

UNIVERSIDADE DE SÃO PAULO
FACULDADE DE ZOOTECNIA E ENGENHARIA DE ALIMENTOS

TALITA ALINE COMUNIAN

SIMULTANEOUS ENCAPSULATION OF ECHIUM SEED OIL (*ECHIUM
PLANTAGINEUM* L.), PHYTOSTEROLS AND PHENOLIC COMPOUNDS:
CHARACTERIZATION AND APPLICATION OF MICROCAPSULES

Pirassununga

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"It is good to go to the fight with determination, embracing life with passion, losing with class and winning boldly, because the world belongs to those who dare and life is too much to be insignificant." (Charles Chaplin).

To my supervisor Carmen, who trusted me so much and who taught me to be the Food Engineer and researcher that I am today. Thank you very much!

BIOGRAPHY

Talita Aline Comunian, daughter of José Eduardo Comunian and Edna Maria Milhorin Comunian, was born in the city of Jaú, São Paulo on December 26, 1985. She is graduated in Food Engineering in the University of São Paulo in 2010. During her undergraduate program, she worked in the Junior Company (Qualimentos Jr) for two years and carried out scientific initiation for a year and a half with FAPESP scholarship. At the end of her undergraduate program, she completed a one-year internship at the Institut für Bio- und Lebensmitteltechnik (KIT) in Karlsruhe, Germany, with scholarship from the German government's PRO-3 program.

At the beginning of 2011, Talita joined the FZEA/ USP Master program in Science of Food Engineering, also with a FAPESP scholarship. During the masters program, she had BEPE scholarship, financed by FAPESP. This benefit enabled Talita to complete a six-month internship at School of Engineering and Applied Sciences, Harvard University, United States, Cambridge in 2012-2013. In 2014, Talita was approved in first place in the FZEA/ USP Doctoral Program in Food Engineering Sciences, with FAPESP scholarship. During her doctorate, Talita worked for eight months in the Department of Food Science at Cornell University in Ithaca, USA, also with a BEPE/ FAPESP scholarship.

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ABSTRACT

COMUNIAN, T.A. **Simultaneous encapsulation of echium (*Echium Plantagineum* L.) seed oil, phytosterols and phenolic compounds: characterization and application of microcapsules.** 2017. 353 f. PhD. Thesis - Faculdade de Zootecnia e Engenharia de Alimentos, Universidade de São Paulo, Pirassununga, 2017.

The consumption of omega-3 fatty acids and phytosterol promotes the reduction of cholesterol and triacylglycerol levels. However, such compounds are susceptible to oxidation, which hampers their application. First, the aim of this work was to encapsulate echium oil (*Echium plantagineum* L.), source of omega-3 fatty acids, with hydrophilic phenolic compounds (sinapic acid and rutin) by double emulsion followed by complex coacervation in order to evaluate the best hydrophilic phenolic compound. In this case, sinapic acid showed better performance as antioxidant. Then, the second objective of this work was to study the microencapsulation of echium oil by complex coacervation using gelatin-arabic gum and gelatin-cashew gum as wall materials and sinapic acid and transglutaminase as crosslinkers. In this step, it was possible to observe that sinapic acid, besides to be an antioxidant, could also act as crosslinker. So, the third objective was to study the effect of sinapic acid in echium microparticles obtained by emulsion followed by spray or freeze drying using arabic gum as carrier agent in order to compare different encapsulation techniques. In addition to these methods, the fourth objective was to compare these techniques already mentioned to the combination of microfluidic devices

and ionic gelation in order to encapsulate echium oil. In this case, sinapic acid and quercetin were also incorporated in the microcapsules. All the microcapsules/ microparticles obtained in the mentioned different techniques presented characteristics feasible for application and also promoted the protection of the oil. However, the encapsulation by complex coacervation and the addition of sinapic acid as crosslinkers was the method chosen for the coencapsulation of echium oil and phytosterols since presented the better results. Moreover, the treatment GA075 (microcapsule with gelatin-arabic gum as wall materials and 0.075g sinapic acid/ g gelatin) promoted the better protection to the encapsulated compounds. In this way, this treatment was applied into yogurt and compared to the one with the compounds non-encapsulated and the yogurt control. The yogurt containing microcapsules, presented a pH range from 3.89–4.17 and titratable acidity range from 0.798–0.826%, with good sensorial acceptance. It was possible to apply the microcapsules in yogurt, without compromising the rheological properties and physicochemical stability of the product, obtaining a functional product rich in omega-3 fatty acids, phytosterols and phenolic compound.

Keywords: ômega-3 fatty acids, complex coacervation, spray drying, microfluidic devices, ionic gelation

LIST OF FIGURES

Figure 1. Schematic representation of each step of this research.....	28
Figure 1. 1. Structures of fatty acids: a) alpha-linolenic acid, b) stearidonic acid, c) eicosapentaenoic acid, d) docosahexaenoic acid.	30
Figure 1. 2. Echium plantation in North Dakota and the echium seed.	32
Figure 1. 3. Chemical structures of major phytosterols.	35
Figure 1. 4. Sinapic acid structural formula.	36
Figure 1. 5. Rutin structural formula.	38
Figure 1. 6. Quercetin structural formula.	39
Figure 1. 7. Scheme of microsphere and microcapsule structure.	43
Figure 1. 8. Illustration of spray drying and ionic gelation methods, respectively.	64
Figure 1. 9. Complex coacervation technique procedure.	67
Figure 1. 10. Structures of α , β and γ -cyclodextrin.	74
Figure 2. 1. Schematic representations of the procedure of the capsules fabrication: a) Samples SIN50, SIN75, SIN100, RUT50, RUT75 and RUT100; b) Sample SIN-GEL; c) Sample Control.....	97
Figure 2. 2. Micrographs obtained by optical microscopy for the samples (a) SIN50 and (b) SIN-GEL; confocal microscopy for the samples (c) SIN50 and (d) SIN-GEL; optical microscopy for the freeze-dried and rehydrated samples (e) SIN50 and (f) SIN-GEL.....	106
Figure 2. 3. Micrographs obtained by scanning electron microscopy for the samples (a) SIN50; (b) SIN75; (c) SIN100; (d) SIN-GEL; (e) RUT50; (f) RUT75; (g) RUT100 and (h) Control.....	107
Figure 2. 4. Spectra obtained by FTIR for (a) gelatin (black) and Arabic gum (red curve); (b) echium oil (black), rutin (blue) and SA (red curve); (c) dried samples SIN50 (black curve), RUT50 (red curve), SIN-GEL (blue curve) and Control (green curve).	109
Figure 2. 5. Thermogravimetry for sample with (a) sinapic acid: samples SIN50 (Black curve), SIN75 (red curve), SIN100 (blue curve) and SIN-GEL (green curve); (b) with rutin: samples RUT50 (black curve), RUT75 (red curve), RUT100 (blue) and control (green curve).....	112

Figure 3. 1. Diagram about the process of complex coacervation and crosslinking for treatments A) GASb and GCSb; B) GASa, GCSa, GAT and GCT; C) GAO and GCO; and D) GA and GC.	128
Figure 3. 2. Optical microscopy of (A) treatment GASb (gelatin-arabic gum as wall materials and sinapic acid added before arabic gum), (B) treatment GCSa (gelatin-cashew gum as wall materials and sinapic acid added after complex coacervation); scanning electron microscopy of (C) treatment GASa (gelatin-arabic gum as wall materials and sinapic acid added after complex coacervation) freeze-dried, (D) treatment GCSa (gelatin-cashew gum as wall materials and sinapic acid added after complex coacervation) freeze-dried, (E) treatment GASa atomized, (F) treatment GCSa atomized; optical microscopy of (G) treatment GASa freeze-dried and rehydrated and (H) treatment GCSa freeze-dried and rehydrated; (I) treatment GASa spray-dried and rehydrated and (J) treatment GCSa spray-dried and rehydrated.	134
Figure 3. 3. Accelerated oxidation behavior of microcapsules by Rancimat: A) treatments with gelatin-arabic gum as wall material; B) treatments with gelatin-cashew gum as wall material.	139
Figure 3. 4. Optical microscopy of the treatments maintained in solution of pH 2, 4 and 8.	145
Figure 3. 5. Optical microscopy of the treatments submitted to temperatures of 2, 20, 60 and 80 °C.	146
Figure 3. 6. Optical microscopy of the treatments in solutions of 1, 3 and 5% (w/w) of salt.	147
Figure 3. 7. A) Sinapic acid (1) and dehydrodisinapic acid derivatives thomasidioic acid (2) and compound 3; B) Oxidative radical coupling dehydrodimerization of sinapic acid to form thomasidioic acid. Adapted from Bunzel et al. (2003).	150
Figure 3. 8. A) Proposed polysaccharide-polysaccharide crosslinkage through a polysaccharide-sinapate ester air-induced oxidative radical coupling dehydrodimerization with a free sinapic acid; B) Proposed polysaccharide-polysaccharide crosslinkage through a polysaccharide-thomasidioate ester..	151
Figure 4. 1. (a) Instability index of each emulsion according to the analyzed time and general instability index of the emulsion; (b) Droplet size distribution of the emulsions in the ratio of 1:2 and (c) 1:3 of oil phase: aqueous phase.....	166

Figure 4. 2. Micrographs obtained by scanning electron microscopy of dried treatments by spray dryer: S0, S200, S600 and S1000 (with 0, 200, 600 and 1000 ppm of sinapic acid, respectively); and treatments dried by freeze dryer: L0, L200, L600 and L1000 (with 0, 200, 600 and 1000 ppm of sinapic acid, respectively).	170
Figure 4. 3. Particle size distribution of the atomized treatments: a) S0 (without addition of sinapic acid); b) S200 (200 ppm of sinapic acid); c) S600 (600 ppm of sinapic acid) and d) S1000 (1000 ppm of sinapic acid).....	176
Figure 4. 4. TG curves for treatments S600 and L600 (treatments with 600 ppm of sinapic acid, submitted to spray drying and freeze drying, respectively). ...	178
Figure 4. 5. X-ray diffraction of particles obtained by (a) lyophilization with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil and (b) spray drying with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil and behavior of accelerated oxidation of pure oil and particles obtained by: (c) lyophilization with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil and (d) spray drying with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil.	182
Figure 5. 1. (a) Graphical representation of the encapsulation process: combination of microfluidic device and ionic gelation; optical microscope image of (b) a glass microfluidic device during the production of double emulsions; (c) Q800-Alg capsules before gelation, (d) Q800-Alg capsules after gelation and (e) scanning electron microscope image of S050-Alg capsules.....	193
Figure 5. 2. a) TG curves for each treatment and pure echium oil; (b) Retention (%) of encapsulated and non-encapsulated quercetin and sinapic acid for the samples stored for 30 days at 40 °C and (c) oxidative stability of the encapsulated and non-encapsulated echium oil by thiobarbituric acid reactive substance (TBARS).....	206
Figure 5. 3. Fourier Transform Infrared Spectrophotometry spectra for the following treatments: (a) control and (b) S050-Alg stored for 30 days at 40 °C.	209
Figure 5. 4. Optical microscope images of the following treatments: (a) Control, (b) S050-Alg and (c) Q800-Alg submitted to different temperatures.	215
Figure 5. 5. Optical microscope image of each treatment freeze-dried and resuspended in water.	216

Figure 6. 1. Spectra obtained by FTIR for (a) treatments with gelatin-arabic gum combination, (b) treatments with gelatin-cashew gum combination and (c) wall and encapsulated materials.	238
Figure 6. 2. X-ray diffraction (a) of the treatments with gelatin-arabic gum combination, (b) of the treatments with the gelatin-cashew gum combination and (c) of the wall materials.	240
Figure 6. 3. Stability of (a) alpha linolenic acid and (b) stearidonic acid present in the encapsulated oil stored for 30 days at 37 °C.....	245
Figure 6. 4. (a) Monitoring of the formation of malonaldehyde in encapsulated materials and stored for 30 days at 37 °C; (b) stability of encapsulated beta-sitosterol during the 30-day period with samples stored at 37 °C.	249
Figure 7. 1. Structure of sinapic acid.....	257
Figure 7. 2. Simulation of the gastric (a and c) and intestinal (b and d) release of sinapic acid over 180 min, from the treatments prepared with gelatin-arabic gum (a and b) and gelatin-cashew gum (c and d).....	271
Figure 7. 3. Simulation of the release of the mixture of oil and phytosterols in (a) gastric fluid and (b) intestinal fluid, during 180 minutes.	274
Figure 7. 4. Optical microscopy of the yogurts (a) T1 (yogurt control - without bioactive compounds added), (b) T2 (yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added), and (c) T3 (yogurt with added microcapsules). Scanning electron microscopy of the yogurts (d) T1 (yogurt control - without bioactive compounds added), (e) T2 (yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added), and (f) T3 (yogurt with added microcapsules).	276
Figure 7. 5. Effect of storage time on apparent viscosity as a function of deformation rate, for treatments T1 (black curve), T2 (red curve), and T3 (blue curve), at (a) 7 and (b) 30 days of storage. T1 refers to the yogurt control (without bioactive compounds added); T2 refers to yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added, and T3 refers to the yogurt with microcapsules added.....	284

LIST OF TABLES

Table 1. 1. Some examples of polymers combinations used as wall materials by complex coacervation and their compounds encapsulated.....	70
Table 1. 2. Characterization of the three cyclodextrins.	74
Table 2. 1. Concentrations and proportions used for each sample in the process of encapsulation.....	96
Table 2. 2. Values of water activity, moisture, particle size, circularity and accelerated oxidation index.	105
Table 2. 3. Concentration and composition of oil and major acids for each sample.	114
Table 3. 1. Composition of each treatment, particle size and circularity values for each treatment in the wet form and accelerated oxidation index for the freeze-dried ones.....	129
Table 4. 1. Values of water activity (A_w), moisture, solubility, hygroscopicity, yield of the drying process, average particle size and oxidation index for each treatment.....	174
Table 5. 1. Composition of each treatment, encapsulation yield for the phenolic compounds and for the oil and average particle size before and after gelation of sodium alginate.....	194
Table 6. 1. Composition of each treatment and yield values of sinapic acid, oil and phytosterol mixture.....	227
Table 6. 2. Stability of encapsulated and free sinapic acid during 30 days-storage at 37 °C.	242
Table 7. 1. Composition of treatments, values of average particle diameter and oxidative stability index for each treatment.....	261
Table 7. 2. Values of L^* , a^* , b^* , pH and titratable acidity for each treatment in the period of 30 days.	281
Table 7. 3. Yield stress, consistency index (K), Complex modulus (G^*) (at frequency of 1 Hz) and apparent viscosity at shear rate of 20 s ⁻¹ ($\eta_a, 20$) for each treatment stored at 4 °C for 30 days.....	285
Table 7. 4. Scores obtained in sensory analysis for the attributes flavor, color, texture, taste and general acceptance.	287

ACRONYMS AND ABBREVIATIONS

Ftir = Fourier Transform Infrared Spectroscopy

TG = Thermogravimetric Analysis

DSC = Differential Scanning Calorimetry

DHA = Docosahexaenoic acid

EPA = Eicosapentaenoic acid

ω -3 = omega-3 fatty acids

ω -6 = omega-6 fatty acids

AS = sinapic acid

PGPR 90 = Polyglycerol polyiricoleate

A/O = Water-in-oil emulsion

A/O/A = Water-in oil-in water double emulsion

w/w = mass ratio

v/v = volume ratio

w/v = Mass in volume ratio

rpm = rotations per minute

SUMMARY

INTRODUCTION	22
CHAPTER 1. MICROENCAPSULATION USING BIOPOLYMERS AS AN ALTERNATIVE TO PRODUCE FOOD ENHANCED WITH PHYTOSTEROLS AND OMEGA-3 FATTY ACIDS: A REVIEW	30
1. OMEGA-3 FATTY ACIDS	30
1.1. Echium seed oil	32
1.2. Stearidonic fatty acids.....	33
2. PHYTOSTEROLS	34
3. PHENOLIC COMPOUNDS	35
3.1. Sinapic acid	36
3.2. Rutin	37
3.3. Quercetin	38
4. MICROENCAPSULATION	39
4.1 Biopolymers used by the encapsulation of phytosterols and omega-3 fatty acids	44
4.1.1. Gelatin	44
4.1.2. Gum Arabic.....	45
4.1.3. Pea protein	47
4.1.4. Pectin.....	48
4.1.5. Casein.....	48
4.1.6. Soy Protein Isolate	50
4.1.7. Chitosan	50
4.1.8. Alginate.....	51
4.1.9. Whey protein	52
4.2. Mechanisms of release	53
5. MICROPARTICLES CHARACTERIZATION.....	55
5.1. Particle size	56
5.2. Encapsulation efficiency.....	57
5.3. Optical, confocal and scanning electron microscopy	58
5.4. Water activity, moisture, hygroscopicity and solubility	58

5.5.	Fourier Transform Infrared Spectroscopy (FTIR).....	59
5.6.	Thermogravimetric analysis (TG).....	60
5.7.	Controlled release and stability of the encapsulated material.....	61
5.8.	Differential scanning calorimetry (DSC).....	61
5.9.	Oxidation analyzes by Thiobarbituric acid reactive substances (TBARS) and Peroxide value.....	62
5.10.	Accelerated oxidation by rancimat.....	63
6.	TECHNIQUES APPLIED FOR ENCAPSULATION OF OMEGA 3 AND PHYTOSTEROLS USING BIOPOLYMERS AND CYCLODEXTRINS.....	63
6.1.	Spray drying.....	63
6.2.	Ionic gelation or ionotropic gelation by extrusion processes.....	66
6.3.	Complex coacervation.....	67
6.4.	Glass microfluidic devices.....	72
6.5.	Complexation.....	74
7.	MICROENCAPSULATION OF PHENOLIC COMPOUNDS.....	77
8.	CONCLUSIONS.....	78
9.	ACKNOWLEDGEMENTS.....	78
10.	REFERENCES.....	79
	CHAPTER 2. PROTECTION OF ECHIUM OIL BY MICROENCAPSULATION WITH PHENOLIC COMPOUNDS.....	92
	ABSTRACT.....	92
1.	INTRODUCTION.....	93
2.	MATERIAL & METHODS.....	94
2.1.	Materials.....	94
2.2.	Methods.....	95
3.	RESULTS AND DISCUSSION.....	102
4.	CONCLUSIONS.....	117
5.	ACKNOWLEDGMENTS.....	117
6.	REFERENCES.....	117

CHAPTER 3. EFFECT OF DIFFERENT POLYSACCHARIDES AND CROSSLINKERS ON ECHIUM OIL MICROCAPSULES.....	121
ABSTRACT	121
1. INTRODUCTION.....	121
2. MATERIAL & METHODS.....	125
2.1. Materials.....	125
2.2. Methods	126
3. RESULTS AND DISCUSSION.....	132
4. CONCLUSIONS.....	151
5. ACKNOWLEDGEMENTS	152
6. REFERENCES.....	152
CHAPTER 4. PREPARATION AND CHARACTERIZATION OF ECHIUM OIL AND SINAPIC ACID MICROPARTICLES BY EMULSION FOLLOWED BY SPRAY AND FREEZE DRYING.....	157
ABSTRACT	157
1. INTRODUCTION.....	158
2. MATERIAL AND METHODS.....	159
2.1. Materials	159
2.2. Study of Emulsions	160
2.3. Incorporation of sinapic acid	160
2.4. Drying.....	161
2.5. Characterization of particles	161
3. RESULTS AND DISCUSSIONS	165
4. CONCLUSIONS	183
6. REFERENCES.....	183
CHAPTER 5. COMBINATION OF MICROFLUIDIC DEVICES, IONIC GELATION AND PHENOLIC COMPOUNDS TO IMPROVE THE OXIDATIVE STABILITY OF ECHIUM OIL.....	188
ABSTRACT	188

1. INTRODUCTION.....	189
2. MATERIAL AND METHODS.....	191
2.1. Materials.....	191
2.2. Methods	191
3. RESULTS AND DISCUSSION.....	199
4. CONCLUSIONS	217
5. ACKNOWLEDGMENTS.....	217
6. REFERENCES.....	218
CHAPTER 6. ENHANCING STABILITY OF OMEGA-3 FATTY ACIDS AND BETA-SITOSTEROL BY THEIR COENCAPSULATION USING DIFFERENT COMBINATIONS OF WALL MATERIALS AND CROSSLINKERS.....	
ABSTRACT	222
1. INTRODUCTION.....	223
2. MATERIAL AND METHODS.....	225
2.1. Material.....	225
2.2. Methods	226
3. RESULTS AND DISCUSSION.....	234
4. CONCLUSIONS.....	250
5. ACKNOWLEDGEMENTS	250
6. REFERENCES.....	250
CHAPTER 7. DEVELOPMENT OF FUNCTIONAL YOGURT CONTAINING FREE AND ENCAPSULATED ECHIUM OIL, PHYTOSTEROL AND SINAPIC ACID.....	
ABSTRACT	255
1. INTRODUCTION.....	256
2. MATERIALS AND METHODS	258
2.1. Material.....	258
2.2. Microencapsulation	259
2.3. Characterization of microcapsules.....	262

2.4. Preparation of yogurt	264
2.5. Characterization of yogurt.....	265
2.6. Statistical analysis	267
3. RESULTS AND DISCUSSION.....	268
4. CONCLUSIONS	287
5. ACKNOWLEDGMENTS.....	288
6. REFERENCES.....	289
CHAPTER 8. GERAL CONCLUSION	293
ATTACHMENTS.....	294

INTRODUCTION

Fatty acids (FAs), compounds composed of an aliphatic chain and a carboxylic group, are the main components of lipids. They are classified as saturated and unsaturated, and the last ones are unstable due to the presence of double bonds in their structure. There are several types of unsaturated fatty acids and those belonging to the omega-3 family are prominent due to their benefits to human health. These fatty acids have double bonds separated by a methylene from the third carbon of the methyl terminus.

Omega-3 fatty acids (ω -3) are widely used in food, pharmaceutical and cosmetic products due to their beneficial effects on health, such as reducing the risk of coronary heart disease (KRALOVEC; ZHANG S.; ZHANG W.; BARROW, 2012), aid in the prevention of allergy, diabetes, Alzheimer's disease and neurodegenerative diseases (LAVIE; MILANI; MEHRA; VENTURA, 2009). There are several sources of omega-3, such as animal origin (marine fish oil), as well as vegetable sources (echium seed oil, chia oil). The oil extracted from the echium seed (*Echium plantagineum* L.) contains from 9 to 16% of stearidonic acid, which belongs to the ω -3 family, is rare in plants, although it is very important in human nutrition because it is an intermediate in biosynthesis of EPA and DHA fatty acids, which in turn, are also extremely important in human nutrition (BERTI; JOHNSON; DASH; FISCHER; WILCKENS, 2007; ZANETTI et al., 2013). In addition to these natural sources of ω -3, there are many food options enriched with these fatty acids such as milk and dairy products, juices, eggs, infant food, meat products and margarine as well as

supplements, representing a large percentage within the market of functional foods.

There is also a class of lipids known as sterol. Sterols are lipids composed of three six-carbon rings and a five-carbon ring also attached to an aliphatic chain and a hydroxyl group (DAMODARAN; PARKIN; FENNEMA, 2010). In the same way as fatty acids, sterols can be of animal origin (zoosterol, such as cholesterol or coprostanol) and vegetable origin (phytosterols, such as β -sitosterol, campesterol and stigmasterol - the most found in the human diet). Phytosterols have beneficial properties for human health, such as reduction of cholesterol absorption (OSTLUND; RACETTE; OKEKE; STENSON, 2002), as well as maintenance of membrane integrity (WATSON; PREEDY, 2004). There are some options for food products enriched with phytosterols, such as bakery products and margarines. However, this number is not as significant as omega-3-enriched foods, resulting in a smaller percentage in the functional foods market.

