"Dunărea de Jos" University of Galați

Doctoral School of Fundamental Sciences and Engineering

PhD THESIS

Summary

Valorisation of biologically active compounds from sea buckthorn by developing value-added food products

PhD student,
Diana ROMAN

Scientific coordinator,
Univer. prof. PhD. ing. Gabriela RÂPEANU

Series I.7: FOOD ENGINEERING Nr. 21

GALAŢI

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CONTENT

Keywords: *Hippophae Rhamnoides L.,* microencapsulation, biologically active compounds, value-added products

Introduction

The concept of "functional foods" originated in Japan in the 1980s and has been practiced in many Asian countries to improve the general population's health. In 1991 the concept of Foods for Specified Health Use (FOSHU) emerged; there are currently over 100 foods recognized as FOSHU. Foods can be considered functional foods if, along with their basic nutritional impact, they also have beneficial effects on one or more body functions. They should improve general and physical health and/or reduce the risk of developing a disease. Food manufacturers and the pharmaceutical industry have become interested in this area. As a result, the so-called grey area has emerged, which describes the overlapping interests of the food and pharmaceutical industries.

Given the significant interest in developing foods with pronounced therapeutic effects, the "design" of functional foods has become widespread in world practice. Such products, not medicines, have a positive physiological impact on the human body. This effect is due to dietary fibers, oligosaccharides, amino acids, glycosides, organic acids, phospholipids, unsaturated fatty acids, minerals, vitamins, antioxidants, bifidobacteria, and enzymes of plant origin in the food. It can eliminate or reduce the negative impact on the human body of real factors such as pollution, stress, and other external factors. At the same time, not only vitamins and minerals, i.e., balanced vitamin-mineral complexes, but also a more comprehensive range of natural food components (natural pigments, oleoresins, biologically active compounds) to which the human body is genetically adapted and which are, therefore nutritional and health factors, are necessary for the normal functioning of the human body. In order to preserve human health, food should improve metabolism and increase the body's resistance to negative environmental influences. In this respect, it is topical to develop specialized products with a balanced composition, with therapeutic and prophylactic effects, considering the physiological needs of different age groups of the population.

The Ph.D. thesis titled **Valorisation of biologically active compounds from sea buckthorn by developing value-added food products** makes original contributions by designing food products supplemented with bioactive compounds from nature's plant resources that have great potential and can be exploited by humanity. The increased interest of scientists worldwide in valorizing plant material and extracts with biologically active substances comes with developing healthy foods by valorizing these compounds. This Ph.D. thesis highlights the potential of biologically active compounds extracted from sea buckthorn with antioxidant effects for the human body and the possibility of obtaining bioingredients with various functions and uses. The study focused on analyzing the phytochemical profile of extracts from sea buckthorn fruits, from which ingredients in the form of powders with antioxidant potential were developed as potential substitutes for artificial colorings in the food industry.

The basis of this work was the curiosity about the phytochemical composition of sea buckthorn fruit, i.e., the lipophilic and hydrophilic compounds in sea buckthorn fruit, and the possibility of exploiting its potential by creating ingredients that can be used in the food industry. Considering that sea buckthorn is a plant that easily adapts to weather, climate, and soil conditions, it would be regrettable for humanity if it were not exploited and used in various branches of the processing industries and beyond. Nature allows us to use all its resources; humans must make the most of them without harming ourselves.

The main **scientific objectives** of the Ph.D. thesis are:

- Comparative evaluation of the phytochemical profile of sea buckthorn fruit extracts obtained by different extraction techniques and correlation of the results with the antioxidant activity of the extracts.
- Microencapsulation of biologically active compounds in different matrices and obtaining value-added ingredients
- Characterization of the obtained ingredients and evaluation of their stability over nine months
- Designing a value-added product with the addition of buckthorn extract powder and characterization of the product from a phytochemical, physicochemical, and sensory point of view.

The PhD thesis is structured in two parts, namely:

i. **The DOCUMENTARY STUDY**, entitled **"Theoretical Aspects of the Functionality of Biologically Active Compounds in Fruit, Leaves, and Seeds of Sea Buckthorn"** has three chapters in which theoretical considerations from articles on the importance of sea buckthorn for humanity and health, extraction, and encapsulation methods and applications of the ingredients obtained in different fields such as food, feed, and cosmetic industry have been systematized.

ii. **EXPERIMENTAL STUDY**, entitled **"Research on the valorization of biologically active compounds in sea buckthorn fruits: Extraction, phytochemical characterization, encapsulation and prospects for use in the food industry"** comprises three chapters, in which the results obtained during the Ph.D. study are presented. The summary of the chapters is as follows:

Chapter 4, entitled **Comparative evaluation of extraction methods with different solvents to obtain extracts rich in biologically active compounds from sea buckthorn fruits,** presents the results obtained using different extraction methods, identification and quantification of biologically active compounds in sea buckthorn (carotenes, polyphenols, and flavonoids) and antioxidant activity using spectrophotometric methods and liquid chromatography (HPLC) techniques.

Chapter 5, entitled **Development of ingredients rich in bioactive compounds extracted with polar solvents from sea buckthorn for food industry applications**, represents the results obtained by encapsulating in different matrices the biologically active compounds extracted from sea buckthorn, such as carotenoids and polyphenols, characterizing the powders obtained from phytochemical, structural, colorimetric, in vitro digestibility and storage stability points of view.

Chapter 6, entitled **Utilization of encapsulated ingredients based on carotenoids extracted from sea buckthorn to obtain value-added mayonnaise sauce** shows the results obtained by developing patent technology for the preparation of value-added mayonnaise sauce with enhanced functionality due to the replacement of preservatives and colorants.

Each chapter of the experimental study includes *Introduction, Objectives of the study, Materials and methods of analysis, Results and discussion, Partial conclusions and Bibliography.*

Chapter 7, Final Conclusions, presents the conclusions of the experiments conducted.

Chapter 8, Personal Contributions and Prospects for Further Study, presents personal contributions and prospects for the continuation of the thesis.

Chapter 9, Valorisation of results, presents a list of publications and national and international conference participations. The research results have been highlighted by the publication of **3 scientific articles** published in ISI-indexed journals (Polymers, The Annals of the University Dunarea de Jos of Galati Fascicle VI - Food Technology, Inventions), as well as **10 communications at scientific** events representative for the field of food engineering. A patent application has been filled for a value-added product (**Mayonnaise-based sauce with added microencapsulated buckthorn extract powder**).

The Ph.D. thesis has 163 pages, including 44 figures,17 tables, and 3 annexes. The documentary study represents 25%, and the experimental part 75%.

The experimental part of the thesis was made possible thanks to the infrastructure of the Integrated Research, Expertise and Technology Transfer Centre (BioAlimentTehnIA) (www.biolaiment.ugal.ro), within the Faculty of Food Science and Engineering, "Dunărea de Jos" University of Galati.

The thesis was carried out under the scientific coordination of the following committee:

- Prof. univ. dr. eng. Gabriela RÂPEANU- PhD supervisor
- Prof. univ. dr. eng. Nicoleta STĂNCIUC- scientific coordinator
- Prof. univ. dr. eng. Gabriela Elena BAHRIM- scientific coordinator
- Prof. univ. dr. ing Iuliana APRODU- scientific coordinator

CHAPTER 4. COMPARATIVE EVALUATION OF DIFFERENT SOLVENT EXTRACTION METHODS FOR OBTAINING EXTRACTS RICH IN BIOLOGICALLY ACTIVE COMPOUNDS FROM SEA BUCKTHORN FRUITS

4.1. General aspects

Sea buckthorn (*Hippophae rhamnoides L*.), also named river buckthorn, belongs to the Order Eleagnales, family Elaeagnaceae, and is a plant native to Europe and Asia, which is now distributed worldwide (Górnaś et al., 2014; Michel et al., 2012). Fruits harvested from these plants are an important source of biologically active compounds such as carotenoids: α and β-carotene, lycopene, lutein and zeaxanthin (Pop et al., 2014), flavones such as isorhamnetin, quercetin, kaempferol (Guo et al., 2017), vitamins (vitamins C and E), organic acids, amino acids, micro- and macro-nutrients, and are considered fruits with high industrial potential (Asofiei et al., 2019).

4.3. Study objectives

In the first chapter of the experimental study the scientific objectives were as follows:

- Comparison of the results obtained by three methods of extraction of biologically active compounds from freeze-dried sea buckthorn fruits (conventional solvent extraction, ultrasound-assisted solvent extraction, microwave-assisted solvent extraction).
- Comparison of sequential and separate extraction results and evaluation of bioactive compounds content and antioxidant activity.
- Evaluation of the content of biologically active compounds in sea buckthorn, such as: total carotenoid content, β-carotene content, lycopene content, total polyphenol content, total flavonoid content and antioxidant capacity of the extracts obtained by the three proposed methods, using spectrophotometric methods of analysis.

4.4. Materials and methods of analysis

4.4.3. Materials used

The sea buckthorn was purchased from a local market in the Galati area. The buckthorn berries were washed with distilled water and dried with a paper towel until completely dry, then frozen and freeze-dried at -42 °C pressure of 0.1 mBar for 48 hours using a CHRIST Alpha 1-4 LD Plus freeze-dryer (Germany).

4.5. Comparative analysis of extraction methods of bioactive compounds from sea buckthorn fruits

In order to enhance the content of bioactive compounds in sea buckthorn fruits, it was decided to develop, integrate and implement three extraction methods (ultrasound-assisted extraction (UAE), microwave-assisted extraction (MAE), conventional extraction (ECS)), with polar (ethanol, acetone, ethyl acetate, formic acid and ultrapure water) and non-polar (hexane) extraction solvents of hydrophilic or lipophilic nature (sunflower oil), as shown in Table 4.1.

The selection of the appropriate solvent considered the polarity of the solvents, the sample matrix and its components, as well as the moisture content which plays an important role, and all samples were freeze-dried to exclude this variable.

The three extraction methods proposed for research: UAE, MAE and ECS are known to obtain extracts rich in biologically active compounds.

Tabel 4.1. Characteristics of the extraction methods

Sample code	Solvents for extractions	Parameters
		Time, minutes(seconds,hours) / Temperature, °C / Frequency, KHz/ Power, W
	UAE	
UAE1	Ethanol-96%	
UAE2	**Ethanol-acetone (4:3)**	
UAE3	Ehtanol:Hexane:Acetone(4:3:1)	
UAE4	**Ethyl acetate:Hexane (2:1)**	
UAE5	Ethyl acetate :Hexane (1:2)	
UAE6	Hexane:Acetone (2:1)	45 minutes / 40 °C/ 40 KHz / 100 W
UAE7	Acetone-80%:Ethanol-70%(1:1)	
UAE8	Ethanol 70%	
UAE9	Sunflower oil	15 minutes / 40 °C/ 40KHz / 100 W
UAE10		30 minutes / 40 °C/ 40KHz / 100 W
UAE11		45 minutes / 40 °C/ 40KHz / 100 W
	MAE	
MAE1	Ethanol-70%	15 seconds / 47÷61 °C / 420W
MAE2	Sunflower oil	30 seconds / 70.6 °C / 1050 W
MAE3		40 seconds / 47.5 °C / 735 W
MAE4		30 seconds / 54.2 °C / 525 W
	MAE-UAE	
MAE-UAE1	Sunflower oil	UAE - 15 minutes / 40-50 °C / 40 KHz /100 W MAE - 30 secondes / 42.1°C / 525W
	ECS	
ECS1	Ethanol-70%	48h / 40°C

MAE is a well known modern extraction technique, but it involves higher costs than UAE, many researchers over the years have optimised this extraction method..

To perform the ultrasound-assisted extraction we monitored the main parameters such as temperature, time and power of the ultrasound installation.

An experiment was also conducted by combining UAE with MAE on the same matrix, using sunflower oil.

It was decided to carry out two extraction methods: separate and sequential, in order to compare the results of the extractions from a phytochemical point of view. Sequential extractions were performed with different solvent mixtures using the same lyophilized matrix as a source of biologically active compounds. The residue that was obtained after the first extraction with ethyl acetate:hexane mixture (2:1), was further extracted sequentially with 20 mL ethanol:hexane

solvent mixture (4:3), 20 mL formic acid:acetone:water solvent mixture (0.35:20:80) and finally extraction with water - 20 mL. After adding the solvent over the initial matrix, EAU extraction was applied each time using the parameters in Table 4.2.

Tabel 4.2. Sample codification according to ultrasound-assisted sequential and separate extraction methods

Code extract	Solvenți utilizați pentru extracții	Extraction parameters
		Time,minutes(seconds,hours)/Temperature, °C/Frequency, KHz/Power, W
SqUAE1 SeUAE1	Ethyl acetat : hexane(2:1) =2:1	20 minutes / 40 °C / 40 KHz
SqUAE2 SeUAE2	Ethanol: Hexane(4:3) = 4:3	20 minutes / 40 °C / 40 KHz
SqUAE3 SeUAE3	**Formic Acide:Acetone:Water (0,35:20:80) = 0,35:20:80**	**20 minutes / 40 °C / 40 KHz**
SqUAE4 SeUAE4	Water	20 minutes / 50 °C / 40 KHz

The liquid part obtained from each extraction step was collected and concentrated under vacuum for subsequent phytochemical and antioxidant analysis. To compare the samples, a separate extraction was also performed under the same conditions and repeated four times.

The aim of this study was to compare the results of sequential extraction and separate extraction through the evaluation of bioactive compounds content and antioxidant activity in freeze-dried sea buckthorn fruits.

4.8. Results and discussion

4.8.1. Comparative phytochemical analysis of sea buckthorn extracts by different extraction methods

For the phytochemical analysis of the extracts obtained, a combination of three methods was used: conventional solvent extraction, ultrasound-assisted extraction and microwave-assisted extraction. A variation of extraction parameters as well as solvents used was performed. The values of the phytochemical components present in the freeze-dried sea buckthorn fruits, analysed and obtained by different extraction methods, are presented in Table 4.3

Statistically significant differences were observed in the selected samples for most of the extraction methods used ($p<0.05$). The extract samples with higher values of bioactive compounds content were UAE3-UAE6. These results may be due to the presence of hexane in the extraction mixture. The combination of a polar solvent (ethanol, acetone in UAE3; ethyl acetate in UAE4 and UAE5; acetone in UAE6) with a non-polar solvent (hexane) in different proportions leads to improved solubilisation of non-polar carotenoids (lycopene and β-carotene) which have a more hydrophobic nature and limited solubility in water (Strati and Oreopoulou, 2011a).

The most suitable results for total carotenoid, β-carotene and lycopene content were obtained for the extraction of UAE4 with the solvent mixture ethyl acetate:hexane (2:1). The

results were due to the polarity of the solvent mixture and the influence of temperature, which allowed solubilization of non-polar carotenoids.

Table 4.3. Phytochemical characterisation of extracts obtained by different extraction methods

Extract cod	Total carotenoids, µg/g d.w.	β-carotene, µg/g d.w.	Lycopene, µg/g d.w.	TPC, mg EAG/g d.w.	TFC, mg CE/g d.w.
UAE1	4,57±0,01^{e}	3,84±0,01^{e}	1,15±0,00^{f}	148,19±0,38^{f}	182,44±0,62^{b}
UAE2	25,08±0,03^{b}	21,01±0,01^{b}	5,97±0,02^{b}	**805,34±5,5^{a}**	**211,65±0,75^{a}**
UAE3	18,14±0,03^{d}	15,12±0,04^{d}	4,17±0,01^{e}	484,67±6,9^{b}	NA
UAE4	**42,43±0,17^{a}**	**35,35±0,06^{a}**	**9,82±0,03^{a}**	61,57±1,76^{g}	NA
UAE5	20,89±0,09^{c}	17,33±0,08^{c}	4,32±0,06^{d}	178,61±9,3^{e}	NA
UAE6	25,20±0,08^{b}	21,17±0,29^{b}	5,52±0,05^{c}	258,03±6,7^{d}	NA
UAE7	0,91±0,01^{f}	0,71±0,02^{f}	0,35±0,01^{g}	339,4±1,05^{c}	72,27±0,62^{f}
UAE8	1,09±0,01^{f}	0,87±0,01^{f}	0,43±0,00^{g}	182,67±0,85^{e}	87,98±4,2^{d}
UAE9	0,23±0,02^{g}	0,20±0,02^{g}	0,05±0,00^{h}	NA	NA
UAE10	0,24±0,03^{g}	0,20±0,03^{g}	0,05±0,01^{h}	NA	NA
UAE11	0,24±0,01^{g}	0,21±0,01^{g}	0,06±0,00^{h}	NA	NA
MAE1	0,97±0,02^{f}	0,77±0,015^{f}	0,38±0,01^{g}	145,04±1,35^{f}	100,16±16,14^{c}
MAE2	0,27±0,02^{g}	0,23±0,019^{g}	0,07±0,01^{h}	NA	NA
MAE3	0,27±0,01^{g}	0,23±0,01^{g}	0,06±0,00^{h}	NA	NA
MAE4	0,22±0,00^{g}	0,18±0,01^{g}	0,05±0,00^{h}	NA	NA
UAE-MAE1	0,22±0,00^{g}	0,20±0,01^{g}	0,05±0,00^{h}	NA	NA
ECS1	1,09±0,02^{f}	0,84±0,02^{f}	0,43±0,01^{g}	149,80±0,86^{f}	79,12±0,51^{e}

Means in each column that do not share a letter in the exponent are significantly different by Tukey's test, $p<0.05$; NA - sample not analyzed or not applicable

At the other extreme is sunflower oil extraction. For both MAE and UAE extraction, the results obtained using sunflower oil as extraction system showed very low extraction efficiency, indicating that vegetable oils are not suitable as solvents for the separation of lipophilic compounds.

