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Objective 2: Obtaining and characterization of sea buckthorn and sour cherries extracts

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

2.1.1. 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 nutritional 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).

2.1.2 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 flavonoids 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.

2.1.3. Results and discussion

Quantitative analysis of sea buckthorn extract compounds

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 9.64 -107.04 mg GA/g d.m.	Kumar <i>et al.</i> (2011) Korekar <i>et al.</i> (2014)
TFC	98±0.01 mg CE/ 100 g	345-854 mg/100 mg 6.79±0.30 mg rutin/g 14.14±1.12- 6.40±2.36 mg rutin/g d.m.	Yuzhen <i>et al.</i> (1997) Chauhan <i>et al.</i> (2012) Kumar <i>et al.</i> (2011)
Antioxidant activity	33.7±0.29%	94.7±3.2%	Papuc <i>et al.</i> (2008)
β-carotene	35.4±1.11 mg/100 g	1.5 – 18.5 mg/100 g 9.4-34.5 mg/100 g 0.53 – 0.97 mg/g d.m.	Andersson <i>et al.</i> (2009) Beveridge <i>et al.</i> (1999) Pop <i>et al.</i> (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 compounds

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
<i>Aspergillus niger</i> MIUG M5	29,5	22,5	26,5	23,72	10,16
<i>Penicillium expansum</i> MIUG M11	32,0	22,5	31,0	29,68	3,12

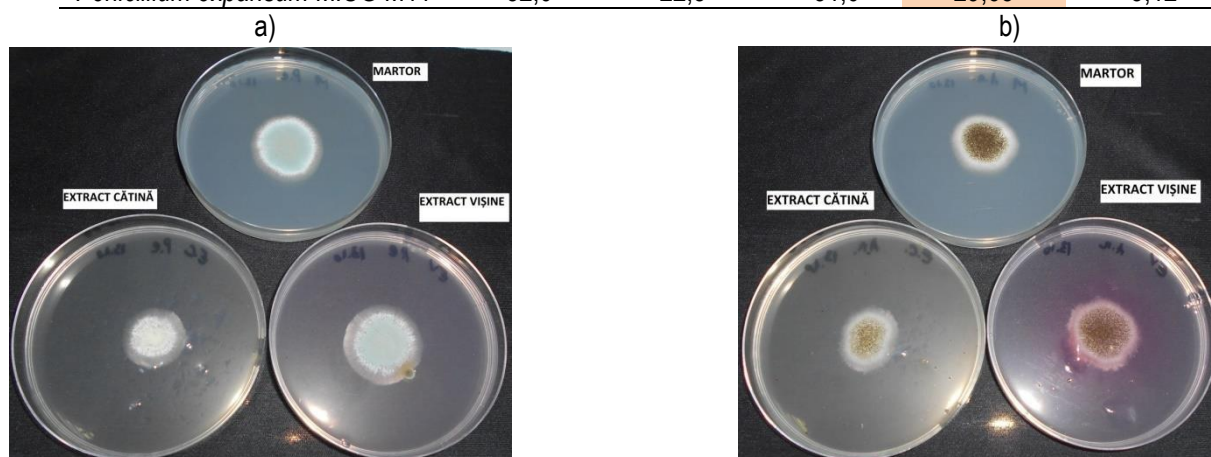


Figure 1. Antifungal activity of sea buckthorn and sour cherries extracts against *Penicillium expansum* MIUG M11 (a) and *Aspergillus niger* MIUG M5 (b)

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

2.2. Carotenoids and anthocyanins identification and quantification

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

Twelve compounds have been identified, as follows: astaxanthin (peak 1), zeaxanthin (peak 2), zeaxanthin-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 compounds 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.

2.3. Extracts purification and characterization of functionality

2.3.1. 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).

2.3.2. 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.

2.3.3. 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 multitude of fluorescent compounds in the extract.