In spite of the high consumer demand and consequently the high industrial interest in foods enriched with these bioactive compounds, the preparation of these foods added of omega-3 fatty acids and/ or phytosterol is not an easy task today, since there are many challenges to overcome; in addition, these compounds are both very unstable, insoluble in water and the omega-3 fatty acids of fish origin - the most common - also present very unpleasant aroma. In this way, two strategies that would solve these limitations would be: (1) addition of a compound with antioxidant function - phenolic compounds - and (2) microencapsulation.

Phenolic compounds, such as sinapic acid, rutin and quercetin, have been of great interest in the food industry due to their beneficial effects on human health, acting as an antioxidant agent (LEE et al., 2013). Sinapic acid is the main phenolic acid of canola (RUBINO et al., 1995; RAWEL; ROHN, 2010) and has been extensively studied in relation to the neuro-protective effect against Alzheimer's disease (LEE et al., 2012), to cardiac hypertrophy and dyslipidemia (PARI; JALALUDEEN, 2011; ROY; PRINCE, 2012; ROY; PRINCE, 2013). Rutin is abundantly found in plants and exhibits various pharmacological activities, including antiallergic, antiinflammatory and vasoactive properties (JANTRAWUT et al., 2013). In the case of quercetin, it is one of the most common flavonoids present in nature that has attracted the attention of many researchers due to its anti-stress, anti-inflammatory and anticancer properties (WATERHOUSE et al., 2014).

In the face of microencapsulation, it has been widely used in the food area. This technique consists in the formation of "small packs", called microcapsules, microspheres or microparticles, which consist in structures with one or more bioactive materials involved or immobilized by one or more polymers or a lipid. According to Favaro-Trindade, Pinho and Rocha (2008), these structures are applied with different objectives, such as reducing the reactivity of the material which is being encapsulated, protection of the encapsulated material against evaporation or loss to another medium, easy handling, storage and application of the encapsulated material, promoting controlled release and masking unpleasant taste and odor.

In the case of omega-3 fatty acids and phytosterols, this technique has been very useful since it can: avoid interaction with other compounds present in

the food; offer protection against light, oxygen and metals; allowing dispersion in water for aqueous formulations, such as beverages; in addition, it is possible to change from liquid to solid state, which facilitates the transport, handling and application of these compounds; moreover, it is possible to maske or reduce the aroma of fish-derived oils.

There are several techniques that have been used or at least studied for microencapsulation of omega-3 fatty acids and phytosterols, such as complex coacervation (BARROW; NOLAN; HOLUB, 2009; CONTO; GROSSO; GONÇALVES, 2013), spray drying (CHEN; MCGILLIVRAY; WEN; ZHONG; QUEK, 2013), high pressure homogenization (SALMINEN; AULBACH; LEUENBERGER; TEDESCHI; WEISS, 2014), emulsion followed by spray drying and lyophilization (ANWAR; KUNZ, 2011), emulsion followed by high pressure homogenization (CHEN; MCGILLIVRAY; WEN; ZHONG; QUEK, 2013), ultrasonic atomizer (KLAYPRADIT; HUANG, 2008), which resulted in more protected omega-3 fatty acids and phytosterols.

The complex coacervation method consists of the spontaneous separation of phases due to the formation of a complex that may be insoluble between two or more polymers resulted from electrostatic interactions (GOUIN, 2004; ROCHA-SELMÍ et al., 2013). In addition to the complex coacervation technique to form resistant microcapsules, the use of a crosslinker is being explored in conjunction with this encapsulation method. The crosslinking process can be used to stabilize and modify the structure and properties of the complexes with the aim of avoiding or promoting the release of bioactive compounds (CHEN et al., 2012). The sinapic acid, due to the presence of charges, can be a compound of great potential as a crosslinking agent, in

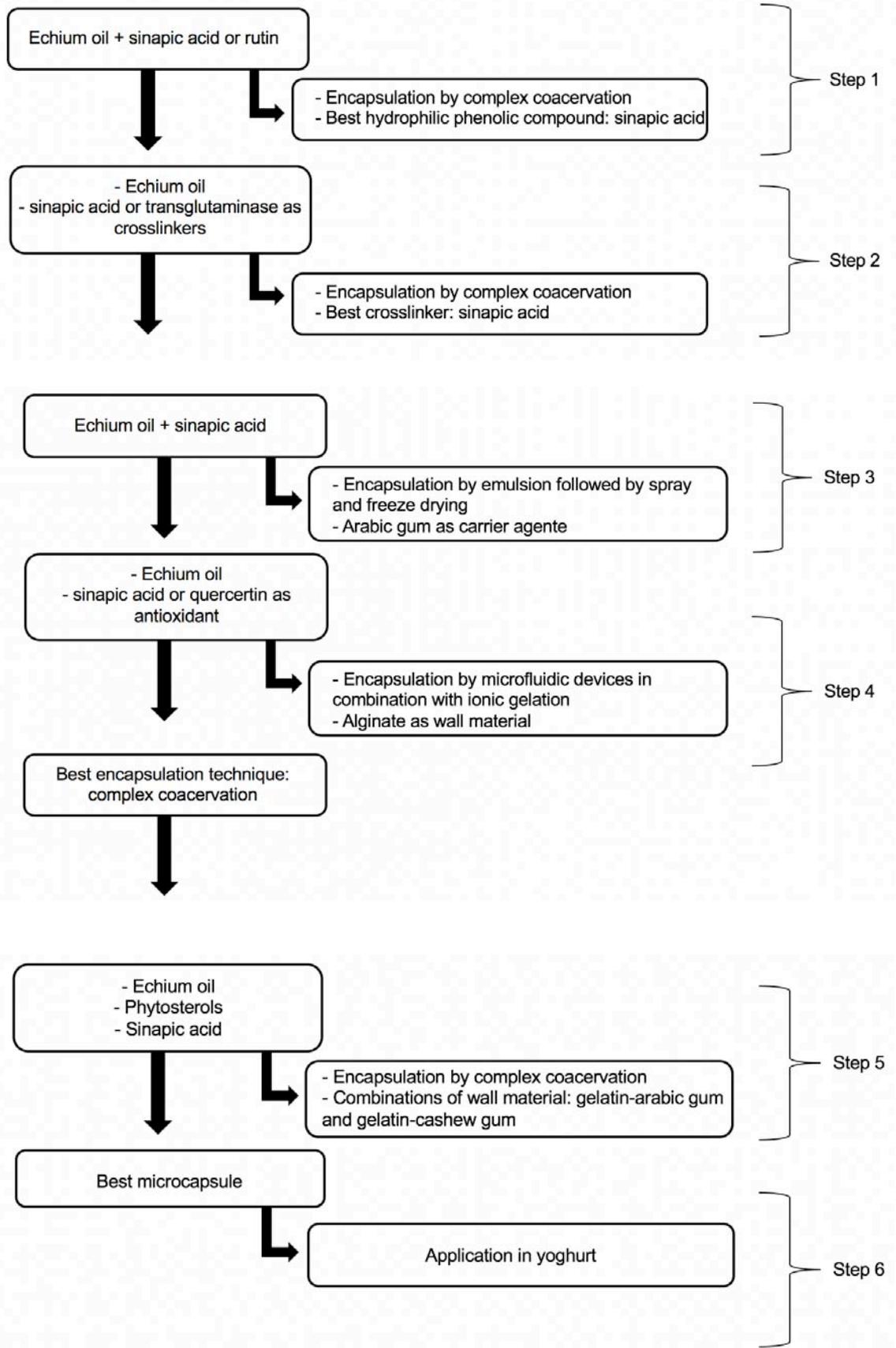
addition to its function as an antioxidant. Moreover, spray drying is the technique most commonly used for the encapsulation of food ingredients and consists in the atomization of a solution, suspension or emulsion; it is also a fast and low cost technology. Ionic gelation is a continuous process and produces matrix or core-shell (reservoir) particles. The most common ionic gelation process uses the biopolymer alginate as wall material and is based on the gelification capacity of this polymer in the presence of ionic calcium, without the need of heating. On other hand, microencapsulation by microfluidic devices is a promising technique for the production of monodisperse microcapsules with uniform mechanical properties, which allows greater control and optimization of encapsulation efficiency and release of the active agents (SUN et al, 2010; ZHAOL et al, 2011).

In this context, the objective of this work was to coencapsulate the echium seed oil, phytosterols and phenolic compounds by different techniques in order to obtain innovative vehicles that will bring benefits to the consumers' health, protection of the compounds and possibility of controlled release.

The first step of this research (Chapter 2 and Attachment B) consisted of the study of encapsulation of echium oil with phenolic compounds sinapic acid and rutin for the determination of the best hydrophilic antioxidant; in this case, sinapic acid was the phenolic compound chosen. Phytosterol was not required for this step. Thus, the second step (Chapter 3 and Attachment C) aimed to compare the effect of the inclusion of sinapic acid as a crosslinking agent on the encapsulation of echium oil, comparing it with transglutaminase (an enzyme commonly used as a crosslinking agent), besides different combinations of wall materials (gelatin-gum arabic and gelatin-cashew gum). Due to the excellent

behavior of sinapic acid as a crosslinking agent, it was also evaluated in echium oil microparticles obtained by spray and freeze drying using gum arabic as a wall material (Chapter 4). For comparison purposes, in addition to the methods already mentioned, the technique of microfluidic devices in combination with ionic gelation was also analyzed in order to study the behavior of sinapic acid in different structures and to compare with the addition of quercetin, a hydrophobic antioxidant (Chapter 5 – Attachment D). With the results obtained until these steps, the best treatment was chosen and used for the addition of phytosterol, controlled release study and stability of the encapsulated bioactive compounds (Chapter 6), besides the application of these microcapsules in yoghurt (Chapter 7 and Attachment E). A schematic representation showing all these steps is shown in Figure 1.

Figure 1. Schematic representation of each step of this research.



**Chapter 1. MICROENCAPSULATION USING BIOPOLYMERS AS AN
ALTERNATIVE TO PRODUCE FOOD ENHANCED WITH PHYTOSTEROLS
AND OMEGA-3 FATTY ACIDS: A REVIEW**

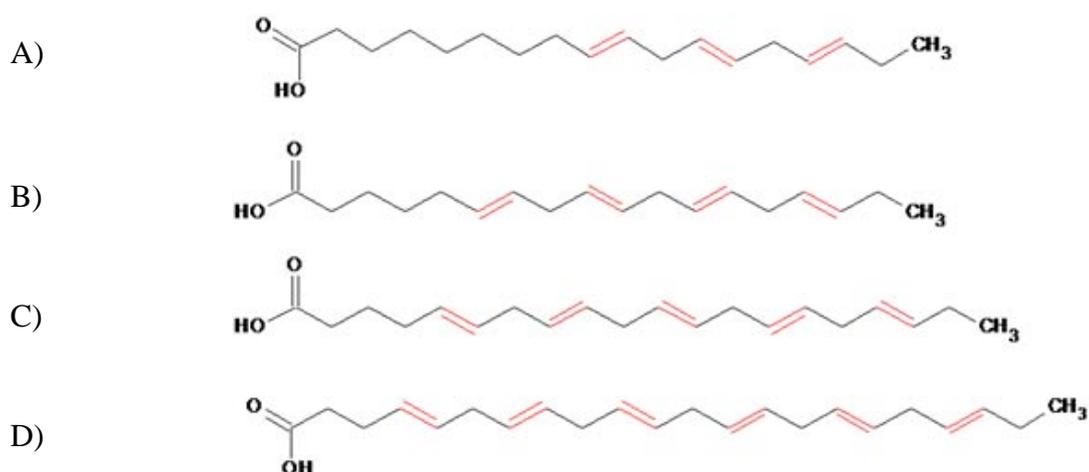
(PART OF THIS CHAPTER WAS PUBLISHED IN FOOD HYDROCOLLOIDS –
ATTACHMENT A)

CHAPTER 1. MICROENCAPSULATION USING BIOPOLYMERS AS AN ALTERNATIVE TO PRODUCE FOOD ENHANCED WITH PHYTOSTEROLS AND OMEGA-3 FATTY ACIDS: A REVIEW

1. OMEGA-3 FATTY ACIDS

As mentioned, the fatty acids are the main constituents of lipids. They can be classified as saturated or unsaturated, having only single bonds and containing at least one double bond, respectively. The fatty acids containing double bond (or unsaturated) in the third carbon counted from the methyl-terminus are known as omega-3 fatty acids (ω -3). The main members of this family are alpha-linolenic acid (ALA) (18:3 n-3), eicosapentaenoic acid (EPA) (20:5 n-3), docosahexaenoic acid (DHA) (22:6 n-3) and stearidonic acid (C18:4 n-3), as shown in Figure 1.1.

Figure 1. 1. Structures of fatty acids: a) alpha-linolenic acid, b) stearidonic acid, c) eicosapentaenoic acid, d) docosahexaenoic acid.



Reference: Own source.

There are some sources of ω -3 viable to be added into food products; they are from animal origin (marine oils) and vegetable origin (as echium, chia, camelina, perilla and flax seed oil). The omega-3 fatty acids from animal origin are derived primarily from certain fish oils and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most important; moreover, they are precursors of anti-inflammatory mediators and have been shown advantages in preventing of diseases inflammatory, allergy, diabetes and Alzheimer's disease (LAVIE; MILANI; MEHRA & VENTURA, 2009; KRALOVEC; ZHANG S.; ZHANG W; & BARROW, 2012). The ω -3 fatty acids from vegetable origin extracted from echium seed oil (*Echium plantagineum* L.) contains 33% of α -linolenic acid and from 9 to 16% of stearidonic fatty acid, which belongs to the ω -3 family and is an intermediate in the biosynthesis of EPA and DHA (BERTI et al., 2007; ZANETTI; MONTI; & BERTI, 2013). The chia (*Salvia hispanica* L.) seeds contain the highest known percentage of α -linolenic fatty acid of any plant source, ranged from 64.8 to 56.9% (AYERZA & COATES, 2011). Camelina (*Camelina sativa* L.), perilla (*Perilla frutescens* L.) and flax (*Linum usitatissimum* L.) seeds containing around of 36, 53, and 57%, respectively, of α -linolenic fatty acid (SULTANA, 1996).

Omega-3 fatty acids are being widely studied due to triglyceride reduction capability, promoting the reduction of risk of cardiovascular disease (POOLE et al., 2013), reducing the risk of coronary heart disease in adults (KHANDELWAL et al., 2013) and in the treatment of people with depression and bipolar disorder (MISCHOULON & FREEMAN, 2013). However, due to the presence of double bonds in their long-chain, these molecules are susceptible

to changes as oxidation, besides being very hydrophobic, what hamper its application in some foods and beverages.

1.1. Echium seed oil

The genus *Echium* has Mediterranean and Macaronesia origin (GUILGUERRERO et al., 2001; BERTI; JOHNSON; DASH; FISCHER; WILCKENS, 2007). *Echium plantagineum* L. has spread all over the world, being an annual or biennial plant. This plant forms a rosette, which gives rise to several stems. The flowers are blue, purple, white and pink, with trumpet shaped (IENICA, 2002; BERTI; JOHNSON; DASH; FISCHER; Wilckens, 2007), while the seeds are dark brown or gray and 3 mm long (NICHOLLS, 2000; BERTI; JOHNSON; DASH; FISCHER; WILCKENS, 2007). The plant grows to about 70 to 120 cm in height (Figure 1.2).

Figure 1. 2. Echium plantation in North Dakota and the echium seed.



Reference: BERTI et al., 2007.

According to Clough (1993), the oil content of the echium seed ranges from 200 to 250 g/Kg and contains from 9 to 16% stearidonic acid. In addition to

this acid, the oil also contains approximately 14% linoleic acid, 10% gamma-linolenic acid and 33% α -linolenic acid (ERDEMOGLU et al., 2004; PAYNE et al., 2014). Thus, echium seed oil is being considered as an alternative in the substitution of fish-derived oils, presenting a single proportion of omega-3 to omega-6 fatty acids that is not found in any other plant (1.8:1 of omega-3 to omega-6) (BERTI; JOHNSON; DASH; FISCHER; WILCKENS, 2007).

There are few works related to this oil, and the ones found presented several health benefits. According to Alhazzaa et al. (2013), Echium plantagineum oil has a better response to immunity than the canola seed oil. Forrest et al. (2012) studied the effect of echium oil on atherosclerosis and reported that this oil reduces significantly triglyceride and cholesterol levels, and consequently atherosclerosis, compared to palm oil. Díaz-López et al. (2010) studied the effects of replacing fish oil by echium oil on the lipid metabolism of enterocytes and hepatocytes of *Sparus aurata* L. and found that the composition of enterocytes and hepatocytes was not affected by the replacement of 50% of the oil, but had an increase in the concentration of healthy fatty acids, such as stearidonic and γ -linolenic acid. In addition, studies also showed that stearidonic and γ -linolenic acids present in echium oil improve the availability of glucose in insulin resistant monkeys (KAVANAGH et al., 2013).

1.2. Stearidonic fatty acids

Stearic acid, a long-chain fatty acid produced by the desaturation of ALA (α -linolenic acid), is found in small amounts in plants, fish and algae, and is very

important in human nutrition because it is an intermediate in the biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). These two fatty acids, present in fish oil, are required in the human diet for cell membrane function and health (COUPLAND; HEBARD 2002; BERTI; JOHNSON; DASH; FISCHER; WILCKENS, 2007; LEMKE et al., 2013). Supplementation of stearidonic acid increases EPA concentrations in red blood cell membranes around 17 to 41% (LEMKE et al., 2013).

This fatty acid is found in other species of Echium (*E. vulgare* L.), hemp (*Cannabis sativa* L., Cannabaceae) (2%-3%) (Callaway et al., 1996), and redcurrant seed (*Ribesnigrum* L., Grossulariaceae) (about 2%) (CLOUGH, 1993). Its structural formula is shown in Figure 1.1 b.

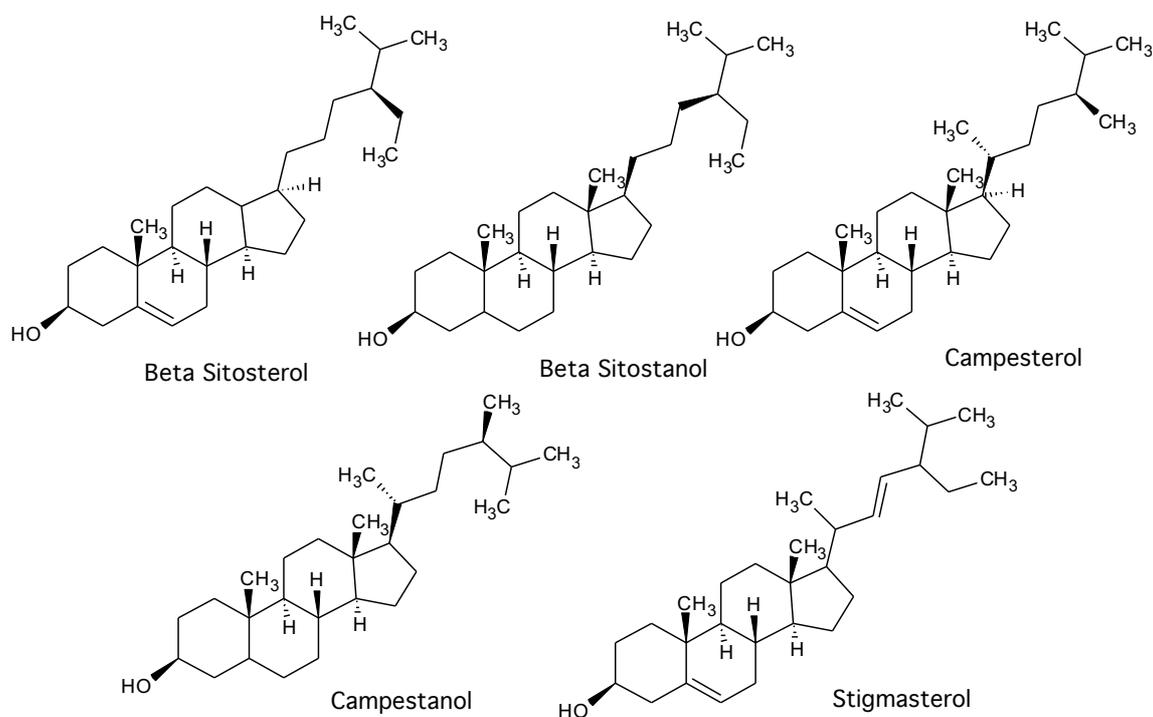
2. PHYTOSTEROLS

In relation to phytosterols, plant sterols, they have structural similarity with cholesterol, differing only in the presence of side chains in the C-24 carbon; the main phytosterols are: beta-sitosterol, beta-sitostanol, campesterol, campestanol and stigmasterol (CARR; ASH; & BROWN, 2010), as observed in Figure 1.3. They are obtained from refined oils of plants, seeds, whole grains and legumes (SANCLEMENT et al., 2012).

In the same way as the omega-3 fatty acids, phytosterols have many benefits, such as the improvement of the profile of serum lipids, reduction of the risk of cardiovascular disease and anticancer effect (AWAD; FINK; WILLIAMS; & KIM, 2001; OSTLUND, 2002; KENDALL & JENKINS, 2004; JU; CLAUSEN, & ALLRED, 2004; WOYENGO; RAMPRASATH, & JONES, 2009; BRADFORD & AWAD, 2010; ITO, 2012; LIU & RUAN, 2013). However, phytosterols are also

unstable when subjected to certain environmental conditions, very hydrophobic and viscous.

Figure 1. 3. Chemical structures of major phytosterols.



Reference: Own source.

3. PHENOLIC COMPOUNDS

The phenolic compounds found in the plant kingdom have been of great interest in the food industry due to their beneficial effects on human health, being important in the growth of plants, responsible for color, astringency and aroma in various foods and also act as antioxidant agent (FIUZA et al., 2004; LEE et al., 2005; ABDALLAH et al., 2011; LEE et al., 2013; SHARMA, 2014; GUINE et al., 2015). It is important to remember that antioxidants are substances that, at low concentrations, decrease or prevent oxidation reactions (GUINE et al., 2015).

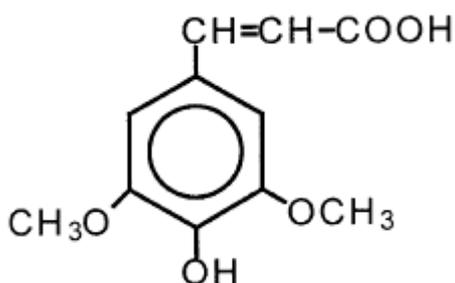
Phenolic compounds offer several benefits to health. McKay et al. (2015) studied the bioavailability of flavonoids and phenolic acids of cranberry juice. It has been observed that phenolics are bioavailable and exert antioxidant action in healthy adults. Fu et al. (2014) evaluated the phenolic composition and skin effects of *Sapium sebiferum* L. leaf extract and found beneficial effects in skin diseases and in the defense system due to the antioxidant activity of the phenolic compounds present in the leaf.

The phenolic compounds highlighted in this research are sinapic acid, rutin and quercetin.

3.1. Sinapic acid

The sinapic acid (SA) is the main phenolic acid of canola (RUBINO et al., 1995; RAWEL; ROHN, 2010), whereas the choline ester of sinapic acid, known as sinapine, is the main phenolic ester of canola (KRYGIER et al., 1982; RUBINO et al., 1996). The structural formula of SA is shown in Figure 1.4.

Figure 1. 4. Sinapic acid structural formula.



Reference: NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY, 2017.