With regard to carotenoid extraction using ECS with 70% ethanol, a low carotenoid content of 1.09 ± 0.02 µg TC/g d.w. was obtained..

The total carotenoid content of extracts obtained by the MAE2-MAE4 methods had similar values. Although microwave-assisted extraction (MAE) is considered a simple, fast and economical method for carotenoid extraction, involving a short extraction time with a small amount of solvent, the oil extraction system was not efficient compared to the solvent system used for UAE.

The results obtained for UAE showed a simpler composition of the extraction solution, an average yield of carotenoid content that was higher in all samples compared to ECS. Therefore, UAE demonstrated to be a simple, efficient, fast, low cost and reliable alternative method for the extraction of these bioactive compounds from the studied matrix or other similar matrices.

The content of phenolic compounds in the extracts analysed is shown in Table 4.3. As expected, the extract or extract mixture in which ethanol is present were generally rich in phenolic compounds. The UAE2 protocol in which the solvent mixture of ethanol:acetone (4:3) was used was the most suitable for the extraction of total polyphenols from sea buckthorn. High values of

805.34 ± 5.5 mg EAG/g d.w. were obtained, probably because both solvents are polar and miscible with water. At the opposite pole is protocol UAE4 in which a mixture of ethyl acetate and hexane (2:1) was used with a total polyphenol content of 61.57 ± 1.76 mg EAG/g d.w.

The highest value for total flavonoid content was 211.65 ± 0.75 mg EC/g d.w. for ultrasound-assisted extraction combined with an ethanol:acetone solvent mixture (UAE2). The lowest value of 72.27 ± 0.62 mg EC/g d.w. was obtained with the ethanol:acetone solvent mixture (UAE7) and is 66.0% lower compared to the most efficient extraction in this extraction series (UAE2).

4.8.2. Comparative phytochemical analysis of sea buckthorn extracts by the EAU method using two different extraction techniques

Another objective of this study was to perform a sequential extraction on the sea buckthorn fruit matrix, based on the concept that the fruit itself is a mixture of lipid- and water-soluble compounds, and stepwise extractions are required to lyse the cell membrane.

Sequential extractions were also carried out on the same matrix by applying different solvent mixtures and the results obtained were compared with separate extractions. The results obtained are shown in Table 4.4, and in Figures 4.2, 4.3 and 4.4.

Table 4.4. Determination of carotenoid compounds in sequential and separate extracts of sea buckthorn fruits

Extract cod	Total carotenes, µg/g d.w.	β-carotene, µg/g d.w.	Lycopene, µg/g d.w.
SeUAE1	**57,81±0,06^{a}**	**47,96±0,04^{a}**	**11,97±0,01^{a}**
SeUAE2	25,94±0,03^{c}	21,54±0,03^{c}	5,30±0,03^{c}
SeUAE3	3,85±0,06^{e}	3,33±0,04^{e}	2,14±0,02^{e}
SeUAE4	3,73±0,05^{e}	3,36±0,04^{e}	2,31±0,03^{e}
SqUAE1	29,05±0,04^{b}	24,19±0,03^{b}	6,41±0,01^{b}
SqUAE2	12,80±0,05^{d}	10,62±0,02^{d}	2,59±0,02^{d}
SqUAE3	3,30±0,03^{e}	2,80±0,02^{f}	1,70±0,01^{f}
SqUAE4	1,61±0,02^{f}	1,39±0,02^{g}	0,87±0,02^{g}

Means in each column that do not share a letter in the exponent are significantly different by Tukey's test, $p < 0.05$; NA - sample not analyzed or not applicable

As expected, the total content of carotenoids, β-carotene and lycopene decreased in both subsequent and separate extractions due to decreased solvent polarity. From Table 4.4 it is evident that the separate extraction had a higher content of carotenoid compounds because the polar solvent mix used resulted in the maximum possible extraction of total carotenes.

From Table 4.4 it can be concluded that the total carotenoid content is high for both the separate and subsequent extraction for the samples treated with ethyl acetate:n-hexane mixture (2:1), respectively 57.81±0.06 µg TC/g d.w. and 29,05±0,04 µg TC/g d.w..

From Figure 4.3, it can be seen that the total polyphenol content increases with sequential extraction, particularly when using the acidic solvent mixture. Thus, the highest polyphenol content was 823.51 ± 5.5 mg EAG/g d.w., and was obtained for aqueous extraction using acetone acidulated with formic acid (SqEAU3) after 20 minutes of extraction at 35-40°C. The developed method is rapid, simple, mild and efficient for the extraction of polyphenolic compounds.

The lowest polyphenol content with 83% less in the sequential extraction series was obtained with the ethanol:hexane solvent mixture (SqUAE2), with values of 143.49 ± 3.2 mg EAG/g d.w.

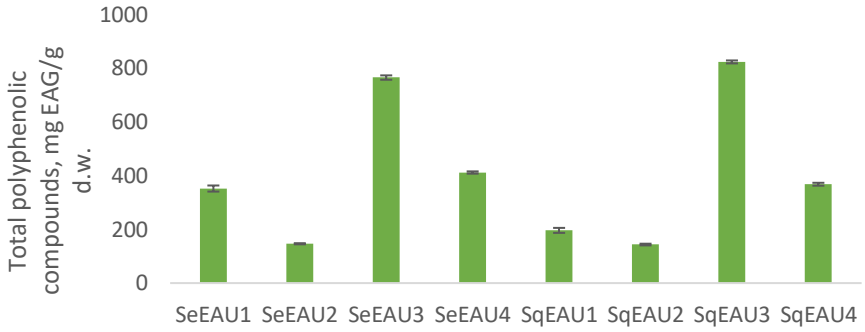


Figure 4.3. Content of total polyphenolic compounds for the analysed samples

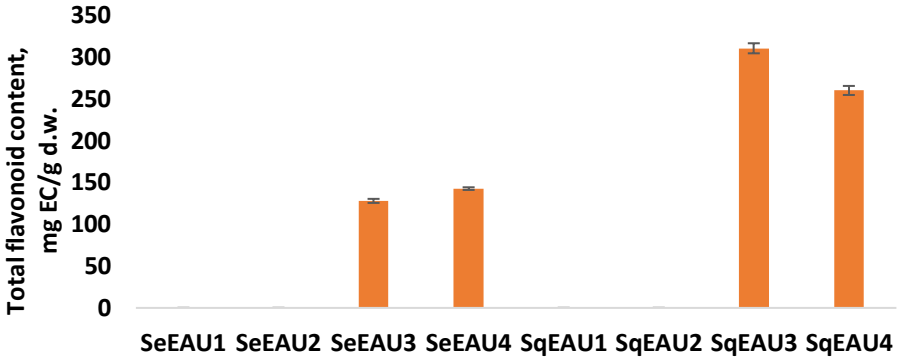


Figura 4.4. Total flavonoid content for samples analysed

In comparison, as revealed by the data shown in Figure 4.4, SqUAE2 aqueous mixture with acidified acetone proved to be the most efficient solvent mixture for the extraction of total flavonoids which showed a value of 310.06 ± 6.00 mg EC/g d.w.

4.8.3. Comparative evaluation of the antioxidant activity of extracts

The antioxidant activity in this study was influenced by several parameters such as: hydrophilic/lipophilic extraction medium, extraction time, etc.

Table 4.5 shows the results of the antioxidant activity of extracts obtained with the method using the free radical DPPH.

The most suitable results were obtained by ultrasound-assisted extraction in combination with the solvent mixture ethanol : acetone.

The highest value of antioxidant activity of 2.15 mM Trolox/g d.w. was obtained using the solvent mixture ethanol:acetone(4:3) - UAE2, this extract also showed an inhibition on the free radical DPPH of 90.75%.

The lowest value of antioxidant activity was obtained with the mixture of ethyl acetate:hexane (1:2) - UAE5 and showed an inhibition of only 1.07 ± 0.05% on DPPH free radicals.

Table 4.5. Antioxidant activity (DPPH) of samples obtained by different extraction methods

Extract cod	AA, mM Trolox/g d.w. (DPPH)	I_{DPPH},(%)
UAE1	1,81±0,01^{b}	76,80±0,47^{b}
UAE2	**2,15±0,01^{a}**	**90,75±0,22^{a}**
UAE3	1,09±0,01^{d}	46,66±0,33^{c}
UAE4	0,59±0,01^{e}	22,21±0,28^{d}
UAE5	0,01±0,00^{f}	1,07±0,05^{e}
UAE6	0,03±0,01^{f}	2,01±0,26^{e}
UAE7	1,84±0,00^{b}	89,56±0,16^{a}
UAE8	1,56±0,01^{c}	75,97±0,61^{b}
UAE9	NA	NA
UAE10	NA	NA
UAE11	NA	NA
MAE1	1,56±0,00^{c}	76,24±0,19^{b}
MAE2	NA	NA
MAE3	NA	NA
MAE4	NA	NA
MAE - UAE1	NA	NA
ECS1	1,54±0,01^{c}	75,21±0,38^{b}

Means in each column not sharing a letter in the exponent are significantly different by Tukey's test, $p < 0.05$NA - sample not analyzed or not applicable

Figure 4.5 and Figure 4.6 show the antioxidant activity values for samples obtained by different extraction methods (ABTS radical method).

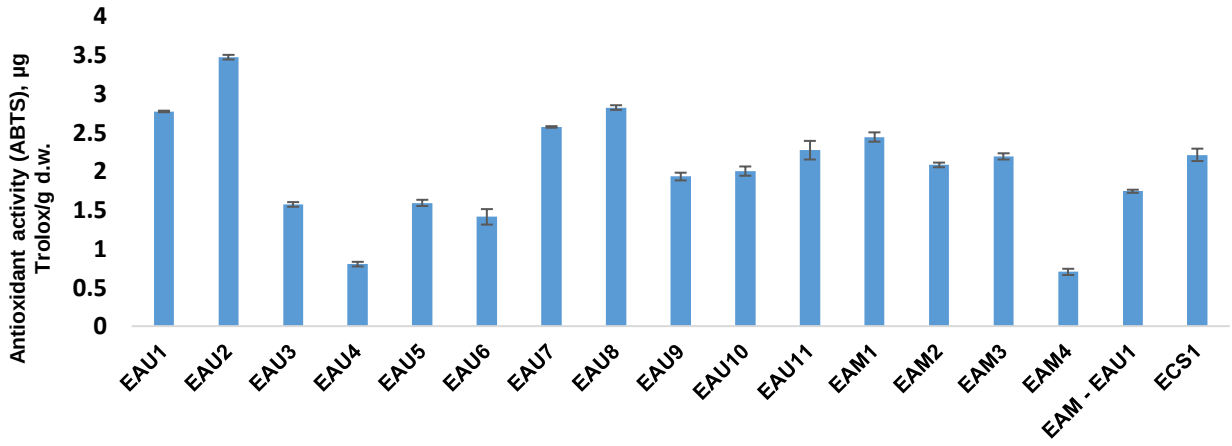


Figure 4.5. Antioxidant activity (ABTS) for samples obtained by different extraction methods

From Figure 4.5 it can be assumed that the antioxidant activity is not influenced by the extraction method, but probably the solvents used influence the antioxidant activity. Thus samples UAE1, UAE8, MAE1 and ECS1 obtained AA values by the ABTS free radical scavenging method of 2.77 µg Trolox/g; 2.88 µg Trolox/g; 2.44 µg Trolox/g and 2.21 µg Trolox/g respectively where alcoholic solutions were used for extraction.

Analysing the data in Figure 4.5 it can be seen that the highest value of antioxidant activity of 3.47 µg Trolox/g d.w. was obtained when the solvent mixture ethanol:acetone in the ratio of 4:3

- UAE2 was used for extraction. Figure 4.6 shows that this extract obtained from sea buckthorn fruit showed an inhibition on ABTS radical of about 85.01%.

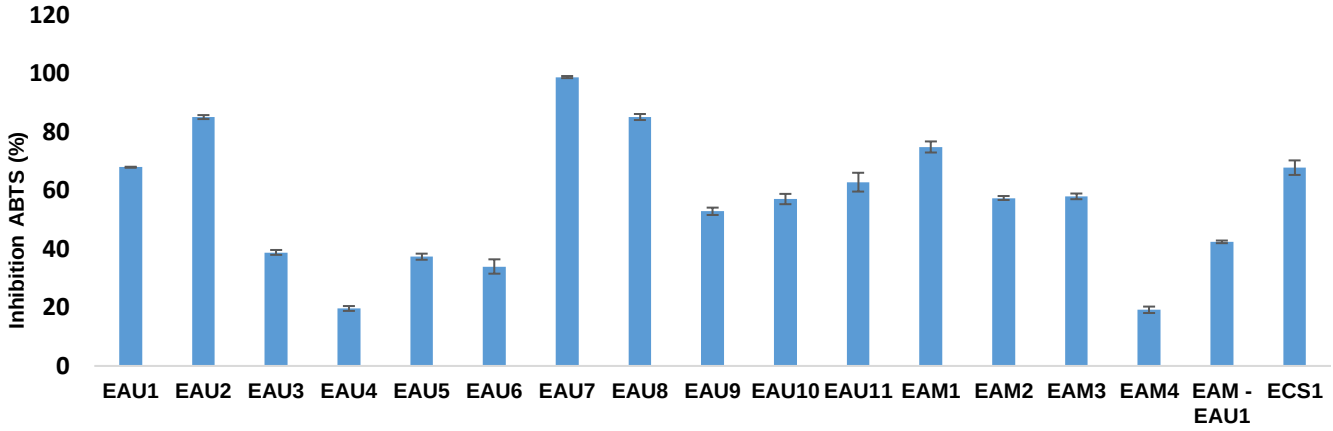


Figure 4.6. Percentage inhibition of ABTS radical for samples obtained by different extraction methods

The lowest value of antioxidant activity for sea buckthorn extract was obtained with sunflower oil - MAE4 and had a value of 0.70 µg Trolox/g d.w. The same extract showed an inhibition of only 19.18% given by ABTS free radicals.

Thus, results obtained by other researchers reveal a clear dependence between extraction solvents and antioxidant activity, and the chosen extraction method (UAE, MAE or ECS) defines the final result.

4.8.4. Comparative evaluation of the antioxidant activity of samples obtained by separate and sequential ultrasound-assisted extraction method

Sequential and separate extraction series were also analyzed, which provided the method for determining the antioxidant activity effective for characterizing the hydrophilic and lipophilic profile of lyophilized sea buckthorn.

Table 4.6 shows the results of the antioxidant activity (DPPH radical method and ABTS radical method) of the samples by sequential and separate ultrasound-assisted extraction of sea buckthorn extracts.

The results obtained for the antioxidant activity given by both free radicals in the sequential extraction are different from the results of the separate extraction.

For sequential extractions using the antioxidant activity evaluation method using the free radical DPPH, rather low antioxidant activity values were obtained in the range 0.03 - 1.73 µg Trolox/g d.w. The highest antioxidant activity value of 1.73 µg Trolox/g d.w. was obtained for the SqUAE4 sequential extraction and the lowest value of 0.03 µg Trolox/g d.w. was obtained for the SqUAE1 sequential extraction.

The lower ABTS radical (ABTS+) inhibition values of sequential and separate extracts (SqUAE2 and SeUAE2) with 70% ethanol indicate that ethanolic mixtures are more appropriate for ABTS radical (ABTS+) inhibition.

