Ph.D. THESIS

The assessment of the *in vitro* bioaccessibility of carotenoids from food matrices-the effect of lipid addition and carotenoids encapsulation

(SUMMARY OF THE DOCTORAL THESIS)

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Introduction

Phytochemicals are associated with numerous health benefits and thought to be responsible for the prevention of many diseases (Zakynthinos &Varzakas, 2016). Carotenoids are a class of lipophilic phytochemicals responsible for the colors of many leaves, fruits and vegetables or some animal organisms. They are synthesized by plants, algae, photosynthetic bacteria and only a few specific animal organisms, while humans cannot synthesize them, their presence in the human body being due to food intake (Rodriguez-Concepcion et al., 2018; Saini et al., 2015). Although they are abundant in food, their effect on human health is limited by their low bioavailability.

The aims of this study were fulfilled by achieving the following objectives

Objective one: Extraction of carotenoids from widely consumed vegetable matrices, namely tomatoes, carrots, baby spinach and sea buckthorn (berries and pomace), using conventional solid liquid extraction (maceration with organic solvents) and the ultrasound-assisted extraction (sea buckthorn pomace). For sea buckthorn pomace, a series of green solvents (ethyl lactate, ethyl acetate, sunflower oil, cyclopenthyl methyl ether, methyl tetrahydrofuran) were tested, compared to a mixture of organic solvents.

Objective two: Identification and quantification of main carotenoids (α-carotene-αC, β-carotene-βC, γ-carotene-γC, lycopene-LYC, lutein-LUT, zeaxanthin-ZEA, and its esters) in the tested food matrices by High Performance Liquid Chromatography (HPLC-PDA) and spectrophotometric analysis. Characterization of fatty acids composition of food matrices and vegetable oils by GC-MS.

Objective three: Determination of the antioxidant activity of the saponified carotenoid extract from sea buckthorn berries, by established antioxidant activity assays: ABTS, DPPH and FRAP.

Objective four: Determination of the *in vitro* bioaccessibility of the main carotenoids in food matrices by using the standardized *in vitro* digestion protocol INFOGEST.

Objective five: Evaluation of the effect of lipid addition (sunflower oil, olive oil, flaxseed oil, avocado pulp, sour cream) on the bioaccessibility of major carotenoids from carrots, baby spinach and cherry tomatoes using the *in vitro* digestion protocol (INFOGEST).

Objective six: Valorization of a by-product resulted from sea buckthorn juice production (sea buckthorn pomace) for the extraction of carotenoids to be encapsulated and further used in food applications.

Objective seven: Obtaining microbeads enriched with carotenoid extract through the technique of gelation by cross-linking with sodium alginate and calcium carbonate; the characterization of the beads through spectroscopic and microscopic techniques, determination of encapsulation efficiency and stability of encapsulated carotenoids in various experimental conditions.

Objective eight: Determining the *in vitro* bioaccessibility of carotenoids encapsulated in alginate microbeads, in the absence/presence of yoghurt.

1st Study- The effect of vegetal oils addition on the bioaccessibility of carotenoids from cherry tomatoes, baby spinach, and carrots

1. Introduction and aims

The aim of this study was to find alternative methods to increase the bioavailability of carotenoids without using processes that can lead to their destruction, therefore the food matrices were analyzed fresh. Knowing that the addition of lipid sources can facilitate carotenoids micellization and also increase their bioaccessibility, in the current study the effect of the addition of different types and quantities of some traditionally used oils addition was investigated. Sunflower and olive oil were selected because of their unsaturated fatty acids profile and because they are the most commonly used oils in alimentation. Flaxseed oil is also a highly unsaturated oil, but having a specific fatty acids profile, with high proportion of n-3 fatty acids.

2.Materials and methods

Cherry tomatoes, baby spinach leaves and carrots were washed and crushed into smaller pieces with a blender. Total carotenoids were extracted from 3g fresh samples using methanol: ethyl acetate: petroleum ether (1:1:1, v/v/v), as extraction solvents, to color exhaustion. The residue was dissolved in methyl-*tert*-butyl-ether (MTBE) and analyzed by C30-HPLC-PDA. Total lipids were extracted from 3 g of fresh samples, as described by Folch et al. (1956). The FAMEs were identified by comparing their retention times with those of known standards and the resulting mass spectra to those in the data-base. The simulated *in vitro* gastrointestinal digestion was performed using the method described by Minekus et al. (2014).

