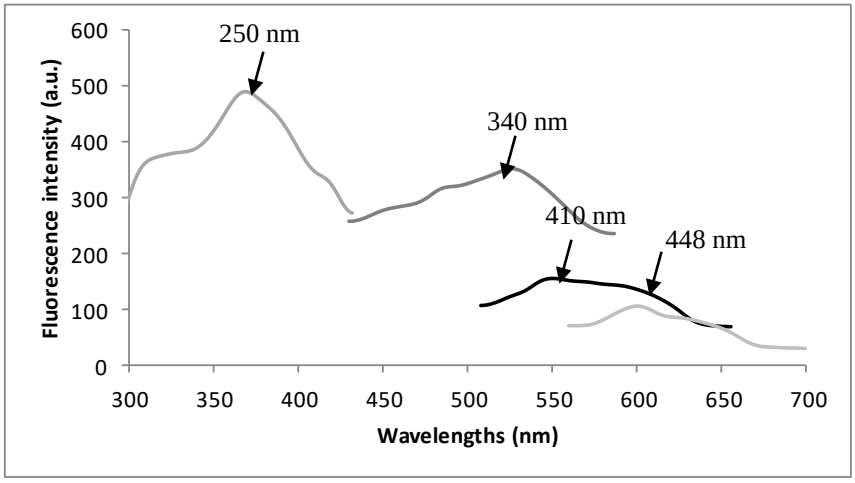


Figure 2. Fluorescence spectra of sea buckthorn extract at different excitation wavelengths. Three independent tests were carried out in each case and SD was lower than 3.5%. (Ursache et al., manuscript submitted to Food Chemistry).

In **Figure 3** are shown the emission spectra obtained after the heat treatment (50-100°C) at excitation wavelength of 250 nm.

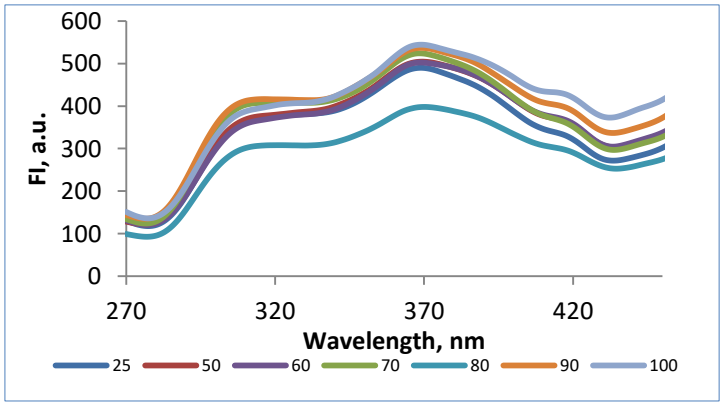


Figure 3. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 250 nm

The carotenoids extract treated at temperatures ranging 70-100ºC showed two peaks with different intensities, reflecting structural changes of fluorescent compounds. **Figure 4** shows the emission spectra of carotenoids extract obtained at excitation wavelength of 340 nm.

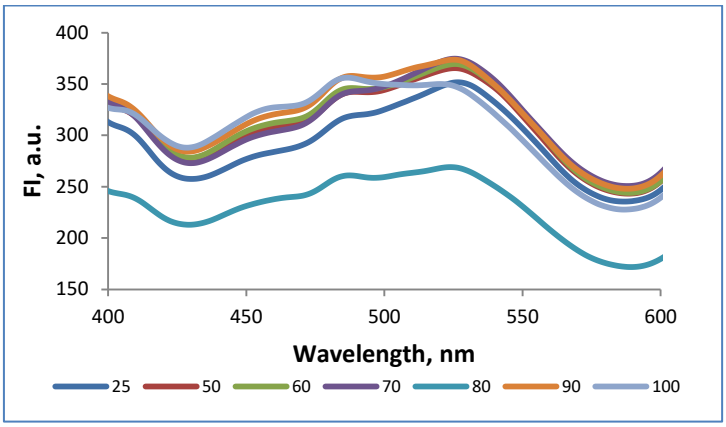


Figure 4. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 340 nm

Heat treatment induced an increase in the fluorescence intensity at temperatures higher than 50ºC, unless the extract was treated at 80ºC. A 6 nm blue-shift was recorded at 100ºC, suggesting significant structural changes. **Figure 5** shows the emission spectra of carotenoids extract obtained at excitation wavelength of 410 nm.