The highest value of antioxidant activity was obtained using the extraction mixture consisting of formic acid, acetone, water in the ratio of 0.35 : 20 : 80, with AA values of 4.01 ±

0.01 µg Trolox/mL for the method using the free radical ABTS and an inhibition of 99.29 ± 0.23%. From the above and the results obtained it can be observed that the lowest values of antioxidant activity were obtained for the extraction mixture consisting of ethyl acetate : hexane in the ratio of 2:1 which is characteristic of lipophilic extracts.

Table 4.6. Antioxidant activity of samples obtained by sequential and separate extraction methods

Sample code	AA, mMTrolox/mL extract (DPPH)	I_{DPPH},(%)	AA, µg Trolox/mL extract (ABTS)	I_{ABTS},(%)
SeUAE1	0,07±0,01^{e}	4,07± 0,57^{e}	0,85±0,03^{e}	25,65±0,85^{c}
SeUAE2	0,64±0,01^{c}	35,29±0,41^{c}	0,78±0,01^{e}	19,28±0,23^{d}
SeUAE3	**1,70±0,00^{a}**	**88,78±0,18^{a}**	**3,66±0,05^{b}**	**98,35±1,24^{a}**
SeUAE4	1,13±0,01^{b}	57,21±0,23^{b}	2,64±0,04^{d}	73,50±1,04^{b}
SqUAE1	0,03±0,02^{e}	2,25±0,77^{e}	0,53±0,04^{f}	12,49±0,96^{f}
SqUAE2	0,17±0,01^{d}	9,84±0,75^{d}	0,623±0,03^{f}	17,65±0,70^{e}
SqUAE3	**1,72±0,01^{a}**	**89,00±0,40^{a}**	**4,01±0,01^{a}**	**99,29±0,23^{a}**
SqUAE4	1,73±0,01^{a}	88,88±0,46^{a}	3,49±0,02^{c}	97,77±0,62^{a}

Means in each column that do not share a letter in the exponent are significantly different by Tukey's test, $p < 0.05$.

As shown in Table 4.6, the highest DPPH radical inhibition value was 88.88 ± 0.46% when using water as SqUAE4 extraction solvent and the lowest DPPH radical inhibition value was 2.25 ± 0.77% when using (2:1) ethyl acetate:hexane mixture as extraction solvent.

The highest ABTS radical inhibition value was 99.29 ± 0.23 % when using the extraction solvent mixture formic acid:acetone:water in the ratio of 0.35:20:80 - SqUAE3, and the lowest ABTS radical inhibition value was 12.49 ± 0.96 % when using the mixture ethyl acetate:hexane in the ratio of (2:1) as extraction medium - SqUAE1.

4.8.5. Correlation of results

Pearson correlation was applied to measure the linear relationship between different groups of bioactive compounds and antioxidant activity. Higher correlation coefficient values suggested a greater contribution for polyphenolic and flavonoid compounds to the antioxidant capacity of extracts.

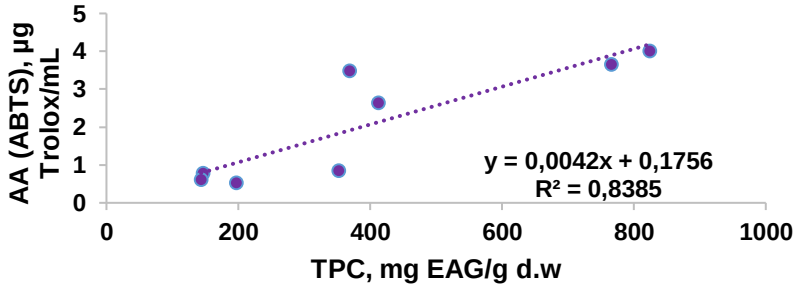


Figura 4.9. Correlation of antioxidant activity on ABTS radical with total polyphenol content (TPC) of extract obtained by UAE

Thus the content of polyphenolic compounds obtained by the method using the Folin-Ciocalteu colorimetric technique showed a stronger correlation with the antioxidant activity obtained by the ABTS radical scavenging method (R2 =0.8385) than the antioxidant activity obtained by the DPPH radical scavenging method (R2 =0.5509) (Figure 4.9).

4.9. Partial conclusions

In this study, several extraction techniques were tested to evaluate the phytochemical profile of sea buckthorn fruits: conventional extraction using 70% ethanol, ultrasound-assisted extraction using a mixture of organic solvents and microwave-assisted extraction with vegetable oil as extraction solvent.

The results showed that ultrasound-assisted solvent extraction provides higher values for the extraction of biologically active compounds using the following parameters: extraction time of 45 minutes; temperature of 40°C, frequency of 40 kHz and power of 100 W.

Ultrasound-assisted ethyl acetate:hexane (2:1) extraction resulted in the separation of more carotenoid compounds (total carotenoids, β-carotene and lycopene).

The highest extraction yield of hydrophilic compounds (total polyphenols and total flavonoids) was obtained with the extraction mixture consisting of formic acid:acetone:water (0.35:20:80), ultrasound-assisted extraction by sequential compound extractions. The highest inhibition of DPPH and ABTS free radicals was obtained with the mixture of ethanol:acetone and formic acid:acetone:water using ultrasound-assisted extraction.

Analysis of phytochemical compounds of extracts obtained from sea buckthorn fruits by the two methods, sequential and separate, indicates that this matrix is rich in both fat-soluble (carotenes) and water-soluble (polyphenols) compounds. The sequential extraction process can be optimised so that bioactive compounds can be extracted with a higher extraction yield.

The Pearson correlation index was calculated for two indicators such as total polyphenol content and antioxidant activity, which indicated that the respective values can be correlated with a correlation index of 0.8385. These results allow us to conclude that in the present study polyphenolic compounds in sea buckthorn extracts have higher antioxidant power than carotenoid compounds extracted from sea buckthorn.

The results demonstrate that the multi-step extraction method is capable of recovering and isolating different fractions of the extract (carotenoids and polyphenols) with biofunctional properties.

CHAPTER 5. DEVELOPMENT OF INGREDIENTS RICH IN BIOACTIVE COMPOUNDS EXTRACTED WITH POLAR SOLVENTS FROM SEA BUCKTHORN FOR APPLICATIONS IN THE FOOD INDUSTRY

5.1. General aspects

The main compounds of scientific interest present in large quantities in sea buckthorn fruits are carotenoids and flavones. Carotenoids are lipophilic pigments with a tetraterpenoid structure and colours such as yellow, orange to red. Flavonoids are secondary metabolites present in sea buckthorn fruits and are part of the polyphenol class, with colours ranging from yellow to orange.

However, the use of carotenoids and flavones as functional ingredients is limited due to their chemical instability, poor bioavailability and bioaccessibility. The microencapsulation technique is a solution to resolve the stability problems of these valued compounds.

Complex coacervation is based on electrostatic attraction between molecules charged with different electrical charges. This attraction produces a complex with two distinct phases. One is a polymer-rich phase (also called coacervate) and the other is the solvent solution (de Souza Simões et al., 2017).

5.2. Objectives of the study

The experimental study had the following scientific objectives:

- Extraction of biologically active compounds using a mixture of solvents (acetone, glacial acetic acid and water) for extract E1 and only water for extract E2, followed by microencapsulation by complex coacervation and lyophilization of biologically active compounds from sea buckthorn fruits using different encapsulation matrices such as: whey protein isolate (WPI) in combination with carboxymethyl cellulose (CMC).
- Characterization of the obtained sea buckthorn powders (coded: powder - P1 and powder - P2) from a phytochemical point of view with emphasis on total carotenoid content, total polyphenol content, antioxidant activity, in vitro digestibility, encapsulation efficiency, colorimetric analysis and stability over time of the obtained powders.
- Analysis of powder structure and morphology using confocal laser scanning microscopy (CLSM) technique.

5.6. Results and discussion

5.6.1. Evaluation of the phytochemical profile of sea buckthorn extracts

Bioactive compounds from freeze-dried sea buckthorn fruits were extracted with different solvent mixtures using the UAE method. Polar solvents such as acetone and water were used for extraction. Two extracts were obtained and the phytochemical content of both extracts is shown in Table 5.2.

The addition of acetone and glacial acetic acid to water led to a significant increase in the extraction yield of phytochemical compounds ($p<0.05$) for both lipophilic and hydrophilic compounds (Table 5.2). This can be explained by the increased breaking strength of the cell walls cu amenste of solvents. Thus, extract E1 showed a lycopene content of 1.69 ± 0.01 mg LC/g d.w. and extract E2 only 0.87 ± 0.01 mg LC/g d.w.

The β-carotene content of E1 extract showed a content of 2.79 ± 0.02 mg βC/g d.w. and extract E2 1,38 ± 0,02 mg βC/g d.w.

Regarding the content of hydrophilic compounds, a total flavonoid content of 310.06 ± 6.01 mg EC/g d.w. was quantified from extract E1. and approximately 127.80 ± 2.41 mg EC/g d.w. in extract E2. The total polyphenol content of the extracts analysed was also 823.50 ± 5.51 mg EAG/g d.w. and approximately 368.12 ± 5.37 mg EAG/g d.w. for extract E1 and extract E2 respectively.

The antioxidant activity of the extracts was quantified by two different free radical scavenging methods DPPH and ABTS. Thus, the antioxidant activity evaluated with percentage values of DPPH radical inhibition was 92% for E1 and 61.01% for E2, and 4.36 and 3.81% for ABTS radical.

Table 5.2. Phytochemical profile of extracts obtained from freeze-dried sea buckthorn fruits

Phytochemical compounds	Extract E1	Extract E2
Lycopene content (mg LC/g d.w.)	1,69 ± 0,01^{d}	0,87 ± 0,01^{d}
β-carotene content (mg βC/g d.w.)	2,79 ± 0,02^{d}	1,38 ± 0,02 ^{d}
Total carotenoid content (mg TC/g d.w.)	3,30 ± 0,03^{d}	1,60 ± 0,01^{d}
Total flavonoid content (mg EC/g d.w.)	310,06 ± 6,01^{b}	127,80 ± 2,41^{b}
Total polyphenol content (mg EAG/g d.w.)	823,50 ± 5,51^{a}	368,12 ± 5,37^{a}
Antioxidant activity ABTS (inhibition, %)	4,36 ± 0,01^{d}	3,81 ± 0,02^{d}
Antioxidant activity DPPH (inhibition, %)	92,00 ± 0,42^{c}	61,01 ± 0,42 ^{c}

Values that do not share a letter on the same line are significantly different ($p < 0.05$) according to the Tukey test, 95% confidence

As expected, the choice of extraction solvent is a decisive step in the extraction of biologically active compounds from sea buckthorn to obtain the high yield of bioactive compounds as well as antioxidant activity using ABTS and DPPH free radicals.

In the present study, the combination of solvents such as acetic acid, acetone and water led to the extraction of a high amount of biologically active compounds with a remarkable antioxidant activity using the free radicals DPPH and ABTS.

Table 5.2 shows the total carotenoid content of E1 and E2 extracts obtained by ultrasound-assisted extraction with solvents such as acetone, glacial acetic acid and water.

The most efficient mixture for the extraction of hydrophilic compounds was the mixture of acetic acid, acetone and water (E1), with a total polyphenol content of 823.50 ± 5.51 mg EAG/g d.w. and a total flavonoid content of 310.06 ± 6.01 mg EC/g d.w. The results obtained for the extracts in the present work show higher values than those reported by other researchers, probably due to the polarity of the solvent mixture used, the extraction method used and the conditions of growth, harvesting of sea buckthorn fruits.

TPC and TFC correlate with the results obtained for antioxidant activity, with values of 92.00 ± 0.42% inhibition against DPPH radical.

As can be seen from the data presented in Table 5.2, the antioxidant activity value of the extracts was different for the two extracts evaluated, the best results being obtained by the

extraction with acetic acid, acetone and water for inhibition of both DPPH and ABTS free radicals. Thus, extract E1 showed an inhibition value of 4.36 ± 0.01% against ABTS free radical and an inhibition value of 92.00 ± 0.42% against DPPH radical.

For the E2 extract, significantly lower inhibition results ($p<0.05$) of 3.81 ± 0.02% versus free radical ABTS and 61.01 ± 0.42% inhibition versus free radical DPPH were shown.

The values obtained by many researchers are different, but they can be cross-checked by correlating the extraction method with the solvent used.

Chromatographic analysis of sea buckthorn extracts

To characterize the phytochemical profile of carotenoids in the extractions obtained, the chromatographic profile was analyzed by the method of high performance liquid chromatography (HPLC) (Figure 5.4).

The HPLC technique allowed the identification of five carotenoid compounds present in both extracts (Figure 5.4). The difference between the chromatographic profiles of the two extracts consists in the amount of quantified compounds.

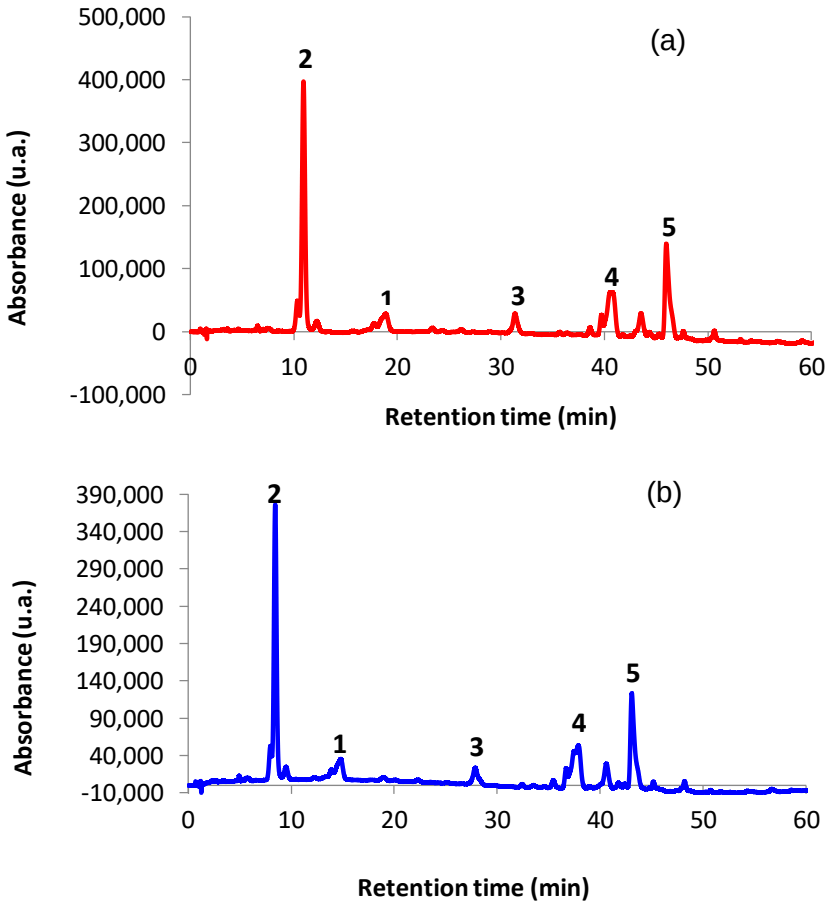


Figure 5.4. Chromatographic profile of carotenoids in lyophilized sea buckthorn fruit extracts E1 (a) and E2 (b): peak 1 — astaxanthin; peak 2 — zeaxanthin; peak 3 — β-cryptoxanthin; peak 5 — β-carotene.

Thus, in the E1 extract were determined: 0.81 mg / g d.w. astaxanthin, 5.11 mg / g d.w. zeaxanthin, 0.43 mg / g d.w. β-cryptoxanthine, 0.96 mg / g d.w. lycopene and 2.71 mg / g d.w. β-carotene (Figure 5.4a).

In the case of E2 extract, the amounts of quantified carotenoids had lower values, as follows: 0.56 mg / g d.w. astaxanthin, 4.74 mg / g d.w. zeaxanthin, 0.55 mg / g d.w. β-cryptoxanthin, 0.98 mg / g d.w. lycopene and 1.31 mg / g d.w. β-carotene (Figure 4b).

5.6.2. Evaluation of the phytochemical profile of sea buckthorn powders

In this study, bioactive compounds in sea buckthorn were encapsulated by complex coacervation, using protein from whey protein isolate and carboxymethylcellulose polysaccharide.