3.Results and discussions

3.1 Total carotenoid content from raw cherry tomatoes, carrots, and baby spinach

Table 1. Total carotenoid content (mg/100g) from raw cherry tomatoes, carrots, and baby spinach

Sample	α-carotene	β-carotene	Lutein	Lycopene	Total content
Baby spinach	0.10±0.003	4.32±0.80	9.58±2.03	-	15.21±1.8*
Carrots	2.13±0.15	6.01 ±0.41	0.07±0.01		11.54±1.8
Cherry Tomatoes	-	1.01±0.36	0.09±0.03	3.67±1.25	5.90±0.74

*Total carotenoids obtained after saponification

**Each value represents the mean ±standard deviation of 3 replicates

3.2 Fatty acid composition of raw samples

Cherry tomato samples had a total lipid content of 0.63 g/100 g fat. Regarding the cherry tomatoes fatty acids profile, polyunsaturated fatty acids were the majority, with a percentage of 63.55%, followed by saturated fatty acids, approx. 23%, and monounsaturated fatty acids, approx. 13%. The predominant fatty acid was linoleic acid, in a concentration of approx. 58%, followed by palmitic and oleic acid, with 20% respectively 12.4%, and, in much lower amounts, α-linolenic (5.64%) and stearic acids (2.56%). Regarding the carrot samples, the total lipid content was 0.35 g/100 g fat and the main identified fatty acid was linoleic acid (68.52%). Polyunsaturated fatty acids were the most abundant ones, with a percentage of 71%, saturated fatty acids represented about 28%, and monounsaturated fatty acids were found in very low amounts (approx. 1%). Palmitic acid was found in significant amounts (26.35%), and in much lower quantities were α-linolenic (2.5%), oleic (0.86%), and stearic acids (0.7%). For baby spinach, the total amount of lipids was 0.39 g/100 g. Polyunsaturated fatty acids

represented the main fraction (88.30%), followed by saturated fatty acids (11.14 %) and very low amounts of monounsaturated fatty acids. The predominant fatty acid was the α-linolenic acid, with 84 %, followed by palmitic acid.

3.3 Characterization of vegetal oils in terms of total carotenoid content and fatty acids composition

The total carotenoid content of oils was sunflower oil - 0.06 ±0.002 mg/100g, linseed oil - 0.2±0.003 mg/100g, and olive oil - 0.4±0.011 mg/100g. The main detected carotenoids in all samples were lutein, zeaxanthin and β-carotene, in various proportions. Sunflower oil had a total content of 8.22% saturated fatty acids, 21.32% monounsaturated fatty acids, and 70.46% polyunsaturated fatty acids. Linoleic acid was the major one (70.46%), followed by oleic (20.53%) and palmitic (5.96%) acids. For linseed oil, the fatty acids profile showed α-linolenic as the major acid (53.72%), and also important amounts of oleic acid (23.3%) and linoleic acid (14.08%). Olive oil is a rich source of oleic acid (86.21%) but also contains lower quantities of palmitic acid (8.82%).

3.4 Carotenoid bioaccessibility

Among all three matrices, carotenoids from carrots had the highest bioaccessibility. The bioaccessibility percentage for lutein was 45.2%, for α-carotene 13.1%, and for β-carotene 12.1%. After the digestion of baby spinach, the bioaccessibility was higher for lutein (6.3%) than for β-carotene (3.2%). The same order was found for carotenoid contribution in the initial extract, before digestion (lutein-74.6%, β-carotene-25.4%). For cherry tomatoes, lutein had the highest percentage of bioaccessibility (40.1%), approximately four times higher than β-carotene (10.2%) and more than ten times higher than lycopene (3.1%). After oil addition, for cherry tomatoes, it can be observed that the bioaccessibility of β-carotene increased from 12.1 % to 16.2 % (olive oil 10 %) and to 25.1 % (sunflower oil 10 %). In the case of carrot samples, the most notable changes in bioaccessibility after the addition of oil were observed for α-carotene. For olive oil (10 %) and for sunflower oil (10 %) the bioaccessibility of α-carotene increased from 13.1 % to 31.4 % and 29.5 %, respectively. For baby spinach samples, the most important effect was observed for olive oil (4.6 %, for 10 % oil) and sunflower oil (4.7 % for 10 % oil), but linseed oil also increased the bioaccessibility, in a lower extent.