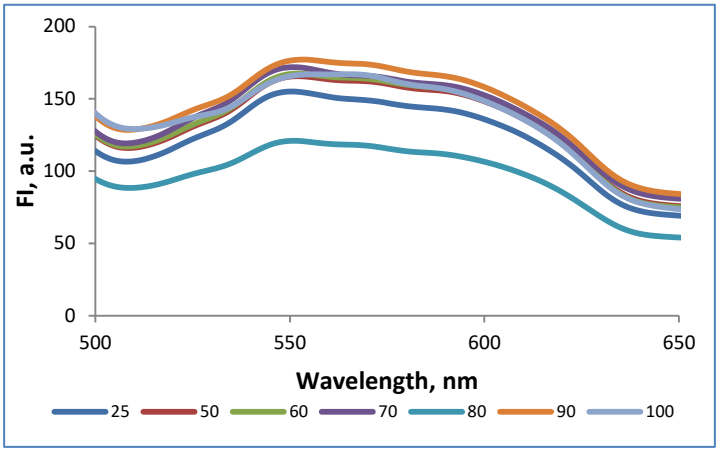
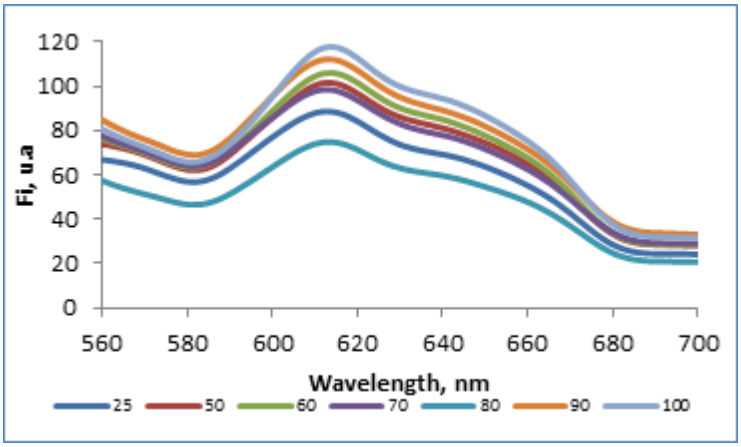


Figure 5. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 410 nm

An increase in the fluorescence intensity can be observed when heating, except at 80ºC. **Figure 6** shows the emission spectra of carotenoids extract obtained at excitation wavelength of 448 nm (a) and 460 nm (b).



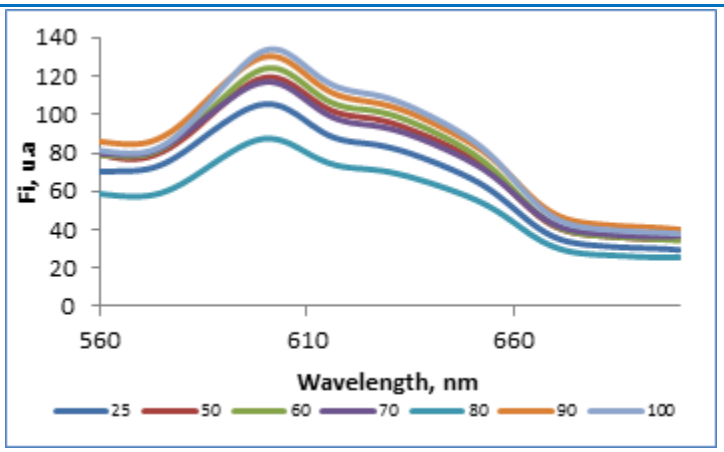


Figure 6. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 448 nm (a) and 460 nm (b)

The lowest florescence intensities in both cases were registered when treated the extract at 80°C, whereas no significant changes were recorded in λ_{max} values. According to Pawlak *et al.*, (2013), the major compound showing fluorescence at these excitation wavelengths is β-carotene in its aggregate form.

Thermal degradation studies on bioactive compounds from *H. rhamnoides* extract

The thermal degradation studies on bioactive compounds from *H. rhamnoides* extract were achieved for the TCC, TPC, TFC and DPPH-RSA, in the temperature range of 60 to 100ºC. In the temperature range studied, the thermal degradation kinetics of TCC followed a fractional conversion kinetic model. **Figure 7** shows the correlation between the experimental and predicted values according to the abovementiomed model.