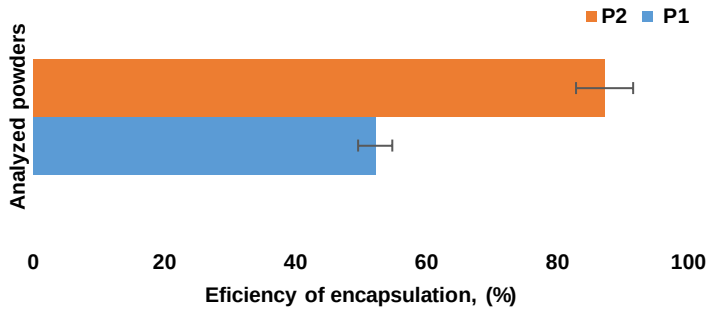


Figure 5.5. Encapsulation efficiency of powders obtained by encapsulating the sea buckthorn fruit extract

For both analyzed powders P1 and P2, the same amount of 0.5 g of extract was used. For a better dispersion of the carotenoid compounds and considering their lipophilic profile, the extracts were mixed with sunflower oil. The characterization of the microencapsulated sea buckthorn extract was carried out by quantifying the lycopene content (LC), β-carotene content (βC), total carotenoid content (TCC), total flavonoid content (TFC), total polyphenol content (TPC) and of antioxidant activity (AA). The results obtained from these experiments are presented in Table 5.3.

In this study the extracts were encapsulated in CMC (carboxymethyl cellulose) and WPI (whey protein isolate) using the complex coacervation method, followed by lyophilization to obtain the most stable powders. From the data presented in Figure 5.5, it is observed that the encapsulation efficiency of P1 powder was significantly lower compared to the encapsulation efficiency of P2 powder ($p < 0.05$). The results obtained in this study are in agreement with those reported by other researchers.

The highest carotenoid encapsulation efficiency was for P2 powder encapsulated in whey protein isolate and carboxymethyl cellulose, with values of 87.23 ± 0.05% for P2 powder and 52.20 ± 1.60% for P1 powder . Both powders were obtained under similar conditions, but with different extracts, respectively E1 (obtained with the mixture of solvents acetic acid, acetone and water) and E2 (obtained with water). Higher carotenoid encapsulation efficiency values for P2 powder are due to the hydrophilic profile of the extract, which demonstrates that the matrices chosen lend themselves to the profile of the obtained extract.

Table 5.3. Phytochemical profile of powders obtained by encapsulating extracts from lyophilized sea buckthorn fruits

Phytochemical characterization of powders	Powder P1	Powder P2
Lycopene content (mg LC/g d.w.)	1,22 ± 0,01^{e}	0,86 ± 0,02^{f}
β-carotene content (mg βC/g d.w.)	0,63 ± 0,03^{f}	0,98 ± 0,05^{f}
Total carotenoid content (mg CT/g d.w.)	2,13 ± 0,03^{e}	1,14 ± 0,04^{f}

Values not sharing a letter on the same row are significantly different ($p < 0.05$) according to Tukey's test, 95% confidence

According to the results obtained, the highest content of total carotenes was reported for the P1 powder being 2.13 ± 0.03 mg CT/g d.w., which is consistent with the values obtained for the E1 extract used for microencapsulation of carotenes (Table 5.2.).

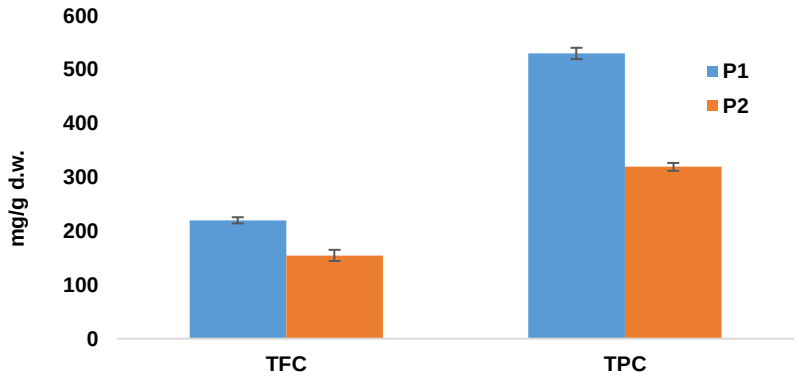


Figure 5.7. The total content of flavonoids (TFC, mg EC/g d.w.) and polyphenols (TPC mg EAG/g d.w.) of P1 and P2 powders

The results of the present study demonstrate that the use of whey protein isolate and carboxymethylcellulose as encapsulation materials are suitable for microencapsulation of extract obtained from lyophilized sea buckthorn.

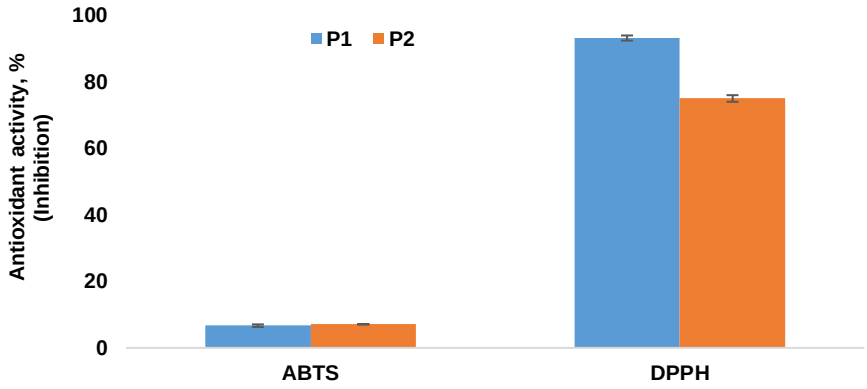


Figure 5.8. Antioxidant activity of sea buckthorn powders P1 and P2 by ABTS free radical and DPPH free radical method

The total content of polyphenols and flavonoids for P1 powder was 529.84 ± 10.54 mg EAG/g d.w and 219.86 ± 5.71 mg EC/g d.w, respectively. (Figure 5.7.) These results being comparatively higher than those obtained for P2 powder with CPT values of 319.14 ± 7.37 mg EAG/g d.w and CFT values of 154.68 ± 10.36 mg EC/g d.w. The values obtained are consistent with the total polyphenol content and the total flavonoid content of the extracts used to encapsulate the biologically active compounds from the sea buckthorn fruits. Thus, this correlation of the total content of polyphenols in the extract with the TPC values in the powders is probably due to the formation of OH bonds in the phenolic aromatic ring with the whey proteins.

The antioxidant activity of the powders was quantified by two different DPPH and ABTS free radical scavenging methods (Figure 5.8.). From the results obtained, it can be stated that the method of determining the antioxidant activity by capturing the DPPH free radical lends itself better to the powders obtained due to the hydrophilic profile of the encapsulates. Thus, by the DPPH free radical capture method, values of 93.05 ± 0.77% inhibition of the DPPH radical and 74.90 ± 0.99% inhibition of the DPPH free radical were obtained for P2 powder.

Powder P1 showed higher values for lycopene, total carotenes, total polyphenol content and total flavonoid content. The β-carotene content, on the other hand, was higher in P2 powder, as well as ABTS free radical scavenging antioxidant activity. Comparatively lower values were obtained for ABTS free radical scavenging antioxidant activity compared to the results obtained for DPPH free radical scavenging antioxidant activity.

The results obtained for antioxidant activity by the DPPH free radical scavenging method were 295.93 and 387.08 µM Trolox/g and 230.61 and 830.36 µM Trolox/g for the ABTS free radical scavenging method. This demonstrates once again that the application of methods for the determination of antioxidant activity using different free radicals depends on the phytochemical profile of the analysed matrix and the encapsulation parameters of the bioactive compounds.

5.6.3. Stability of encapsulation efficiency of sea buckthorn powders

Encapsulation efficiency values can predict the stability over time of compounds within the encapsulation matrix. Table 5.4. shows the variation in encapsulation efficiency of carotenoids during 180 and 270 days of storage at 4°C and in the dark.

Table 5.4. Encapsulation efficiency (EE) is a primary indicator for the shelf life of carotenoid-containing powders.

	Stability of encapsulation efficiency, %		
Powder	0 day	after 180 days	after 270 days
P1	52,20 ± 1,60^{b}	49,20 ± 0,02^{b}	48,88 ± 0,01^{b}
P2	87,23 ± 0,05^{a}	80,45 ± 0,03^{a}	76,43 ± 0,01^{a}

Values that do not share a letter on the same line are significantly different ($p < 0.05$) according to Tukey's test, 95% confidence

Analysis of the data presented in Table 5.4 shows a slight decrease in carotenoid encapsulation efficiency over time for both powders analysed. Thus, the encapsulation efficiency for P1 powder is 7% lower after 270 days of storage at 4°C in the dark and the encapsulation

efficiency for P2 powder showed a 12% decrease in encapsulation efficiency after 270 days of storage at 4°C in the dark.

Encapsulation efficiency (EE) is a primary indicator for the shelf life of carotenoid-containing powders.

P1 and P2 powders, during storage at room temperature without packaging (glass containers with stanol), released oil, and its oxidation could lead to degradation of the structure of the powders and decrease their encapsulation efficiency.

5.6.4. Colorimetric analysis and colour stability over time of sea buckthorn powders

The results of the colorimetric analysis of the powders are shown in Figures 5.8., 5.9. and 5.10. In the colorimetric analysis the following parameters were determined: brightness (L*), parameter a* (red) and parameter b* (yellow), which were monitored during 270 days of storage at 4°C in the dark.

The experiment lasted 270 days, during which P1 and P2 powders were stored under refrigerated conditions at 4°C in hermetically sealed brown glass tubes. From the results obtained we can conclude that both powders during the 270 days had an increase in lightness index, i.e. the powders became lighter in colour. This process being irreversible and is explained by the degradation of carotenoids embedded in the WPI and carboxymethylcellulose matrices and probably oxidation of the oil used for encapsulation. Carotenoid degradation is closely related to the size of the carotenoid spherosomes embedded in the encapsulation matrices, the storage conditions of the powders and the oxidation processes. The smaller the size of the spherosomes and the smaller the encapsulating material, the faster the degradation takes place, leading to discoloration of the powders.

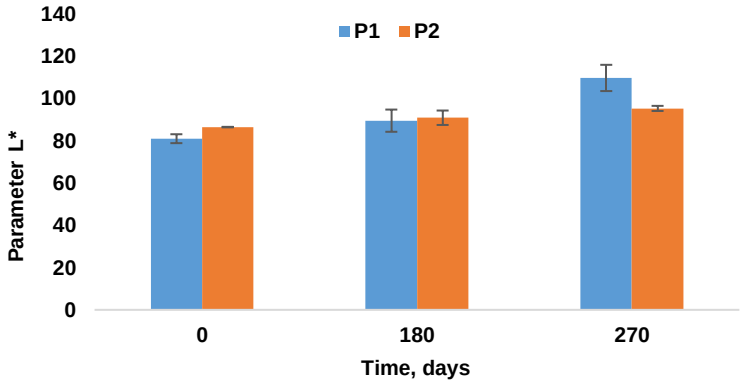


Figure 5.9. Luminosity stability for 270 days for P1 and P2 powders

From Figure 5.9. it can be concluded that P1 powder has approximately 29% higher brightness and P2 powder 9% higher lightness on day 270 of storage compared to the day of encapsulation.

In conclusion we can state that the discoloration process of powders is closely related to the encapsulation matrix and the encapsulated material.

Parameter a* represents the red/green colour and reflects oxidation reactions in the powders during storage. For P1 powder the total carotenoid content shows values of 2,13 ± 0,03

mg TCC/g d.w., being higher than for P2 powder of 1,14 ± 0,04 mg TCC/g d.w. Probably the higher content of carotenoid compounds in the powder resulted in a slower deterioration of the colour of the powders by about one unit during 270 days of storage.

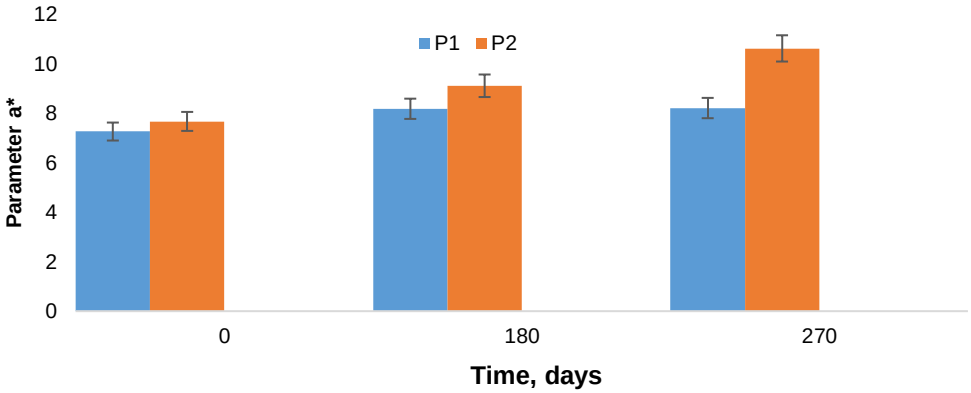


Figure 5.10. Stability of parameter a* for 270 days for P1 and P2 powders

As with the L* colour parameter, the definition of the a* parameter depends on the encapsulation materials and encapsulated compounds.

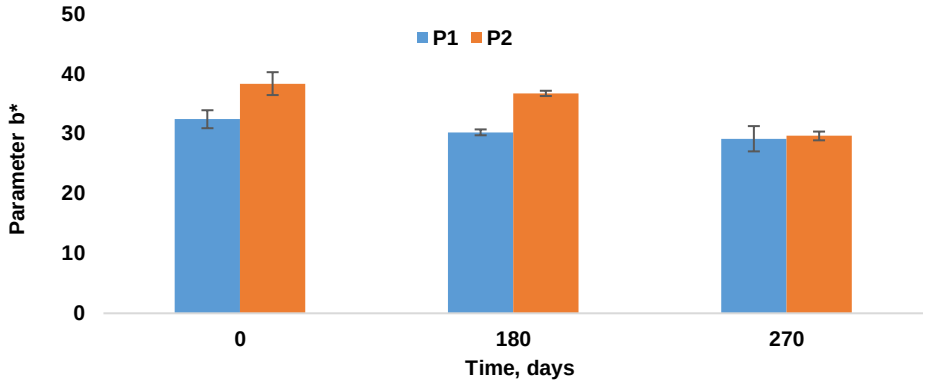


Figure 5.11. Stability of parameter b* for 270 days for P1 and P2 powders

Measurements of colour parameters over time show a significant decrease in b* ($p<0.05$), which can be correlated with the change in encapsulation efficiency. This may be due to the release of compounds from the matrix that led to the degradation of the yellow colour intensity.

On the other side the increase in the intensity of the L*-luminosity parameter may also influence the yellow colour shade of the microencapsulated powders.

The values of the b* parameter presented in Figure 5.11 show a high intensity of the initial yellow colour for both P1 and P2 powders due to the presence of carotenoids and flavonoids in the extract, with P2 powder having a slightly higher value.

5.6.5. Morphological and structural analysis of sea buckthorn powders using confocal laser microscopy

Confocal laser microscopy analysis of two samples (Figure 5.12 and Figure 5.13) aimed to clarify the morphological and structural appearance of the powders according to the extraction method. The following lasers were used for the analysis of the samples: Ar laser (458, 488 and 514 nm) and DSSP (pumped solid state diodes with λ=561 nm, as literature data indicate carotenoid absorption ranges of 448, 476 and 505 nm (Katoh et al., 1991; Marinova et al., 2007).

The carbohydrates used, in our case CMC, introduced into the microencapsulation matrices showed absorbance between 300 and 400 nm which were measured by the diode laser (405 nm).