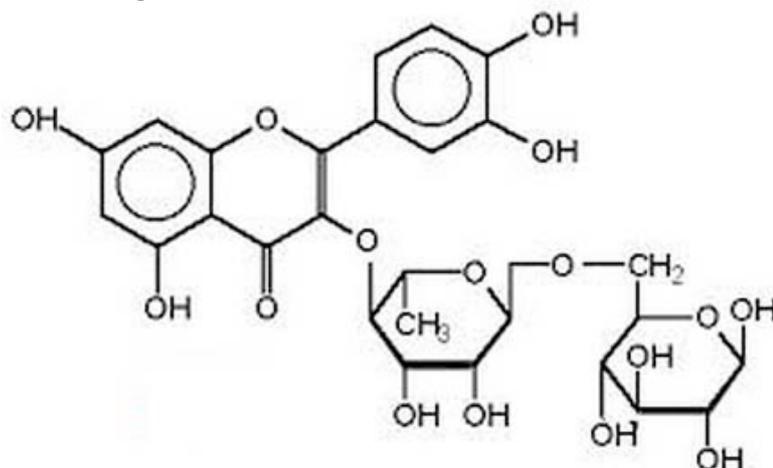
The SA has been extensively studied in relation to the neuroprotective effect against Alzheimer's disease (LEE et al., 2012), cardiac hypertrophy and dyslipidemia (PARI; JALALUDEEN, 2011; ROY; PRINCE, 2012; ROY; PRINCE,

2013). Marinova et al. (2003) studied the antioxidant activity and mechanisms of action of different phenolic acids at room and high temperature and verified that the antioxidant activity of sinapic acid and caffeic acid is higher than that of benzoic acid derivatives. Silambarasan et al. (2015) studied the antioxidant and cardioprotective potential of the sinapic acid against ischemia/ reperfusion injury (I/R). Results showed that sinapic acid protects cardiac cells and their I/R functions induced by oxidative stress.

3.2. Rutin

The rutin (quercetin-3-O-rutinoside), a conjugated glycoside belonging to the class of flavonols (rutinose = rhamnose+glucose), is abundantly found in plants and food such as onions, grapes, beans, apples and tomatoes (WANG et al., 2010), and presents various pharmacological activities, including antiallergic, anti-inflammatory and vasoactive properties (KIM et al., 2011; JANTRAWUT et al., 2013), as well as antioxidant activity, aid in the inhibition of lipid peroxidation, and cytoprotective activity NEGRÉ-SALVAYRE et al., 1991; IHME et al. 1996; BOYLE et al., 2000; POTAPOVICH; KOSTYUK, 2003; SHEU et al., 2004; ZIAEE et al., 2009; TANG et al., 2011; SANTOS et al., 2011; ARAUJO, 2012). Its structural formula is shown in Figure 1.5.

Figure 1. 5. Rutin structural formula.



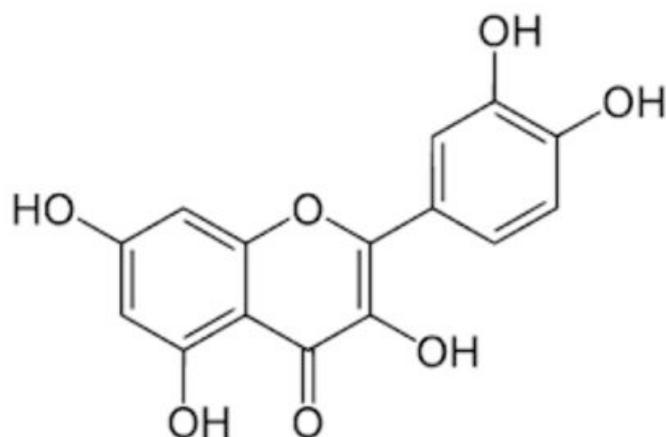
Reference: NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY, 2017.

Horcajada et al. (2015) have shown that oleuropein and rutin, when combined, induce interesting metabolic effects on osteoarthritis (OA) in pigs, protecting against pro-inflammatory and catabolic processes involved in the development of OA. Rutin may also serve as a hepato-protective agent and its supplementation assists in attenuation of hepatic injury-related cholestasis (PAN et al., 2014). In addition, rutin may also aid in the treatment of inflammatory disease (MASCARAQUE et al., 2014).

3.3. Quercetin

Quercetin (3,5,7,3'-4'-pentahydroxyflavone) is one of the most common flavonoids present in nature that has attracted the attention of many researchers due to its anti-stress, anti-inflammatory and anticancer properties. Quercetin can be obtained from onion, pod, broccoli, cabbage and tomato, as well as apple (WATERHOUSE et al., 2014). Its structure can be seen in Figure 1.6.

Figure 1. 6. Quercetin structural formula.



Reference: NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY, 2017.

In the same way as sinapic acid and rutin, quercetin has been extensively studied. Vrolijk et al. (2015) found that the interaction of quercetin and tamsulosin increases the potential impact of drug-supplement interactions for prostate diseases, besides inducing vessel relaxation. Xia et al. (2015) studied the effects of quercetin on hippocampus and memory-dependent learning of rats with different diets and found that an appropriate dose of quercetin may attenuate oxidative stress and improve hippocampal-dependent cognition.

4. MICROENCAPSULATION

Microencapsulation is a technique in which one or more compounds (core or internal phase) are surrounded or immobilized by one or more materials (shell, carrier or wall material) in order to be protected from external factors such as light, high concentration of oxygen, heat, moisture, preventing evaporation of volatiles compounds, masking unpleasant tastes and odors, and for development of value-added products. So, this technology has been used in

the food industry since it facilitates the application of unstable ingredients, in addition to improving the processing and texture of ingredients due to lower hygroscopicity, increased solubility and dispersibility in different kind of materials. Besides, there is the possibility of release control in the site of action of the compounds of interest. The cited advantages can be seen in various applications, such as the cited one by Gallardo, Guida, Martinez, López, Bernhardt, et al. (2013), who microencapsulated linseed oil by spray drying and applied it into bread manufacturing. In a recent research, Marsanasco, Piotrkowski, Calabró, Alonso, & Chiaramoni (2015) encapsulated omega-3, omega-6 and tocopherol by liposomes and incorporated it orange juice as new functional food. Marsanasco, Márquez, Wagner, Chiaramoni, & Alonso (2015) encapsulated omega-3, omega-6 and vitamin E by liposomes and applied it into chocolate milk, obtaining a functional food product.

There are several techniques that have been used or at least studied for microencapsulation of different food ingredients. Moreover, these techniques are classified into three different methods: (1) Physical methods, such as, spray drying, spray chilling/cooling/congealing, supercritical fluids encapsulation processes, process with microfluidic devices and spray coating; (2) Chemical methods, as polymerization; and (3) Physico-chemical methods, such as, complex coacervation, liposomes, micelles, emulsions, process with nanostructured lipid matrices, solvent evaporation, and molecular inclusion. All of these techniques can be adapted for the encapsulation of omega 3 fatty acids (CAO; HE; ZHANG, & WANG, 2011; CONTO et al., 2013; ERATTE et al., 2015; UMESHA; MANOHAR; INDIRAMMA; AKSHITHA, & NAIDU, 2015; MARSANASCO; MÁRQUEZ; WAGNER; CHIARAMONI, & ALONSO, 2015;

GÓMEZ-MASCARAQUE & LÓPEZ-RUBIO, 2016) and phytosterols (MENG; PAN; & LIU, 2012; CHEN; ZHONG; WEN; MCGILLIVRAY, & QUEK, 2013), although, this chapter focuses on the methods that use biopolymers and cyclodextrins.

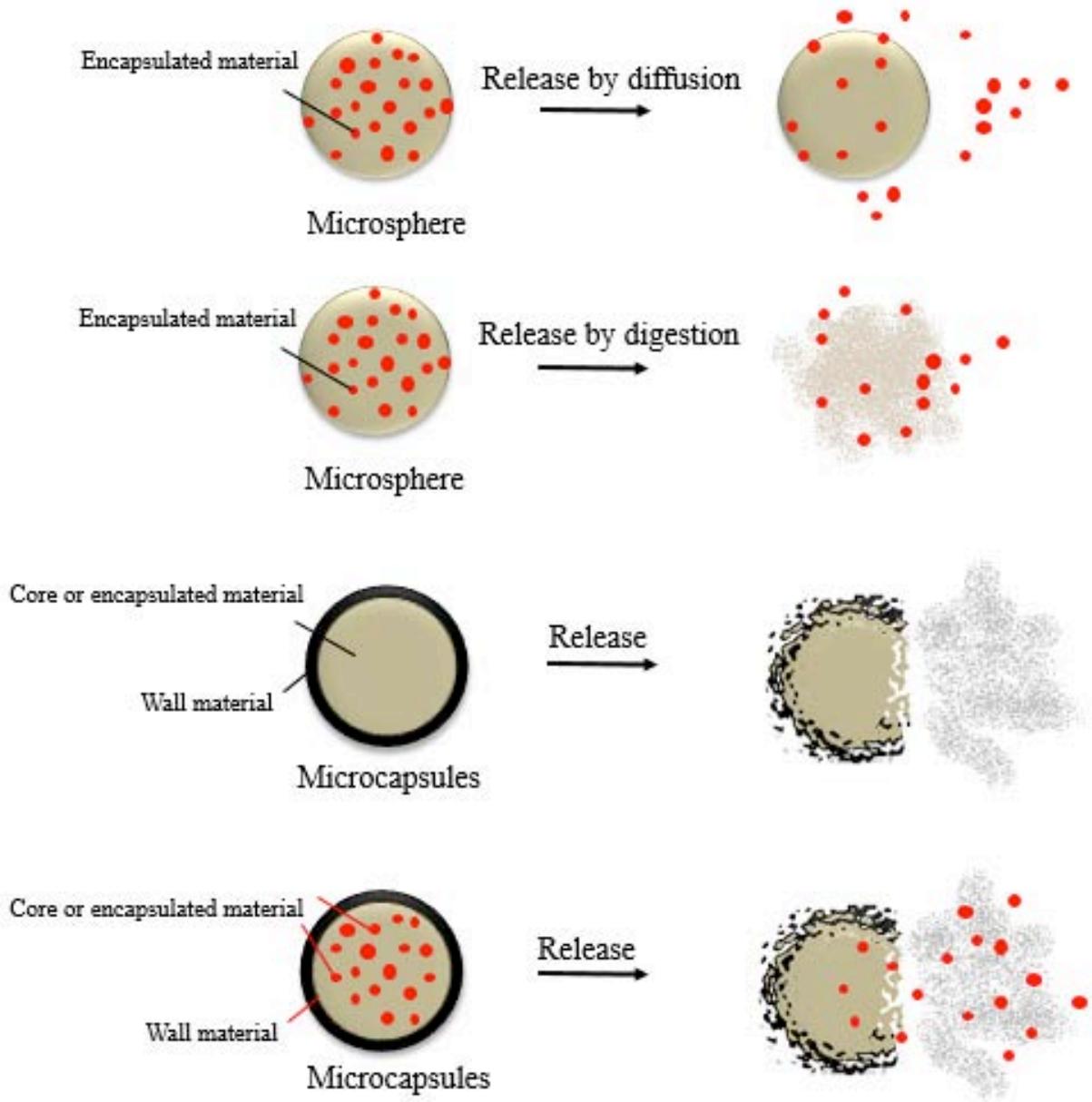
For the choice of technique to be used, it is necessary to analyze the final product in which it will be applied, the desired particle size, release mechanisms and the physicochemical properties of the material used as the encapsulating agent and core (FAVARO-TRINDADE; PINHO; & ROCHA, 2008). Besides, in the case of a food ingredient, the cost and safety of the method and wall material also must be considered in the selection.

According to Coupland & Hayes (2014), there are different formulation challenges for food compounds encapsulation. One of them is the choice of materials to be used in the encapsulation process. There are many kinds of polymers that cannot be applied in food products, limiting the use of various compounds. In addition, the economics and marketing concerns of food need to be taken into consideration. However, even with several challenges mentioned, the encapsulation technique has been increasingly explored, achieving excellent results in obtaining ingredients with functional appeal.

The particles produced by encapsulation methods are known as microcapsules or microspheres, as can be seen in Figure 1.7. Both structures (microcapsules and microspheres) are very important for practical applications and can have similar and different utilities or functionalities; the controlled release of the active material, for example, depends on the location of this compound inside the microcapsule and/or microsphere. The obtainment of microcapsules or microspheres depends mainly on the encapsulation technique

used, as mentioned following. Microcapsules are reservoir type particles, where the core is surrounded by a shell (SUAVE et al., 2006); moreover, it can have one or multiple nucleus. Liposomes, complex coacervation, microfluidic devices, spray coating and molecular inclusion are examples of techniques of encapsulation that produce microcapsules (XIAO; LIU; ZHU; ZHOU; & NIU, 2014; COMUNIAN et al., 2014). Microspheres are matrix type particles, i.e., structures where the bioactive compound is spread by all the volume of the particle, sometimes including in its surface (SUAVE et al., 2006). Spray drying and spray chilling are examples of methods of encapsulation that produce microspheres (OKURO et al., 2013; JIMÉNEZ-MARTÍN; GHARSALLAOUI; PÉREZ-PALACIOS; CARRASCAL; & ROJAS, 2015). In relation to the biopolymers that have been used, studied or could be useful for the encapsulation of omega 3 fatty acids and phytosterols, the next section will illustrate some of them.

Figure 1. 7. Scheme of microsphere and microcapsule structure.



Reference: Own source.

4.1 Biopolymers used by the encapsulation of phytosterols and omega-3 fatty acids

4.1.1. Gelatin

Gelatin, one of the most used hydrocolloids in food industry, may be obtained from pig skin and bones (46% and 23.1%, respectively), from cowhide (29.4%) and from fish (1.5%). Moreover, it is produced from the partial hydrolysis of collagen (acidic or basic hydrolysis). In relation to the gelatin charge, it has a negative charge above its isoelectric point (IEP) and positive charge below it. According to Mishra (2015), IEP is the pH at which the charge of gelatin is zero. Furthermore, its isoelectric point (IEP) varies according to its obtainment; due to minor changes in the amide groups, the gelatin obtained by acid hydrolysis (gelatin type A) has IEP value in alkaline medium (it ranges from pH 7 to 9). In the case of gelatin obtained by alkali hydrolysis, due to the large amount of free carboxylic acid, it has IEP value in acid medium (it ranges from 4 to 5). Knowing the isoelectric point of the gelatin is essential to understand the complex coacervation process. Different IEP result in different ranges of coacervation pH values, which can vary from 3.2 to 5.50 for the combination of gelatin and gum arabic, for example (Mishra, 2015). In addition to IEP, the encapsulation of omega-3 fatty acids and phytosterols can be affected by other factors such as pH and ionic strength, mainly in the complex coacervation, which is a technique that uses gelatin as wall material very often (BURGESS, 1990; KAUSHIK; DOWLING; BARROW; & ADHIKARI, 2014; MISHRA, 2015). Furthermore, gelatin has tensoactive properties, acting as a surfactant in food applications (DIAZ-CALDERÓN; CABALLERO; MELO; & ENRIONE, 2014;

DUCONSEILLE; ASTRUC; QUINTANA; MEERSMAN, & SANTE-LHOUELIER, 2015).

Gelatin is one of the most used biopolymers as wall material, as it is shown in Table 1.1. Besides its low cost, gelatin gel melts at temperatures around 30-40 °C, what helps in the controlled release of the encapsulated compounds. This temperature range is similar to body temperature; however, this kind of material may take up to 30 minutes to melt, depending on the concentration of the solution and also on the interactions to its neighbors. Thus, gelatin is an important biopolymer in the microencapsulation of omega-3 fatty acids and phytosterol, since it has suitable properties for its use in various encapsulation techniques such as spray drying and complex coacervation.

4.1.2. Gum arabic

Gum arabic, highly water-soluble polymer, is an acacia tree secretion, and it is found in Arab countries such as Sudan and also African countries (ISLAM; PHILLIPS; SLJIVO; SNOWDEN, & WILLIAMS, 1997). According to Damodaran, Parkin, & Fennema (2010), the gum arabic is a material composed of two fractions: (1) polysaccharides chain, corresponding to greater part of its structure (D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid), and (2) protein content, which corresponds to about 2% of the total weight. As well as all protein, the protein fraction of gum arabic also changes the charge, however, due to its small proportion, this charge change has slight influence on the total charge of gum arabic. Also, according to Damodaran, Parkin, & Fennema (2010), the polysaccharide structures are highly ramified and the uronic acid

units occur more often as non-reducing ends, having acid character and being responsible for the hydrophilic part of the polymer, whereas the protein structure is responsible for the hydrophobic part of the gum arabic. These amphiphilic character allows gum arabic to act as emulsifier.

In the food industry, gum arabic is widely used as a stabilizer, emulsifier and thickener, and also acts as flavor fixative (RIBEIRO & SERAVALLI, 2004). As mentioned in this review, the process of complex coacervation makes use of different wall material combinations (item 4.3), however the pair gelatin-gum arabic is the most traditional one due to several factors such as: (1) the hydrophobic material is totally coated by polymers, ensuring the protection of omega-3 fatty acids and phytosterols against adverse environmental conditions; (2) producing microcapsules of rounded shape (characteristic not observed in the most other wall material combinations) and this morphology helps in the application of microcapsules in a food product due to the easy flow. Moreover, according to Eastoe & Leach (1977), Ducel, Richard, Saulnier, Popineau, & Boury (2004), Schmitt, Sanchez, Banon, & Hardy (2010) and Kaushik, Dowling, Barrow, & Adhikari (2014), due to the appropriate amino acid profile and charge density of gelatin, and the polysaccharide configuration of gum arabic, which preserves the charges and allows the water to be blocked between the chains, the combination gelatin and gum arabic helps the formation of the complex coacervate due to the increased interaction between these two polymers.

Additionally, gum arabic is negatively charged, whereas gelatin has a negative charge above its isoelectric point (IEP) and positive charge below it. When pH of the combination of gelatin and gum arabic is below the IEP of the gelatin, the coacervate is formed due to the interaction of these polymers in

suitable temperature, ionic strength, concentrations and polymer ratios, becoming therefore, a feasible combination with gelatin to form microcapsules by complex coacervation, as noted in Table 1.1.

Gum arabic is also very useful for encapsulation of hydrophobic compounds by spray drying since it has emulsifying ability. Besides, it forms low viscosity solutions in high concentration, what is very important to facilitating pumping and to improving the productivity of the process.

Thus, according to the above, in the same way as gelatin, gum arabic is considered a viable biopolymer for the encapsulation of omega-3 and phytosterols, especially by spray drying and complex coacervation techniques.

4.1.3. Pea protein

According to Kent & Doherty (2014), the peas are used worldwide as sources of starch, fiber and protein because of its economic viability, including easy obtainment and peeling. Pea protein has several characteristics such as high temperature stability and the ability to act as an emulsifier.

The pea protein has some limitations to its application as wall material in complex coacervation due to a relatively broad range of the isoelectric point from pH 4 to 6, depending on raw material and process history. Therefore, the source has to be chosen well to avoid solubility problems at certain pH-dependent applications. However, even with these limitations, researchers have been able to use this protein as encapsulating agent (Table 1.1).

Thus, due to the mentioned limitations, pea protein may be considered a biopolymer little explored in the encapsulation field; however, not less important than gelatin and gum arabic.

4.1.4. Pectin

Pectin, a linear polysaccharide obtained from the extraction of the albedo of citrus fruits and apple pulp, is considered a good material to be used as encapsulating agent since it is degradable by the microbiota, what facilitates the controlled release of the encapsulated compounds. It is classified according to the degree of methoxylation (methyl groups esterified to the carboxylic group) and it is widely used by the food industry due to its gelling power (RIBEIRO & SERAVALLI, 2004; FATHI; MARTIN; & MCCLEMENTS, 2014). As pectin is an anionic polysaccharide, an inexpensive and abundant material, it has been used to encapsulate lots of compounds by complex coacervation (Table 1.1).

Pectin can be considered a good alternative for the encapsulation of omega-3 and phytosterols, since it is easy to obtain and can be applied as wall material for various encapsulation techniques (spray drying, complex coacervation and ionic gelation).

4.1.5. Casein

Casein, protein derived from milk (representing around 80% of milk proteins), has been of interest to the food industry due to its composition (it contains all the common amino acids and it is also rich in essential amino acids) and its interactions with ions like calcium phosphate, providing an additional role

for nutritional value. Besides its ability to form micelles, it is into three subgroups, including α -casein, β -casein and κ -casein (DAMODARAN; PARKIN & FENNEMA, 2010; KOURY; SCHEED-BERGDAHL; & BERGDAHL, 2014). According to Sgarbieri (2005), the α -caseins are divided in α_{S1} and α_{S2} , depending on the presence of fast migration proteins designated α_S -caseins. The α_{S1} -casein is the main and consists of a polypeptide chain with 199 amino acid residues. In the case of β -caseins, they represent from 30 to 35% of total caseins. As opposed to α_{S1} -casein, β -casein forms colloidal suspensions in the presence of Ca^{++} , besides presenting temperature, concentrations and pH that equilibrium (association - dissociation) occurs. In the case of κ -casein, it is the only glycosylated casein and consists of three monosaccharides (galactose, N-acetyl-galactosamine or N-acetyl neuraminic acid). In the encapsulation process, the functionality of caseins depends on their process history, that means in which constitutional form are used: as salt (e.g. sodium caseinate), as micelles (native casein entities like in milk) or as protein particle suspension (e.g. like in acidified milk drinks).

Although the mean charge of a casein micelle at pH 6.8 is negative, in its surface spots with different partial charges from negative to positive values are found depending on the casein type and its role in the micellar structure. Therefore, it depends on the processing or selecting of casein type if casein presents a charge interaction functionality like gum arabic for example, and thus, might be expected to be a fitting combination to encapsulate with gelatin due to the electrostatic interactions resulted from the opposite charges. If casein is applied in an acidified system in non-micellar form, some types of pectin

might be the adequate interaction partner like shown in the literature and seen in Table 1.1.

4.1.6. Soy Protein Isolate

Soybean is a commonly cultivated product in the world of significant economic value. As mean value, it contains about 40% of protein and 20% of oil on a dry basis. According to Damodaran & Arora (2013), the soy protein isolate used in food for humans is obtained from defatted soy flour, which contains about 50% of protein on a dry basis. It is an abundant, inexpensive and renewable raw material (ORTIZ et al. 2009).

The soy protein isolate, because it is a protein of low cost and wide availability, is being very explored as encapsulating material when the complex coacervation technique is used, however not as much as gelatin and gum arabic (Table 1.1). Soy protein isolate has been also used to encapsulate by spray drying (ORTIZ et al. 2009).

4.1.7. Chitosan

Chitosan, polysaccharide derived from chitin, is a cationic and biodegradable polymer, found mainly in crustacean shell and considered highly renewable and economically viable material. It has been used by food industries mainly in health area, presenting antimicrobial and antioxidant activities (CANELLA & GARCIA, 2001; JANEGITZ; LOURENÇÃO; LUPETTI, & FATIBELLO-FILHO, 2007; FATHI; MARTIN, & MCCLEMENTS, 2014). In addition, studies have shown the relation of chitosan to body weight reduction (TRIVEDI et al., 2015).

In relation to its use as encapsulating material, its solubility only at low pH values may limit its application, however, due to its mucoadhesive nature, the controlled release of the encapsulated material in the gastrointestinal medium can be facilitated. To control the release by mucoadhesive nature of an encapsulating partner is possible to use an insoluble coating together or modify the material into suitable viscosity and chemistry according to the desired place to release the active compound.

For complex coacervation process, it is necessary that the used polymers have opposite charges. Therefore, if one polymer is protein, which can change the charge due to pH, the way how to do the encapsulation depends on the sign of the charge of the polysaccharide. In the case of chitosan, the process would begin with the protein in an acid medium, followed by raising the pH in order to obtain a negative charged protein binding to chitosan. Thus, chitosan, as well as having its beneficial properties to health, it can be considered a feasible biopolymer to be applied in various encapsulation techniques, especially in coacervation techniques.