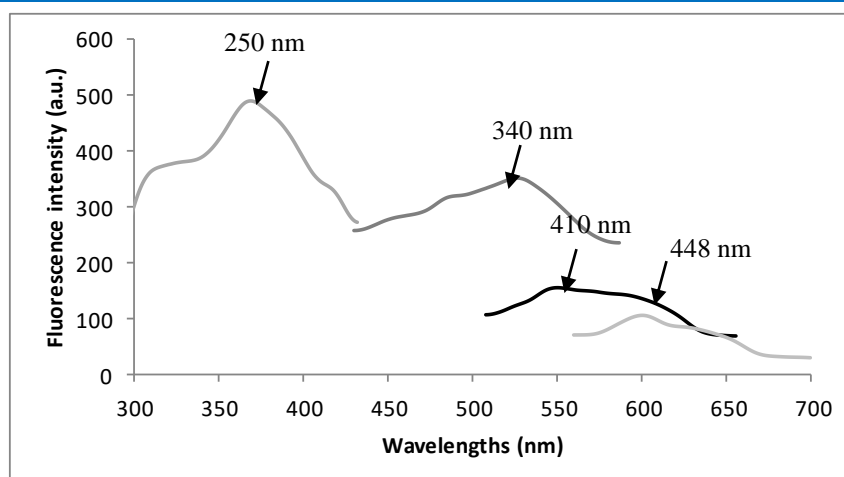


Figure 2. Fluorescence spectra of sea buckthorn extract at different excitation wavelengths. Three independent tests were carried out in each case and SD was lower than 3.5%. ([Ursache et al., manuscript submitted to Food Chemistry](#)).

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

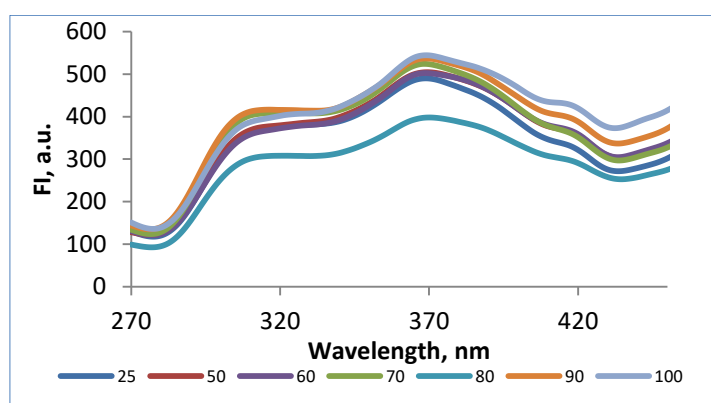


Figure 3. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 250 nm

The carotenoids extract treated at temperatures ranging 70-100°C 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.