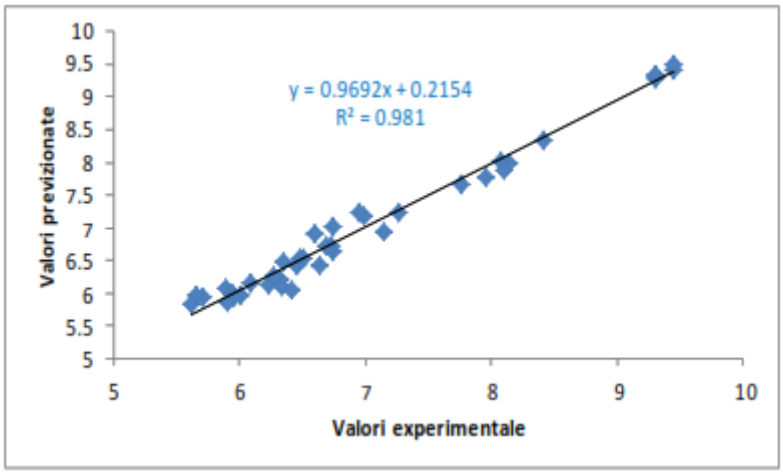


Figure 7. The correlation between the experimental and predicted values for the heat-induced changes in TCC according to the fractional conversion kinetic model

A significant reduction between 22% and 42% was observed for the TCC in the studied temperature range. In the literature, the kinetics of degradation of β-carotene depends on the product matrix. Thus, for β-carotene from pumpkin heat-treated in the temperature range 60 -100ºC, the *k* values ranged from 0.004 to 0.008 min^{-1}, with an estimated value for E_a of 27 kJ / mol^{-1} (Dutta *et al.*, 2006). For papaya β-carotene, the E_a value was 21 kJ/mol^{-1} (Ahmed *et al.*, 2002). For orange juice treated in the temperature range 50-90ºC, *k* values ranged from 0.02 and 0.57 min^{-1}, with an activation energy of 79 kJ / mol^{-1} (Meléndez-Martínez *et al.*, 2003).

Assessment of the effect of heat treatment on fluorescence intensities sour cherries extract

In **Figure 8** are given the emission spectra of sour cherries extract at excitation wavelengths of 270 nm and 340 nm. As it can be observed, sour cherries extract showed fluorescence with a single absorbtion maximum at λ_{max} = 320 nm when excited at 270 nm, and two absorbtion maxima at excitation of 340 nm, one with a lower intensity at λ_{max} of 386 nm, and the second at λ_{max} of 440 nm (**Figure 8**).

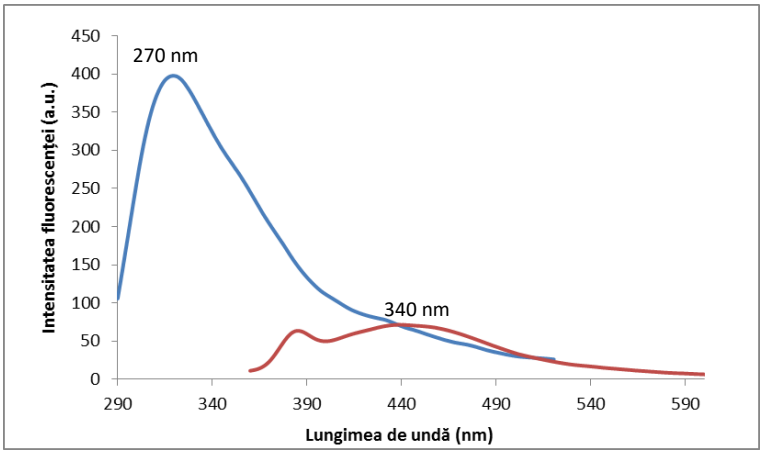


Figure 8. The emission spectra of sour cherries extract at different excitation wavelengths

Heat treatment caused structural changes leading to a decrease in fluorescence intensity. When excited at 270 nm, the fluorescence intensity decreased at temperature higher than 130°C, whereas the maximum emission was at 320 nm. At higher temperature, a secondary emission peak, around 375 nm was observed.

The effect of heating on TAC

Heating caused a significant decrease in TAC, varying between 42% and 96% at temperature at 100°C and 150°C, respectively.

The effect of heating on TFC

In case of TFC, an increase in flavonoid content with increasing temperature from 100°C to 160°C up to 30 min was observed, from 153.06±1.90 to 211.03±9.52 mg CE/100 g, respectively. However, after prolonged heating time, at temperature higher than 120°C, the TFC decreased.