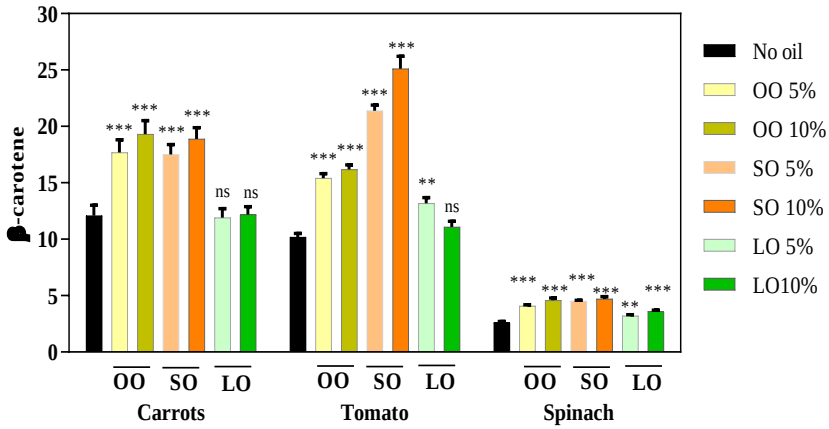

Figure 1. Bioaccessibility of β-carotene from carrots, cherry tomatoes and spinach, without and with lipid addition.

2nd Study – The effect of addition of rich sources of lipids on the bioaccessibility of lutein and β-carotene from raw baby spinach leaves

1.Introduction and aims

In this study, we aimed to investigate the effect of both the amount of added lipids and the type of lipid on the bioaccessibility of the major carotenoids from baby spinach. In this regard, two types of lipid sources were chosen, namely avocado - a plant lipid source rich in monounsaturated fatty acids, and sour cream - an animal lipid source rich in saturated fatty acids. Moreover, prior to the *in vitro* digestion, all the food samples were characterized in terms of carotenoid and lipid compositions.

2.Materials and methods

Fresh baby spinach (*Spinacia oleracea*), fresh avocado (*Persea americana, Cv. Hass*), and sour cream were subjected to carotenoids and lipids extraction. The extraction, HPLC-PDA analysis and GC-MS analysis were performed in similar conditions as described in section 1. The *in vitro* gastrointestinal digestion was performed according to the INFOGEST method on baby spinach samples without and with the addition of lipids from avocado (5% and 10%) or sour cream (10% or 20%).

3.Results and discussions

3.1. Carotenoid composition of baby spinach, avocado and sour cream

Table 1. Carotenoid content (µg /100g) * of fresh samples

Sample	β-carotene	Lutein	Total carotenoids
Baby spinach	2848.82 ± 40.71	8407.43 ± 143.10	16104.44 ± 291.00
Avocado	33.73 ± 0.80	102.34 ± 2.71	182.04 ±12.00
Sour cream	44.54 ± 0.70	4.73 ± 0.52	75.10±3.00

*Mean ± SD (n = 3)

3.2. Fatty acids profile and total lipids in baby spinach, avocado and sour cream

Although lipids constitute a minor fraction of baby spinach leaves, they contain, as all photosynthetic tissue, a high proportion of polyunsaturated fatty acids. In the analyzed samples, the total lipid represented 0.25 g/100g F.W. The major fatty acids were α-linolenic (84.1%) and palmitic acid (11%). Polyunsaturated fatty acids (PUFA) represented the major class, with more than 88% of total fatty acids, while saturated fatty acids (SFA) were 11%. Avocado samples had a total lipid content of 10.8 g/100g lipids and the most important acid was oleic acid (52.8%), followed by palmitic (23.7%) and linoleic acids (11.7%). Avocado is an important source of MUFA (63.3%) but with lower proportion of polyunsaturated fatty acids. Sour cream had the highest lipid content (24.5 g lipids/100 g), with saturated fatty acids representing over 77%, while MUFA and PUFA represent 22%, and respectively, below 1%. The representative fatty acids were palmitic (36%), stearic (15%), myristic (13%), as well as small quantities of medium and short chain saturated fatty acids (SMCFA), C4-C12. The only unsaturated fatty acid present in significant amount was oleic acid (22%).

3.3. Bioaccessibility of lutein and β-carotene from baby spinach without and with lipid addition

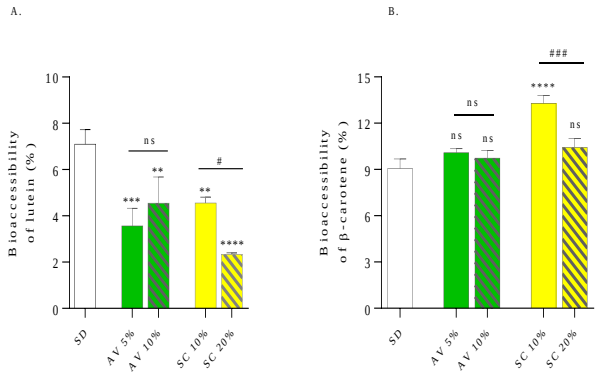

Figure 1. Bioaccessibility of lutein (A) and β-carotene (B)