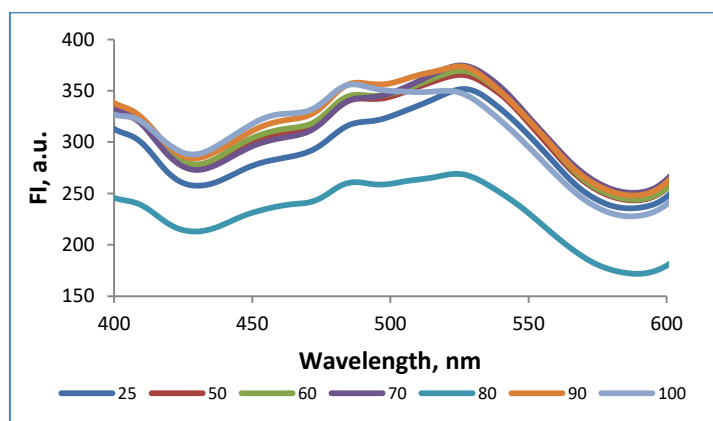


Figure 4. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 340 nm

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

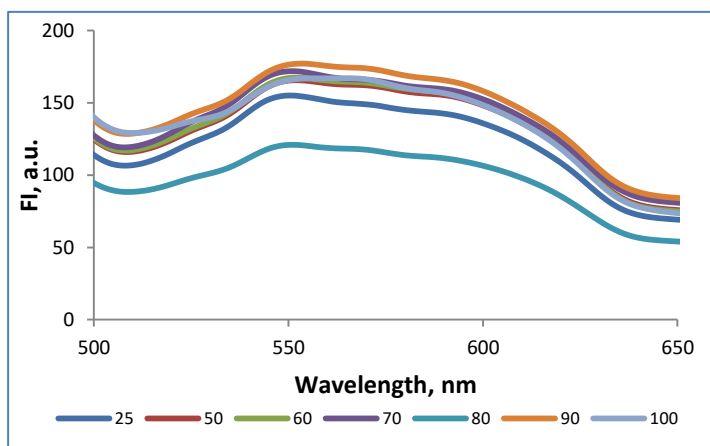


Figure 5. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 410 nm

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

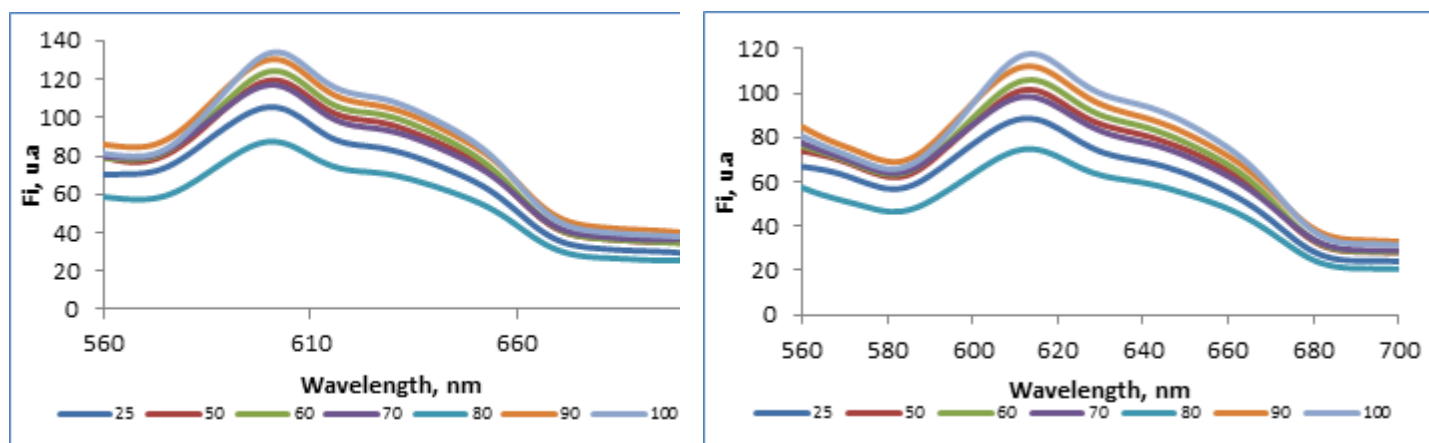


Figure 6. The emission spectra of the heat-treated carotenoids extract at the excitation wavelength of 448 nm (a) and 460 nm (b)

The lowest fluorescence 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 100°C. 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 abovementioned model.

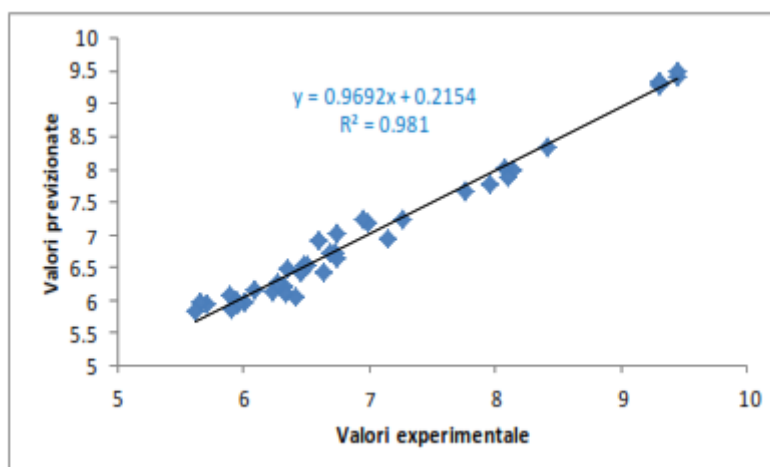


Figure 7. The correlation between the experimental and predicted values for the heat-induced changes in TCC according to the fractional conversion kinetic model

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 -100°C, 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 50-90°C, 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 absorption maximum at λ_{max} = 320 nm when excited at 270 nm, and two absorption 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).