The effect of heating on TPC

After 30 minutes at 120°C, the TPC decreased to198.32±1.03 mg GAE/100 g d.m., whereas heating at 160°C for 60 minutes lead to an increase in polyphenols concentration to 263.3±1.55 mg GAE/100 g d.m. The increase in TPC in sour cherries extract after heat-treatment is given either by the breaking of esterified and glycosylated bonds or may be due to the formation of Maillard compounds (Maillard *et al.*, 1996).

The effect of heating on antioxidant activity

Heating between 100–150°C for 60 minutes caused a decrease with 10-17% in antioxidant activity. After prolonged holding time, a gradual decrease in antioxidant activity was found; for example at 150°C, after 120 minutes a decrease by 30% was observed, whereas a significant decrease of 99% was found at 160°C. The first order kinetic model was used to quantitatively decribe the effect of heating on the measured paramteres. Ahmed *et al.* (2004) reported activation energy value of 37.48 kJ · mol^{-1} for the degradation of anthocyanins in plum puree at a temperature ranging from 50 to 90°C.

4.3.4. Selective references

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Chapter 5. Protein-anthocyanins and protein-carotenoids complexes

5.1. Evalutions on β-LG capacity to bind anthocyanins in model systems

5.1.1. Introduction

β-LG is a small globular, protein, composed of 162 amino acid residues with a molecular mass of 18,400 Da. The protein belongs to the lipocalin family due to its high capacity to bind hydrophobic low molecular ligands (Li *et al.* 2013). Harvey *et al.* (2007) suggested that β-LG containing three binding sites for hydrophobic molecules: the first is located in the central cavity, known as *calyx*, the second is located at the molecule surface from α-helix and the barrel, and the third is located at the interface betweem monomers.

The anthocyanins are a group of naturally occurring polyphenols, responsible for the attractive color of many fruits and products. The anthocyanins are of particular importance because of their antioxidant properties (Wang & Xu, 2007), but these compounds readily degrade when exposed to environmental factors such as light, pH, temperature, presence of oxygen and enzymes. Moreover, development of food colorants or other functional ingredients based on anthocyanins is limited due to their low stability under environmental conditions and interaction with other compounds from food matrices. Attempts to improve the bioavailability of polyphenols were made by using different matrices, such as β-LG (Gholami & Bordbar, 2014), bovine serum albumin, whey protein, etc.

5.1.2. Objectives

The aim of this study was to assess the interaction between anthocyanins from sour cherries extract and bovine β-LG based on quenching experiments.

5.1.3. Results and discussion

The interaction between β-LG and sour cherry extract was examined by investigation the influence of increased extract concentration on (un)-heat treated β-LG fluorescence intensity (25°C-100°C for 15 min). The fluorescence intensity was significantly influenced by increasing the concentration of ligand, at all tested temperatures.

The Stern-Volmer plots at each temperature were linear (data not shown). The lowest K_{SV} was calculated at 100°C (0.23 ± 0.03 × 106 M^{-1}), whereas the highest was found at 80°C (0.59 ± 0.15 × 106 M^{-1}), suggesting a higher Trp exposure as the temperature increases up to 80°C and a decrease at higher temperatures. It has been observed that K_{SV} values decreased with increased temperature, suggesting a static quenching mechanism.

The K_a values varied from 1.25±0.22 x 10^{14} M^{-1} la 25°C la 1.18±0.16 x 10^{14} M^{-1} la 100°C, whereas the binding number (*n*) had values lower than 1, suggesting that the weak binding of sour cherries extract to β-LG may be due to the presence of a multitudine of compounds in the extract, competing for the binding sites. However, an increase of *n* at 100°C, from 0.60 ± 0.11 to 0.88 ± 0.03 was observed, showing therefore an increased in affinity of β-LG for anthocyanins.

5.2. Evalutions on α-LA capacity to bind carotenoids in model systems

5.2.1. Introduction

Due to their structural particularities, milk proteins are used as encapsulating agents, being exploited as carriers for the hydrophobic molecules to transport biologically active compounds, therefore facilitating their functionality in various systems. Whey proteins have the ability to form complexes via covalent or electrostatic bonds with the different molecules of interest (Tavares *et al.*, 2014). Data from the literature refer to the ability of α-lactalbumin (α-LA) to interact with metal ions, co-solvents, surfactants, fatty acids and polyphenolic compounds. Recently, it has been demonstrated that the conformational changes induced by removal of Ca ions from the protein molecule facilitate the protein interaction

with oleic acid and palmitic (Barbana *et al*., 2008, 2006). Mohammadi & Moeeni (2015) suggested a high affinity of α-LA for kaempherol and genistein.