4.1.8. Alginate

The alginate, an anionic and hydrophilic polysaccharide of natural origin, has been used in the pharmaceutical and food field, acting in microencapsulation processes as well as building membranes with regulated permeability. This biopolymer can be obtained from marine plants (ZIA K.M.; ZIA F.; ZUBER; REHMAN; & AHMAD, 2015).

The alginate is composed by carboxylic acid, hydroxyl functional groups, glycosidic and glycolic bonds. Due to the presence of charges, this polymer has been used as wall materials for the encapsulation technique of complex coacervation. This polysaccharide is also very useful for the immobilization of thermal unstable materials, as omega 3 and phytosterol, by ionic gelation, because it is able to jellify at room temperature. The gelling capacity of a polymer is related to its composition. The difference between alginate and other polymers is that alginate consists of α -L-guluronics and β -D-manuronics acids, varying in relation to manuronics and guluronics residues and its level of polymerization. In this case, alginate gels are easily formed when in contact to cations Ca^{++} or Mg^{++} .

Moreover, alginate, besides being a good choice as wall material for complex coacervation technique, it has similar properties to pectin, being also applied in the ionic gelation method.

4.1.9. Whey protein

The whey is considered a byproduct of cheese production, it is rich in proteins (β -lactoglobulin, α -lactoglobulin, immunoglobulin and serum albumin), and it is being widely used by the food industry due to their nutritional and functional properties.

The whey proteins (β -lactoglobulin and α -lactoglobulin) have negative charge at a pH of 6.8 like found in fresh milk, making its use as wall materials possible in complex coacervation technique, since it can participate in the electrostatic interactions in the same way as the other biopolymers cited in this review. In addition, due to their globular structure if native in a solution, these

proteins provide only low viscosities even at high concentrations. They present also properties of emulsifiers and therefore provide this functionality process where encapsulation is done by emulsification. In other words, the whey protein is a polymer suitable to provide needed functionality when applied as wall material.

It is important to highlight that heat-induced changes the whey protein conformation due to disulfide bonds, being an essential information to control the functionality of the material. According to Sikorski (2002), thermal changes are responsible by loss of solubility due to aggregation of the proteins, β -lactoglobulin and immunoglobulin for example, besides the increase in solubility as a resulting of the breaking down of structures, as collagen for example. In the case of proteins that present low number of S-S-bonds, the heat-induced leads to an increased solubility. However, even with these changes in the properties, these changes probably will not influence on the activity as wall materials of these proteins; in the case of whey protein for example, several researches on encapsulation with this type of material are found in the literature (VAN LEUSDEN et al., 2016; WANG; LIU; CHEN; & SELOMULYA, 2016), showing that encapsulated materials were protected.

4.2. Mechanisms of release

Besides the protection of the encapsulated material, the microencapsulation techniques also aimed optimal release conditions of the active material in appropriate locations and times in order to obtain better effectiveness and availability of the compounds of interest. It is important to

note that the release of the omega-3 and phytosterol should be avoided during processing and storage of food products, occurring in the consumer's body.

According to Xu et al. (2013), Zhang, Decker, & McClements (2014) and Moomand & Lim (2015), it is possible to analyze how these cited conditions are applied in the controlled release study due to the microcapsules and/or complex subjected to in vitro gastrointestinal conditions using simulated gastric and intestinal fluids. These release mechanisms depend on various conditions, such as diffusion, solvents, pH and temperature.

The release by diffusion is a mechanism that varies according to the biopolymer used as wall material and the encapsulation technique. The active material diffusion rate also depends on conditions of microcapsule wall (integrity) or distribution of the material in the microspheres, besides the presence of pores, which facilitates the diffusion and, consequently, the release of the compounds of interest. Moreover, the state in which the wall materials are, also determines the diffusion of active material. According to Ré (1998), compounds in glassy state are more impermeable than the gummy state, then showing the importance of studying the temperatures and heat flow associated with thermal transition of the compounds. In this case, due to the presence of pores formed in the wall material, this type of release is most noticeable when the spray chilling technique is used (method not shown in this chapter).

In relation to the solvent, it is a potential factor in the release of active materials since it assists in the disruption or dissolution of the wall and consequently in the desired release of the compound of interest. Any kind of molecular interaction that occurs during the encapsulation process (spray drying, complex coacervation, ionic gelation or complexation) can be undone

with the addition of solvents. At the same time, the pH values determine the release of the encapsulated compounds since the variations in these values change the solubility of the wall materials. This method is widely used for microcapsules obtained by complex coacervation.

In relation to release by temperature, these release method is one of the methods most commonly used due to a change of the physical state of the wall materials and/or fusion of these materials when applied temperature changes. This method is not recommended when sensitive compounds to high temperature are encapsulated, such as omega-3 fatty acids and phytosterols, however can be applied to capsules formed with gelatin as wall material, for example. Moreover, the combination of these factors can also be done aiming the best conditions for the desired controlled release.

5. MICROPARTICLES CHARACTERIZATION

There are several types of analyzes used for the characterization of these kind of materials. These analyzes can be classified in (1) characterization methods and quality control for the microcapsules, (2) characterization methods and quality control for the fatty acids and phytosterols encapsulated, (3) methods for the right combination or interaction capability of biopolymers. For the first classification, particle size, optical, confocal and scanning electron microscopy, water activity, moisture, hygroscopicity and solubility analyses can be cited. For the second classification, the methods are controlled release, stability of the encapsulated material, oxidation analyzes by thiobarbituric acid reactive substances (TBARS), peroxide value and accelerated oxidation by rancimat. In relation to the third classification, encapsulation efficiency, Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TG) and

differential scanning calorimetry (DSC) can be mentioned. All these methods are cited below to give an overview about their importance in the omega-3 fatty acids and phytosterols encapsulation.

5.1. Particle size

The particle size is one of the most decisive factors in the application of the microcapsules obtained by complex coacervation into food products, since certain sizes may interfere on the texture and final flavor. The particles can be measured by scattering and refractive index, or even by optical microscopy, aided by software available online ImageJ (ImageJ, Image Processing and Analysis in Java), depending on the structure available for each researcher (Comunian et al., 2011; Comunian et al., 2014).

If the analysis is performed by scattering and refractive index, it is possible to measure the particle size prior to drying step (in the case of complex coacervation technique for example), but several characteristics of the dispersant like ionic strength have to be chosen well to avoid dilution related changes of the microstructure. For some systems, alternatively a dispersion of the sample in a solution or solvent in which the capsules and/or spheres do not solubilize (in the case of spray drying technique, for example) is used. In this case, the type of encapsulation technique and the wall material used is decisive in the choice of solvent (CHEN; ZHONG; WEN; MCGILLIVRAY; & QUEK, 2013).

In the case of complexes formed by the complexation technique, this analysis may be difficult due to obtainment of samples with undefined format.

5.2. Encapsulation efficiency

The amount of encapsulated material depends on several factors, including the encapsulation technique employed, the compounds used as encapsulating agents and as the core and the process conditions (time, temperature and pH). The encapsulation efficiency is necessary to know how much of the active material is inside and outside of the microcapsule and/or complex and thus know how much sample is necessary to add in the final food product. The lower the amount of capsule (and higher the encapsulation efficiency), the better the texture and flavor of the food. In other words, the less capsule applied to the food, the more difficult is to be perceived by the consumer, not affecting the sensory characteristics of the product. It is not possible affirm a limit of microcapsule to be applied in the final product, since it depends on the concentration of active material encapsulated and regulations of each country for each type of compound.

The encapsulation efficiency can be measured in several ways, depending on the encapsulated material (High-performance liquid chromatography, gas chromatography coupled to mass spectrometry, spectroscopy or titration) (LAMPRECHT; SCHÄFER; & LEHR, 2001; CONTO; GROSSO; & GONÇALVES, 2013; COMUNIAN et al., 2013; CARNEIRO; TONON; GROSSO; & HUBINGER, 2013; SANTOS et al., 2014). In the case of oil encapsulation, it is often made only the measurement of oil by the technique of Bligh & Dyer (BLIGH & DYER, 1959).

5.3. Optical, confocal and scanning electron microscopy

The study of the morphology of the microcapsules and/or complexes is important to define the format, location of compounds within the structure and physical characterization. Depending on the encapsulation and drying techniques used, it can be obtained microcapsules with rounded shape or with no defined format, an important feature for the application of the material and which can be analyzed by these various microscopy techniques.

According to Joye & McClements (2014), optical microscopy is usually used to study the microstructure in general, while the confocal, with the help of dyes, it is possible to view certain compounds and study their behavior depending on the location that they are within the microcapsule and/or complex. Comunian et al. (2016) studied the morphology of microcapsules prepared with echium oil (source of omega-3) and phenolic compounds by complex coacervation using gelatin and gum Arabic as wall materials and applied Nile red as fluorophore to dye the oil and verify its location inside the capsule. The micrographs were analyzed by confocal microscopy. Moreover, with confocal microscopy is also possible to determine 3D images. When it comes to nano-sized samples or there is a need to study the surface characteristics, the use of scanning electron microscopy is more recommended.

5.4. Water activity, moisture, hygroscopicity and solubility

Besides the size and morphology of the microcapsules, physicochemical characteristics are also important in the characterization of the components and their ability to form a biopolymer complex. Future applications depend directly

on the hygroscopicity, solubility, moisture and water activity of the capsule and the complex formed.

Analysis of water activity for omega-3 and phytosterols microcapsules is important to assess the microbiological stability of the material. According to Damodaran, Parkin & Fennema (2010), water activity values below 0.6 ensure microbiological safety. In the case of hygroscopicity and solubility values, they help to determine the storage conditions and the type of product which the capsules would be applied, respectively, since the hygroscopicity corresponds to the amount of water that the material can absorb from the environment and the solubility corresponds to the material capacity of dissolving in a specific media. The obtaining of the material moisture values is also essential, since this value assists in determining the capsule composition on a dry basis, a decisive factor for the encapsulation efficiency calculations, for example. Thus, these properties can be controlled according to the desired application.

5.5. Fourier Transform Infrared Spectroscopy (FTIR)

During the encapsulation process, reactions or interactions can occur, affecting the structure of the encapsulated material, wall materials and/or the microcapsule and complex. The Fourier Transform Infrared Spectroscopy (FTIR) can be used for the determination of possible compounds or radicals formed by these reactions or interactions and consequently to analyze if the encapsulated material suffered any influence during the encapsulation process and/or if the wall of the capsule (or complex) was actually formed.

One example for this analysis is shown by Torres-Giner, Martinez-Abad, Ocio, & Lagaron (2010), when they studied the stabilization of a nutraceutical omega-3 fatty acid by encapsulation in ultrathin electrosprayed zein prolamine. According to these authors, by ATR-FTIR was possible to verify that the encapsulation was more efficient against degradation under ambient conditions.

5.6. Thermogravimetric analysis (TG)

Thermogravimetric analysis (TG) is important when it is necessary to evaluate the behavior of the material in relation to weight loss according to the applied temperature which can vary from 0 to 800°C. For example, the cross-linking process is widely used for the purpose of forming more rigid capsules. In this case, the TG analysis is important to evaluate the range of temperature that the capsule is stable and/or hard enough to protect the encapsulated material. Peng et al. (2014) studied the chemical composition, antimicrobial property and microencapsulation of Mustard (*Sinapis alba*) seed essential oil with genipin by complex coacervation and carried out thermogravimetric analysis of the microcapsules at a heating rate of 20 °C/min for 25 to 550 °C. With the aid of this analysis, they observed that low concentration of genipin (compound used as crosslinker) resulted in greater weight loss, in other words, less stable capsules. Recently, Comunian et al. (2016) studied the encapsulation of echium oil by complex coacervation using gelatin and gum Arabic as wall material and performed the termogravimetric analysis of the microcapsules at a heating rate of 10 °C/min in the temperature range of 25 to 800 °C (data presented in chapter 2). These authors observed that the higher the concentration of the

phenolic compound (sinapic acid) added in the microcapsule, the lower the weight loss in the range from 250 to 430 °C.

5.7. Controlled release and stability of the encapsulated material

The main objectives of microencapsulation are to promote the controlled release and protect the compounds against adverse environmental conditions (light, oxygen, moisture), increasing their stability. According to Quirós-Sauceda, Ayala-Zavala, Olivas, & Gonzalez-Aguilar (2014), for the controlled release analysis, it is necessary to study parameters such as moisture, temperature, pH variations and mechanical disruption (KISER; WILSON; & NEEDHAM, 2000; ANDERSON; HANSEN; LUKIANOVA-HLEB; HAFNER; & LAPOTKO, 2010) to obtain the best conditions for the release of drugs or active material in predetermined place and time in the organism or in the food in which the microcapsules and/or complexes were applied. With the same principle, the study of stability is extremely important to obtain a material resistant to environmental conditions and therefore feasible for applications.

Thus, it can be inferred that the characterization of the microcapsules and/or complex obtained by the technologies cited in this chapter has to be chosen in a way that allows differentiating several independent aspects regarding all functionalities needed to obtain a product of quality and with functional appeal.

5.8. Differential scanning calorimetry (DSC)

The differential scanning calorimetry is an essential method for evaluating the physical state of the wall material and core, associating the

behavior of heat flow and different temperatures, obtaining information such as glass transition temperature (T_g), melting and crystallization temperature. According to Nollet (2004), this is a technique often used when information related to conformational changes are needed. One example of this application is shown by Awad, Helgason, Weiss, Decker, & McClements when they studied the effect of omega-3 fatty acids on crystallization, polymorphic transformation and stability of tripalmitin solid lipid nanoparticle suspension.

5.9. Oxidation analyzes by Thiobarbituric acid reactive substances (TBARS) and Peroxide value

In the case of microencapsulation of omega-3 fatty acids, analysis of oxidation of these compounds is essential, since one of the goals of encapsulation of these materials is their protection against oxidation. The evaluation of the oxidation by Thiobarbituric acid reactive substances (TBARS) and peroxide value are the main methods for measurement of oxidation. Usually, to carry out these analyzes, the microcapsules (or microspheres) are submitted to different storage conditions for a certain period. However, according to Kaushik, Dowling, Barrow, & Adhikari (2014), these methods present some disadvantages such as unstable measurements and intermediate products (peroxide value) and is not a good indicator at lower oxidation levels (TBARS).

5.10. Accelerated oxidation by rancimat

In addition to the analyzes mentioned in item 5.9, the oxidation can also be assessed by rancimat. In this case, the samples are subjected to conditions that accelerate the oxidation process, thus obtaining the results in function of oxidation time. According to Kaushik, Dowling, Barrow, & Adhikari (2014), the advantage of this method is the assessment of a large number of samples with good reproducibility. However, it needs high-cost equipment, making the analysis expensive.

6. TECHNIQUES APPLIED FOR ENCAPSULATION OF OMEGA 3 AND PHYTOSTEROLS USING BIOPOLYMERS AND CYCLODEXTRINS

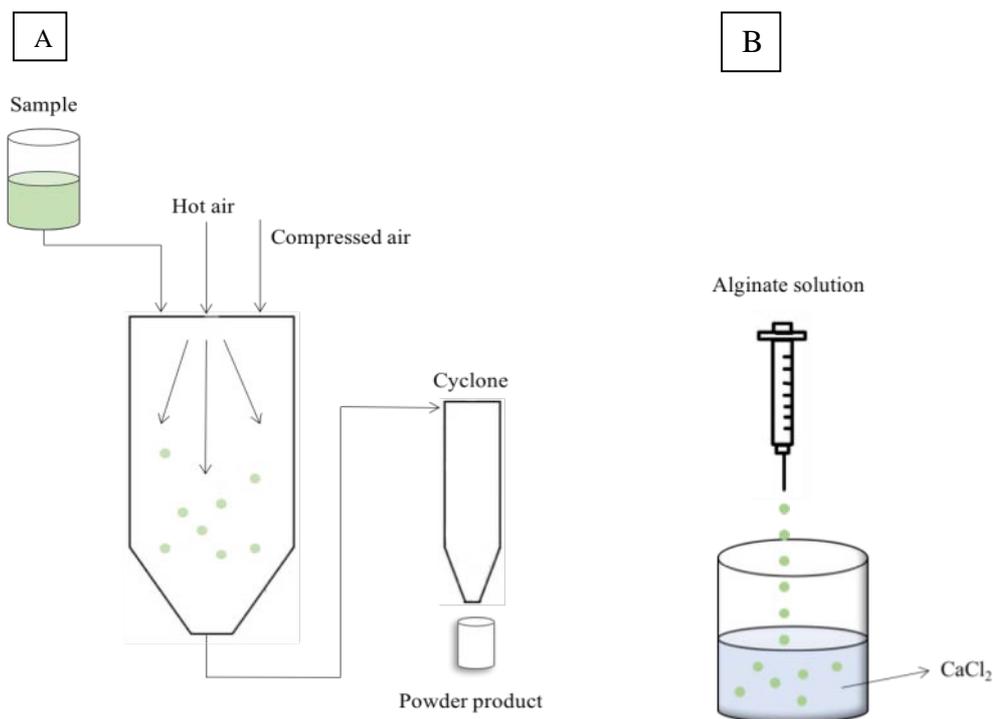
6.1. Spray drying

Spray drying is the technique most commonly used for the encapsulation of various food ingredients and consists in the atomization of a solution, suspension or emulsion, formed between the active ingredient and an encapsulating material (carrier), in a drying chamber where it comes into contact with a heater medium (usually hot air) and dry almost instantaneously (Figure 1.8a). Generally, this technique produces matrix type microparticles, or microspheres, that are hollow structures (SUBTIL et al. 2014), with size ranged from 10 to 120 μm .

It is a low cost technology, fast, continuous process, that produces free-flowing powders and despite of the fact that is necessary to use high temperatures in this process, the time of contact is too short (few seconds)

meaning the energy is used for evaporation only and not for rising the temperature of the powder particles, enabling the application of this technology to encapsulate heat sensitive materials with low thermal degradation. So, this is interesting for the encapsulation of bioactive compounds such as omega 3 and phytosterols, which are often heat-sensitive.

Figure 1. 8. Illustration of spray drying and ionic gelation methods, respectively.



Reference: Own source.

Among the carriers used in this technique, can be cited the polysaccharides maltodextrin (RUBILAR et al., 2012), gum Arabic (RUBILAR et al., 2012), chitosan (KLINKESORN; SOPHANODORA; CHINACHOTI; MCCLEMENTS, & DECKER, 2005) and some types of modified starches and proteins, as gelatin (CASTRO-MUÑOZ; BARRAGÁN-HUERTA; & YÁÑEZ-FERNÁNDEZ, 2015), whey protein (UMESHA; MANOHAR; INDIRAMMA;

AKSHITHA, & NAIDU, 2015) and soy protein (AUGUSTIN et al., 2014). These materials have relatively low costs and do have no taste, aroma or color which facilitates the application of the product obtained in different systems.

Umesha, Manohar, Indiramma, Akshitha, & Naidu (2015) encapsulated garden cress seed oil, which is rich in α -linolenic acid (ALA), using whey protein concentrate as wall material by spray drying method. The encapsulated oil was applied to biscuits and stored in different conditions. Moreover, in all the conditions, the oxidation rate of ALA was high when it was free compared to when it was encapsulated, indicating that this encapsulation method protected the fatty acid in biscuits. In other study, Chen, Mcgillivray, Wen, Zhong, & Quek, (2013) coencapsulated fish oil with phytosterol ester and limonene by spray drying and freeze drying using whey protein and soluble corn fiber as wall materials. According to these authors, the oxidation of the oil was similar for both methods during the accelerated storage, so either freeze-drying or spray drying provided protection to the core materials. Recently, Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas (2015) encapsulated omega-3 fatty acids by spray drying using lecithin and lecithin-chitosan fish oil emulsions with maltodextrin as wall material. These authors observed that microcapsules with lecithin-chitosan protected the material against lipid oxidation during storage at 30 and 60 °C, showing that this method is suitable to encapsulate omega-3 fatty acids.

Thus, according to the results reported in many studies, it can be said that the spray drying technique is suitable and feasible for encapsulating of phytosterols and oils containing omega-3 fatty acids, providing protection to these compounds of interest and possible application in food products.

6.2. Ionic gelation or ionotropic gelation by extrusion processes

Ionic gelation or ionotropic gelation by extrusion processes produce matrix or core-shell (reservoir) particles, depending on nozzle design (Figure 1.8b). It produces narrow size distribution of particles with sizes from 1 to 10000 μm . These processes are continuous and have high production capacity. The most common extrusion process uses the biopolymer alginate as wall material and is based on the gelification capacity of this polymer in the presence of ionic calcium, without the need of heating. Due to the mild process conditions needed, it is suitable for the encapsulation of omega 3 and phytosterols.

However, few studies addressing the encapsulation of omega-3 fatty acids by ionic gelation were found in the literature, which makes it an innovative idea for the encapsulation and, therefore, protection of these compounds. Mwangi, Ho, Ooi, Tey, & Chan (2016) studied a method for forming ionically cross-linked chitosan microcapsules from Pickering emulsion templates using the same idea of ionic gelation technique. These authors obtained microcapsules suited for the rapid controlled release of lipophilic compounds. In the case of phytosterols, stigmasterol was encapsulated by Fujiwara et al. (2013) by the external ionic gelation technique using sodium alginate, corn starch and chitosan as wall materials. According to these authors, the method was feasible due to the high yield and encapsulation efficiency values.

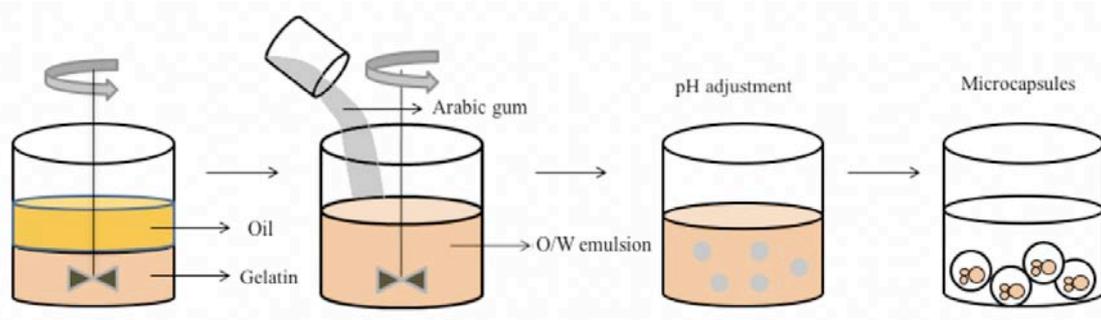
According to the above, it is observed that extrusion or ionic gelation processes, methods which guarantee the protection of the encapsulated compounds, are rarely explored in relation to the encapsulation of omega-3 fatty acids and phytosterols. It can be explained because the polymers used as wall material for ionic gelation process, as alginate and carrageen, have no surface

property and are hydrophilic compounds, so, for encapsulation of hydrophobic materials, it is necessary to include an emulsifier and to prepare an emulsion before the gelation process. So, it is an innovative idea, which gives a quite promising field for future research and applications of interest in the food industry.

6.3. Complex coacervation

Complex coacervation is an encapsulation technique based on the electrostatic interaction between two polymers with opposite charges (Figure 1.9). It is a relatively simple methodology and useful to elaborate water and heat resistant microcapsules, with size about 0.1 to 500 μm . One disadvantage of this method is that it typically yields particle dispersions that most of the time need a post-process, such as spray drying or freeze drying, in order to obtain a more stable material and of simple application. This method is feasible for the encapsulation of hydrophobic compounds, since that the first step is an oil in water (O/W) emulsion, so it is perfect for the encapsulation of omega-3 fatty acids and phytosterols.