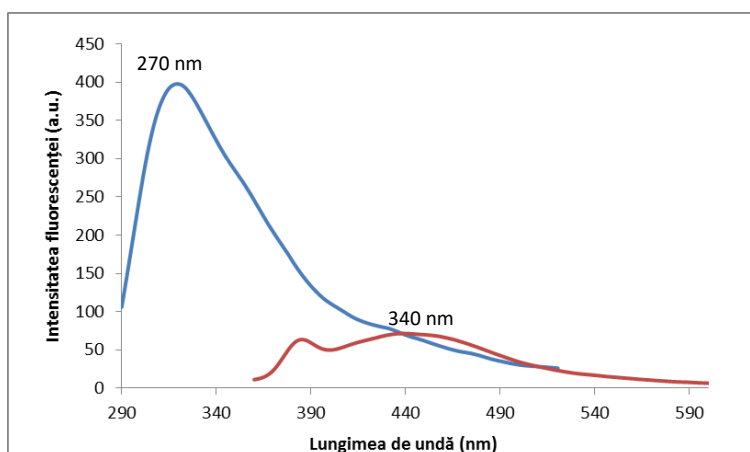


Figure 8. The emission spectra of sour cherries extract at different excitation wavelengths

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 to 198.32 ± 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 describe the effect of heating on the measured parameters. Ahmed *et al.* (2004) reported activation energy value of $37.48 \text{ kJ} \cdot \text{mol}^{-1}$ for the degradation of anthocyanins in plum puree at a temperature ranging from 50 to 90°C.

2.3.4. Selective references

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2.3.5. Project results

ISI papers:

Ursache, M.F., Ghinea, I.O., Turturică, M., Aprodu, I., Râpeanu, G., Stănciuc, N. Phytochemicals content and antioxidant properties of sea buckthorn (*Hippophae rhamnoides* L.) as affected by heat treatment – quantitative spectroscopic and kinetic approaches. Submitted to **Food Chemistry (FOODCHEM-D-16-00827R1)**

Oancea, A.M., Turturică, M., Bahrim, G., Râpeanu, G., Stănciuc, N. Polyphenols and antioxidant activity degradation kinetics during thermal treatments of sour cherry extract. Submitted to **LWT-Food Science and Technology (LWT-D-17-00090)**.

International conferences

Ursache, M.F., 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.

Oancea, A.M., Turturică, M., Râpeanu, G., Stănciuc, N. **2016**. Anthocyanins thermal degradation in sour cherry extract on fluorescence spectroscopy and kinetic modeling basis. 8th Congress Pigments in Food "Coloured food for health benefits", 28 June – 1 July, Cluj Napoca, Romania, poster presentation.

PhD student conferences

Oancea, A.M., Stănciuc, N., Râpeanu, G., Aprodu, I., Bahrim, G. **2016**. Thermal degradation of phytochemicals and the effect on the antioxidant activity of sour cherry. PhD student conference CSSD-UDJG, Dunărea de Jos University of Galați, Romania, 2nd-3rd of June 2016, poster presentation.

3. Protein-anthocyanins and protein-carotenoids complexes

3.1. Evaluations on β -LG capacity to bind anthocyanins in model systems

3.1.1. 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 between 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.

3.1.2. 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.

3.1.3. 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 ($0.23 \pm 0.03 \times 10^6 \text{ M}^{-1}$), whereas the highest was found at 80°C ($0.59 \pm 0.15 \times 10^6 \text{ M}^{-1}$), 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 $1.25 \pm 0.22 \times 10^{14} \text{ M}^{-1}$ la 25°C la $1.18 \pm 0.16 \times 10^{14} \text{ M}^{-1}$ 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 multitude 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.

3.2. Evaluations on α -LA capacity to bind carotenoids in model systems

3.2.1. 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 kaempferol and genistein.

3.3.2. 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.

3.2.3. 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 occurred. 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 at 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 ($1.54 \pm 0.12 \times 10^{-7} \text{ L mol}^{-1}$), and the highest at 100°C ($2.29 \pm 0.12 \times 10^{-7} \text{ L mol}^{-1}$), 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.

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

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

3.3.1.1. 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).

3.3.1.2. 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.

3.3.1.3. 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.