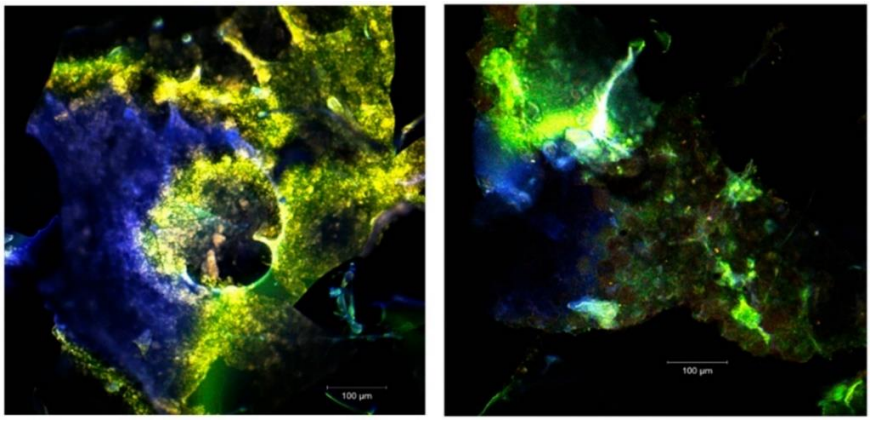


Figure 5.12. Confocal laser scanning microscope (CLSM) images of unprocessed native powders

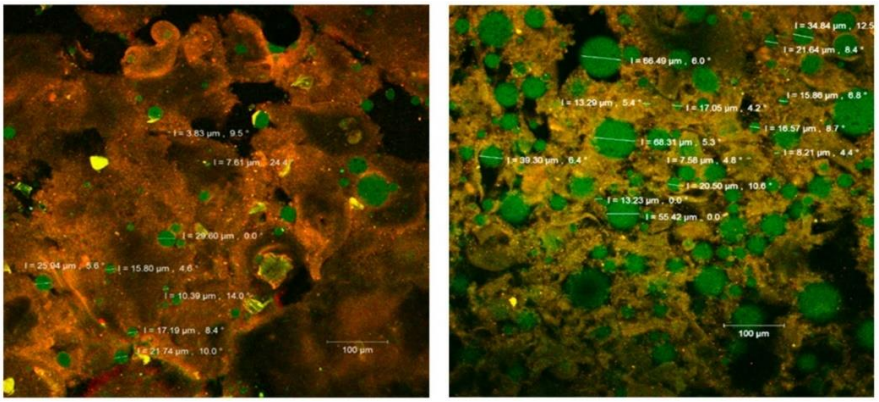


Figure 5.13. Confocal laser scanning microscope (CLSM) images of fluorophore dye powders

Fine biofilms (marked in blue or green depending on the ratio of WPI to polysaccharide biopolymer) were formed (Figure 5.12). Within the biopolymer matrix, several pigmented (in yellow-orange) microspherosomes (1-2μm) can be seen anchored in the microencapsulated powder. The interaction of plant pigments in sea buckthorn fruit with polysaccharide compounds in the microcapsules did not cause any changes on spectral absorbance.

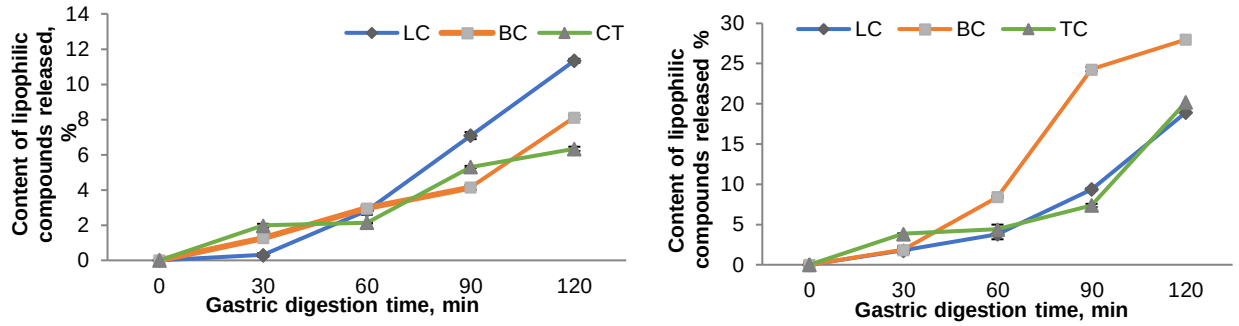
Confocal analysis of the samples after fluorescent staining with Congo Red dye revealed the presence of biologically active compounds in sea buckthorn fruit extracts in the form of spherosomes (marked in green) with different diameters ranging from 3.83 to 215.78 μm. Congo

Red dye binds intact β-D-glucans in biopolymer matrices (McDonald et al., 2012), as well as the peptides from the IPZ (whey protein isolate), marking them in the figure below in orange (Figure 5.13).

Small spherosomes (<25 µm) are visualized in P1 powder, while in P2 powder they are of medium size. P2 powder showed a large number of medium sized, well individualized spherosomes with a tendency to cluster and well stabilized in the matrix used by WPI and CMC.

5.6.6. *In vitro* digestibility of powders obtained from sea buckthorn extract

The digestibility of encapsulated sea buckthorn powders was studied under in vitro digestion conditions. Figure 5.14 and Figure 5.15 show the percentage release of CT(total carotenes), βC(β-carotenes) and LC(lycopene) from P1 and P2 powders in the gastrointestinal tract.



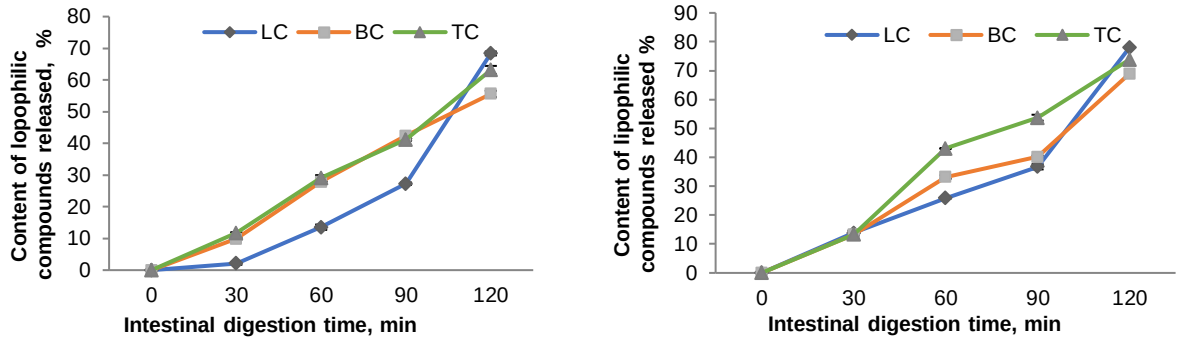
(a) (b)

Figura 5.14. Content of lipophilic compounds released from P1 powder (a) and P2 powder (b) during *in vitro* gastric digestion

Results obtained for in vitro digestibility in simulated gastric juice showed that encapsulation materials show a protective effect on lipophilic compounds, especially in the case of P1 powder. However, a slight release of lipophilic compounds was observed for both powders in simulated gastric juice. The lycopene content in P1 powder after 120 min of digestion showed the highest release of all compounds analysed being 11.33 ± 0.11% (Figure 5.14a).

β-carotene and total carotenoids released after 120 min of gastric digestion had values of 8.12 ± 0.1% and 6.33 ± 0.12%, respectively. In the case of P2 powder, β-carotene showed the highest release of all compounds analysed with values of 27.97 ± 0.26% after 120 minutes (Figure 5.14b).

Under simulated intestinal digestion conditions, the results show that a maximum amount of lipophilic compounds is released after 120 minutes of digestion (Figure 5.15). Thus, P1 powder showed values of 55.59 ± 0.99%, 63.14 ± 1.3% and 68.36 ± 0.62% for βC, TC and LC release after 120 minutes of digestion (Figure 5.15a). P2 powder showed higher compound release values of 68.98 ± 0.41% for β-carotenes, 73.86 ± 1.03% for total carotenes and 78.01 ± 0.91% for lycopene after 120 min of digestion (Figure 5.15b). The results are in agreement with other studies present in the literature.



(a) (b)

Figure 5.15. Content of lipophilic compounds released from P1 powder (a) and P2 powder (b) during *in vitro* intestinal digestion

5.8. Partial conclusions

The main objective of this study was to exploit extracts rich in bioactive compounds from sea buckthorn fruits, as well as to configure ways to increase the stability and bioavailability of bioactive compounds in sea buckthorn fruits. Biologically active compounds from the fruits of sea buckthorn Hippophae rhamnoides L. were extracted using ultrasound-assisted extraction method. The results obtained allowed the following conclusions to be elaborated:

Different polar solvents were used to extract as much lipophilic and hydrophilic bioactive compounds as possible. Extraction of the bioactive compounds was performed with a mixture of solvents such as glacial acetic acid, acetone, water for E1 and water for E2 extract. By adding acetic acid and acetone to water, higher values of total carotenoid and polyphenol contents were obtained in E2 extract.

The chromatographic profile of both extracts revealed the presence of five lipophilic compounds, zeaxanthin being in the highest concentration. Following the separation and chromatographic identification of carotenoids in sea buckthorn fruit extract, the two compounds with the highest concentration were zeaxanthin and β-carotene. Lycopene, β-cryptoxanthin and astaxanthin were also identified in both extracts in decreasing order of concentration

To increase the stability and bioavailability of the bioactive compounds, both extracts were encapsulated in an encapsulation matrix composed of CMC and IPZ. The complex coacervation encapsulation technique was used to obtain the buckthorn extract powders, followed by freeze-drying. Characterization of phytochemical compounds showed that both powders presented a high content of lipophilic and hydrophilic bioactive compounds. Although P2 powder showed the highest encapsulation efficiency, P1 powder was noted for the highest phytochemical content.

P2 powder showed the highest intensity of yellow colour, correlating with high content of bioactive compounds. Confocal microscopy images revealed that the complex coacervation and freeze-drying technique resulted in scalariform microcapsules. From the MCSL analysis it can be stated that the bioactive compounds in sea buckthorn have complex structures in the form of yellow-orange microspherosomes (after staining). The spherosomes identified in P1 powder had

a small size of 25 µm, but in P2 powder, the spherosomes showed medium size, evidenced by the tendency to form clusters in the matrix of whey protein isolate and carboxymethyl cellulose.

The in vitro digestibility of sea buckthorn powders was also performed to test their functionality. Both powders showed a substantially higher release of lipophilic compounds in the simulated intestinal juice, but the P2 powder showed the highest release of bioactive compounds. These results suggest that, regardless of the differences between the powders, both could be used in food applications as possible delivery agents for target bioactive compounds (carotenoids and polyphenols).

The results may provide evidence on how the targeted biologically active compounds in sea buckthorn can be used in the development of functional ingredients for multiple applications. The obtained powders have biological activities demonstrated in this study and these make them suitable for multiple applications as value-added ingredients and can be successfully used in the food, textile, cosmetic and pharmaceutical industries.

CHAPTER 6. UTILIZATION OF ENCAPSULATED INGREDIENTS BASED ON CAROTENOIDS EXTRACTED FROM SEA-BUCKTHORN TO OBTAIN VALUE-ADDED MAYONNAISE SAUCE

6.1 Introduction

For all the advantages and benefits of fortifying foods with plant extracts, incorporating sea buckthorn plant extracts directly into processed foods is still a challenge. Current food processing conditions as well as interaction with other food constituents can lead to carotenoid degradation. Encapsulation technique is the best approach to increase their stability and bioavailability of biologically active compounds. Encapsulation has been defined as a process that provides stability to a specific substance by encapsulating it in another material, thus avoiding contact with any external factors that may lead to degradation(de Freitas Santos et al., 2021).

Sauces are consumed all over the world and are associated with various foods because of their positive influence on taste and colour. The best known sauce is mayonnaise. Mayonnaise is a cold oil-in-water (O/W) emulsion composed mainly of egg yolk and vegetable oil. Nutritionally, it has a high energy value due to the fat used, but also provides vitamins, proteins and minerals (Khalid et al.,2021).

Mayonnaise is rich in unsaturated fatty acids, which makes it susceptible to oxidation. This process could release toxic compounds such as free radicals, which can affect consumer health, sensory properties and stability during storage. Oxidation of mayonnaise could be prevented by adding natural antioxidants from plant extracts (Khalid et al.,2021).

6.2 Objectives of the study

The scientific objectives of this study were:

- Extraction of carotenoids from sea buckthorn fruits and characterization of phytochemical content and antioxidant activity.
- Encapsulation of sea buckthorn extracts by complex coacervation and freeze-drying using different encapsulation matrices. The obtained powders were characterized in terms of encapsulation efficiency, phytochemical content, antioxidant activity, storage stability, colour, morphological structure and *in vitro* digestibility.
- Obtaining a value-added mayonnaise sauce by incorporating sea buckthorn powder and its characterization. The obtained mayonnaise samples were characterized in terms of phytochemical compounds and antioxidant activity as well as in terms of their textural, colour and sensory properties and the stability of phytochemical compounds during storage.

6.8 Results and discussion

6.8.1 Phytochemical characterisation of sea buckthorn fruit extract

Carotenoids from sea buckthorn fruits were extracted by ultrasound-assisted method using a combination of ethanol and hexane as solvents. The resulting extract (E) was analyzed phytochemically and further encapsulated in two different encapsulation matrices composed of

WPI and CMC on and alginate, agar and chitosan on one side. This resulted in two powders, coded P1 and P2 respectively.

The total carotenoid content of sea buckthorn fruit extract was quantified with a value of 32.33 ± 1.52 mg TC/g d.w. and an antioxidant activity using the ABTS free radical scavenging method of 422.03 ± 1.33 µM Trolox/g d.w. extract.

6.8.2 Phytochemical characterisation of sea buckthorn powders

In the present study, the biologically active compounds in the extract were encapsulated by complex coacervation followed by freeze drying. The obtained powders were characterized phytochemically and in terms of encapsulation efficiency.

Total carotenoid content

From the analysis of the obtained results shown in Figure 6.2, it can be observed that the use of the combination of encapsulation matrices such as alginate, agar and chitosan (P2) captured higher amounts of carotenoids from the sea buckthorn extract, showing also significantly higher encapsulation efficiency (EE) values compared to the P1 variant with whey protein isolate and carboxymethyl cellulose ($p < 0.05$).

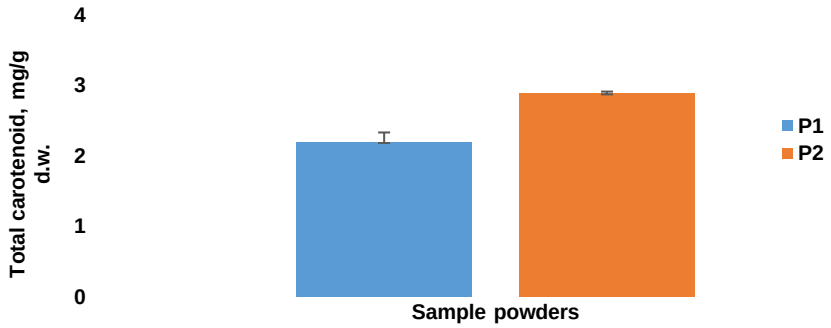


Figure 6.2. Total carotenoid content of the two powders P1 and P2

This resulted in values of 2.20 ± 0.13 mg TC/g d.w. and 2,89 ± 0,02 mg TC/g d.w. for total carotenoids.

Antioxidant activity

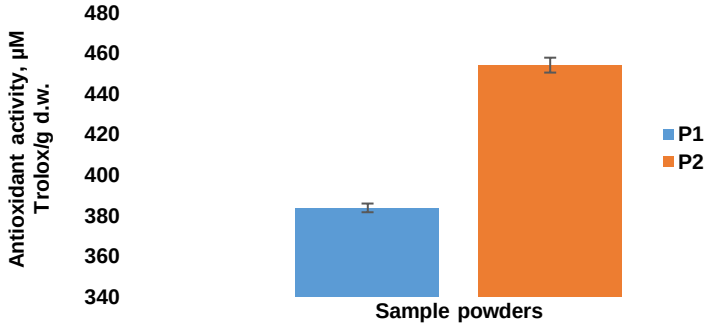


Figure 6.3. Antioxidant activity of the two powders P1 and P2 obtained from sea buckthorn extract

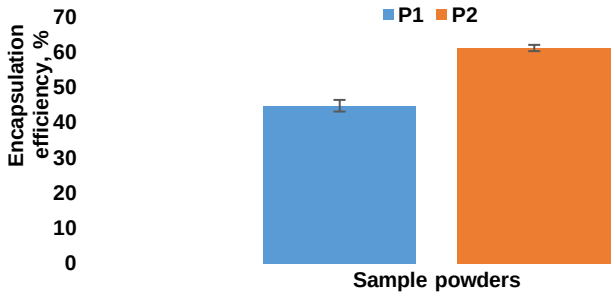


Figure 6.4. Encapsulation efficiency of the two powders P1 and P2 obtained from sea buckthorn extract

This difference in carotenoid content between the two powders is also reflected in the antioxidant activity of the powders (Figure 6.3). In terms of antioxidant activity, P1 and P2 powders showed values of 383.73 ± 2.14 µM Trolox/g d.w. for P1 powder and 454.04 ± 3.67 µM Trolox/g d.w. for P2 powder, respectively. Both powders showed values of 44.78% for P1 powder and 61.17% for P2 powder, respectively, for carotenoid encapsulation efficiency (Figure 6.4).