Figure 1. 9. Complex coacervation technique procedure.



Reference: Own source.

There are several combinations used as wall material for complex coacervation encapsulation, as it is shown in Table 1.1; however, as already mentioned, the protein gelatin and the polysaccharide gum Arabic are the most widely used biopolymers due to the perfect combination of their charges and low cost (ALVIM & GROSSO, 2010). However, this perfect combination is not achieved with all kinds of combination of the oppositely charged polymers. A preliminary study analyzing the zeta potential of the biopolymer is important in order to obtain the best relationship between the charge and concentration of these materials.

Omega-3 fatty acids are some of the compounds more encapsulated by complex coacervation because of their importance and instability, as already mentioned in the course of this review and also because this technique is widely used to encapsulate lipophilic compounds. It is not possible to encapsulate hydrophilic materials by complex coacervation without any prior step due to the process occur in an aqueous medium, hindering the aggregation of the material by polymers. Furthermore, the multilayer coating obtained by complex coacervation provides better protection to omega-3 fatty acids and phytosterols against oxidation. This technique also produces more stable and heat resistant microcapsules, of high encapsulation efficiency and of rounded shape; this last characteristic helps in the future application due to the easy flow (BARROW; NOLAN; & JIN, 2007; TANEJA & SINGH, 2012; KAUSHIK; DOWLING; BARROW, & ADHIKARI, 2014). Eratte, Wang, Dowling, Barrow, & Adhikari (2014) studied the encapsulation of omega-3 rich tuna oil by complex coacervation using whey protein isolate and gum Arabic as wall materials and

obtained capsules stable against oxidation with potential application into food products.

Recently, the idea of coencapsulation was used by Eratte et al. (2015) when they encapsulated omega-3 fatty acids and probiotic bacteria by complex coacervation followed by spray and freeze drying. Protein whey isolate and gum Arabic were used as wall materials, tuna oil as a source of fatty acids and *Lactobacillus casei* 431 as probiotics. According to these authors, the materials were successfully encapsulated and the oxidative stability of the tuna oil was improved both in the presence and absence of bacterial cells, showing that these capsules may be useful in case of release of these two important ingredients in functional foods and nutraceutical applications. Comunian et al. (2016) studied the coencapsulation of echium seed oil, vegetable source of omega-3 fatty acids, with phenolic compounds sinapic acid or rutin by complex coacervation using gelatin and gum Arabic as wall materials, obtaining rounded microcapsules, with good values for encapsulation process yield for the oil and for the phenolic compounds (data presented in chapter 2).

Tamjidi, Nasirpour, & Shahedi (2014) studied the rheological characteristics of yogurt enriched with fish oil microcapsules obtained by complex coacervation using gelatin and acacia as wall materials. The yogurt with microcapsules presented higher apparent viscosity than the control and a non-Newtonian shear-thinning flow behavior; moreover, it was possible to obtain a yogurt which provides the fish oil health effect.

Table 1. 1. Some examples of polymers combinations used as wall materials by complex coacervation and their compounds encapsulated.

Polymers combination	Encapsulated material	Source
Gelatin and chitosan	Limonene oil	Prata & Grosso, 2015
Gelatin and OSA	--	Wu & McClements, 2015
Gelatin and gum Arabic	Xylitol	Santos, Bozza, Thomazini, & Favaro-Trindade, 2015
Lactoferrin and gum arabic	--	Gulão, Souza, Silva, Coimbra, & Garcia-Rojas, 2014
Whey protein and pectin	<i>Lactobacillus acidophilus</i>	Ribeiro et al., 2014
Gelatin and gum arabic	Vitamin C	Comunian et al., 2013
Gelatin and gum arabic	Aspartame	Rocha-Selmi, Theodoro, Thomazini, Bolini, & Favaro-Trindade, 2013 ^a
Gelatin and gum arabic	Sucralose	Rocha-Selmi, Theodoro, Thomazini, Bolini, & Favaro-Trindade, 2013b
Pea protein isolate and alginate	--	Klemmer, Waldner, Stone, Low, & Nickerson, 2012
Casein and pectin	Acetaminophen	Baracat et al., 2012
Soy protein isolate and pectin	--	Chen, Ding, & Duo, 2012
Hydrolyzed collagen and chitosan	Lavender oil	Ocak, 2012
Soybean protein isolate and pectin	propolis	Nori et al. 2011
Soy globulins and chitosan	--	Liu et al., 2011
Gelatin and gum Arabic	Peppermint oil	Dong et al., 2011
Gelatin and gum arabic	Paprika oleoresin	Alvim & Grosso, 2010
Gelatin and sodium carboxymethylcellulose	Polyisobutylene Succinimide	Dai et al., 2010
Soybean protein isolate and pectin	Casein hydrolysate	Mendanha et al., 2009
Casein and pectin	probiotics	Oliveira et al. 2007a, b
Gelatin and polyphosphate	Fish oil (source of Omega-3 fatty acids)	Barrow, Nolan, & Holub, 2009
Soy protein isolate and gum arabic	Omega-3 ethyl ester	Conto, Grosso, & Gonçalves, 2013
Whey protein isolate and gum arabic	Tuna oil (source of omega-3 fatty acids) and probiotic bacteria	Eratte et al., 2015
Gelatin and acacia	Eicosapentaenoic acid ethyl ester	Lamprecht, Schäfer, & Lehr, 2001

Differently of omega-3 fatty acids, no work on encapsulation of phytosterols by complex coacervation was found in the literature. Many studies on the encapsulation of this material are being done, however with other encapsulation methods, mainly with the formation of liposomes (not mentioned in this chapter) and spray drying. This shows that there are still many possibilities for encapsulation of phytosterols, since the study of their stability and controlled release in the organism are extremely important for obtaining quality products, and hence the maintenance of health. In this way, it can be said that the microencapsulation by complex coacervation technique, besides being the most widely used for encapsulation of omega-3 fatty acids, still needs to be explored in relation to encapsulation of phytosterols. As already mentioned, it is a technique that uses inexpensive materials, besides it ensures the oxidative protection of compounds of interest as observed in the studies cited.

Furthermore, along with the complex coacervation technique, the cross-linking process has been widely used for the purpose of forming more resistant capsules. This process consists in the addition of a compound (anionic or cationic) in the wall material in order to bind themselves and then, stabilize and modify the structure of the complex (CHEN; DING; & DUO, 2012). The traditional crosslinking agents used in this process are formaldehyde and glutaraldehyde, which are toxic and prohibited in food applications. For this reason, in order to replace them, the crosslinking function of specific natural compounds are of interest. Ferulic acid (CAO; FU & HE, 2007) and genipin (LI et al., 2015), are being studied for this reason; transglutaminase, an enzyme very applied in cheese, meat products and edible films, and tannic acid are the

compounds most used with this function (DONG et al., 2008; PENG et al., 2014). To briefly summarize, the major difference between the process of complex coacervation and crosslinking is that the first one is the encapsulation technique itself, while the second one consists of the addition of a compound in the coacervate microcapsule in order to stabilize its structure.

The crosslinking technique has been widely used for the encapsulation of omega-3 fatty acids by complex coacervation. Conto, Grosso, & Gonçalves (2013) evaluated the encapsulation of omega-3 fatty acids by complex coacervation using soy protein isolate and gum Arabic as wall materials, varying the polymer concentration, the core and crosslinker agent (transglutaminase). According to these authors, it was possible to obtain the microcapsules and, in addition to that, to obtain a final functional food, it would be necessary to add 0.4 g of microcapsules in 100 g of final product portion. In other study, Wang, Adhikari, & Barrow (2014) microencapsulated tuna oil by complex coacervation using the combination gelatin-sodium hexametaphosphate as wall materials and transglutaminase as crosslinking agent. These authors obtained high values of encapsulation efficiency and samples more stable than non-encapsulated oil.

6.4. Glass microfluidic devices

Microencapsulation by microfluidic devices is a promising technique for the production of monodisperse microcapsules with uniform mechanical properties, as well as multifunctional capsules with multiple compartments, overcoming the limitations of variability in production, which allows greater

control and optimization of encapsulation efficiency and release of the active agents (SUN et al, 2010; ZHAOL et al, 2011).

Due to the excellent flow control achieved in microfluidic devices, emulsion droplets are generated with extreme precision. Although it is only one drop at a time, this allows the formation of highly monodisperse emulsions. The degree of control provided by the microfluidic device is enhanced by the ability to generate double, triple or even higher order emulsions in a controlled manner in which the size and number of encapsulated droplets can be handled with great precision (ZHAOL et al., 2011). The glass capillaries are a class of device that allows the production of monodisperse emulsions. These devices consist of coaxial assemblies of a series of glass capillaries, which have inherent advantages such as their wetting capacity that can be easily and precisely controlled, are rigid and resistant to chemicals and their three-dimensional geometry allows controlled fabrication of multiple emulsions.

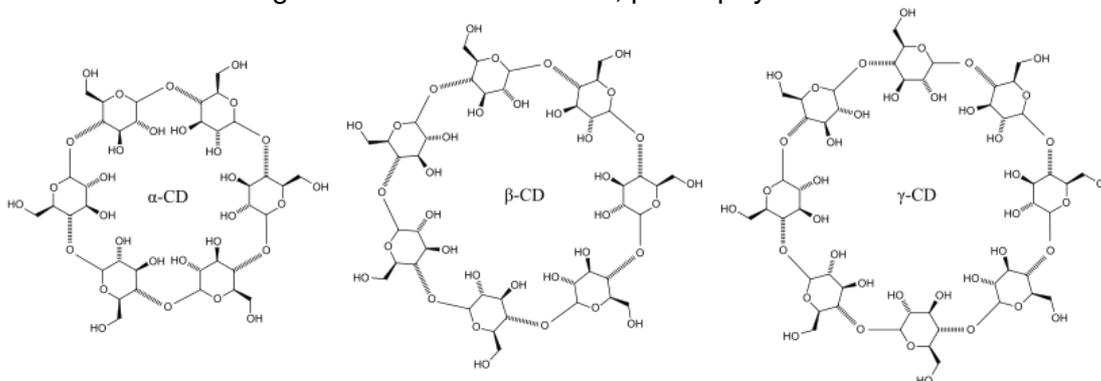
Simple emulsion devices are constructed from a round capillary that is inserted into a square glass capillary tube. In co-flow geometry, the flow of the two phases flow in the same direction, with the flow of the inner phase within the round capillary and the flow of the continuous phase between the square and the round capillary. Alternatively, in flow focusing geometry, the phases flow in opposite directions, generating emulsions in the collection tube. Simple emulsions can be produced using both types of geometry, whereas double emulsions are generally produced using the combination of the two geometries. The device used to produce double emulsions consists of two round capillaries arranged within a square capillary. The inner phase is pumped through inside of one of the round capillaries while the middle fluid is in the same direction

through the square capillary. The outer phase also flows through the square capillary, however in the opposite direction to the other two fluids. The double emulsion is formed when all three fluids are in the collection tube (ZHAOL et al., 2011).

6.5. Complexation

Complexation is a technique used for different purposes; however, it is considered one of the simplest methods of encapsulation which consists of only a mixture of cyclodextrin (α -cyclodextrin (α -CD), β -cyclodextrin (β -CD) and γ -cyclodextrin (γ -CD) (Figure 1.10 and Table 2) with the hydrophobic material that will be encapsulated. The β -cyclodextrin is the most used of the three mentioned because of the purification cost.

Figure 1. 10. Structures of α , β and γ -cyclodextrin.



Reference: NATIONAL INSTITUTE OF STANDARDS AND TECHNOLOGY, 2017.

Table 1. 2. Characterization of the three cyclodextrins.

Types of cyclodextrins	Glucopyranose units	Solubility in water	Rigidity of the structure	Size of the largest internal diameter (nm)
Alfa-cyclodextrin	6	medium	medium	0.57
Beta-cyclodextrin	7	low	strong	0.78
Gama-cyclodextrin	8	high	weak	0.95

Reference: Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara, 2009.

Cyclodextrins (CDs) are oligomers in the form of truncated cone; the outer region of the structure is composed by hydrogen atoms and glycosidic oxygen bridge, while the interior by free electrons. The CDs are formed by glycosidic bonds and produced from the processing of starch by bacteria, which help in the protection of flavors, vitamins and natural dyes (FATHI; MARTIN; & MCCLEMENTS, 2014). According to Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara (2009), the obtainment of the cyclodextrin is composed by four steps, including: (1) the culture of the microorganism which produces the cyclodextrin glucosyl transferase enzyme (CGT-ase); (2) the separation, concentration and purification of the enzyme; (3) enzymatic conversion and (4) separation, purification and crystallization of the cyclodextrins.

The CDs have been very used for encapsulation, because its shape allows to form complex, involving hydrophobic compounds inside. In this case, there is no formation of round microcapsules as in the case of complex coacervation technique; however, it is also a method that allows the protection and controlled release of lipophilic and sensitive compounds when exposed to various environmental conditions. According to Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, & Simal-Gandara (2009), the complexes formed by CDs are also known as inclusion complexes, since CDs can involve the encapsulated compound partially or completely.

Omega-3 fatty acids and phytosterols are materials often encapsulated by this technique. Yoshii, Furuta, Yasunishi, Linko Y-Y., & Link P. (1996) studied the complexation of omega-3 polyunsaturated fatty acids eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) using CD. The

obtained samples were stable to oxidation and most EPA remained unoxidized. Recently, Vestland, Jacobsen, Sande, Myrset, & Klaveness (2015) produced compactible powders of omega-3 and β -cyclodextrin. These authors analyzed the powders produced by vacuum drying, freeze drying or spray granulation of aqueous mixtures of omega-3 oil and β -cyclodextrin, confirming the true complexation.

The encapsulation of phytosterols with the use of CDs by the complexation technique was studied by Meng, Pan, & Liu (2012) when they evaluated the preparation and properties of phytosterols in their inclusion in complexes of hydroxypropyl- β -cyclodextrin. The inclusion efficiency values (it is also known as encapsulation efficiency for other authors) were in the range from 92 to 98% and, according to these authors, the formed complexes presented high solubility in water, which facilitates the application of the final product.

Besides the CDs, spring dextrans are also used for the complexation technique for encapsulation of lipophilic compounds. Xu et al. (2013) studied the stability and controlled release of polyunsaturated fatty acids ω 3/ ω 6 encapsulated by spring dextrin. According to these authors, the complex improved the stability of α -linolenic fatty acid (ALA) and linoleic (LA) and enabled the controlled release of 21.7 and 18.5% of ALA and LA of its complex when the gastrointestinal tract was simulated.

No application of complex of omega-3 fatty acids or phytosterols with CDs into food products was found in the literature. Thus, according to the above, it can be said that complexation technique, besides being a simple method of encapsulation, has potential in protecting omega-3 fatty acids and

phytosterols and is also a technique which can provide a promising field for the future since the application of this material has been little explored.

7. MICROENCAPSULATION OF PHENOLIC COMPOUNDS

Unlike the omega-3 fatty acids, few works are found in the literature that address the encapsulation of sinapic acid, rutin and quercetin. In the case of sinapic acid, no work was found.

Regarding the rutin, Cui et al. (2014) evaluated the effects of the incorporation of it on the physical and oxidative stability of emulsions and verified that it can be used as a stabilizer of emulsions with proteins due to droplet size reduction. Akhtar et al. (2014) encapsulated rutin in double emulsion using a spinning disc reactor. Rutin was successfully encapsulated within the inner aqueous phase of the W/O/W emulsion, resulting in encapsulation efficiency around 80%. Kerdudo et al. (2014) studied the encapsulation of rutin and naringenin in multilamellar vesicles in order to optimize antioxidant activity, obtaining encapsulation efficiency bigger than 60% and retention of rutin of 16% after 30 days of storage.

Park et al. (2013) studied the encapsulation of quercetin and rutin in liposomes and the effect of the release on the liposome-hydrogel system. It has been found that the liposome-hydrogel system can act as an excellent release system for the encapsulated material, enhancing the transdermal permeation of the phenolic compounds.

8. CONCLUSIONS

The potential of the microencapsulation technique has greatly increased, since this process has been one of the best alternatives to improve the stability of phytosterols and omega-3 fatty acids and also control the release of these compounds to the maintenance of health. These materials have often been encapsulated by techniques which use biopolymers such as complex coacervation, complexation, spray drying, ionic gelation and microfluidic devices, as they allow the formation of microcapsules and/or microparticles and/or complex of low cost and easy handling. Moreover, the encapsulated phytosterols and/or omega-3 fatty acids are applied to food products in order to provide better nutritional attributes with functional appeal, and thereby help in preventing of cardiovascular diseases.

Encapsulation of omega-3 fatty acids has been more explored than phytosterols. Many studies on the encapsulation of phytosterols are found, however there is no study using complex coacervation and only one using complexation in the literature, showing an area that needs to be explored in the future. Moreover, microcapsules and/or complex composed by biopolymers have wide application, not just in the food field, but also in the pharmaceutical and cosmetics industries.

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**Chapter 2. PROTECTION OF ECHIUM OIL BY MICROENCAPSULATION
WITH PHENOLIC COMPOUNDS**

(PAPER PUBLISHED IN FOOD RESEARCH INTERNATIONAL –
ATTACHMENT B)

CHAPTER 2. PROTECTION OF ECHIUM OIL BY MICROENCAPSULATION WITH PHENOLIC COMPOUNDS

ABSTRACT

The consumption of omega-3 enables the reduction of cardiovascular disease risk; however, they are unstable. The aim of this work was to encapsulate echium oil (*Echium plantagineum* L.), a rich source of omega-3 fatty acids, with phenolic compounds (sinapic acid and rutin) by double emulsion followed by complex coacervation or by complex coacervation with sinapic acid in the capsule wall. Analyzes of morphology, particle size, circularity, water activity, moisture, Fourier transform infrared spectroscopy, thermogravimetry, process yield, accelerated oxidation and identification and quantification of fatty acids present in the encapsulated oil were performed. Samples presented values of encapsulation process yield of phenolics and oil in the range of 39-80% and 73-99%, respectively. Moreover, all samples protected the oil against oxidation, obtaining induction time (accelerated oxidation) of 5 hours for pure oil and values in the range from 10 to 18 hours for samples. Thus, better protection to the oil was possible with sinapic acid applied in the capsule wall, what enhances its protection against lipid oxidation.

Keywords: omega-3 fatty acids, sinapic acid, rutin, complex coacervation, rancimat, stearidonic acid

1. INTRODUCTION

There are several sources of omega-3 fatty acids (ω -3 FA) that can be added to food products. These sources can be from animal origin (marine oils extracted from cold water fish) or vegetable origin, such as echium oil (WHELAN, 2009). *Echium plantagineum* L. has a Mediterranean and Macaronesian origin (BERTI, JOHNSON, DASH, FISCHER, & WILCKENS, 2007) and contains 33% of α -linolenic acid and from 9 to 16% stearidonic acid, an intermediate in the biosynthesis of EPA and DHA, (CLOUGH, 1993; PAYNE, LAD, FOSTER, KHOLA, & GRAY, 2014), which are important omega-3 FA. Besides, the echium oil is being used in replacement of fish derived oils, since it presents unique ratio of omega-3 to omega-6 FA of 1.8:1, suitable for nutraceutical applications (BERTI et al., 2007).

Omega-3 FA have been assessed for reducing the risk of cardiovascular disease (POOLE et al., 2013); however, they are very susceptible to oxidation, which hinders their application in food. Lipid oxidation could be delayed by different factors such as packaging and modified atmosphere. Moreover, other two strategies that could minimize these limitations are: (1) adding a compound with antioxidant function and (2) microencapsulation.

Phenolic compounds, such as sinapic acid and rutin, have been of great interest in the food industry, as they act as antioxidant agents (LEE et al., 2013). Sinapic acid, the main phenolic acid of canola, has been studied regarding its neuroprotective effect against Alzheimer's disease (LEE et al., 2012), cardiac hypertrophy and dyslipidemia (ROY & PRINCE, 2013). In relation to rutin, phenolic compound found in plants and food sources such as onions, grape, bean, apple and tomato, it has several pharmacological

activities, including anti-allergenic, anti-inflammatory and vasoactive properties (KIM, KWON, & JANG, 2011; JANTRAWUT, ASSIFAOU, & CHAMBIN, 2013). Espinosa, Inchingolo, Alencar, Rodriguez-Estrada, & Castro (2015) studied the effect of eleven compounds on oxidative stability of emulsions prepared with echium oil and it was observed that sinapic acid and rutin were the most efficient to delay lipid oxidation, so they were distinguished from the compounds analyzed, which make them potential alternative to be applied as antioxidant in products, specially with echium oil.

The encapsulation is a process in which retains a bioactive (solid, liquid or gas) inside another (wall material) in order to protect the material against adverse environmental conditions, thereby increasing the shelf-life and promoting the controlled release of the active compound in the microcapsule (SHAHIDI & HAN, 1993; NEDOVIC, KALUSEVIC, MANOJLOVIC, LEVIC, & BUGARSKI, 2011).

In this context, the aim of this work was to protect echium oil. In order to achieve this aim, the echium oil was encapsulated in the presence of the phenolic compounds rutin and sinapic acid.

2. MATERIAL & METHODS

2.1. Materials

As core were used echium oil (NEWmega™ Echium Oil, Ref.15200, from De Wit Specialty Oils (De Waal, Tescel, The Netherlands), sinapic acid and rutin from Sigma Chemical Co. (St. Louis, MO, USA). As wall material were used gelatin and Arabic gum from Gelita South America (Mococa/SP, Brazil)

and Nexira (São Paulo/SP, Brazil), respectively. Polyglycerol ricinoleic acid (PGPR 90) (Danisco, Denmark) was used as an emulsifier.

2.2. Methods

2.2.1. Microencapsulation

For the production of microcapsules, a primary water-in-oil emulsion (W/O) consisting of sinapic acid or rutin solution in the internal aqueous phase (0.50 mg of phenolic compound/ mL of water) and echium seed oil in the external oil phase was prepared at a ratio of 2:1 (v:v oil phase: aqueous phase). Polyglycerol ricinoleic acid (PGPR 90) was used as the emulsifier in the concentration of 0.5% (w/w) and added in the oil phase.

The primary emulsions (W/O) were emulsified in the gelatin solution (7.5%-w/w) with pH 6.0 to obtain a double emulsion water-in-oil-in-water (W/O/W) with different concentrations of phenolic compounds as showed in Table 2.1. The homogenization steps were performed at 12,000 rpm for 4 min (primary emulsion – W/O) and 10,000 rpm for 3 min (double emulsion – W/O/W) with an Ultra-Turrax apparatus (Ika, Germany). The Arabic gum solution (7.5% - w/w) was added to these emulsions by magnetic stirring at 40 °C for 3 min.

Besides the application of sinapic acid in the internal phase of the primary emulsion, a sample was also obtained where it was added directly to the primary emulsion, before the addition of Arabic gum solution (SIN-GEL). In this case, the double emulsion was not prepared, but only the primary emulsion composed of echium oil and gelatin solution. Moreover, the control sample composed of only echium oil and the wall materials (gelatin and Arabic gum) was also prepared.

Samples with different concentrations of the primary emulsion (50, 75 and 100% - SIN50, SIN75 and SIN100 and RUT50, RUT75 and RUT100, respectively) in relation to the total mass of the polymer (7.5% - w/w) were prepared, in addition to samples in which sinapic acid was used in the capsule wall and the oil was encapsulated without a phenolic compound (SIN-GEL and Control, respectively), resulting in eight samples (Table 2.1). The ratio of polymers (gelatin and Arabic gum) was fixed at 1:1.

Table 2. 1. Concentrations and proportions used for each sample in the process of encapsulation.