Different simbols indicate a significant difference between the simple digesta and the digesta with avocado pulp and sour cream determined by Ordinary one-way ANOVA Multiple Tukey comparison test: extremely significant****; p = 0.0001 to 0.001, extremely significant ***; p = 0.001 to 0.01, very significant**; p = 0.01 to 0.05, non-significant – ns; to compare digesta with avocado pulp 5% with 10%, respectively digesta with sour cream 5% with 10% -different simbols were used to indicated the significant difference by Ordinary one-way ANOVA Multiple Tukey comparison test: extremely significant ###; p = 0.001 to 0.01, significant #; p ≥ 0.05, non-significant – ns. (SD- simple digesta; AV 5% - digesta with avocado pulp 5%; AV 10% - digesta with avocado pulp 10%; SC 5% - digesta with sour cream 5%; SC 10% - digesta with sour cream 10%).

The bioaccessibility of both carotenoids without lipid addition is low (under 10%). In the case of lutein, the lipid addition had a negative impact for both lipid sources, while for β-carotene it had a positive effect, especially when saturated animal origin fat was used, at lower concentration. We can conclude that the lipid addition is more beneficial for the nonpolar carotenes than for the xanthophylls from spinach, regardless the type of lipid. At the same time, the addition of a high amount of lipids (10% avocado, 20% cream) generally had a negative impact on the bioaccessibility of investigated carotenoids.

3rd Study- Carotenoids from Sea buckthorn berries – composition, antioxidant activity and in vitro bioaccessibility

1. Introduction and aims

All carotenoids, to different degrees, can act as antioxidants by free radical quenching properties. Considering the multitude of beneficial properties of sea buckthorn, the current study aimed to determine the carotenoid composition of berries, the antioxidant activity and the *in vitro* bioaccessibility using a standardized protocol (Minekus et al., 2014).

2. Materials and methods

Carotenoids were extracted and analyzed (HPLC-PDA) from wild type sea buckhthorn berries as previously described (section 1). The antioxidant activity of LSBE was determined using: ABTS (Tocopherol equivalent antioxidant capacity), DPPH and FRAP (Ferric reducing antioxidant power) methods. The bioaccessibility of carotenoids was determined using the slightly modified INFOGEST protocol (Minekus et al., 2014). Briefly, 2.5 g of sea buckthorn berries (deseeded) were subjected to oral, gastric and intestinal phase.

3.Results and discussions

3.1. Carotenoid composition of sea buckthorn berries

Table 1. The carotenoid content (mg/100 FW) of saponified and unsaponified sea buckthorn berries extract

ID	Identification	UV-Vis maxima	Concentration (mg/100 g F.W)	
			Saponified extract	Unsaponified extract
1	Neoxanthin	416,439.468	0.40 ± 0.07	nd
2	not identified	400, 422, 448	0.39 ± 0.04	nd
3	*cis*-Lutein	330, 420, 441, 472	0.80 ± 0.16	0
4	*all-trans*-Lutein	422, 444, 473	1.80 ± 0.43	0.29 ± 0.04
5	Zeaxanthin	427, 450, 477	8.61 ± 0.81	1.22 ± 0.31
6	β-Cryptoxanthin	428, 451, 476	0.94 ± 0.22	0.15 ± 0.05
7	*cis*-β-Carotene	338, 420, 449,472	0.49 ± 0.19	0.18 ± 0.05
8	*all trans* β-Carotene	421, 451, 478	4.14 ± 0.23	3.92 ± 0.23
9	*cis*-β-Carotene	345, 421, 447, 473	0.39 ± 0.13	0.35 ± 0.04
10	not identified	420, 441, 465	0.28 ± 0.11	nd
11	*cis*-γ-Carotene	361, 433, 460, 491	0.26 ± 0.09	nd
12	*all trans* γ-Carotene	434, 461, 492	1.65 ± 0.21	0.60 ± 0.08
13	*cis*-γ-Carotene	358, 431, 458, 489	0.04 ± 0.03	nd
14	Zeaxanthin 14:0; 16:0	426, 450, 475	0	1.95 ± 0.24
15	Lutein 16:0;16:0	421, 446, 474	0	1.98 ± 0.31
16	Zeaxanthin 16:0; 16:0	427, 450, 476	0	7.44 ± 0.55
	Other esters	-	0	6.04 ± 0.48
	Total		20.19 ± 2.72	24.12 ± 2.81

*The results are presented as the mean of three measurements ± SD of the same sample (mean ± SD, n =3)

3.2. The antioxidant activity of LSBE

Table 2. The percent* of DPPH and ABTS inhibition induced by ascorbic acid (AA) as compared to LSBE and zeaxanthin (ZEA). *mean ± SD, n =3