6.8.3 Morphological structure analysis of microcapsules by CLSM

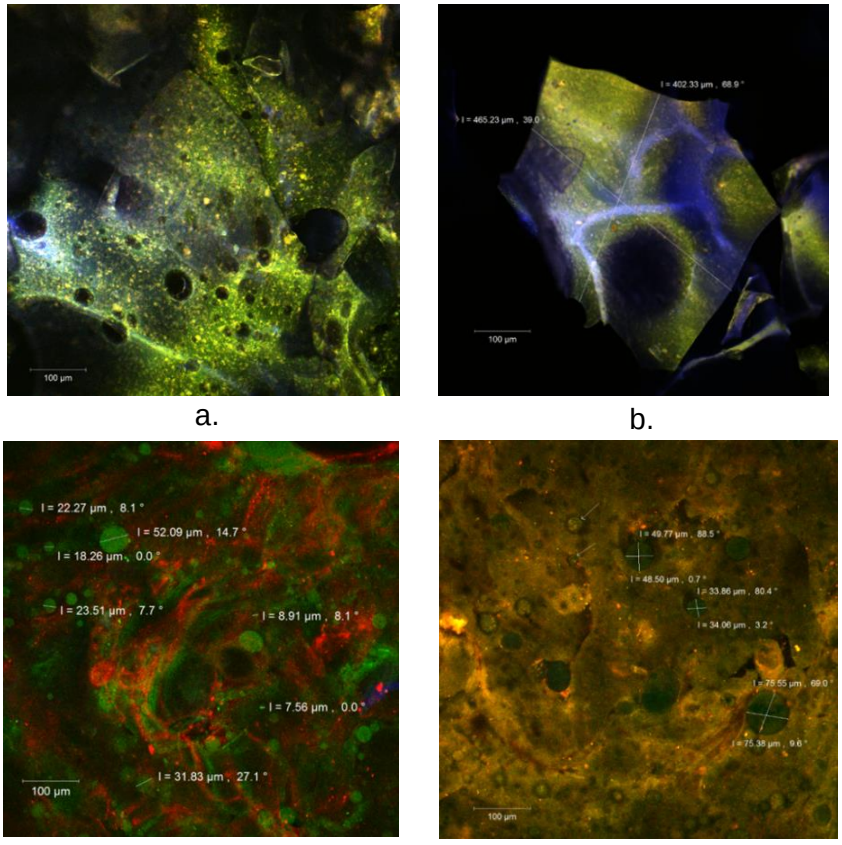


Figure 6.5. Confocal laser scanning microscopy (CLSM) images of native, unstained (a, b) and fluorophore-stained (c, d) powders.

The morphology of the microcapsules was assessed by point-by-point laser scanning microscopy and the resulting 3D images are shown in Figure 6.5.

In the native form, fine blue or green biofilms were formed depending on the ratio of IPZ or polysaccharides. Carotenoids from the sea buckthorn extract were anchored in the encapsulating matrix as yellow to orange microspherosomes (1-2 µm) (Figure 6.5 a, b).

After coloration of the powders with Congo Red fluorophore (Figure 6.5 c, d), the carotenoids in the extract appear as green spherosomes of average size 7.56 µm to 75.55 µm in both powders. The carotenoid compounds are well individualized and stable in the encapsulating matrices, but with a tendency to cluster. The results are consistent with the carotenoid encapsulation efficiency for P2 powder, from which it is observed that the matrices used revealed new hydrophobic attachment sites for the carotenoids, resulting in a more efficient encapsulation of the biologically active compounds of sea buckthorn.

6.8.4 Colour analysis of sea buckthorn powder

The colour parameters of the powders are presented in Table 6.1. Significant differences were observed between powders for all the colourimetric parameters analysed ($p < 0.05$). These results suggest that the encapsulation matrix had a notable influence on the colour of the powders ($p < 0.05$).

The L* and b* values suggest that both powders have a more intense lightness and yellow colour typical of carotenoid compounds. It can be affirmed that the use of the combination of polysaccharides such as alginate, agar and chitosan as encapsulation material conferred a significantly higher yellow color shade compared to whey protein isolate and carboxymethylcellulose ($p < 0.05$).

Table 6.1. Colorimetric parameters for the two powders tested

Samples	Colorimetric parameters		
	L*	**a***	**b***
P1	$73,58 \pm 0,04^{b}$	$8,99 \pm 0,02^{a}$	$52,41 \pm 0,59^{b}$
P2	$92,09 \pm 0,64^{a}$	$4,28 \pm 0,05^{b}$	$62,45 \pm 1,47^{a}$

L* -lightness, a* -red, b* -yellow; Means in each column not sharing a letter in the exponent are significantly different by Tukey's test, $p < 0.05$.

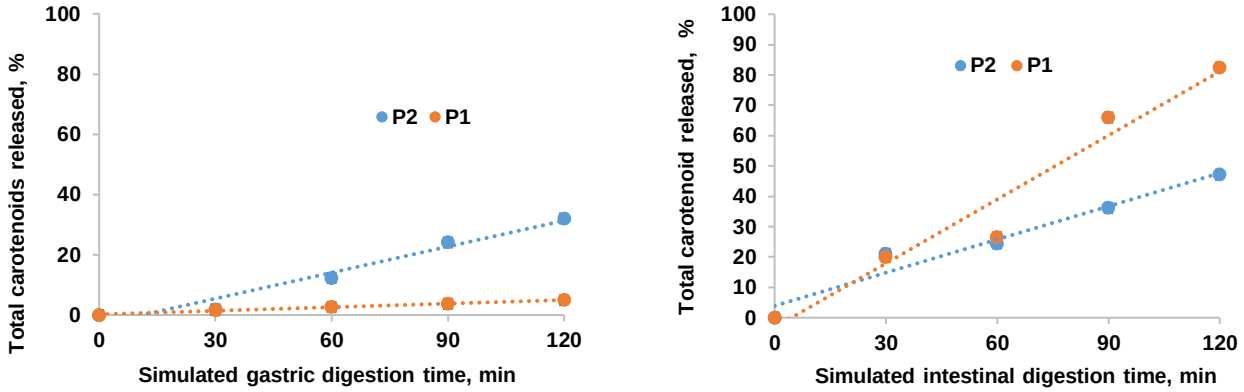
Thus, P2 is the lighter and yellower of the two samples obtained, although the parameter a* (red colour) has a value of only 4.28 ± 0.05. In addition, the value of parameter b*(yellow) of P2 powder compared to P1 powder can be correlated with the higher concentration of carotenoids ($p < 0.05$). The positive value of the b* parameter can be associated with the carotene content that gives the sea buckthorn fruit a yellow-orange colouring, a hue imparted by the sea buckthorn extract also in the sea buckthorn fruit powder and later in the mayonnaise sauce.

6.8.5 Simulated *in vitro* digestibility of total carotenoids in powders

The percentage of carotenoids released during simulated in vitro gastrointestinal digestion for two hours is shown in Figure 6.6. A significant release of carotenoids from the matrix was

observed in the gastric phase for P2 powder, with a maximum of 32.07 ± 0.05% after 60 min of digestion (Figure 6.6a). P1 powder showed the highest stability in the simulated gastric phase, with a maximum carotenoid release of 4.94 ± 0.07% after 120 min of digestion (Figure 6.6.b). It is observed that although P2 powder showed the highest encapsulation efficiency, it has little lower stability in SGS (simulated gastric juice) compared to P1 powder.

Carotenoids from both powders were gradually released into the intestinal environment (Figure 6.6.b). P1 powder showed a maximum release of 82.47 ± 0.22%, while P2 showed a release of only 47.14 ± 0.44% after 2 h of digestion. Therefore, microencapsulation of carotenoids in a mixture of proteins and polysaccharides resulted in retarded release of the compounds in the gastric phase and improved it in the intestinal phase.



a. b.

Figure 6.6. Total release of carotenoid compounds from powders during gastric (a) and intestinal(b) digestion simulated *in vitro*

6.8.6 Stability of phytochemical compounds and colour of sea buckthorn powders

To determine the stability of the microencapsulated carotenoids in sea buckthorn, the powders were stored in the dark at 4 °C for 90 days. Every 30 days, total carotenoid content, antioxidant activity and colour parameters were measured. Figures 6.8. and 6.9. show the data obtained from the phytochemical characterization of the powders stored for 90 days in the dark at 4 °C.

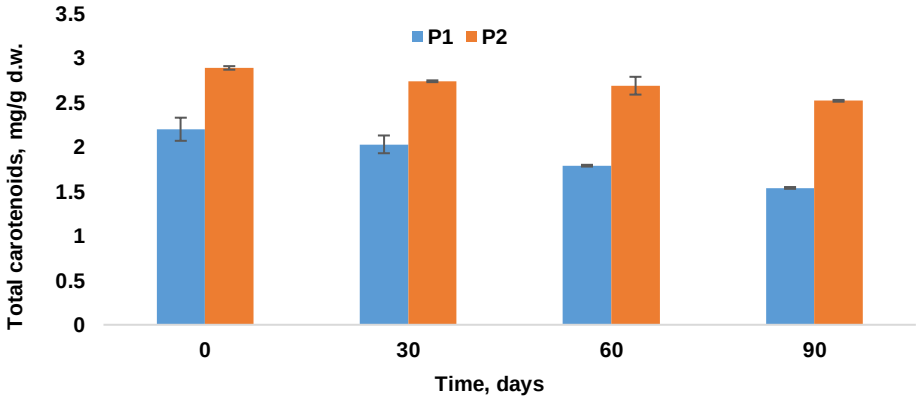


Figure 6.7. Storage stability of total carotenoid content of tested powders (P1 = powder with 2% WPI and 2% CMC; P2 = powder with 4% alginate, 1% agar and 4% chitosan)

Analysing the data in Figure 6.7. and those in Figure 6.8. shows that there is a variation in total carotenoid content and antioxidant activity value during the 90 days of storage.

Significant carotenoid degradation occurred in samples of both ($p < 0.05$) (Figure 6.8.). Thus, in P1 powder a decrease in TC content of 30% and 13% in antioxidant activity was observed, probably due to the oxidation process. A significant decrease in TC content and antioxidant activity was also observed in P2 powder of about 13% for TC content and 11% for antioxidant activity, respectively ($p < 0.05$).

From the data presented in Figure 6.8. it can also be seen that the mixture of polyglycosides in the encapsulation matrix of P2 powder had higher stability compared to the mixture consisting of proteins and polysaccharides of P1 powder. The results obtained are consistent with other studies that have reported carotenoid degradation after storage of microencapsulated plant extracts.

Storage stability of powders is consistent with colorimetric degradation of the b* parameter and decreased antioxidant activity.

From Figure 6.7. and Figure 6.8. it can be seen that after 90 days of storage, P2 powder remains the powder with the highest antioxidant activity and total carotenoid content.

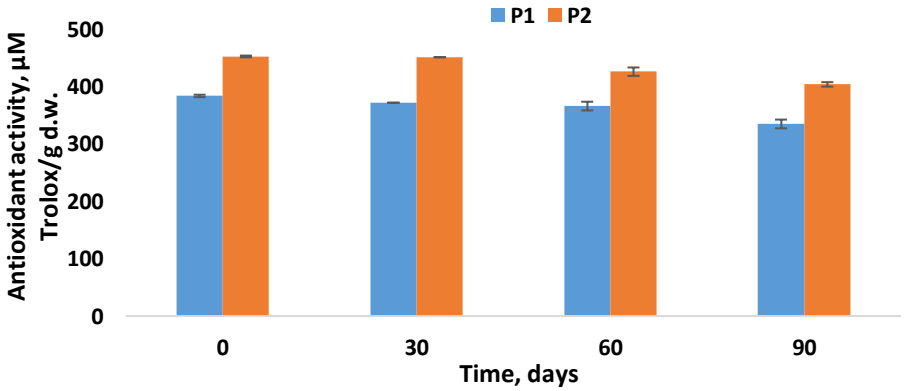


Figure 6.8. Evolution of the antioxidant activity of the tested powders (P1 = powder with 2% WPI and 2% CMC; P2 = powder with 4% alginate, 1% agar and 4% chitosan) during storage

Evaluating the antioxidant activity of P1 and P2 powders during the storage period (90 days) at 4°C in the dark, a reduction in AA was observed for P1 powder of 13% and 11% for P2 powder, respectively.

The changes in colour parameters during storage of P1 and P2 powders were also measured and are presented in Table 6.2. The data presented in Table 6.2. suggest that both the storage period and the nature of the encapsulating agent significantly influenced the change in colour parameters in both powders analysed. A gradual degradation of the yellow colour in both powders was also visually observed during 90 days of storage, evidenced by a significant decrease in the b* parameter values ($p < 0.05$).

However, the L* brightness and the a* parameter specific to both powders increased significantly during the storage period ($p < 0.05$). In the present study for P1 powder, the value of the b* (yellow) parameter decreased by 13%, but for P2 powder the reduction was only 7%. The

degradation of the b* (yellow) parameter is directly correlated with the degradation of carotenoid compounds in the powders and antioxidant activity.

Table 6.2. Colour stability of powders tested after 90 days of storage

Powder	Storage period, days	Colorimetric parameters		
		L*	a*	b*
P1	0	73,58 ± 0,04^{c}	8,99 ± 0,02^{b}	52,41 ± 0,59^{a}
	30	78,02 ± 0,14^{b}	9,35 ± 0,36^{a}	50,37 ± 0,51^{ab}
	60	90,53 ± 0,05^{a}	9,43 ± 0,35b	48,61 ± 0,55^{b}
	90	91,30 ± 0,52^{a}	10,93 ± 0,89^{a}	45,64 ± 0,47^{c}
P2	0	92,09 ± 0,64^{b}	4,28 ± 0,05^{d}	62,45 ± 1,47^{a}
	30	92,88 ± 1,08^{b}	5,32 ± 0,31^{c}	60,67 ± 0,38^{ab}
	60	92,40 ± 0,55^{b}	6,43 ± 0,19^{b}	59,42 ± 0,31^{ab}
	90	98,82 ± 1,01^{a}	6,94 ± 0,04^{a}	58,26 ± 0,50^{b}

L* - lightness, a* - parameter responsible for red colour, b*- parameter responsible for yellow colour; Means in each column not sharing a letter in the exponent are significantly different according to Tukey's test, $p < 0.05$.

In contrast, the lightness and parameter a* (red) showed higher values by 20% and 18% in P1 powder and by about 9% and 38% in P2 powder. The total colour change (ΔE) during storage of the powders is shown in Figure 6.9. from which it can be concluded that both powders changed colour during storage of the samples.

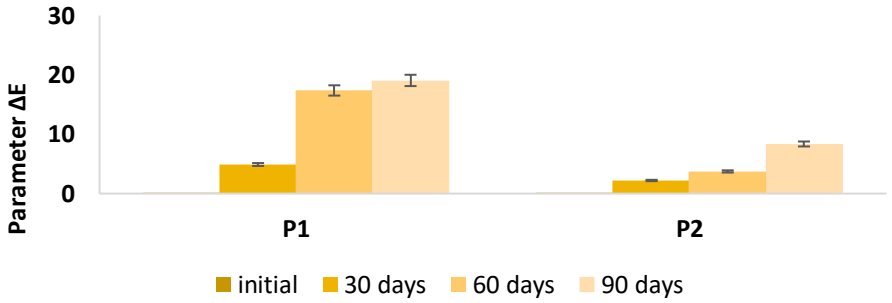


Figure 6.9. Evolution of the total colour change (ΔE) of the powders analysed during storage

This can be correlated with the oxidation process of the carotenoids in the powders and the encapsulation efficiency of the bioactive substances. The P2 powder encapsulated with alginate, agar and chitosan was much more colorimetrically stable upon storage.

6.8.7 Physico-chemical characterisation of value-added mayonnaise

Mayonnaise is definitely one of the most famous, popular and popular sauces in the world. In our study, mayonnaise sauce was obtained by adding different percentages of microencapsulated sea buckthorn extract to the classic sauce recipe. The powder selected for introduction into the innovative product was P2 powder. Thus, three variants of powdered mayonnaise sauces were obtained: 0%, 2.5% and 5%, coded as follows: M - 0% powder; M1 - 2.5% powder; M2 - 5% powder. All samples were analysed for physico-chemical and phytochemical characteristics, the results of which are presented in Table 6.3 and Table 6.4

respectively.The results presented in Table 6.3. indicate that mayonnaise sauces obtained with the addition of sea buckthorn powder are characterised by a significantly lower protein content compared to mayonnaise sauce obtained conventionally ($p < 0.05$). In contrast, there is no change in the carbohydrate and lipid content of the value-added sauces, which are similar to the carbohydrate and lipid content of the control sample. As for the energy value of the samples analysed, there is a decrease of only 1.53% in the samples with higher powder concentrations. However, the energy values of all variants are very close.