Sample	Concentration of phenolic ($\mu\text{g/g}$ of capsule)	Proportion of phenolic in relation to the oil (ppm)	Proportion of core in relation to total amount of polymer (%)	Concentration of oil (g/g of capsule)
SIN50	65.1	200	50	0.24
SIN75	87.0	200	75	0.32
SIN100	104.8	200	100	0.39
SIN-GEL	88.9	200	50	0.33
RUT50	65.1	100	50	0.24
RUT75	87.0	100	75	0.32
RUT100	104.8	100	100	0.39
CONTROL	--	--	50	0.33

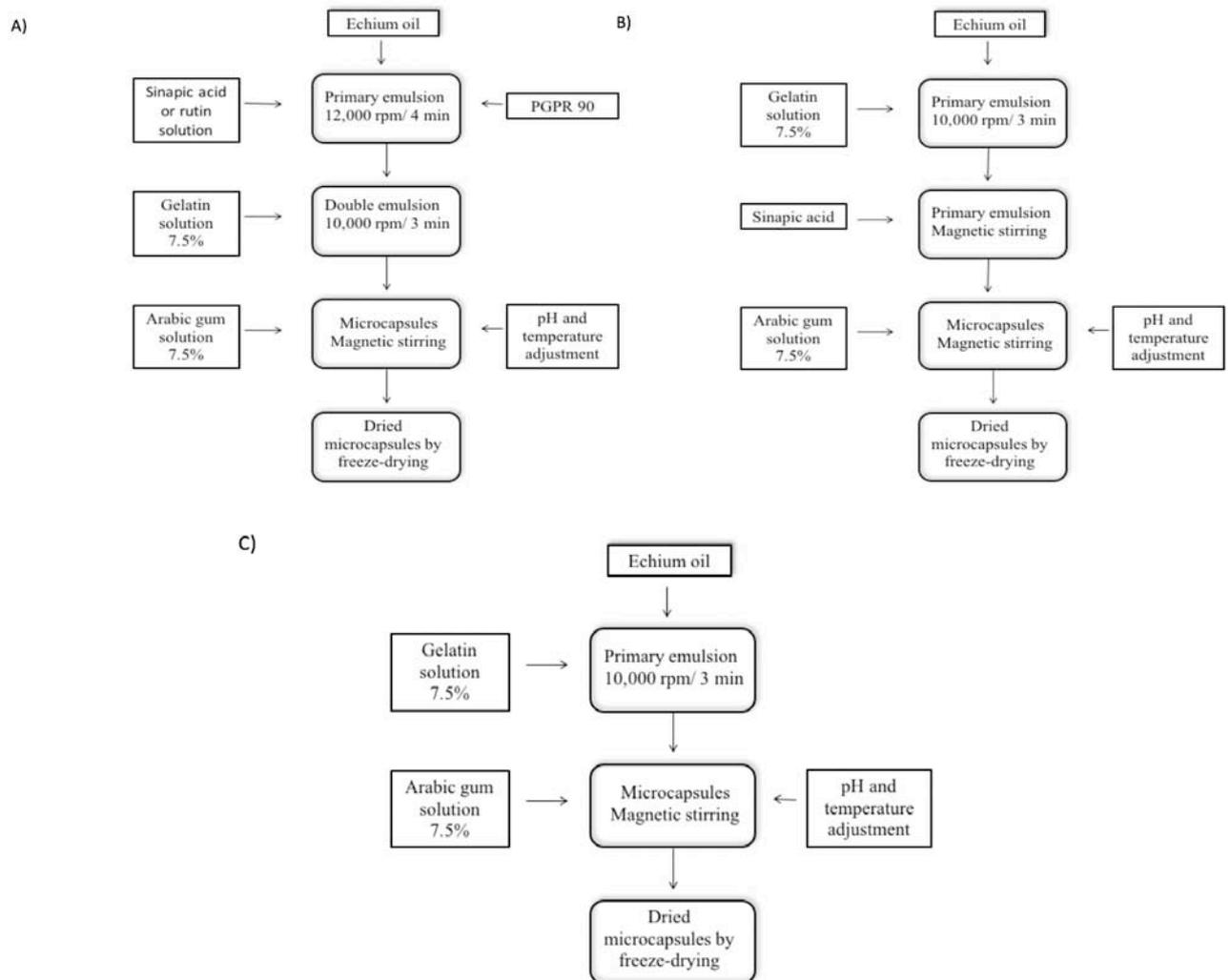
Reference: Own source.

- SIN50 and RUT50: Sample with a 1:1:0.5 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN75 and RUT75: Sample with a 1:1:0.75 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN100 and RUT100: Sample with a 1:1:1 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN-GEL: Sample with a 1:1:0.5 proportion of gelatin, Arabic gum and echium oil with sinapic acid as a cross-linker
- CONTROL: Sample with a proportion of 1:1:0.5 of gelatin, Arabic gum and echium oil.

To promote complex coacervation, the pH was adjusted to 4.0 at 40 °C under magnetic stirring and the temperature was gradually lowered to 10 °C in an ice bath. The material was stored for 24 h at 7 °C to promote decantation. The coacervates were frozen (-18 °C) and freeze-dried (Terroni/ São Carlos - SP, Brazil) for 24 h at a pressure of 1 to 0.1 kPa pressure, at a temperature of -

20 °C and a final temperature of 30 °C. All the procedure of capsules fabrication is presented in a schematic representation in Figure 2.1.

Figure 2. 1. Schematic representations of the procedure of the capsules fabrication: a) Samples SIN50, SIN75, SIN100, RUT50, RUT75 and RUT100; b) Sample SIN-GEL; c) Sample Control.



Reference: Own source.

2.2.2. Characterization of coacervates samples

2.2.2.1. Yield of encapsulation process of phenolic compounds

The yield was determined by the mass difference of the phenolic compound contained in the microcapsules and the total quantity used in the encapsulation process. For the rupture of the capsule, 0.2 g of sample was diluted in 5 mL of methanol, 5 mL of ZnSO₄ solution (5%-w/w) and 0.8 g of KCl, subjected to ultrasound for 10 minutes and centrifuged at 4000 rpm at 25 °C for 10 min. The supernatant was filtered and analyzed by High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, Japan). A C18 column was used with water:methanol as the mobile phase at a ratio of 45:55 (water at pH 3.5), flow of 1 mL/min. The detection wavelength was 325 and 356 nm and the injection volume was 10 and 20 µL for sinapic acid and rutin, respectively. The yield of the encapsulation process was calculated from Equation 1.

$$Yield = \frac{(Phenolic\ added - phenolic\ in\ the\ capsule) \times 100}{Phenolic\ added} \quad (1)$$

2.2.2.2. Yield of encapsulation process of echium oil

The yield was determined by the difference in the mass of the oil contained in the microcapsules and the total quantity used in the encapsulation process. For the rupture of the capsule, the same method mentioned in section 2.2.2.1 was used. After the rupture of the capsule, the procedure continued with the Bligh Dyer method with some modifications for oil extraction (BLIGH & DYER, 1959). In the broken capsules, it was added 5 mL of chloroform, 10 mL

of methanol and 4 mL of distilled water. The mixture was lightly stirred for 1 minute and then 5 mL of chloroform and 5 mL of sodium sulfate solution (1.5% w/w) were added to it, stirring gently in the vortex for 1 minute more. The samples were allowed to stand for 30 minutes and a known volume of the chloroform phase was withdrawn and kept in oven at 60 °C to evaporate the solvent. The quantification of the oil was made from the remaining mass after evaporation of the chloroform. Thus, knowing the mass of oil added to the microcapsule and the volume of evaporated chloroform, it was possible to obtain the yield of encapsulated oil.

2.2.2.3. Morphological characterization of the microcapsules by optical microscopy, scanning electron microscopy (SEM) and confocal microscopy

Wet microcapsules were analysed by optical microscopy using Bio3 equipment (Bel Photonics, Italy) and by confocal microscopy, using an Axio Observer Z.1 and an LSM 780-NLO Zeiss microscope, with a 40x objective. A laser with a wavelength of 561 nm was used for excitation along with a 569-691 nm emission filter for the fluorophore Nile Red. The pinhole was set to 1 Airy unit in each channel and the image size was 1024×1024, with an optical zoom of 1.7x. Lyophilized microcapsules were analysed by SEM using a TM 3000 table-top microscope (Hitachi, Tokyo, Japan), and TM 3000 software.

2.2.2.4. Particle size analysis

To obtain the particle size, 200 microcapsules of each sample were individually measured using ImageJ software. A BIO3 microscope (Bel Photonics, Italy) was used to obtain the images.

2.2.2.5. Circularity

To determine the circularity the ratios between the smallest and the largest diameter of the capsules were used. These measurements were obtained from 100 capsules for each sample using ImageJ software. A BIO3 microscope (Bel Photonics, Italy) was used to obtain the images.

2.2.2.6. Water activity and Moisture

Water activity and moisture content were determined using AQUALAB equipment (Decagon Devices, Pullman, WA) and moisture analyser (Ohaus model MB 35, Ohio, USA).

2.2.2.7. Fourier transform infrared spectroscopy

The spectra of the ingredients and the microcapsules were obtained in the region of 4000 to 600 cm^{-1} using a Perkin Elmer FTIR spectrometer (Massachusetts, USA) with Spectrum One software version 5.3.1.

2.2.2.8. Thermogravimetric analysis

The thermogravimetric curves (TG) were obtained using Shimadzu TGA-50 equipment, which had been calibrated at a heating rate of 10 $^{\circ}\text{C min}^{-1}$ with calcium oxalate monohydrate. Sample masses of 8 mg were used, which were placed in a platinum sample holder. The furnace atmosphere was saturated with super-pure nitrogen at a flow rate of 50 mL/min. The analysis temperature range was 25 to 800 $^{\circ}\text{C}$.

2.2.2.9. Identification and quantification of fatty acids (FA) in encapsulated oil

FA concentrations were monitored by gas chromatography (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara, United States), with a J&W DB-23 column (60 m x 250 μ m x 0.15 μ m; Agilent 122-2361). The analyses were performed in triplicate. After the rupture of the capsules, 150 mg of oil extracted from the microcapsules was weighed; 5 mL of hexane was added to this sample and mixed on a vortexer. For the esterification of FA, 50 μ L of this diluted oil (corresponding to 1.5 mg of oil) was added to 15 mL of a standard solution (C23:0) in dichloromethane and 1 mL of 0.5 M NaOH in methanol was added. This mixture was mixed on a vortexer for 15 seconds and placed in a water bath at 100 °C for 5 minutes. After cooling, 2 mL of BF₃-methanol 14% was added, then the sample was shaken and placed in a water bath at 100 °C for 5 minutes. One milliliter of isooctane was added, shaken vigorously for 30 seconds and added of saturated NaCl solution. Then, the sample was homogenized. After phase separation, the isooctane layer was transferred to a 1.5 mL microtube and dried completely with nitrogen gas. The residue was resuspended in 500 μ L of isooctane and 200 μ L of this mixture was transferred to a vial with an insert for chromatographic analysis.

2.2.2.10. Accelerated oxidation

Accelerated oxidation tests for the pure and for echium oil encapsulated were performed with the aid of Rancimat equipment (model 873, Metrohm, Switzerland). Samples were subjected to heating under a purified air flow rate of 20 L /h at 90° C. The induction time of sample was used as oxidative stability

index. Besides, it was used 5 g of pure oil and 1.5 g of microcapsule, and this analysis was performed in duplicate.

2.2.2.11. Statistical analysis

All experiments were done three times, except Rancimat, which was done twice. Data were statistically analyzed using SAS statistical software (Statistic Analysis System), version 9.2, by ANOVA and Tukey's test at 5%.

3. RESULTS AND DISCUSSION

3.1 Yield of encapsulation process of phenolics and oil

The values obtained for the process yield are shown in Table 2.2. In relation to the yield of the process for rutin and sinapic acid, the obtained values were within the range from 39 to 81%. There was a significant difference for the samples RUT75 and RUT100 (75 and 100% core in relation to the total amount of polymer). The yield values of these samples were considered good and were approximately double compared to those obtained for samples with sinapic acid (SIN75 and SIN100) with the same concentration of core. The sample in which sinapic acid was applied in the capsule wall (SIN-GEL) showed a yield of 50%. The expected would be that the higher the concentration of phenolic compound in the microcapsule, the greater its effective action as antioxidant, in other words, RUT75 and RUT100 samples would present better oxidative protection to the oil than the other samples. However, that is not what happened, as shown in the accelerated oxidation test (item 3.7). This can be explained due to the polarity of the sinapic acid – a compound more polar than rutin. According to

Shahidi & Zhong (2011), polar antioxidants are more effective in less polar media, what happened to sinapic acid in this oil system.

In the case for the yield of the process for oil, values between 73 and 99% were obtained, with no significant difference among the samples. So, different concentrations of the phenolics and oil, and different position of sinapic acid, did not affect the mass of oil that was encapsulated. Oil losses are related to the amounts that were in the beaker or in the Ultra-Turrax during the process.

Rocha-Selmi, Theodoro, Thomazini, Bolini, & Favaro-Trindade, (2013a) and Rocha-Selmi, Bozza, Thomazini, Bolini, & Favaro-Trindade, (2013b) studied the encapsulation of aspartame and sucralose, hydrophilic compounds, by double emulsion followed by complex coacervation using gelatin and Arabic gum as wall materials; they obtained yields ranging from 43 to 89% and from 45 to 71%, respectively; these values corroborate those obtained for sinapic acid and rutin.

3.2 Morphological characterization of the microcapsules by optical microscopy, SEM and confocal microscopy

The micrographs are shown in Figures 2.2 and 2.3. For optical microscopy, there were no morphological differences among all samples, showing round and multinucleated microcapsules. It was also noted that the use of sinapic acid in the microcapsule wall did not affect the morphology, showing that it can be incorporated in different positions without damaging the structure.

In the same way, Santos et al. (2015), Rocha-Selmi et al. (2013a) and Rocha-Selmi et al. (2013b) obtained round, multinucleated microcapsules when xylitol, sucralose and aspartame were encapsulated, respectively, using the

double emulsion technique followed by complex coacervation. Regarding to the SEM results (Figure 2.3), it was observed that there was increased surface roughness with an increasing concentration of the core in relation to the total mass of the polymer; in other words, samples SIN100 and RUT100 were rougher than SIN75 and RUT75. However, samples SIN50, RUT50 and control showed smooth surfaces. Regarding the sample SIN-GEL, it was observed that the presence of sinapic acid in the capsule wall made the surface rougher than when it was located within the microcapsule. However, this feature did not affect the functionality of the material. The micrographs showed that the microcapsules were linked by "bridges" due to the freeze-drying method. This structure complicates applications in food products where good flow is necessary; however, it becomes a positive factor regarding wettability due to the presence of canals in these agglomerates, through which water infiltrates. Besides, the dehydration process facilitates the microcapsules preservation, its packaging and transport; and the microcapsules circularity can be recovered putting or applying the microcapsules in wet products.

The micrographs obtained from confocal microscopy are also presented in Figure 2.2. The red dye Nile Red was applied to the oil in order to verify the location of the oil within the microcapsule. In these micrographs, it is possible to observe that the sample in which the double emulsion was used as one of the steps presented a light red coloration due to the lower amount of oil compared to samples in which the core was only oil.

Table 2. 2. Values of water activity, moisture, particle size, circularity and accelerated oxidation index.

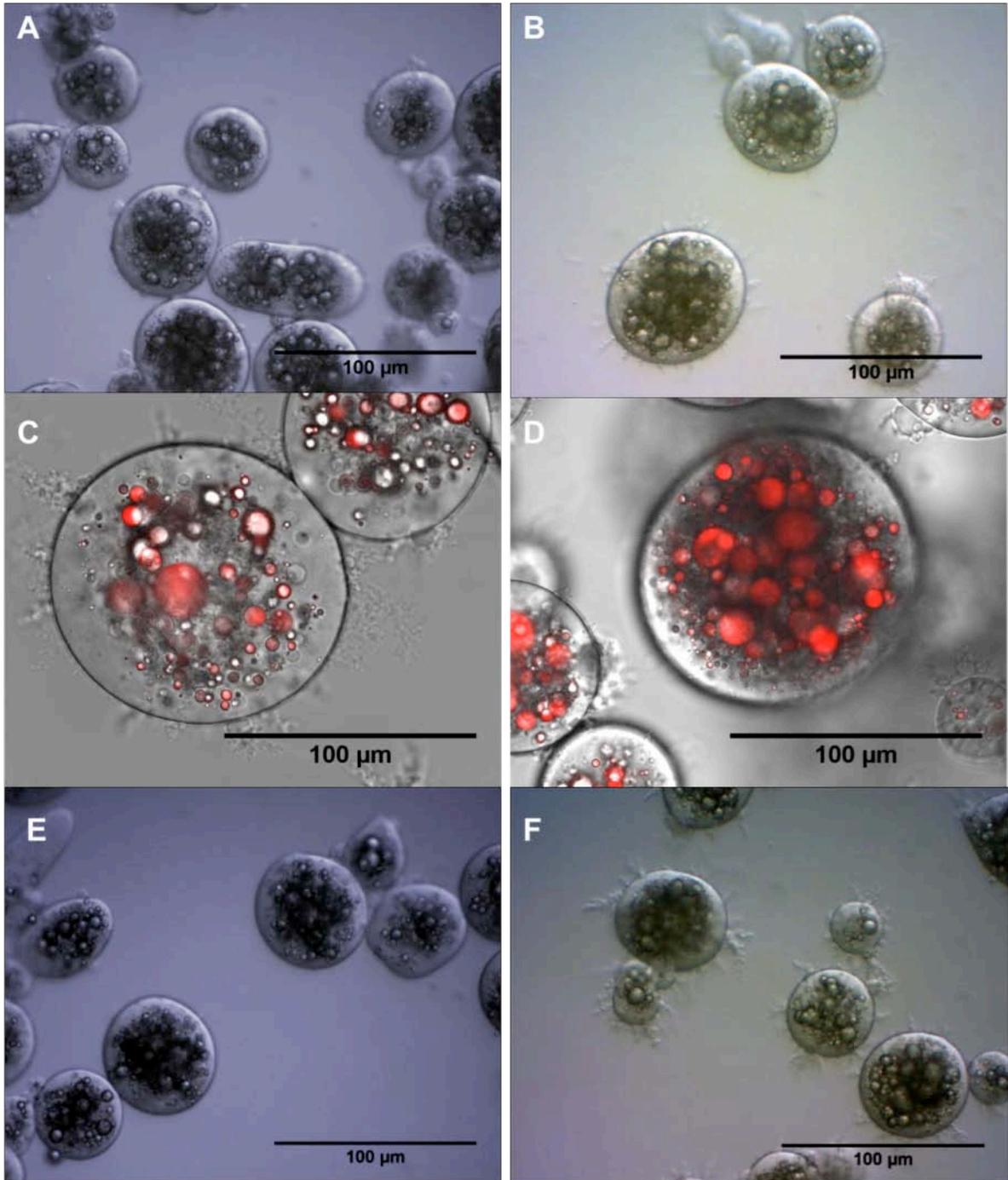
Samples	Yield for phenolics (%)	Yield for oil (%)	Water activity	Moisture (%)	Particle size (um)	Circularity	Accelerated oxidation index (hours)
SIN50	39.48±5.99 ^b	99.80±0.34 ^a	0.51 ± 0.08 ^{ab}	9.12 ± 1.30 ^a	45.14 ± 13.94 ^{bc}	0.89± 0.12 ^{ab}	10.18 ± 0.00 ^b
SIN75	43.37±10.18 ^b	95.69±7.46 ^a	0.49 ± 0.03 ^{ab}	9.43 ± 0.36 ^a	42.39 ± 12.96 ^c	0.90 ± 0.11 ^{ab}	9.97 ± 0.00 ^b
SIN100	46.35±4.21 ^b	94.37±6.37 ^a	0.53 ± 0.02 ^a	8.89 ± 0.62 ^{ab}	45.01 ± 14.71 ^{bc}	0.87 ± 0.12 ^{ab}	10.06 ± 0.28 ^b
SIN-GEL	50.55±5.98 ^b	87.08±12.95 ^a	0.32 ± 0.07 ^c	7.43 ± 0.23 ^{ab}	42.22 ± 13.13 ^c	0.90 ± 0.14 ^{ab}	18.77 ± 2.42 ^a
RUT50	58.16±7.88 ^b	73.16±4.89 ^a	0.43 ± 0.13 ^{abc}	8.98 ± 1.92 ^{ab}	47.31 ± 14.86 ^{ab}	0.92 ± 0.08 ^a	10.04 ± 0.05 ^b
RUT75	78.70±18.03 ^a	91.31±10.78 ^a	0.49 ± 0.17 ^{abc}	8.71 ± 1.85 ^{ab}	51.75 ± 17.97 ^a	0.90 ± 0.12 ^{ab}	10.26 ± 0.19 ^b
RUT100	80.97±20.17 ^a	85.61±15.01 ^a	0.43 ± 0.07 ^{a^{bc}}	8.14 ± 1.07 ^{ab}	42.55 ± 18.11 ^c	0.87 ± 0.12 ^{ab}	10.06 ± 0.05 ^b
CONTROL	--	79.49±18.22 ^a	0.36 ± 0.04 ^{bc}	6.93 ± 0.52 ^b	44.86 ± 13.75 ^{bc}	0.86 ± 0.12 ^b	10.05 ± 0.05 ^b
PURE OIL	--	--	--	--	--	--	5.15 ± 0.04 ^c

Reference: Own source.

* Equal letters in the same column do not differ significantly by the Tukey test at 5% probability.

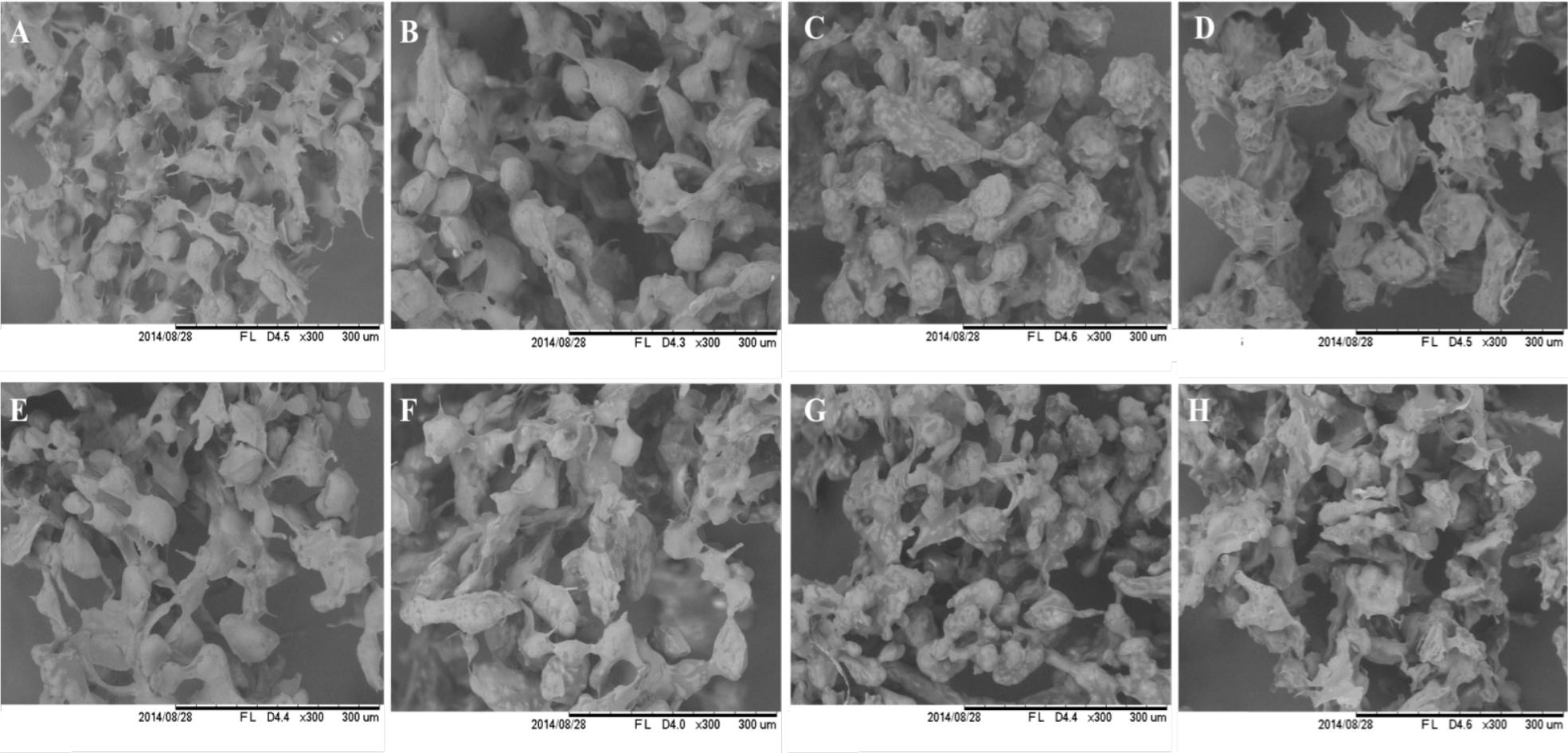
- SIN50 and RUT50: Sample with a 1:1:0.5 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN75 and RUT75: Sample with a 1:1:0.75 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN100 and RUT100: Sample with a 1:1:1 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN-GEL: Sample with a 1:1:0.5 proportion of gelatin, Arabic gum and echium oil with sinapic acid as a cross-linker
- CONTROL: Sample with a proportion of 1:1:0.5 of gelatin, Arabic gum and echium oil.