5.3.2. Objectives

The main aim of this study was to demonstrate the formation of complexes between carotenoids from sea buckthorn extract (CSE) and bovine α-LA based on quenching experiments.

5.2.3. Results and discussion

Quenching mechanism

The interaction between α-LA and CSE was examined by investigating the influence of increase in CSE concentration on protein intrinsic fluorescence intensity. Previously, the protein solutions were thermally treated in the temperature range of 25°C to 100°C for 15 minutes. The quenching process was independent of temperature, demonstrating that regardless of the temperature applied, the interaction between ligand and protein occured. The λ_{max} values of α-LA in the absence of the ligand was shifted from 326 nm at 25°C nm to 329 nm la 100°C, suggesting the exposure of the Trp residues at temperatures higher than 80°C. Under these conditions, the protein loses its compact subdomain structure containing hydrophobic residues. The red-shift is an indicator of the Trp microenvironment nature, the increase in temperature resulting in increased hydrophilicity in the vicinity of Trp. The same behavior was observed when ligands were used, suggesting hydrophilic interaction between the two components. K_{SV} values for CSE quenching were calculated at 50˚C (1.54±0.12 × 10^{-7} L mol^{-1}), and the highest at 100˚C (2.29±0.12× 10^{-7} L mol^{-1}), highlighting that the exposure degree of Trp residues increases with increasing temperature. The increases in quenching constant values at high temperatures suggest alteration of tertiary α-LA, with a greater exposure of Trp residues. The calculated *kq* values are higher than the maximum collision constant describing the interaction with different polymers, suggesting that the quenching process is initiated by a static mechanism.

Chapter 6: Comparative analysis of the ability of whey protein to bind biologically active compunds in various forms (unfolded, aggregates, etc) induced by altering different variables (pH, temperature, crosslinking)

6.3.1. Assessment of the thermostability of the complex formed between α-LA and CSE

6.3.1.1. Introduction

Adding bioactive ingredients in food systems are simple ways of developing functional foods that can provide physiological benefits and reduce the risk of some diseases (Tavares *et al*. 2014).

6.3.1.2. Objectives

The main aim of this study was to assess the thermostability of the complex formed between α-LA and CSE based on fluorescence spectroscopy and molecular modeling investigations.

6.3.1.3. Results and discussion

Due to the heat treatment, the unfolding of the protein may allow to carotenoids to bind to regions which are not available in the native state. **Figure 9** shows the structural changes of the complex formed between α-LA and CSE monitored by emission spectra. It can be seen that heating had a minor effect on Trp fluorescence, with two maxima at 60°C and 100°C, suggesting a maximum degree of exposure to solvent.

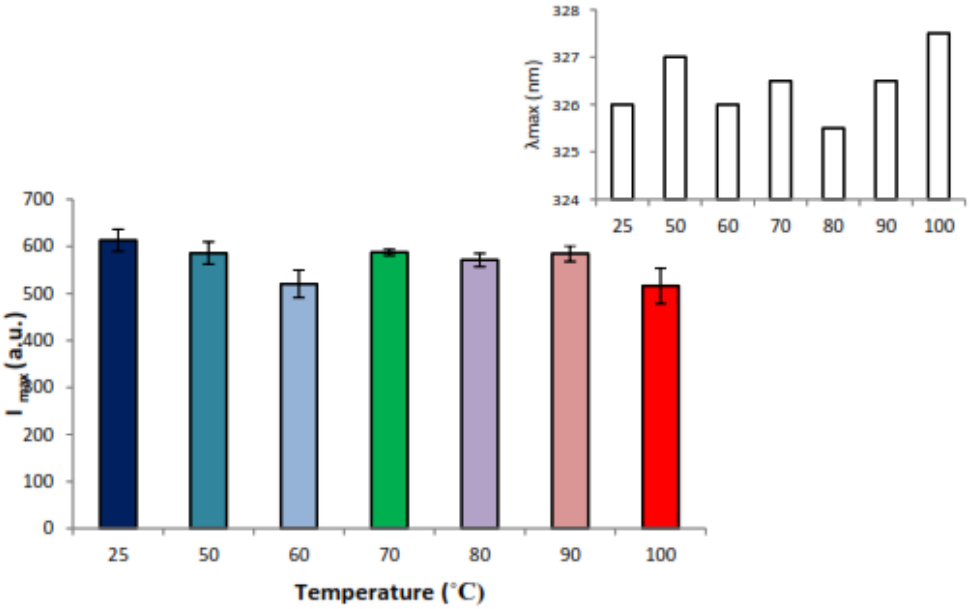

Figure 9. Heat induced structural changes of the α-LA-CSE complex monitored by fluorescence intensity. The excitation wavelength was 292 nm. Inset: The heat induced changes in λ_{max} (Dumitrașcu et al., 2016)