Table 6.3. Physico-chemical characteristics of value-added mayonnaise

Physical and chemical characteristics	Witness (M)	M 1 (2,5%)	M 2 (5%)
Protein, g/100 g	8,06 ± 0,07^{c}	6,94 ± 0,02^{c}	6,93 ± 0,04^{c}
Lipid, g/100 g	72,02 ± 0,20^{a}	72,72 ± 0,32^{a}	71,30 ± 0,24^{a}
Carbohydrates, g/100 g	2,45 ± 0,21^{d}	2,09 ± 0,21^{d}	2,61 ± 0,05^{d}
Moisture, g/100 g	15,58 ± 0,04^{b}	16,28 ± 0,10^{b}	16,98 ± 0,12^{b}
Ash, g/100 g	1,84 ± 0,02^{d}	1,96 ± 0,04^{d}	2,16 ± 0,21^{d}
Energy value, %			
Kcal	713,24 ± 0,74^{b}	713,34 ± 2,30^{b}	702,29 ± 2,71^{b}
Kj	2984,20 ± 3,13^{a}	2984,61 ± 9,62^{a}	2938,38 ± 11,33^{a}

Means on each line that do not share a letter in the exponent are significantly different by Tukey's test, $p < 0.05$.

Moisture is an important factor to be determined in food analysis as it affects the final result and the performance of the mayonnaise emulsion. In addition, moisture is of great economic value to producers, as it is a quality factor in the preservation of some products that affects their stability, and is an essential parameter for determining the nutritional value of foods (Nielsen et al., 1998). According to the literature, the maximum moisture content in mayonnaise is 30%. As can be seen from Table 6.3 these values are lower and increased with increasing concentration of added powder in mayonnaise samples M1 and M2 compared to the control sample M, while the energy value decreased by about 60 kJ per 100g product, due to the decrease in the amount of lipids and proteins in the mayonnaise samples.

6.8.8 Phytochemical characterisation of value-added mayonnaise

The phytochemical characteristics of the mayonnaise sauces for both the control and value-added samples are shown in Figure 6.10 and Figure 6.11. Thus, it can be seen that the addition of sea buckthorn powder in the obtained mayonnaise sauces resulted in increased total carotenoid content compared to the control mayonnaise sauce.

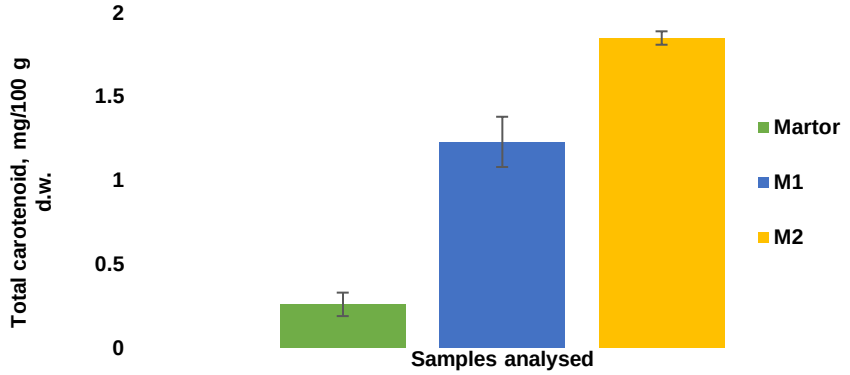


Figure 6.10. Total carotenoid content of mayonnaise sauce samples analysed

As predicted, there is a significant increase in carotenoid concentration and antioxidant activity value as the amount of added powder increases ($p < 0.05$). Thus, the mayonnaise variants showed a TC content with values between 0.26 ± 0.07 mg TC/100 g d.w. and 1,85 ± 0,04 mg TC/100 g d.w. As regards antioxidant activity, it increased from 9.99 ± 0.60 µM Trolox/g d.w. to 293.38 ± 2.77 µM Trolox/g d.w. The content of phytochemical compounds (total carotenoids) and antioxidant activity determined by the ABTS free radical scavenging method of the value-added mayonnaise sauce samples are shown in Table 6.4.

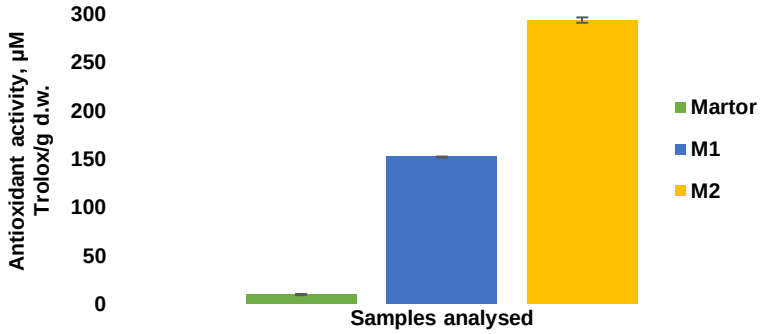


Figure 6.11. Antioxidant activity of mayonnaise sauce samples analysed

Table 6.4. Phytochemical characteristics of value-added mayonnaise samples

Phytochemical characteristics	M	M1 (2,5%)	M2 (5%)
TC, mg /100 g d.w.	0,26 ± 0,07[b]	1,23 ± 0,15[b]	1,85 ± 0,04[b]
Antioxidant activity, µM Trolox/g d.w.	9,99 ± 0,60[a]	152,05 ± 0,41[a]	293,38± 2,77[a]

Means on each line that do not share a letter in the exponent are significantly different by Tukey's test, $p < 0.05$.

The results presented in Table 6.4. confirm the added value of mayonnaise sauces with buckthorn powder by increasing total carotene content and antioxidant activity, respectively.

6.8.9 Colour analysis of value-added mayonnaise

The sauces were also analysed in terms of CIELab colorimetric parameters. The results were expressed as L* (lightness), a* (tendency towards red or green) and b* (tendency towards yellow or blue) parameters. Table 6.5. shows the CIELab parameter values for the mayonnaise sauces analysed.

The significant increase in b* value with the amount of powder added suggests a tendency towards yellow offered by the biologically active compounds in the powders used as a functional ingredient ($p < 0.05$). However, a significant decrease in lightness is observed with increasing powder concentration and a tendency to green up (Table 6.5). Also the L* luminosity showed a slight decrease.

The increase in yellow colour intensity with increasing powder concentration can also be observed in Figure 6.12.

Table 6.5 Colour characteristics of value-added mayonnaise samples

Sample	L*	a*	b*
M	65,67 ± 0,62^{a}	-1,01 ± 0,13^{a}	30,23 ± 1,25^{c}
M1 (2,5%)	61,08 ± 1,07^{a}	-0,53 ± 0,02^{b}	42,93 ± 0,81^{b}
M2 (5%)	54,34 ± 1,23^{b}	-0,34 ± 0,02^{c}	57,36 ± 1,50^{a}

L* -lightness, a* -red, b* -yellow; Means in each column not sharing a letter in the exponent are significantly different by Tukey's test, $p < 0.05$.

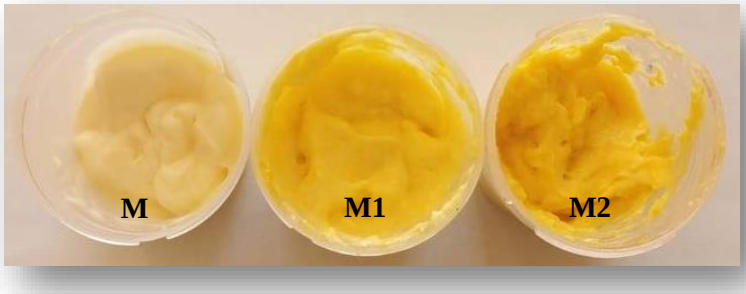


Figure 6.12. Mayonnaise sauce samples: M - sauce without added powder, M1 and M2 - mayonnaise sauce with 2.5 and 5% (w/w) added powdered raisin extract

The graphical representation of the total colour change (ΔE) is shown in Figure 6.13.

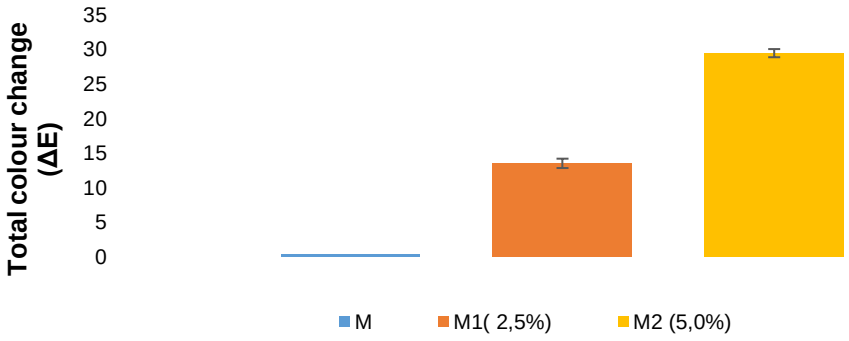


Figure 6.13 Total colour change (ΔE) of mayonnaise sauce samples analysed

When ΔE > 1 means that the colour difference is perceptible to the human eye (Drozłowska et al.,2020). The value of colour difference (ΔE) in the obtained mayonnaise samples increased with the concentration of added powder, thus ΔE was higher for the mayonnaise with 5% added sea buckthorn powder.

Our observations showed that the addition of sea buckthorn powder in samples M1 and M2 caused a perceptible colour change in the mayonnaise samples. In addition, these results were confirmed with the sensory evaluation, as sample M2 had the highest score from tasters in terms of colour.

6.8.10 Texture analysis of value-added mayonnaise

To estimate the impact of the powders on the texture of the mayonnaise sauce, firmness, adhesion, cohesiveness and masticability were tested using the TPA method. The results of these parameters are presented in Table 6.6.

The addition of powder resulted in higher firmness values in the mayonnaise sauce compared to the control sample (Table 6.6.), due to the stabilising compounds in the encapsulating matrix. The control sample showed a higher porosity compared to the other variants, resulting in a significantly lower compressive strength ($p < 0.05$). Cohesivity, which expresses the strength of the internal bonds that give consistency to the product, was in a slight decrease compared to the control sample and is due to the increase in the concentration of powder incorporated in the mayonnaise sauce.

Table 6.6. Textural characteristics of value-added mayonnaise

Texture parameters	M	M 1 (2,5%)	M 2 (5%)
Firmness, N	0,22 ± 0,01^{d}	0,23 ± 0,03^{d}	0,34 ± 0,03^{d}
Adherence, mJ	1,63 ± 0,15^{b}	2,32 ± 0,12^{b}	3,41 ± 0,38^{b}
Cohesivity	0,68 ± 0,02^{c}	0,65 ± 0,03^{c}	0,62 ± 0,05^{c}
Elasticity, mm	11,13 ± 0,21^{a}	10,67 ± 0,74^{a}	10,98 ± 0,32^{a}

Means on each line that do not share a letter in the exponent are significantly different by Tukey's test, $p < 0.05$.

In our study, it was also noted that the addition of powder led to improved adhesion. In terms of cohesion and elasticity, no significant differences were observed with the addition of powder ($p > 0.05$).

Adherence is important for a sauce in order for the sauce to stick less to the cutter and be easily spreadable, or for salads as a garnish.

6.8.11 Sensory analysis of value-added mayonnaise

From a sensory point of view, the sauces were analyzed using a 10-point hedonic scale based on unit numbering. Attributes tracked were: colour, aroma, taste, consistency, texture,

odour, aftertaste, spreadability and acceptability. Sensory analysis was carried out at 20 °C and 45-47 % relative humidity. The average scores obtained from the sensory analysis are shown in Figure 6.14.

Panellists reported differences in colour between the sauces, with M2 sauce being the most intensely yellow coloured. Mayonnaise sauces with added sea buckthorn powder were evaluated as having a balanced taste, odour and colour. The smooth, creamy and fluffy consistency of the mayonnaise sauces was also appreciated. All samples of mayonnaise obtained were positively evaluated by the panel team, with no perceptible sea buckthorn flavour.

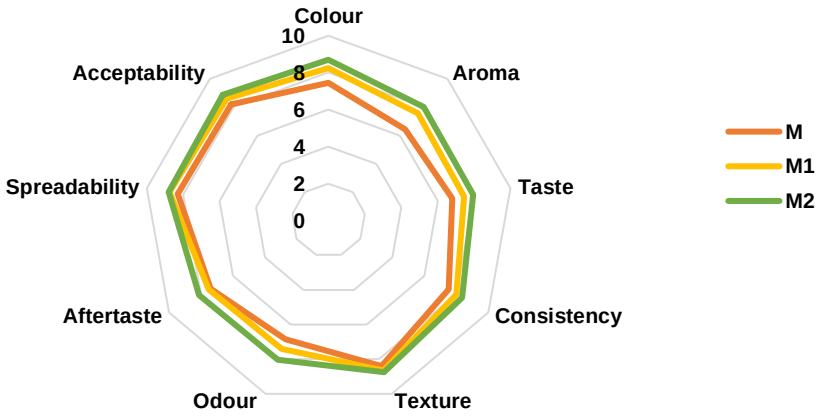


Figure 6.14. Comparative diagram of the specific sensory attributes of the sauces: M - mayonnaise containing no added powder, M 1 and M 2 - mayonnaise with 2,5 and 5 % added buckthorn powder

Acceptability can be correlated with texture, spreadability, sauce colour and rheological parameters of sauces.

There were no significant variations in overall acceptability between the three mayonnaise samples analysed. Aftertaste is the intensity of the taste of a food that is perceived immediately after the food is removed from the oral cavity. Aftertaste values can be related to the flavour, aroma and taste of the product. Using a 10-point hedonic scale to measure food preferences, all attributes assessed for the mayonnaise samples fortified with raisin extract powder were rated as very much liked "I like it very much" (Wichchukit et al., 2014).

6.9 Correlation of results

Powder stability is an index of the shelf life of bioingredients. From the results, a correlation can be made between colorimetric stability and phytochemical stability of sea buckthorn powders, which can provide us with a sensory index of powder degradation.

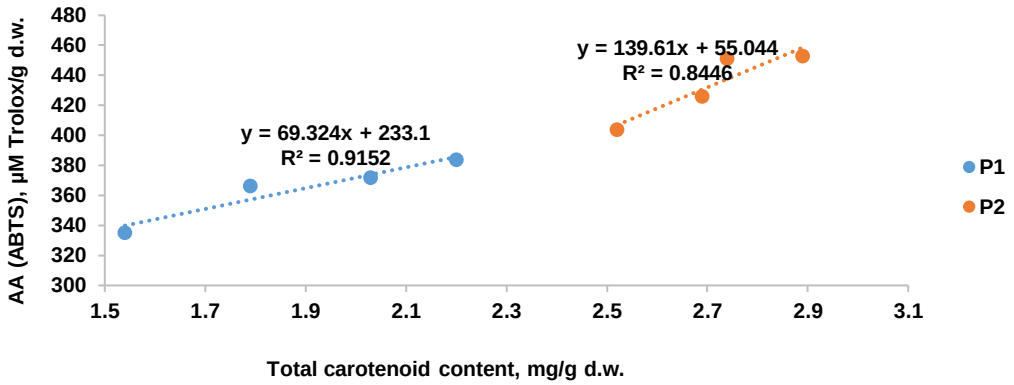


Figure 6.15 Correlation of antioxidant activity (ABTS) and total carotenoid content during 90 days of storage of sea buckthorn powders

From Figures 6.15, 6.16 and 6.17 it can be seen that there is a strong correlation between the results of the stability of the powders during 90 days of storage for the indices: total carotenoid content, antioxidant activity determined by the ABTS free radical scavenging method and the colour parameter b*(yellow). This means that during 90 days of storage all the above indices and parameters deteriorated slightly.

From Figures 6.15, 6.16 and 6.17 it can be concluded that there is a strong correlation between the three parameters (total carotene content, antioxidant activity and the colour parameter b* (yellow).

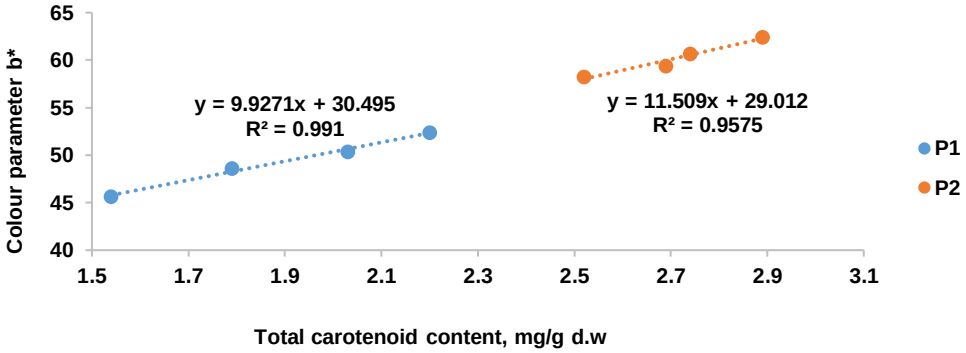


Figure 6.16 Correlation of total carotenoid content and colour parameter b*(yellow) during 90 days of storage of sea buckthorn powders

This correlation depends on storage conditions (temperature, humidity, dark/light storage), but also on the oxidation process of fat-soluble compounds, more specifically enzymatic oxidation. Also, during storage P2 powder was influenced less by degradation processes compared to P1 powder (Pearson's coefficient indicates a lower correlation with P2).