Figure 2. 2. Micrographs obtained by optical microscopy for the samples (a) SIN50 and (b) SIN-GEL; confocal microscopy for the samples (c) SIN50 and (d) SIN-GEL; optical microscopy for the freeze-dried and rehydrated samples (e) SIN50 and (f) SIN-GEL.



Reference: Own source.

Figure 2. 3. Micrographs obtained by scanning electron microscopy for the samples (a) SIN50; (b) SIN75; (c) SIN100; (d) SIN-GEL; (e) RUT50; (f) RUT75; (g) RUT100 and (h) Control.



Reference: Own source.

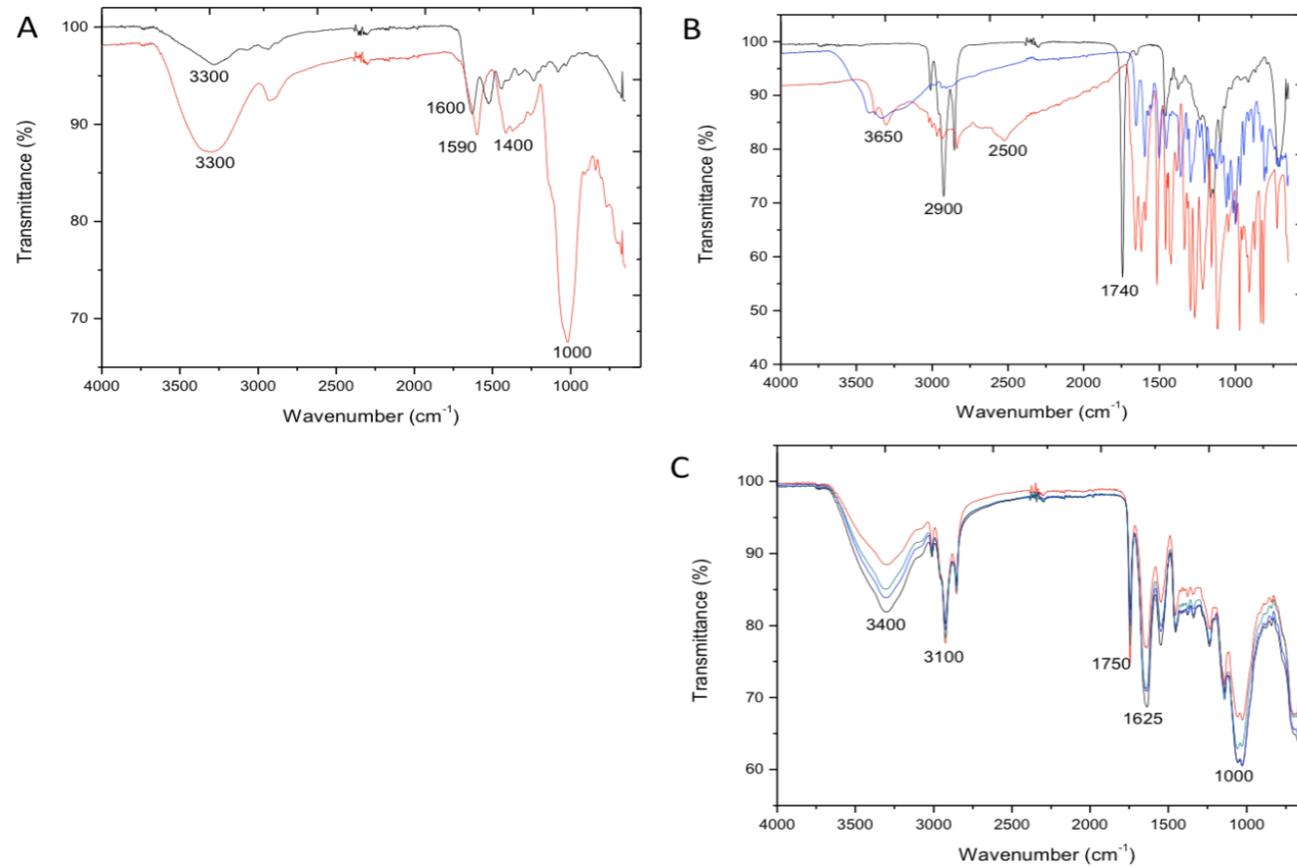
3.3. Fourier transform infrared spectroscopy

For analysis by FTIR, spectra were obtained for the wall materials, the cores and for the samples (Figure 2.4).

In relation to the wall material, according to Santos et al. (2015), the peaks around 3300 cm^{-1} present in the spectrum of gelatin represent amino groups (when the protein is positively charged), while peaks about 2900 cm^{-1} present in the spectrum of Arabic gum represent carboxyl groups (negatively charged); these peaks were also present in the samples.

The spectrum of sinapic acid showed intense vibrations between 3650 and 2500 cm^{-1} and between 1750 and 600 cm^{-1} . A similar spectrum was obtained by Tan & Shahidi (2013). According to these authors, peaks around 1700 cm^{-1} represent carbonyl groups (C=O) and between 3300 and 3500 cm^{-1} represent hydroxyl groups in the phenolic portion. These peaks present in the spectrum of sinapic acid were also present in the spectra of the samples, showing that the structure of this compound remained intact. For rutin, very intense vibrations between 1700 and 1000 cm^{-1} were obtained. According to Yan, Li, Zhao, & Yi, (2011), peaks around 1450 cm^{-1} represent CH_2 or CH_3 bonds. It is observed that all samples with rutin showed similar behavior, differing only in the intensity of the peaks, since the difference is related to the concentration of the compounds.

Figure 2. 4. Spectra obtained by FTIR for (a) gelatin (black) and Arabic gum (red curve); (b) echium oil (black), rutin (blue) and SA (red curve); (c) dried samples SIN50 (black curve), RUT50 (red curve), SIN-GEL (blue curve) and Control (green curve).



Reference: Own source.

3.4. Analysis of particle size, circularity, water activity and moisture

The values obtained for water activity, moisture, average particle size and circularity are shown in Table 2.2. The analyzes of water activity and moisture were performed on freeze-dried capsules and particle size and circularity were performed in the wet ones. The values of water activity were between 0.36 and 0.51, which can be considered low and, since these values are below than 0.6, they ensure microbiological stability (FENNEMA; DAMODARAN & PARKIN 2010). Regarding the moisture, they were within the range from 6.9 to 9.4% and are also considered suitable for materials dried by freeze-drying. These values facilitate the storage, handling and application of the powders.

Regarding the average particle size values for wet microcapsules, the mean diameter ranged from 42 to 51 μm , showing significant differences among the samples, although it was not verified a clear trend. According to Fávares-Trindade et al. (2008), capsules obtained by complex coacervation can present particle sizes in the range from 1 to 500 μm , which is within the range obtained in this work. Furthermore, they were smaller than 100 μm , which can facilitate their application in food products due to the low sensory perception in the mouth of consumer (THIES, 1995). Regarding the circularity, values were in the range from 0.86 to 0.92. As already mentioned, the value closer to 1, the more rounded the capsule is, which facilitates application in food due to easy flow. The values obtained in this work, can be considered close to 1 and, consequently, indicate a rounded shape. This result was confirmed by optical and confocal microscopy. It is important to highlight that this analysis was

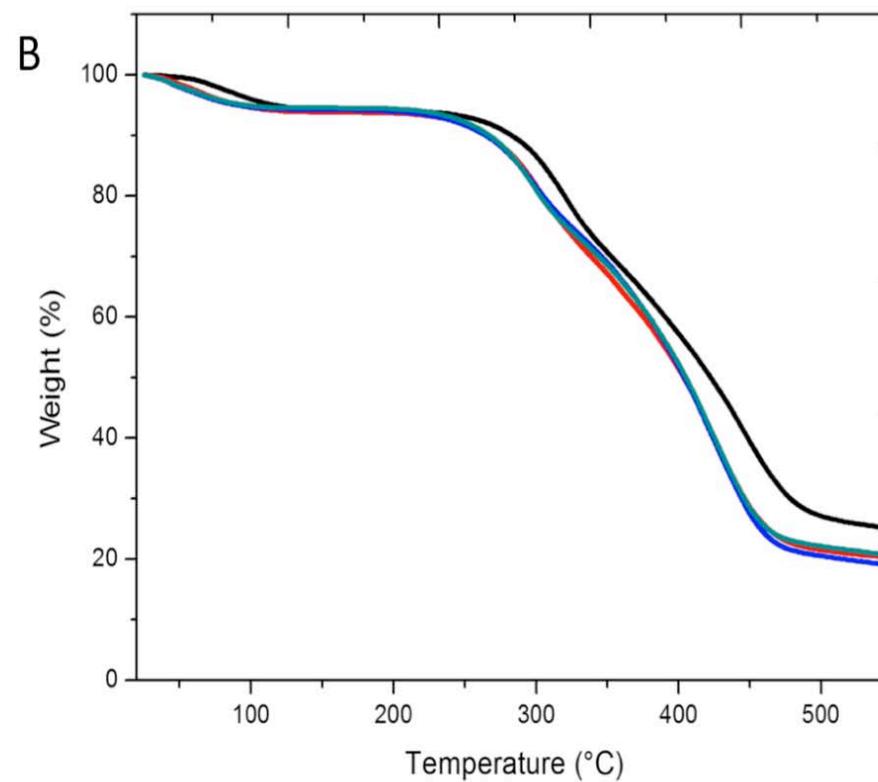
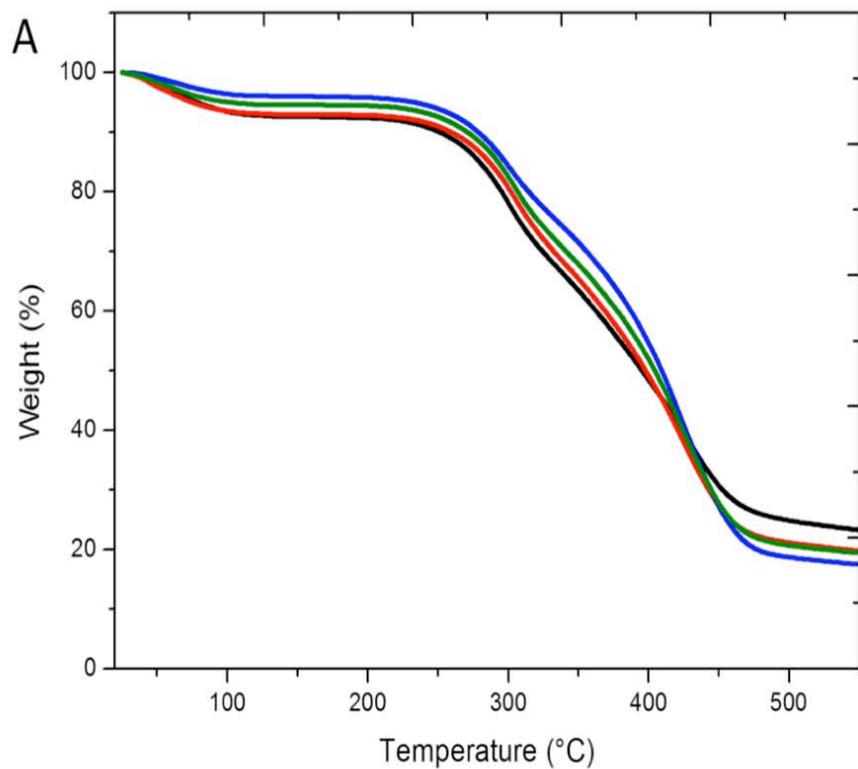
performed with wet microcapsules. If they are applied in a dried food, maybe there is no advantage, since the lyophilized microcapsules lose the circularity, as seen in SEM micrographs (Figures 2.2 and 2.3).

3.5. Thermogravimetric analysis

To carry out the thermogravimetric analysis, the behavior in relation to the mass loss according to the temperature applied for each sample was analyzed (Figure 2.5). It was observed small weight loss with the increase of temperature up to 100 °C; this first thermal degradation of the dried microcapsules is owed to the weight loss of remained water. After this first thermal degradation, the samples weight kept constant in the temperature range from 100 to 250 °C. However, this was followed by the second thermal degradation in the range from 250 to 430 °C; according to Xiao, Li, & Zhu (2015), degradation in this range is related to the evaporation of oil and partial decomposition of the wall materials. In this case, approximately 80% of the total mass was decomposed.

In Figure 2.5, it can be seen that SIN100 was more stable than SIN75, which was more thermally stable than SIN50, in other words, the higher the concentration of sinapic acid (ug of sinapic acid/g of capsule) (Table 2.1), the lower the weight loss in the range from 250 to 430°C. This small difference can be attributed to the low concentration of sinapic acid in relation to the content of gelatin (0.001 g/g of gelatin). According to Peng, Zhao, Zhang, Huang, Chen, & Zhao, (2014), who studied the encapsulation of mustard essential oil by complex coacervation with genipin, low concentrations of genipin such as 0.025 g of genipin/g of gelatin resulted in less stable microcapsules, which decomposed at temperatures below 300 °C.

Figure 2. 5. Thermogravimetry for sample with (a) sinapic acid: samples SIN50 (Black curve), SIN75 (red curve), SIN100 (blue curve) and SIN-GEL (green curve); (b) with rutin: samples RUT50 (black curve), RUT75 (red curve), RUT100 (blue) and control (green curve).



Reference: Own source.

The same relation cannot be applied to the samples with rutin. It is observed in Figure 2.5 that the sample RUT50 was more stable than the other samples (RUT75 and RUT100), which showed similar behaviors, including the control sample (encapsulated oil without phenolic compound); moreover, in this case, sinapic acid was more effective than rutin.

3.6. Identification and quantification of fatty acids (FA) present in encapsulated echium oil

Nine FA were identified in the microcapsules, including palmitic (C16:0), stearic (C18:0), oleic (C18:1 n9 cis), elaidic (C18:1 n9 trans), linoleic (C18:2 n6 cis), alpha (C18:3 n3) and gamma linolenic (C18:3 n6), stearidonic (C18:4 n3) and gondoic acid (C20:1 n9), where alpha linolenic and stearidonic are the omega-3 FA.

The FA composition for each sample are presented in Table 2.3. It was observed that with an increasing of oil concentration, the FA content also increased, which was expected. The SIN-GEL and control samples, i.e. microcapsules in which the core was composed only of oil, presented oil concentrations similar to the SIN100 sample. For this reason, the concentrations of FA were also similar.

As already mentioned, the echium oil has a ratio of 1:1.8 of omega-6 to omega-3 FA. This ratio is considered suitable for nutraceutical applications and it is not found in other vegetable oil. The encapsulation process used in this study has maintained this ratio, since the concentrations of omega-3 and omega-6 FA presented in Table 2.3 for each sample are in agreement with that expected.

Table 2. 3. Concentration and composition of oil and major acids for each sample.

Sample	Oil (g oil/g of capsule)	Palmitic acid C16:0 (mg/g of capsule)	Stearic acid C18:0 (mg/g of capsule)	Oleic acid C18:1 n9 cis (mg/g of capsule)	Elaidic acid C18:1 n9 trans (mg/g of capsule)	Linoleic acid C18:2 n6 (mg/g of capsule)	α -linolenic acid C18:3 n3 (mg/g of capsule)	γ -linolenic acid C18:3 n6 (mg/g of capsule)	Stearidonic acid C18:4 n3 (mg/g of capsule)	Gondoic ac C20:1 n9 (mg/g of capsule)
SIN50	0.24	19.71±1.39 ^{bc}	16.89±7.06 ^a	26.44±2.46 ^{cd}	2.48±1.19 ^a	25.80±2.40 ^c	50.13±7.12 ^{cd}	25.63±11.70 ^a	31.05±11.68 ^{ab}	1.90±0.94 ⁱ
SIN75	0.32	28.22±1.70 ^{ab}	17.55±0.86 ^a	39.63±3.50 ^{ab}	1.54±0.26 ^a	38.75±3.11 ^{ab}	74.23±2.01 ^b	24.64±1.07 ^a	33.09±0.30 ^{ab}	1.78±0.12 ⁱ
SIN100	0.39	32.43±0.53 ^{ab}	19.05±0.60 ^a	49.83±3.08 ^a	2.05±0.65 ^a	48.27±2.91 ^a	102.50±7.78 ^a	34.55±2.56 ^a	44.61±3.34 ^a	2.39±0.25 ⁱ
SIN-GEL	0.33	24.67±2.27 ^{abc}	18.72±5.42 ^a	32.54±0.12 ^{bcd}	2.20±1.51 ^a	31.17±0.09 ^{bc}	65.93±0.89 ^{bc}	28.96±9.58 ^a	37.19±12.40 ^{ab}	1.64±1.40 ⁱ
RUT50	0.24	13.33±2.63 ^c	7.93±1.82 ^a	19.97±3.54 ^d	0.75±0.16 ^a	19.39±4.37 ^c	43.72±1.77 ^d	13.42±2.54 ^a	18.06±3.18 ^b	0.95±0.06 ⁱ
RUT75	0.32	20.54±2.24 ^{bc}	12.23±1.58 ^a	28.21±1.76 ^{bcd}	0.90±0.03 ^a	28.16±0.84 ^{bc}	59.97±2.01 ^{bcd}	20.03±0.52 ^a	24.61±2.77 ^{ab}	1.21±0.08 ⁱ
RUT100	0.39	28.82±2.12 ^{ab}	14.12±4.12 ^a	49.60±6.05 ^a	1.50±0.11 ^a	46.52±4.81 ^a	93.09±10.39 ^a	25.29±7.60 ^a	31.15±9.32 ^{ab}	2.03±0.01 ⁱ
CONTROL	0.33	36.92±9.54 ^a	20.21±2.15 ^a	37.46±6.24 ^{abc}	2.44±1.48 ^a	40.11±6.54 ^{ab}	95.96±0.76 ^a	28.12±4.35 ^a	36.67±5.46 ^{ab}	2.13±0.46 ⁱ

Reference: Own source.

The analyzes were performed in triplicate. Equal letters in the same column do not differ significantly by the Tukey test at 5% probability.

- SIN50 and RUT50: Sample with a 1:1:0.5 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN75 and RUT75: Sample with a 1:1:0.75 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN100 and RUT100: Sample with a 1:1:1 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively
- SIN-GEL: Sample with a 1:1:0.5 proportion of gelatin, Arabic gum and echium oil with sinapic acid as a cross-linker
- CONTROL: Sample with a proportion of 1:1:0.5 of gelatin, Arabic gum and echium oil.

3.7. Accelerated oxidation by Rancimat

The samples and pure oil were submitted to accelerated oxidation by Rancimat in order to compare the effect of encapsulation, the presence of phenolic compounds in the microcapsules and the position of sinapic in the microcapsules to protect echium oil against oxidation. Table 2.2 shows the induction time values, values used as oxidative stability index, measured in hours, for all samples and for the pure echium oil.

In comparison to control sample (oil encapsulated without phenolic compound), it was observed that the pure echium oil took about 5 hours to oxidize, while the encapsulated oil without phenolic compounds took approximately 10 hours, in other words, the encapsulation of echium oil by complex coacervation offered twice more stability to the oil.

In relation to the oxidative stability of pure echium oil compared to samples in which the oil was encapsulated in the presence of phenolic compound within the microcapsule, it can be stated that the encapsulation process together with the addition of an antioxidant compound (rutin or acid sinapic) also provided twice more stability for the echium oil (Table 2.2). However, as noted, control sample and the samples with phenolic compounds within the microcapsule had the same induction time, in other words, the addition of phenolic compounds was not more effective than just encapsulation. This can be explained due to the position of the phenolic compounds in the microcapsule, protected and with little contact to the oil, which hindered its action as antioxidant.

However, for the sample in which sinapic acid was applied to the

microcapsule wall, it took about 18 hours to oxidize, so, the encapsulation of echium oil by complex coacervation with the addition of the phenolic compound in the microcapsule wall offered almost four times more stability for the oil when compared to the pure echium oil and almost twice when compared to the encapsulated oil without antioxidants or with them within the microcapsule. This can be explained due to sinapic acid position in the microcapsule wall, enhancing its contact with the oil and so its antioxidant function. Wang, Adhikari, & Barrow (2014) studied the microencapsulation of tuna oil by complex coacervation using gelatin-sodium hexametaphosphate as wall material and genipin as crosslinking agent and compared the action of nine different antioxidants, added in the tuna oil, by accelerated oxidation (Rancimat). According to these authors, the addition of antioxidant also increased the oxidative stability of tuna oil, obtaining great values of induction time (29.27 ± 1.04 hours).

4. CONCLUSIONS

With these results, it can be concluded that encapsulation of echium oil by complex coacervation improves its chemical stability. In this case, the use of double emulsion before the coacervation procedure was not as effective as the complex coacervation process with the addition of the phenolic compound in the capsule wall. In other words, the encapsulation of this oil with sinapic acid added directly in the gelatin solution is the most effective sample and offers almost four times more stability for the oil.

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**Chapter 3. EFFECT OF DIFFERENT POLYSACCHARIDES AND
CROSSLINKERS ON ECHIUM OIL MICROCAPSULES**

(PAPER PUBLISHED IN CARBOHYDRATE POLYMERS – ATTACHMENT C)

CHAPTER 3. EFFECT OF DIFFERENT POLYSACCHARIDES AND CROSSLINKERS ON ECHIUM OIL MICROCAPSULES

ABSTRACT

Microencapsulation by complex coacervation using gelatin and arabic gum (AG) as wall materials and transglutaminase for crosslinking is commonly used. However, AG is only produced in a few countries and transglutaminase is expensive. This work aimed to evaluate the encapsulation of echium oil by complex coacervation using gelatin and cashew gum (CG) as wall materials and sinapic acid (S) as crosslinker. Treatments were analyzed in relation to morphology, particle size, circularity, accelerated oxidation and submitted to different stress conditions. Rounded microcapsules were obtained for treatments with AG (45.45 μm) and microcapsules of undefined format were obtained for treatments with CG (22.06 μm). The S incorporation for 12 hours improved the oil stability by three fold compared to oil encapsulated without crosslinkers. Treatments with CG and S were resistant to different stress conditions similar to treatments with AG and transglutaminase, making this an alternative for delivery/application of compounds in food products.