The molecular modeling suggested the fact that Trp exposure increased with increasing temperature. Therefore, the total accessible surface of Trp to solvent was 53.27Å2 at 60°C and 68.26Å2 at 90°C. The λ_{max} value for the un-treated complex was 326 nm. Stănciuc *et al.* (2012) studied the effect of pH and temperature on bovine α-LA structure and showed that at neutral pH, α-LA had a λ_{max} at 331 nm. The 5 nm blue-shift can be correlated with the addition of CSE, therefore blocking the Trp residues in the protein cavity. The heat-treatment leads to a 2 nm red-shift, suggesting an increase of hydrophilicity in the vicinity of Trp residues. Molecular modeling results showed that the binding of carotenoids did not occur near Trp residues.

Quenching experiments

These experiments were conducted in order to evaluate the conformational changes induced by heat treatment on the complex formed between α-LA and CSE. The titration experiments were conducted by sequentially addition of acrylamide or/and KI to heat treated complex at different temperatures. Acrylamide and KI are external quenchers usually used to investigate the accessibility of solvent and of Trp residues microenvironment polarity.

For acrylamide, the maximum value for K_{SV} (4.53 ± 0.08×10^{-2} mol^{-1} L) was calculated at 80°C, whereas the lowest value was found at 60°C (3.52±0.20×10^{-2} mol^{-1} L), suggesting that Trp residues have a higher accessibility to solvent at 80°C, and are less accesibile at 60°C. In case of quenching with KI, the lowest value was obtained at 25°C (1.42±0.05×10^{-2} mol^{-1} L) and the highest at 70°C (2.14±0.01×10^{-2} mol^{-1} L). The higher accessibility at 70°C is associated with an increased degree of exposure of Trp residues, followed by a decrease at higher temperatures, due to the folding of polypeptide chains. It was appreciated that the presence of sea buckthorn extract affected mostly the fluorescence of Trp^{118} and Trp^{104} residues.

3D Spectra

The three-dimensional spectroscopy was used in this study to get more information regarding the heat-induced changes in α-LA-CSE complex. The peak 1 (λ_{ex} = 280 nm, λ_{em} = 329 nm) refers to changes associated with the Trp and Tyr residues microenvironments, whereas peak 2 (λex = 230 nm, λem = 329 nm) reveals the spectral behavior of the polypeptidic chains, thereby given information about the protein secondary structure. The fluorescence intensity of the

peak 1 decreased by 30% between 25°C and 70°C, reaching a maximum at 100°C. These results suggested that the protein structure was modified without altering the microenvironment of Trp and Tyr residues.

6.3.1.4. Conclusions

This study has highlighted a number of interesting information regarding the possibility of obtaining bioactive ingredients by interaction between α- LA and CSE. Quenching studies have shown a greater accessibility for acrylamide and KI at 80°C and 70°C, respectively. The heat treatment favored a series of molecular events which have influenced the interaction between ligand and protein and the stability of the complex. These results can be used in food industry in terms of new formulations with high functionality.

6.3.2. Assessment of the thermostability of the complex formed between β-LG and CSE

6.3.2.1. Objective

The main aim of this study was to investigate the heat induced structural changes of the complex formed between ß-LG and CSE (ß-LG-CSE complex), in correlation with protein structural changes monitored by fluorescence spectroscopy. The used methods were extrinsic and intrinsic fluorescence, phase diagram, synchronous spectra, 3D spectra and quenching experiments with acrylamide and KI. Binding sites for ß-LG-CSE complex and its effect on conformational stability and the secondary structure of ß-LG were evaluated using quenching experiments with ß-carotene and molecular docking and molecular dynamics simulations.

6.3.2.3. Results and discussion

In the present study, in order to obtain a complete picture of the influence of thermal treatment on the intrinsic fluorescence of the complex, three excitation wavelengths were used: 274 nm, 280 nm and 292 nm. By excitation at 274 nm, 280 nm and 292 at 25°C, the peak emission of the corresponding β-LG hydrophobic residues were located at 336 nm, 340 nm and 327 nm, respectively.