Concentration				% ABTS inhibition			% DPPH inhibition		
(μM)	(μg/mL)			AA	LSBE	ZEA	AA	LSBE	ZEA
	AA	LSBE	ZEA	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
1000	176	557.75	568.90	92.80±0.57	56.80±0.28	58.60±0.85	87.13±1.24	68.65±0.07	41.75±1.06
750	132	418.31	426.70	65.70±0.99	46.88±0.45	42.55±0.78	60.73±0.39	52.90±0.28	32.13±1.59
500	88	278.87	284.49	43.30±0.42	37.21±0.30	30.60±0.85	42.50±0.71	37.05±0.07	23.75±0.35
250	44	139.42	142.22	27.20±0.28	13.80±0.64	14.65±0.49	23.85±0.21	23.57±0.62	14.25±0.49
100	17.6	55.77	56.89	12.20±0.28	7.48±0.25	7.58± 0.11	10.90±0.57	10.87±0.47	5.55± 0.35
50	8.8	27.88	28.45	5.30±0.14	3.38±0.11	3.63± 0.11	6.13±0.18	5.68±0.11	3.00±0.28

3.3. Bioaccessibility of carotenoids from sea buckthorn berries

In this study we characterized the carotenoid composition of sea buckthorn berries and demonstrated that the saponified lipophilic extract rich in zeaxanthin (LSBE) exerts in vitro antioxidant activity, in a dose dependent manner. Moreover, we demonstrated that the carotenoid fraction from sea buckthorn is highly bioaccessible, providing a significant amount of health promoting compounds, such as zeaxanthin (42 % bioaccessibility), lutein (39.8 %) and β-Carotene. Our results provide experimental evidence supporting the potential use of sea buckthorn berries as a functional bioactive ingredient for food or pharmaceutical applications.

4th Study- Valorization of sea buckthorn pomace carotenoids through green extraction and encapsulation

1. Introduction and aims

Following the concept of the circular economy in food science, our purpose was to valorize the sea buckthorn pomace through the green extraction and encapsulation of its rich

carotenoid fraction. The aims of the present work were: (1) to test the efficacy of five green solvents and two extraction methods for the carotenoid extraction from sea buckthorn pomace resulted from juice production; (2) to obtain and characterize alginate beads as encapsulating agents for sea buckthorn pomace carotenoids; (3) to determine the carotenoids stability and bioaccessibility from alginate beads added to a food product.

2. Materials and methods

Sea buckthorn pomace was obtained after separation of juice from fresh berries. The obtained pomace was weighed, dried and powdered. Carotenoid extraction from pomace was conducted comparatively using an organic solvent mixture and, respectively, green solvents (ethyl acetate-ETA, ethyl lactate-ETL, cyclopentyl methyl ether-CPME, methyl tetrahydrofuran-METHF and cold pressed sunflower oil), used individually, by two extraction methods, maceration and ultrasound assisted extraction. The carotenoid extract was utilized for the subsequent encapsulation, resulting alginate-based microspheres. The bright-field and conventional fluorescence images of the microbeads were collected using an inverted microscope. The beads samples were subjected to stability tests for 30 days, in three different conditions: wet and refrigerated (WRF), wet at room temperature (WRT) and dry at room temperature (DRT). The standardized INFOGEST protocol was used for the *in vitro* gastrointestinal digestion.

3. Results and discussions

3.1. Carotenoids extraction from sea buckthorn pomace

Carotenoids were extracted through conventional solid-liquid extraction (maceration), and, respectively UAE (Table 1).

Table 1. Major carotenoid yields (mg/100 dry powder) obtained using ultrasound-assisted extraction from sea buckthorn pomace powder with different solvents

Carotenoid	OS	MeTHF	CPME	ETL	ETA	SFL oil
Zeaxanthin	3.76±0.43^{a}	2.55±0.21^{a}	4.69±0.35^{ac}	7.23 ±0.75^{b}	6.29±0.85^{b}	4.62±0.51^{abc}
ZMP	8.13±0.62^{a}	9.84±1.63^{a}	9.20±2.11^{a}	8.10±1.14^{a}	7.02±1.23^{a}	7.23±0.63^{a}
ZDP	20.97±1.90^{a}	26.76±3.52^{a}	24.49±3.81^{a}	18.24±2.70^{ab}	18.63±2.14^{ab}	16.17±1.42^{ab}
β-carotene	14.83±1.40^{a}	16.65±3.24^{a}	15.45±1.83^{a}	13.69±2.45^{a}	11.10±1.72^{a}	14.42±1.00^{a}
γ-carotene	3.52±0.50^{a}	5.82±0.91^{a}	8.41±2.34^{b}	3.24±0.40^{a}	2.78±0.32^{ac}	3.20±0.24^{a}
Lycopene	3.94±0.43^{a}	3.54±0.61^{a}	2.58±0.25^{ab}	1.54±0.30^{b}	1.63±0.40^{b}	2.77±0.33^{ab}

Data represents the arithmetic mean and standard deviation (SD) of 3 different experiments. Statistically significant differences were determined using Ordinary one-way ANOVA (Tukey's Multiple Comparison Test; GraphPad Prism Version 9.3). Different superscript letters (a–d) in the same row indicate a significant difference between the solvents used for ultrasound assisted extraction (UAE).