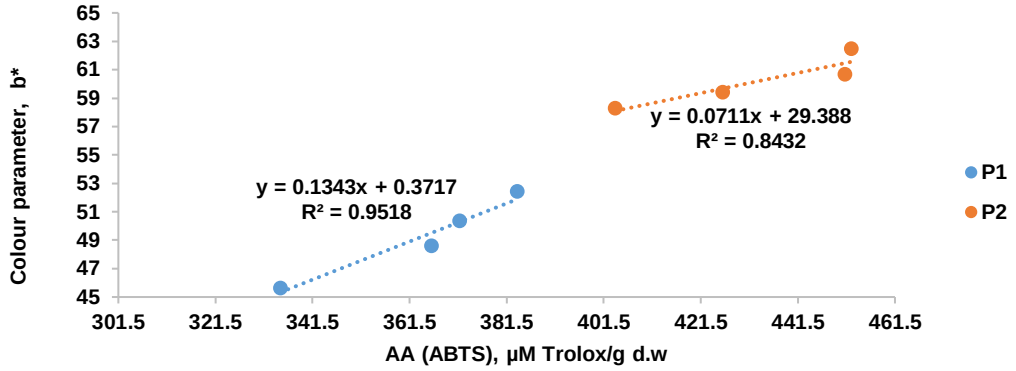


Figure 6.17 Correlation of antioxidant activity (ABTS) and colour parameter b* (yellow) during 90 days of storage of sea buckthorn powders

Principal component analysis (PCA) is a statistical method to find relationships between variables (Barbu et al., 2015). It is used to check the relationship between different variables. PCA was used to find the relationship between the variables of microencapsulated buckthorn powder (ABTS, ΔE, L*,CT, b*).

Table 6.7 Correlation matrix of main components for microcapsule powder during storage for 90 days

	ΔE	L*	CT	b*	ABTS
ΔE					
L*	0,9967				
CT	0,8293	0,8937			
b*	0,7171	0,8353	0,991		
ABTS	0,4947	0,6567	0,9152	0,9518	

The aim of correlating the obtained results was to find the relationships between the following variables: antioxidant activity on the free radical ABTS; colorimetric parameters L*-luminosity, b*-yellow colour, ΔE- total colour change during 90 days of storage and the amount of total carotenoids by principal component analysis.

From Table 6.7 it can be seen that all the components are well correlated which would suggest a more thorough investigation of the interdependence of these parameters to explain the biochemical processes in the storage process of rattan powders.

The results encourage further studies on the bioavailability of carotenoids encapsulated in alginate, agar and chitosan matrices.

6.10 Partial conclusions

In this study, carotenoids in sea buckthorn extract were successfully encapsulated using complex coacervation and freeze-drying as encapsulation techniques.

Two different combinations of whey proteins and polysaccharides were used as encapsulating agents. The highest carotenoid encapsulation efficiency was obtained for P2

powder, which was encapsulated only with polysaccharide-type biopolymers such as agar, chitosan, and alginate.

Both powders showed high phytochemical content with remarkable antioxidant activity. During storage, a slight degradation of the phytochemicals was observed, with an increase in the brightness of the powder and a decrease in the parameter b*, which reflects the yellow color.

The CLSM revealed the presence of carotenoids anchored in the encapsulation matrices, well individualized, but with a tendency to form clusters. Both encapsulation matrices used increased the bioavailability of sea buckthorn carotenoids in the digestive system in vitro. To demonstrate the functionality of sea buckthorn powders, different percentages of P2 powder were evaluated and added as an ingredient in a mayonnaise-type sauce.

The obtained mayonnaise sauces had a satisfactory total carotenoid content as well as an antioxidant activity. Analysis of the texture of the mayonnaise sauces suggested that the addition of sea buckthorn powder caused an increase in firmness and stickiness. The sensory analysis of the mayonnaise sauces showed that the panelists appreciated the mayonnaise sauces obtained.

Therefore, the microencapsulation of sea buckthorn extracts and their incorporation into mayonnaise presented a promising method for the stabilization of carotenoids in the obtained value-added products. Thus, sea buckthorn extracts can be considered valuable ingredients for the development of food products with added value.

CHAPTER 7. FINAL CONCLUSIONS

Current research in the specialized literature substantiates the information regarding the structure and functionality of the biologically active compounds in sea buckthorn, which vary from a geographical point of view, genetic variability, crop area, species, etc. These aspects are important and require in-depth studies due to the diversity, different composition, which depend on exogenous factors of growth and development of the plant in general. The studied fruits from wild sea buckthorn varieties are rich in biologically active compounds: polyphenols and carotenoids with special functional potential. It is important to evaluate the stability over time of the bioactive potential of the compounds and ingredients obtained from the extracts of these fruits, so that they can be applied in the development of composites for use in different branches of industry.

The doctoral thesis concerned the study of bioactive compounds from sea buckthorn, from the perspective of phytochemical extraction and characterization, their encapsulation, the evaluation of the stability of the obtained powders, as well as their integration into food products, giving added value to the obtained products. Based on the experimental results and the partial conclusions presented at the end of each chapter of the doctoral thesis entitled "Valorization of some biologically active compounds from sea buckthorn through the development of food products with added value", all the objectives proposed at the beginning of the thesis were fulfilled, and the results they are highlighted in the part of partial conclusions and final conclusions as follows:

- The extraction and comparative evaluation of the biologically active compounds from sea buckthorn proved once again that it is a poly-compositional fruit, due to the fact that it is rich in both lipophilic compounds - carotenoids and hydrophilic compounds - polyphenols. This unique property already demonstrated makes this fruit competitive in the market of plant products, so it deserves to be promoted through article publications, invention patents and derivative commercial products with a major impact on the market.
- By comparing the three extraction techniques, it was observed that UAE combined with various polar and non-polar solvents provides a higher release yield of the bioactive compounds of interest. For the extraction of lipophilic compounds (carotenoids) the optimal variant was the mixture of ethyl acetate and n-hexane in the ratio (2:1), and for the hydrophilic compounds (polyphenols) it was the mixture of ethanol and acetone in the ratio (4:3). The extraction of the compounds was carried out for 45 minutes at the temperature of 40°C and the frequency of 40 kHz.
- In order to observe the selectivity of the extractions from the phytochemical point of view, the separate and sequential extraction was performed with different solvent mixtures using the same lyophilized matrix as a source of biologically active compounds. The best results for the extraction of polyphenolic compounds were obtained in the sequential extraction with acidified solution of formic acid, acetone and water (0.35:20:80).
- The correlation of the content of hydrophilic compounds, specifically polyphenols, with the antioxidant activity measured by the neutralization capacity of ABTS and DPPH

free radicals was demonstrated. The best correlation between TPC was for antioxidant activity measured by ABTS free radical neutralizing capacity, and the best value was for sequential extraction. It was also confirmed that the antioxidant activity does not depend on the extraction method, but depends on the extraction solvents and the phytochemical compounds released.

- Chromatographic analysis of carotenoids from the analyzed extracts revealed the presence of five main carotenoid compounds: astaxanthin, zeaxanthin, β-cryptoxanthin, lycopene and β-carotene.
- A series of microencapsulated variants of sea buckthorn fruit extract have been developed, through lyophilization and complex coacervation encapsulation techniques, using polysaccharides (CMC, agar, chitosan, alginate) and whey proteins (WPI) as encapsulation materials. The highest encapsulation efficiency was obtained for the variant WPI(2%):CMC (2%)1:1(v/v) and had a value of 87.23%.
- The powders with the best phytochemical characteristics, in terms of antioxidant activity and encapsulation efficiency, were chosen for analysis by confocal laser microscopy and showed values of the order of micrometers uniformly distributed in the form of well-individualized spherosomes sometimes forming clusters (but well fixed) depending on the matrices used.
- The powders with the highest carotenoid content and encapsulation efficiency were selected to evaluate their storage stability. Thus, during 270 days of storage, the encapsulation efficiency of the powders decreased on average by 10% compared to the initial encapsulation efficiency. The color degradation of the sea buckthorn powder variants during 270 days (9 months) of storage was also tested, which showed values of 16% for the b* parameter.
- The in vitro digestibility studies showed that the polysaccharide and protein matrices protect the encapsulated biologically active substances, compared to other variants analyzed, and under conditions of simulated intestinal digestion, the maximum amount of lipophilic compounds is released after 120 minutes of simulated intestinal digestion. The amount of compounds released under conditions of simulated gastric digestion was small, their release being greater in simulated intestinal juice.
- The functional properties of the powders were tested in mayonnaise-type sauce variants with the addition of sea buckthorn. Mayonnaises obtained with the addition of microencapsulated sea buckthorn powders have been shown to have added value in terms of antioxidant activity, carotenoid content, improved color, nutritional value and rheological properties similar to the control sample which represents a beneficial advantage for consumers who are used to classic mayonnaise.
- The energy values for all variants of mayonnaise as well as the control sample were approximately equal, we can say that mayonnaises with added value are richer in carotenoid compounds, have a higher antioxidant activity at the same energy values as the control.

- The texture analysis of the samples indicated that the addition of powder resulted in higher firmness values in value-added mayonnaise variants and improved stickiness. From the point of view of cohesion and elasticity, no significant differences were observed when adding powder compared to the control sample. These results are consistent with the sensory analysis attributes such as: spreadability and texture of the analyzed samples.
- The colorimetric analysis of the mayonnaise sauce variants revealed that the sauce with the highest concentration of added powder (5%) had the highest value of the parameter b* which is also consistent with the sensory attribute color.
- The maximum concentration of powder added in the mayonnaise sauce was 5%, since higher amounts lead to the deterioration of the textural and sensory qualities of the value-added product.
- The results obtained in this study reveal the fact that by extracting the bioactive compounds fromsea buckthorn, colored extracts are obtained, which have a high potential of compoundsphytochemicals with remarkable antioxidant activity, and their encapsulation allows obtaining food products with added value that can replace synthetic dyes and additives in some food products.

CHAPTER 8. PERSONAL CONTRIBUTIONS AND PERSPECTIVES FOR FURTHER STUDIES

With the help of the applied and fundamental instrumental techniques in the doctoral thesis entitled "Valorisation of biologically active compounds from sea buckthorn by developing value-added food products", the stages of recovery, stabilization and incorporation of bioactive compounds from sea buckthorn were established. Thus derive the following original contributions:

- Comparative evaluation of different extraction techniques and methods from the perspective of establishing the optimal parameters for obtaining lipophilic (carotenoids) and hydrophilic (polyphenols) compounds from freeze-dried sea buckthorn fruits.
- Global and advanced characterization of the phytochemical profile of the extracts using spectrophotometry and chromatography techniques.
- The development of variants of sea buckthorn powders by complex coacervation and lyophilization obtained from sea buckthorn extracts and the evaluation of their phytochemical profile, color and storage stability, with the aim of use in the technology of obtaining food products with added value.
- The valorization of sea buckthorn extract powders, to obtain economically and ecologically sustainable products by applying the clean shelf label concept.

In the perspective of future research, the following are outlined:

- Determination of the antimicrobial activity of the sea buckthorn extracts obtained, of the prebiotic and cytotoxic potential of the powders from sea buckthorn;
- The use of sea buckthorn powders obtained to obtain other food products: fermented dairy products, Feta cheese, thermostable fillings - thanks to the accentuated colorimetric and phytochemical profile, pasta - possibility of coloring without the addition of dyes and the patenting of the products with added value made.

CHAPTER 9. VALORISATION OF RESULTS

I. Articles published in ISI rated journals

1. Roman D., Condurache (Lazar) NN, Stănciuc N., Andronoiu G., Aprodu I., Enachi E., Barbu V., Bahrim GE, Stanciu* S., Râpeanu* G. Advanced composites based on sea buckthorn carotenoids for mayonnaise enrichment, Polymers (2022),*14*(3), 548.https://doi.org/10.3390/polym14030548,Q1, Impact Factor = 4.8 (2021)

II. Articles published in indexed journals WoS

1. Roman D., Constantin O., Stănciuc N., Râpeanu G. Bioactive compounds and antioxidant activity in different extracts of Sea Buckthorn (Hippophae rhamnoides L) berries. The Annals of the University Lower Danube of Galati Fascicle VI – Food Technology (2020), 44(1), 178-192.

2. Roman D.,Condurache (Lazar) N.,Aprodu I., Enachi E. Barbu V., Bahrim G.E., Stănciuc N., Râpeanu G. Insights into Sea Buckthorn Extract's Encapsulation Coacervation Technique.*Inventions*(*2021),6*(3), 59.https://doi.org/10.3390/inventions6030059

III. Patent applications

1. Roman D., Râpeanu G., Condurache (Lazăr) N.N., Stănciuc N., Andronoiu D.G., Aprodu I., Bahrim G.E., Dunărea de Jos Galați University, Mayonnaise-based sauce with the addition of powder from microencapsulated sea buckthorn extract - product with added value and its production technology - no. of OSIM registration (A00657/1.11.2021), Derwent accession number 2023-59087U .

IV. Participation in national and international conferences

1. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim, G.E. 2019 Extraction and characterisation of bioactive compounds from lyophilized sea buckthorn, poster, 7th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.
2. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim G.E., 2019 Extraction and characterisation of bioactive compounds from sea buckthorn (Elaeagnus rhamnoides(L.)A. Nelson), poster, 9th International Symposium EuroAliment of Faculty of Food Science and Engineering, „ Dunarea de Jos" University of Galati.
3. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim G.E., 2020 Phytochemical characterization of sea buckthorn hydrophilic and lipophilic extracts, poster, 8th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.
4. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim G.E.,2020. Evaluation of biological active compounds found in sea buckthorn fruits. Poster, Scientific Symposium

"Young people and multidisciplinary research in applied life sciences",7th edition, Section: Food Engineering, Banat USAMVB „King Michael I of Romania" Timișoara.

5. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim, G.E. 2021. Sea buckthorn berries as a wide source of bioactive compounds. Poster. 9th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.
6. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim, G.E. 2021. Encapsulation of oleoresins from sea buckthorn in different matrix, Poster. 9th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.
7. Roman D., Horincar G., Stănciuc N., Aprodu I., Râpeanu G. 2021 Stability of encapsulated carotenoids extracted from sea buckthorn. Poster. 10th International Symposium EuroAliment, Faculty of Food Science and Engineering, „Dunarea de Jos" University of Galati.
8. Roman D., Aprodu I., Bahrim G., Stanciuc N., Rapeanu G., 2022 Hippophae rhamnoides – potential application in food industry. Poster, 10th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.
9. Roman D., Aprodu I., Bahrim G., Stanciuc N., Rapeanu G., 2022 Carotenoids based sauce as a current trend on sea buckthorn valorization. Poster, 10th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.
10. Roman D., Rapeanu G., Bahrim G., Aprodu I., Stanciuc N., 2023 Designing a new healthy and functional mayonnaise sauce. Poster, 11th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati.

V. Awards

1. Honorable Mention. Poster. Phytochemical characterization of sea buckthorn hydrophilic and lipophilic extracts. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim G.E., 2020 7th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati 18-19 iunie 2020 Galați.

2. Premiul III, Poster, Evaluation of biological active compounds found in sea buckthorn fruits. Roman D., Râpeanu G., Stănciuc N., Aprodu I., Bahrim G.E Scientific Symposium "Young people and multidisciplinary research in applied life sciences",7th edition, Banat University of Agricultural Sciences and Veterenary Medicine „King Michael I of Romania" 27 November 2020 Timișoara.

3. Honorable Mention. Poster. Carotenoids based sauce as a current trend on sea buckthorn valorization. Roman D., Aprodu I., Bahrim G., Stanciuc N., Rapeanu G., 10th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati 7-9 iunie 2022 Galați.

4. Honorable Mention. Poster. Designing a new healthy and functional mayonnaise sauce. Roman D., Râpeanu G., Bahrim G., Aprodu I., Stanciuc N., 11th edition of Scientific Conference of Doctoral Schools SCDS – UDJG, „Dunarea de Jos" University of Galati 8-9 iunie 2023 Galați.