The fluorescence intensity increased with 118% and 147% when the samples were excited at 280 nm and 292 nm, respectively. When excited at 280 nm, 25 nm blue-shifts have been observed in the temperature range of 50°C-80°C, followed by 3-5 nm red-shifts at higher temperatures. The decrease in fluorescence intensity at 100°C may be explained by the effect of heating on the unfolding properties of the protein (Borkar *et al*. 2012), which may favour aggregation throught intermolecular bonds (Rodrigues *et al.* 2015).

Red-shifts were registered in the whole temperature range, varying from 7 nm at 60°C to 17 nm at 100°C, when the complex was excited at 292 nm. From our results, it can be concluded that in the untreated states, Trp and Tyr residues are located inside of protein core, when λ_{max} is equal or lower than 330 nm and exposed to solvent when λ_{max} is higher than 330 nm. The changes observed in the emissive properties of ß-LG-CSE complex are associated with the structural changes occurring in the tertiary structure of ß-LG.

Quenching experiments

For acrylamide, the K_{SV} values slight increase in the temperature range of 25-70°C, followed by a significant increase at higher temperature, which can be attributed to the accessibility of Trp to acrylamide. The maximum value ($5.88\pm0.007\cdot10^{-3}$ mol^{-1} L) was calculated at 100°C, whereas the minimum value ($2.29\pm0.25\cdot10^{-3}$ mol^{-1} L) was estimated at 60°C. In case of quenching with KI, the minimum K_{SV} value was found at 25°C ($2.27\pm0.26\cdot10^{-3}$ mol^{-1} L) and the highest at 50°C ($4.18\pm0.44\cdot10^{-3}$ mol^{-1} L).

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Chapter 7. Evaluation of the composites functionality

7.1. Studies of the stability of the complexes in different environmental conditions in model system (pH changes, temperature, concentration of ions and carbohydrates etc)

7.1.1. Introduction

Nowadays, there is a high demand for foods with positive health effects, such as antidiabetic, anti-inflammatory, antioxidant properties, etc. These products should present a better alternative as dietary supplements in terms of safety, consumption, and delivery efficiency of bioactive compounds (Šaponjac *et al*., 2016). All the natural pigments are unstable when extracted from their natural environment. Natural phenolic compounds degrade easily during processing and storage due to their sensitivity to heat treatment, light and oxygen (Zhang *et al*., 2014). Additionally, the direct use of polyphenols in foods is limited due to their unpleasant taste. However, it was observed (Luca *et al*., 2014) that natural polyphenols are unstable at pH and enzymes, which significantly influences their health effects after oral administration. Encapsulation of polyphenols may be a viable alternative to improve their stability and solubility.

Gharsallaoui *et al.* (2007) defined micro-encapsulation as the technique in which a bioactive compound is encapsulated in a biopolymer, which protects it from oxygen, water, light and other agents, in order to improve its stability and also to change the presentation from the liquid to solid (powder) for ease of use. In the literature it has been reported that due to the interaction with proteins, polyphenols can be supplied in the lower part of the gastrointestinal tract, thereby improving their bioavailability, while retaining the anti-proliferative properties (Jakobek, 2015). The whey proteins are suitable matrices for encapsulation of anthocyanins. Given their structural and functional diversity, several strategies have been described for use as encapsulating agents (Tavares *et al.* 2014).

7.1.2. Objectives

The aim of the present study was to understand the feasibility of encapsulating of sour cherries anthocyanins extract (SCA) by using β-LG as encapsulating agent in various forms, using lyophilization as microencapsulation technique. The microparticles obtained were analyzed for anthocyanin content, encapsulation efficiency and color parameters. The microstructures of the particles were characterized by confocal microscopy. FT-IR spectroscopy was used as a complementary technique to study the changes in the secondary structure of the β-LG in all the experimental conditions. Microparticles functionality was tested as antioxidant activity, antimicrobial activity and *in vitro* digestibility.

7.1.4. Results and discussion

Encapsulation efficiency

β-LG has been studied extensively for its ability to bind hydrophobic and amphiphilic compounds such as flavor compounds, vitamins, fatty acids and polyphenols (Tavares *et al*. 2014). Encapsulation efficiency of the carrier material indicates its ability to seal and maintain the target molecule (Murali *et al*. 2015). All experimental samples contained anthocyanins of 0.13-0.14 CGE / g powder. Encapsulation efficiency (EE) was 54.14% for P1, 44.79% for P2 and 64.69% for P3, with a productivity of microencapsulation of 64.24%, 67.97% and 70.22%. Therefore, crosslinking reactions appear to improve EE of anthocyanins from sour cherries, while in the unfolded state (P2), β-LG showed the lowest value.