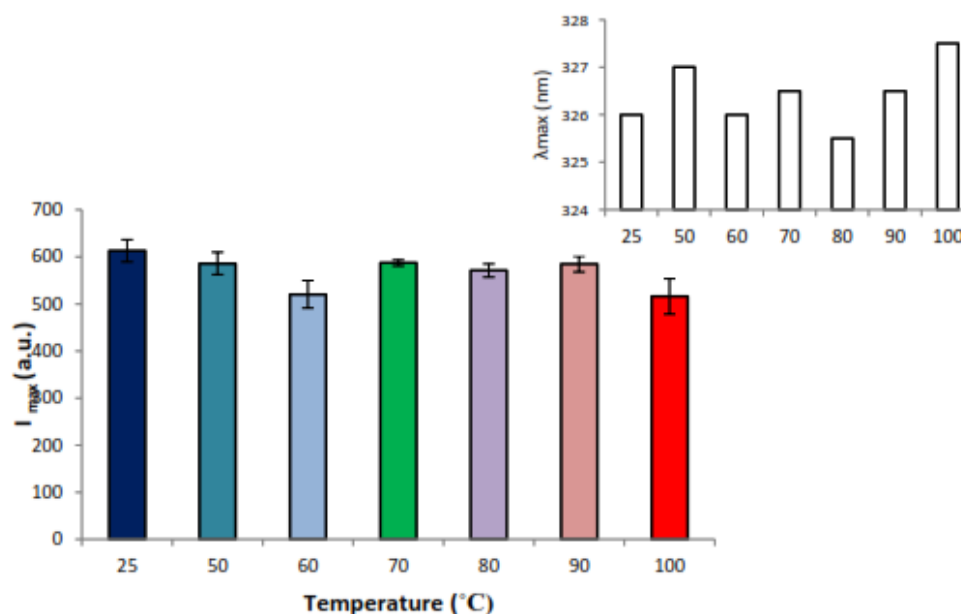


Figure 9. Heat induced structural changes of the α -LA-CSE complex monitored by fluorescence intensity. The excitation wavelength was 292 nm. Inset: The heat induced changes in λ_{max} (Dumitrașcu et al., 2016)

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 sequential 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} ($4.53 \pm 0.08 \times 10^{-2} \text{ mol}^{-1} \text{ L}$) was calculated at 80°C, whereas the lowest value was found at 60°C ($3.52 \pm 0.20 \times 10^{-2} \text{ mol}^{-1} \text{ L}$), suggesting that Trp residues have a higher accessibility to solvent at 80°C, and are less accessible at 60°C. In case of quenching with KI, the lowest value was obtained at 25°C ($1.42 \pm 0.05 \times 10^{-2} \text{ mol}^{-1} \text{ L}$) and the highest at 70°C ($2.14 \pm 0.01 \times 10^{-2} \text{ mol}^{-1} \text{ L}$). 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 Trp¹¹⁸ and Trp¹⁰⁴ 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 ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 329$ nm) refers to changes associated with the Trp and Tyr residues microenvironments, whereas peak 2 ($\lambda_{ex} = 230$ nm, $\lambda_{em} = 329$ 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.

3.3.1.4. 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.

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

3.3.2.1. Objective

The main aim of this study was to investigate the heat induced structural changes of the complex formed between β -LG and CSE (β -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 β -LG-CSE complex and its effect on conformational stability and the secondary structure of β -LG were evaluated using quenching experiments with β -carotene and molecular docking and molecular dynamics simulations.

3.3.2.3. 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 through 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 β -LG-CSE complex are associated with the structural changes occurring in the tertiary structure of β -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 ($5.88 \pm 0.007 \cdot 10^{-3} \text{ mol}^{-1} \text{ L}$) was calculated at 100°C, whereas the minimum value ($2.29 \pm 0.25 \cdot 10^{-3} \text{ mol}^{-1} \text{ L}$) was estimated at 60°C. In case of quenching with KI, the minimum K_{SV} value was found at 25°C ($2.27 \pm 0.26 \cdot 10^{-3} \text{ mol}^{-1} \text{ L}$) and the highest at 50°C ($4.18 \pm 0.44 \cdot 10^{-3} \text{ mol}^{-1} \text{ L}$).

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4. Evaluation of the composites functionality

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

4.1.1. 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).

4.1.2. 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.

4.1.4. 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 $75.35 \pm 0.23\%$ ($480.23 \pm 0.15 \mu\text{mol TE/g d.m.}$), whereas microencapsulation lead to a decrease in DPPH-RSA for P1 of $70.33 \pm 0.33\%$, $73.21 \pm 1.76\%$ for P2, și $64.56 \pm 0.33\%$ for P3.

In vitro digestibility

The microparticles digestion was favored in sample P1, with a maximum release of $31.27 \pm 0.81\%$, whereas for P2 the digestion was limited to a release of maximum $8.20 \pm 0.26\%$ 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.

4.1.5. 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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Project results

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5. Obtaining a yogurt with functional composites (protein-carotenoids and/or protein-anthocyanins)

5.1. Testing various technological alternatives and selecting the optimal variant

5.1.1. 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.

5.1.2. 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.