3.2. Encapsulation of carotenoid extract

The morphology and size of the final microbeads were analyzed by bright-field (Fig.1.A) and conventional fluorescence imaging (Fig.1.B), taking benefit of the its label-free intrinsic fluorescence capabilities. The microbeads deposited onto a glass microscope slide presents a spherical shape, which measures a mean diameter of 700 ± 50 μm. The confocal laser scanning microscopy confirmed the size and the shape of the particles (Fig. 2).

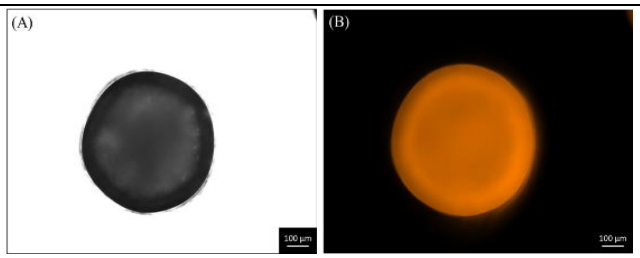

Figure 1. A representative bright-field (A) and conventional fluorescence (B) images of the obtained microbeads. The scale bar is 100 µm.

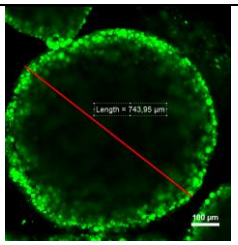

Figure 2. The confocal laser scanning microscopy (CLSM) images of the alginate microbeads containing.

Sea buckthorn pomace resulted from juice production is a valuable source of carotenoids, mainly zeaxanthin and its esters, and β-carotene. Carotenoids from sea buckthorn pomace can be efficiently recovered using a variety of green solvent, CPME and MeTHF (especially with UAE) being the most efficient. Although sunflower oil had a lower extraction yield, it can be used for extraction of carotenoids due to their lower costs and the possibility to further use the extracts without the need of evaporation and the cost associated with this process.

3.3. Stability of encapsulated carotenoid extracts

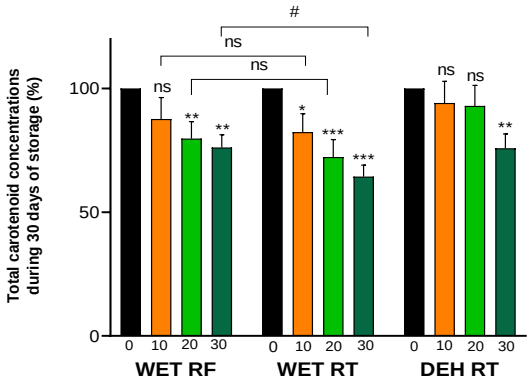

Figure 3. Retention of total carotenoids from alginate microbeads containing carotenoids from sea buckthorn powder during 30 days of storage in three different conditions: WRF - wet microbeads kept at refrigerator, WRT - wet microbeads kept at room temperature and DRT- dehydrated microbeads kept at room temperature. Statistically significant differences between the same type of microbeads, at different storage times, were determined using Ordinary one-way ANOVA Dunnett´s multiple comparisons test: extremely significant ***; p = 0.001 to 0.01, very significant**; p = 0.01 to 0.05, non-significant – ns,p≥ 0.05. The differences between the same type of microbeads (wet) kept at different temperatures were determined using Unpaired t test and the symbols represent extremely significant ###; p = 0.0001 to 0.001, very significant ##, p= 0.001 to 0.01, significant #; p ≥ 0.05, non-significant – ns.