Color parameters

No significant differences (P> 0.05) were found in terms of *L* value between samples, suggesting that the encapsulating agent had no significant effect on surface color powders. This can be explained by the stability of the matrix during the freeze drying process due to adequate protection of basic materials, which increase the amount of encapsulated material on the surface of the particles. *a** values were characteristic for red color in P1, indicating that encapsulation of anthocyanins in the β-LG showed a greater amount of red compared to samples P2 and P3. The largest amount of yellow corresponded to P2. The P3 samples showed the lowest Hue value (0.67 ± 0.01), followed by P1 (0.80 ± 0.01) and P2 (0.90 ± 0.05). Therefore, all samples had values Hue closed to the pure red. The highest value for Chroma was determined for samples P1 and P3, and the lowest for P2.

Antioxidant activity

The SCA showed an antioxidant activity of 75.35±0.23% (480.23±0.15 μmol TE/g d.m.), whereas microencapsulation lead to a decrease in DPPH-RSA for P1 of 70.33±0.33%, 73.21±1.76% for P2, și 64.56±0.33% for P3.

In vitro digestibility

The microparticles digestion was favored in sample P1, with a maximum release of 31.27 ± 0.81%, whereas for P2 the digestion was limited to a release of maximum 8.20 ± 0.26% after 120 minutes. Therefore, these results showed that the heat treatment induced aggregation of β-LG molecules, which become less sensitive to peptic hydrolysis. The results obtained from stomach digestion were examined in intestinal phase, and showed that the anthocyanins were faster digested in P1 after 120 minutes when compared with P2 and P3.

7.1.5. Conclusions

The results show that β-lactoglobulin is a promising matrix for encapsulation of the sour cherry anthocyanins. Encapsulation efficiency ranged between 45 and 65%, with a maximum value recorded for the protein treated with transglutaminase. The antioxidant activity of the microparticles showed satisfactory values.

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Chapter 8: Obtaining a yogurt with functional composites (protein-carotenoids and/or protein-anthocyanins)

8.1. Testing various technological alternatives and selecting the optimal variant

8.1.1. Introduction

Fermented milk products are extremely popular worldwide due to their pleasant sensory characteristics and the potential to maintain and even to improve the consumer's health. Consumption of dairy products in general and fermented dairy products in particular has reached a new dimension in recent years due to the demonstrated beneficial effects on health. The correlations between consumption of yoghurt and proper functioning of the digestive, immune, and even circular systems are just some of the reasons that consumers worldwide are increasingly attracted by these foods.

8.1.2. Technology of yogurt with functional composites

The classical technology for yogurt involves the following steps:

- Qualitative and quantitative reception of milk
- Standardization
- Pasteurization
- Homogenization
- Cooling at the inoculum optimum temperature
- Inoculation
- Packaging
- Incubation
- Precooling
- Cooling
- Storage

The technological scheme used in the project for testing various alternatives for incorporating the microcapsules is protected by a patent application.

8.2. Other added-value products

The obtained results allowed us to develop 3 new products with added microencapsulated carotenoids as follow:

1) Desert dairy product based on whey protein concentrate and sea buckthorn and method for making it off. OSIM registration number A/00045/2017,
2) Appetizing biscuits with sea buckthorn extract obtained by extraction with supercritical fluids and appetizing biscuits with sea buckthorn extract micro-encapsulated in whey proteins and technologies for their production. OSIM registration number A/00289/2017 and

3) Muffins with sea buckthorn extract microencapsulated in whey protein and the technology of thereof. OSIM registration number A/00509/2017.

The development of these technologies is based on epidemiological evidence that carotenoid-rich diets are associated with a low risk of developing cancers, age-related macular degeneration, cataracts, inhibition of macrophage-mediated LDL oxidation (Perez-Galvez, Martin, Sies, & Wilhelm, 2003, Yeum, & Russell, 1999; Nishino *et al*., 1999, Keri *et al*., 1997). In order to obtain the added-value food products, the following raw materials and ingredients were used: whole rice flour, wheat flour, coconut butter, egg yolk, white sugar, cow's milk and microencapsulated powder, salt, baking powder. The described processes were simple, assuming the mixing of the above ingredients, the microencapsulated powder being added as an ingredient.

The technological schemes used in the project for testing various alternatives for incorporating the microencapsulated powders are protected by a patent application.