3.4. Bioaccessibility of carotenoids from alginate microbeads

Table 3. Carotenoids bioaccessibility from hydrated and dehydrated microcapsules (%)

Carotenoid	Hydrated capsules		Dehydrated capsules	
	Control	With yoghurt	Control	With yoghurt
Zeaxanthin	6.31±2.42	4.73±2.12	28.67±3.24	41.64±3.90
ZMP	64.26± 4.32	50.87± 3.91	42.10±4.13	49.13±3.70
ZDP	66.05±4.11	46.85±2.32	39.58±3.81	45.93±5.30
β-carotene	51.42±3.60	36.24±2.24	41.48±3.90	43.66±2.71
γ-carotene	51.38± 3.90	36.47±2.32	40.85±2.53	38.83±1.22
Lycopene	11.08±1.90	6.98±0.71	29.58±3.70	24.12±1.32
Total Bioaccessibility	42.11±4.60	29.50±3.91	40.80±4.00	40.70±3.82

Carotenoid extract from sea buckthorn pomace can be efficiently encapsulated in alginate microbeads showing a good stability during storage, especially in hydrated and refrigerated conditions. The bioaccessibility of total and individual carotenoids was good, but depends on the type of carotenoid and the storage form of microbeads. Yoghurt addition had a

positive effect on the bioaccessibility of carotenoids only in dehydrated microbeads, and the impact was higher for free zeaxanthin and its esters.

8. Conclusions and Recommendations

The aims of this thesis were to investigate the *in vitro* bioaccessibility of the main carotenoids found in human blood and tissues, from some common vegetal matrices, without thermal preparation of the matrix, in order to study the effect of the addition of different lipid sources on the carotenoid bioaccessibility, Moreover, we aimed to valorize a by-product of berries processing (sea buckthorn pomace), rich in bioactive compounds, by green extraction of carotenoids and their encapsulation for further use in food industry.

As final conclusions:

In chapter four (Study 1), the bioaccessibility of major carotenoids from carrots, cherry tomato and baby spinach was investigated, in the presence or in the absence of vegetable oils (5 and 10 %), using the standardized INFOGEST *in vitro* digestion protocol. The results showed that the bioaccessibility of carotenoids is strongly affected by both the type of carotenoid and the matrix. Thus, regardless of the samples analyzed, lutein (a xanthophyll) showed better bioaccessibility than all the investigated carotenes. Among the carotenes, α-carotene had the highest bioaccessibility, followed closely by β-carotene, while lycopene had the lowest one. Moreover, for the same compound, the bioaccessibility is strongly influenced by the type of matrix (e.g., β-carotene and lutein are more bioaccessible from raw carrots and tomato, compared to baby spinach). The addition of lipids had a positive impact on the bioaccessibility of all carotenoids from carrots and cherry tomatoes (with a more significant effect for carotenes), but almost no effect on their bioaccessibility from spinach. Regarding the type of oil, better results were obtained after addition of olive oil (monounsaturated, n-9 major FA) and sunflower oil (polyunsaturated, n-6 major FA), while the addition of linseed (polyunsaturated, n-3 major FA) oil had no a consistent positive effect.

In chapter five (Study 2), the bioaccessibility of lutein and β-carotene was determined from fresh baby spinach leaves, in the presence or in the absence of two lipid sources, avocado pulp and sour cream, at 5 %, 10 % or 20 % - sour cream. A low bioaccessibility of lutein and β-carotene was observed from raw samples with no added lipids. In the presence of lipids, for lutein, both the addition of avocado pulp and the addition of sour cream had a negative impact on bioaccessibility, leading to a lower micellization percentage compared to control samples, regardless the amount of added lipids. However, for β-carotene the presence of lipids had a positive effect on bioaccessibility, especially in the presence of sour cream, an important source of saturated fatty acids. At the same time, an increase in the amount of added lipids, led to a negative impact on the micellization of the investigated compounds. Based on our results, we could affirm that the lipid addition is more beneficial for the nonpolar carotenes than for the xanthophylls from spinach, regardless the type of lipids, and a high quantity of lipids does not lead to a higher micellization for lutein and β-carotene.

In chapter six (Study 3), the carotenoid composition of sea buckthorn berries was determined in saponified and unsaponified extracts, as well as the antioxidant activity and the bioaccessibility of major carotenoids. We found that the zeaxanthin and its esters, together with

β-carotene were the major constituents in the unsaponified extract. The saponified lipophilic extract (LSBE), having free zeaxanthin as major compound, exerts *in vitro* antioxidant activity, in a dose dependent manner, as resulted from all the three assays, ABTS, DPPH and FRAP. Moreover, we found good bioaccessibilities of carotenoids from sea buckthorn berries, with the highest values being recorded for xanthophylls (zeaxanthin, lutein and β-cryptoxanthin). The high content, high bioaccessibility ant antioxidant activity of carotenoids from sea buckthorn berries advocates for the necessity of wider studies on the benefits of these fruits.

In chapter seven (Study 4), firstly we compared the efficiency of five green solvents (ethyl lactate, ethyl acetate, sunflower oil, cyclopenthyl methyl ether, methyl tetrahydrofuran) and two extraction methods (maceration-CE and ultrasound assisted extraction-UAE) for the recovery of carotenoids from sea buckthorn berries, compared with a mix of organic solvents. When using UAE, the extraction yield for all the tested solvents was better, except for sunflower oil. In terms of solvents used, for classical extraction CPME had the best results, while for UAE, the best results were obtained with CPME and MeTHF. Even though sunflower oil had a lower extraction yield, its use for the extraction of carotenoids presents numerous advantages. Regarding the type of carotenoids analyzed, CPME was the most efficient in extracting zeaxanthin esters and carotenes, while ethyl lactate was the most efficient solvent for free zeaxanthin. Further, the obtained carotenoid extract was successfully encapsulated (99 %) in alginate microbeads (700 nm diameter). The retention of carotenoids during storage was higher for the microbeads stored in the hydrated form, at low temperature, and the microbeads were stable in acidic pH, releasing the carotenoids at neutral pH. The highest bioaccessibility of total carotenoids was obtained from hydrated capsules, while for the individual carotenoids, the highest micellization percentages were obtained for zeaxanthin esters, β-carotene and γ-carotene. Yoghurt addition had a positive effect on the bioaccessibility of carotenoids only in dehydrated microbeads, and the impact was higher for free zeaxanthin and its esters.

As the bioaccessibility of carotenoids is influenced by so many factors, advanced statistic methods must be applied to fully understand the complex interactions between carotenoid structure, matrix and deposition form, and the type of lipid added. Additionally, it would be important to determine the extent of hydrolysis of lipids during the i*n vitro* digestion, as it could be helpful for understanding the mechanisms of carotenoid micellization. However, a major finding of our study was that, generally, the addition of an unsaturated lipid source during *in vitro* digestion facilitates the transfer of carotenoids (mainly carotenes) into mixed micelles, thus enhancing their bioaccessibility.

As future recommendations, a special attention should be given to the extraction of phytochemicals (especially carotenoids) using green solvents, as they can be an effective method for the recovery of plant bioactive compounds, with a reduced impact on the environment and on human health.

The by-products resulting from the food industry deserve increased attention, due to their high content of nutritionally important molecules which can be extracted and further reused as food ingredient/ supplements, or by cosmetic and pharmaceutical industry.

9. Originality and personal contributions

In the frame of the current PhD thesis, the bioaccessibility of carotenoids from various food matrices was investigated, using different approaches. Moreover, the use of a standardized in vitro digestion protocol unable us to compare out results with the existing literature in the field.

New relevant information was obtained regarding the carotenoid and fatty acids composition of some common foods in human diet, in raw state. At the best of our knowledge, this is the first study to investigate, in the same study and in identical experimental condition, the effect of olive, sunflower, linseed, avocado pulp and sour cream on the bioaccessibility of major carotenoids from carrots, cherry tomatoes and baby spinach. Due to the complexity of the experimental design, we could underline the interactions between the chemical nature of carotenoids (polarity), the matrix and the type of lipid, as well as their influence on the bioaccessibility of carotenoids. Thus, we find out that regardless the matrix, xanthophylls are more bioaccessible than carotenes, but the addition of lipids, especially in higher concentration has a stronger positive effect on carotenes than in xanthophylls. We also proved that olive oil and sunflower oil enhance the bioaccessibility of all carotenoids, at 5 % concentration.

Regarding sea buckthorn berries, we characterized a lipophilic extract in terms of carotenoid composition and demonstrated its better antioxidant activity compared to pure compounds, using a battery of antioxidant tests. As in the case of vegetables, we found a better bioaccessibility of xanthophylls, especially zeaxanthin from sea buckthorn berries, thus demonstrating their high nutritional value.

To the best of our knowledge, this is the first study that combines several new elements in terms of analysis of carotenoid compounds, respectively the use of green solvents for extraction, the use of ultrasound-assisted extraction as an extraction method, but also the valorization of a by-product (sea buckthorn pomace) through encapsulation. The findings presented in this thesis bring new and relevant information about new methods of extracting carotenoid compounds, much less expensive and as effective or more effective than those traditionally used. Concerning ultrasound-assisted extraction, we can say that it is a method that offers an extraction yield as good or even better than classical extraction, but in a much shorter time.

Also, the advantages of using green solvents for the extraction of carotenoids was emphasized, by obtaining results as good or better than those obtained by using traditional organic solvents.

From an economic point of view, the originality of this study consists in the valorization of a by-product, by using it as a matrix for the efficient and green extraction of carotenoids, which are later used to obtain microcapsules for nutritional enrichment of various products.