Keywords: complex coacervation, sinapic acid, cashew gum, transglutaminase, crosslinking

1. INTRODUCTION

Echium oil is a plant-based oil which contains 9 to 16% stearidonic acid, 14% linoleic acid, 10% gamma-linolenic acid and 33% alpha-linolenic acid. It is being considered as an alternative to fish oils, as it presents a unique ratio of

omega-3 to omega-6 fatty acids that is not found in any other plant (BERTI et al., 2007). However, this oil is very unstable, and is also a hydrophobic material which hampers its application in beverages, for instance. Thus, one alternative to minimize the problems related to echium oil is the microencapsulation process.

The microencapsulation technique can be defined as a process in which one or more materials are surrounded by a membrane, in order to protect against environmental conditions, and also to promote the controlled release of the materials in specific locations and conditions. The complex coacervation encapsulation technique consists of the electrostatic interaction between oppositely charged macromolecules. Microcapsules are obtained by this interaction, where the formation of a shell around the encapsulated material is possible under specific conditions of pH and temperature (XIAO; LIU; ZHU; ZHOU, & NIU, 2014). Many wall material combinations are used in the complex coacervation technique, including casein and pectin (BARACAT et al., 2012), gelatin and arabic gum (COMUNIAN et al., 2013; SANTOS; BOZZA; THOMAZINI; & FAVARO-TRINDADE, 2015), alginate and pea protein isolate (KLEMMER; WALDNER; STONE; LOW; & NICKERSON, 2012), pectin and soy protein isolate (Mendanha et al., 2009) and gelatin and chitosan (PRATA & GROSSO, 2015). The combination of gelatin and arabic gum is the most common and effective one.

Gelatin, a hydrocolloid majorly obtained from the bones and skins of mammals and fish, has amphoteric character and cationic properties at pH below its isoelectric point (IEP) and anionic characteristics at pH values above its IEP, which makes it a great polymer to be used as wall material in the

complex coacervation technique (XIAO; LIU; ZHU; ZHOU; & NIU, 2014). On the other hand, arabic gum has some limitations. It is produced in just a few countries (such as Senegal and Sudan), and variations in its quality and composition make its obtainment and standardization difficult. The use of new polysaccharides in the complex coacervation process is important to increase the range of choices and, consequently, to decrease the cost of the final product, in order to obtain innovative and excellent products. A new option is the cashew gum, a macromolecule similar to arabic gum due to its thermal and rheological behavior (MOTHÉ & RAO, 2000). It is an exudate from the *Anacardium occidentale* tree and is composed of galactose, arabinose, glucose and rhamnose (ABREU; OLIVEIRA; PAULA; & PAULA, 2012). Additionally, some studies have shown health benefits such as antitumor and antihypertensive activities (CARESTIATO; AGUILA; & MOTHÉ, 2009). The cashew gum has a negative charge, which makes it a feasible and effective alternative as a wall material in the complex coacervation technique.

Microcapsules obtained by complex coacervation are also known to be fragile under certain conditions. For this reason, specific compounds are used as crosslinkers in order to obtain more resistant structures. The traditional crosslinkers are formaldehyde and glutaraldehyde, which are toxic and prohibited in the food industry. Transglutaminase has also been widely used, however it is an expensive material which limits its application (PENG; ZHAO; HUANG; CHEN; & ZHAO, 2014). Furthermore, countries such as Spain do not allow the use of transglutaminase in food products.

The sinapic acid compound, extracted from fruits (lemons, oranges, tangerines, strawberries, blueberries), vegetables (onions, garlic, broccoli),

cereal grains (rye, rice and oat) and herbs and spices (borage, thyme, nutmeg) (NICIFOROVIC & ABRAMOVIC, 2014), is a phenolic compound with antioxidant functions and has been studied regarding its neuroprotective effect against Alzheimer's disease (LEE et al., 2012) and cardiac hypertrophy and dyslipidemia (ROY & PRINCE, 2013). Sinapic acid also has possible reactions with proteins and enzymes, which lead to covalent bonds and thereby crosslinking reactions (RAWEL & ROHN, 2010). Moreover, the chemistry of hydroxycinnamic acid derivatives concerning polysaccharide-polysaccharide and lignin-polysaccharide crosslinking in grass cell walls (RALPH; QUIDEAU; GRABBER; & HATFIELD, 1994) and in soluble and insoluble dietary fibers of many cereal grains (BUNZEL et al., 2003) seems to be a clue towards understanding the enhanced mechanical properties of microcapsules formed through a coacervation with sinapic acid as a crosslinker. In grasses, it is well-known that ferulic acid is esterified to grass cell wall polysaccharides (arabinoxylans) at the C-5 position of α -L-arabinofuranoside moieties (HATFIELD; RALPH; & GRABBER, 1999). Dimerization of such polysaccharide-ferulate esters provides a pathway for crosslinking polysaccharide chains (RALPH; QUIDEAU; GRABBER; & HATFIELD, 1994). Similarly, another hydroxycinnamic acid derivative, sinapates, were also claimed to have a similar role to ferulates in crosslinking polysaccharides in cereal grains and presumably in grass cell walls in general (BUNZEL et al., 2003). Thus, the use of sinapic acid as a crosslinker instead of the traditional crosslinkers can be an alternative for achieving more rigid and stable microcapsules.

The current research therefore aimed to compare the use of cashew gum and sinapic acid, instead of arabic gum and transglutaminase, as the polysaccharide-encapsulant and crosslinker, respectively, to encapsulate echium oil by complex coacervation and submit the capsules to different temperatures, pH levels, and salt and sucrose concentrations. To the authors' best knowledge, there are no published studies regarding the use of cashew gum as a wall material and sinapic acid as a crosslinker, making this an innovative idea.

2. MATERIAL & METHODS

2.1. Materials

Echium oil (NEWmega™ Echium Oil, Ref.15200/ De Wit Speciality Oils, Tescel, Netherlands) was used as the core. The encapsulants were gelatin, purchased from Gelnex (Santa Catarina, Brazil), arabic gum (composed by protein (0.99% - w/w), rhamnose (4% - w/w), arabinose (46% - w/w), galactose (38% -w/w) and glucuronic acids (6.5% - w/w), purchased from Nexira (São Paulo / SP, Brazil), and cashew gum (composed by β -D-galactose (72-73% - w/w), α -D-glucose (11-14% - w/w), arabinose (4-6.5%-w/w), rhamnose (3.2-4%-w/w) and glucuronic acids (4.7-6.3% -w/w)) (De Paula, Heatley, & Budd, 1998) which was obtained from EMBRAPA Tropical Agribusiness (Fortaleza/Ceará, Brazil). The crosslinkers used were sinapic acid (Sigma, St. Louis, MO, USA) and transglutaminase (Ajinomoto, São Paulo, Brazil) with an activity of 100 U/g. Lycopene (LycoVit Dispersion 20%) from BASF (Ludwigshafen, Germany) was used to dye the oil.

2.2. Methods

2.2.1. Purification and characterization of cashew gum

For purification of cashew gum, it was dispersed in 96% ethanol and the precipitate was dried at 60 °C for 24 hours. An aqueous solution of 6% (w/w) of the obtained powder was prepared and centrifuged at 5000 rpm for 15 min at 20 °C with the equipment Centrifuge 5430R, Eppendorf AG (Hamburg, Germany). The supernatant was filtered to obtain the purified cashew gum solution. This solution was stored in Petri dishes and maintained at 65 °C for 24 hours in a kiln (Fanem, Model 315 SE, São Paulo/ Brazil) for complete drying and obtaining powder material. The purified cashew gum is referred to throughout the text as cashew gum. The study was adapted from Torquato et al. (2004).

For the characterization of cashew gum, analysis of protein content (Baethgen & Alley, 1989), ash content (AOAC, 2005) and solubility (Cano-Chauca, Stringheta, Ramos, & Cal-Vidal, 2005) were carried out.

2.2.2. Microencapsulation process

2.2.2.1. Complex coacervation

The microcapsules were produced according to Nori et al. (2011), with some modifications. A concentration of 50% (w/w) of oil in relation to the polymer mass, with 8% (m/m) of lycopene in relation to the oil, was added to a 5% (w/w) gelatin solution and homogenized at 10,000 rpm for 3 min with an Ultraturrax T25 (IKA, Germany), obtaining an oil in water emulsion. Then, in order to perform the complex coacervation, a 5% (w/w) arabic or cashew gum solution was added to the oil in water emulsion under magnetic stirring at 40 °C. The pH was adjusted to 4.0 and the temperature reduced to 10 °C via an ice

bath. After preliminary tests, the proportions of gelatin:arabic gum and gelatin:cashew gum were fixed at 1:1 and 1:2.5, respectively.

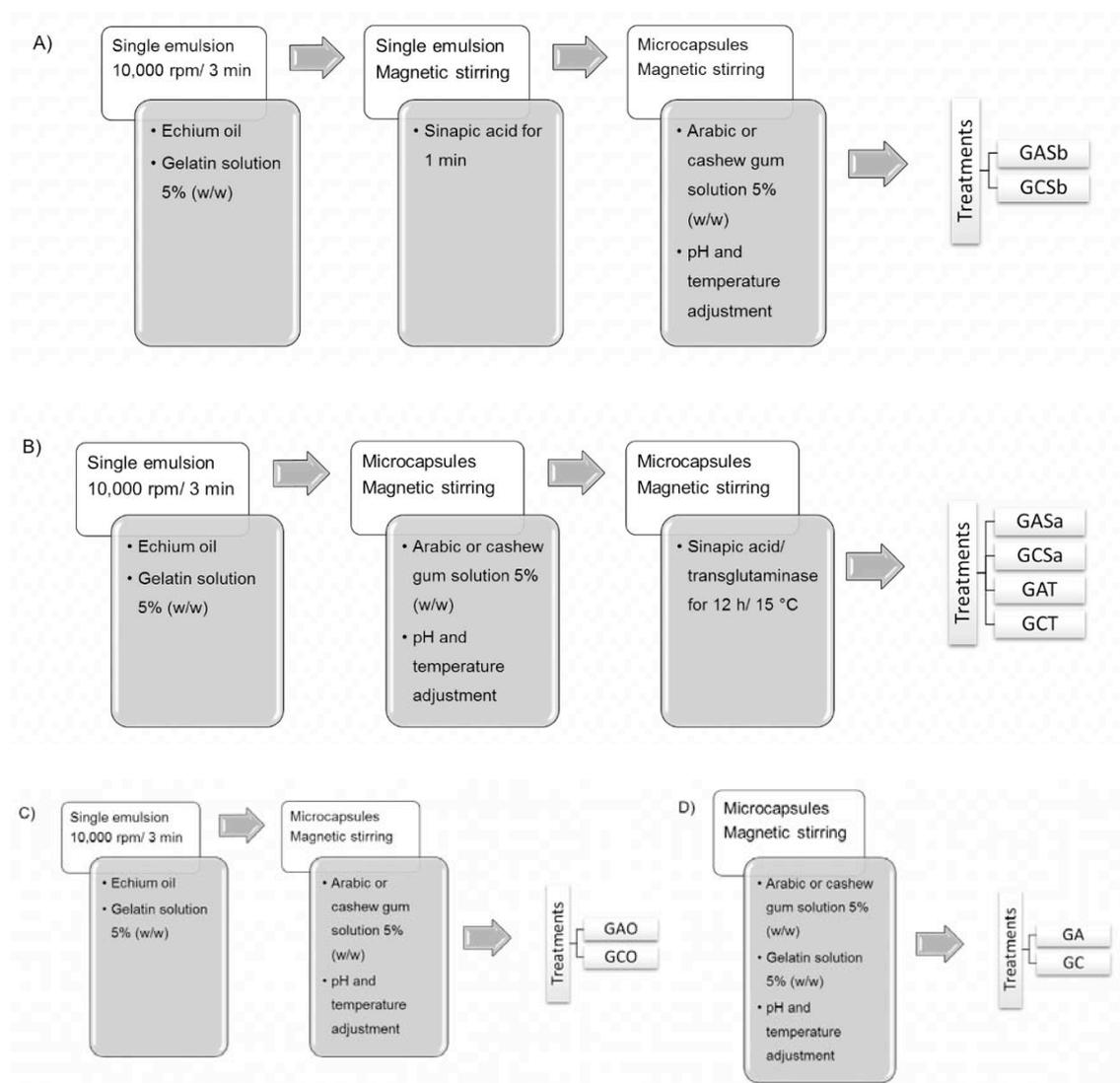
2.2.2.2. Crosslinking

Sinapic acid (0.05 g/g of gelatin) was added in two different ways: (1) After preparing the simple oil in water emulsion (oil in gelatin solution) and before the addition of the arabic gum solution, sinapic acid was added while using a magnetic stirrer over 1 min at 40 °C; (2) After the complex coacervation process, following the pH adjustment and temperature reduction, sinapic acid was added to the solution with the microcapsules and maintained under low magnetic stirring for 12 h at 15 °C in the BOD TE-391 incubator (Tecnal/ Piracicaba, São Paulo - Brazil).

For the crosslinking with transglutaminase, a 100 mL aqueous solution was prepared containing the enzyme at a concentration of 15 U/g of gelatin and a pH of 6.0. The solution was added to the microcapsules after the coacervation and maintained under low magnetic stirring for 12 hours at 15 °C in the BOD incubator.

In addition, the control treatments were prepared, making up ten treatments in total (Table 3.1 and Figure 3.1).

Figure 3. 1. Diagram about the process of complex coacervation and crosslinking for treatments A) GASb and GCSb; B) GASa, GCSa, GAT and GCT; C) GAO and GCO; and D) GA and GC.



Reference: Own source.

Table 3. 1. Composition of each treatment, particle size and circularity values for each treatment in the wet form and accelerated oxidation index for the freeze-dried ones.

Treatments	Wall material	Crosslinking agent	Incorporation of crosslinkers	Average particle size (μm)	Circularity	Accelerated oxidation index (hours)
GASb	Gelatin-arabic gum	Sinapic acid	Before arabic gum*	48.72 ± 16.09^a	0.88 ± 0.14^a	20.99 ± 2.87^b
GASa	Gelatin-arabic gum	Sinapic acid	After complex coacervation**	46.18 ± 13.41^a	0.92 ± 0.11^a	26.50 ± 1.60^a
GCSb	Gelatin-cashew gum	Sinapic acid	Before arabic gum*	24.21 ± 9.53^b	--	19.88 ± 2.99^b
GCSa	Gelatin-cashew gum	Sinapic acid	After complex coacervation**	21.45 ± 5.43^b	--	20.15 ± 3.50^b
GAT	Gelatin-arabic gum	Transglutaminase	After complex coacervation**	44.87 ± 14.37^a	0.88 ± 0.14^a	9.86 ± 0.63^c
GCT	Gelatin-cashew gum	Transglutaminase	After complex coacervation**	20.53 ± 4.83^b	--	9.48 ± 0.06^c
GAO***	Gelatin-arabic gum	--	--	45.49 ± 19.18^a	0.87 ± 0.15^a	9.39 ± 0.06^c
GCO***	Gelatin-cashew gum	--	--	21.89 ± 4.77^b	--	8.73 ± 0.04^d
GC****	Gelatin-cashew gum	--	--	--	--	--
GA****	Gelatin-arabic gum	--	--	42.06 ± 13.19^a	0.90 ± 0.14^a	--
Echium oil	--	--	--	--	--	5.10 ± 0.09^e

Reference: Own source.

* stirring for 1 minute

** stirring for 12 hours

*** control treatments: with wall materials and echium oil without crosslinkers

**** with wall materials only

Equal letters in the same column do not differ at 5% of significance by Tukey test.

2.2.2.3. Drying processes of microcapsules

The coacervates were stored for 24 h at 7 °C to allow the microcapsule precipitate to settle. The supernatant (water) was removed with a dispenser. Then, the samples were frozen and lyophilized (Freeze drier Terroni, São Carlos, SP - Brazil). For comparison, the coacervates were also atomized using a spray dryer, model LM MSD 1.0 (Labmaq, Ribeirão Preto/SP, Brazil), with a heating air flow of 2.50 m/s, inlet air temperature of 150 °C, feed flow rate of 0.6 L/h and a nozzle diameter of 1.2 mm. In this case, the capsules were suspended in water and atomized right after the encapsulation and crosslinking process.

2.2.3. Microcapsule characterization

2.2.3.1. Morphology

The morphology of wet microcapsules was analyzed by optical microscopy (BIO3 equipment - Bel Photonics, Italy), and dehydrated microcapsules were analyzed by scanning electron microscopy (SEM) using the Tabletop Microscope TM 3000 (Hitachi, Tokyo, Japan).

2.2.3.2. Particle size and circularity

To obtain the particle size, 100 microcapsules per treatment were individually measured using the ImageJ software. The BIO3 microscope (Bel Photonics, Italy) was used to obtain the images. To calculate the circularity of the microcapsule, the smallest and the biggest diameters of 100 capsules were

measured individually for each treatment. These measurements were made with wet microcapsules.

2.2.3.3. Accelerated oxidation by Rancimat

Accelerated oxidation analysis for the encapsulated oil was performed with the Rancimat equipment (model 873, Metrohm, Switzerland). Dried capsule samples (1.5 g) were submitted to heating under a purified air flow rate of 20 L/h at 90 °C. The induction times (hours) of the samples were used as the oxidative stability index (Comunian et al., 2016).

2.2.3.4. Stress conditions submitted

The analyses of stress conditions were performed according to Chung & McClements (2015), with modifications. The wet coacervates were submitted to different stress conditions, such as different temperatures (2, 10, 20, 30, 40, 50, 60, 70 and 80 °C) during 5 minutes, pH values (2, 3, 4, 5, 6, 7 and 8), NaCl solutions (1, 3 and 5%) (w/w) and sucrose solutions (1, 10 and 20%) (w/w). To evaluate different temperatures, the wet coacervates were dispersed in a beaker containing 50 ml of an aqueous solution at 2 °C which was maintained on a heater plate under constant stirring, while controlling the increase of temperature up to 80 °C with a thermometer. To test different pH values, the wet coacervates were dispersed in an aqueous solution, the pH was adjusted with a solution of NaOH 0.1 M or H₃PO₄ 0.1M and the mixtures were stirred for 2 min at room temperature. To test different concentrations of NaCl and sucrose, the coacervates were again dispersed in aqueous solutions with the respective concentrations, and the mixtures were stirred for 2 min at room temperature. After being submitted to each one of these conditions, the

microcapsules structures were evaluated using a BIO3 optical microscope (Bel Photonics, Italy).

2.2.3.5. Statistical analysis

Data were statistically analyzed using SAS statistical software (Statistic Analysis System), version 9.2, by ANOVA and the Tukey's test at 5%.

3. RESULTS AND DISCUSSION

3.1. Characterization of cashew gum

In order to characterize the cashew gum, analyzes of protein, ash and solubility were performed, obtaining values of $0.26 \pm 0.03\%$, $0.6 \pm 0.09\%$ and $71.11 \pm 0.08\%$, respectively. Even the cashew gum presenting similar composition to gum arabic, its content of protein, ash and the solubility are different, obtaining approximated values of 2%, 3.39 % and 50%, respectively (WEINBRECK; VRIES; SCHROOYEN; & KRUIF, 2003; DAMODARAN; PARKIN; & FENNEMA, 2010). This can be considered a small difference, however enough to influence on the structure of the capsules, as it will be shown following.

3.2. Morphology by optical and scanning electron microscopy

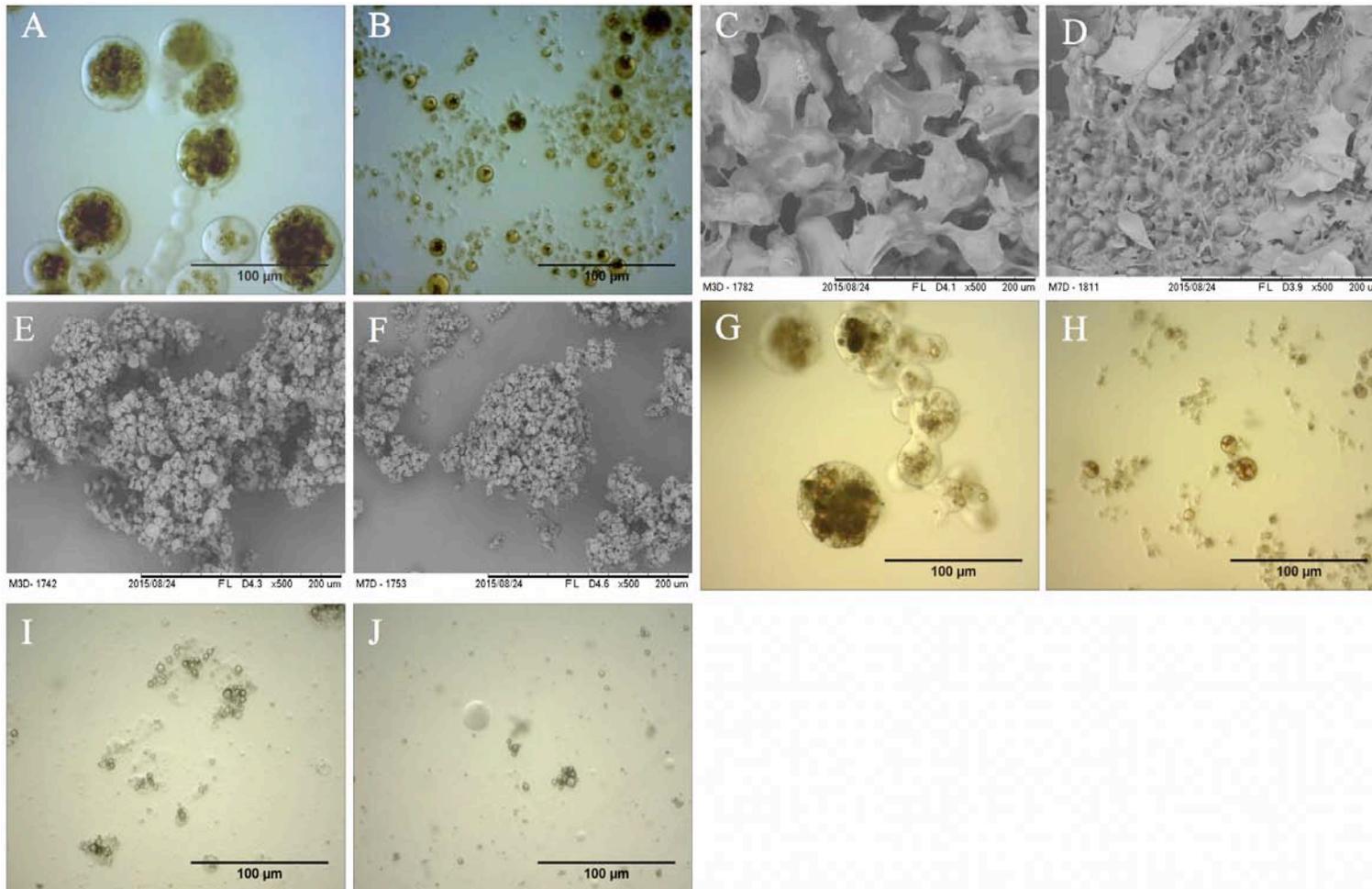
The complex coacervation process using gelatin and arabic gum as wall materials is known for producing multinucleated microcapsules with a rounded shape (COMUNIAN et al., 2016). Furthermore, as these wall materials are commonly used, researchers have sought new polymers for application in the food, cosmetic and pharmaceutical industries, ensuring the substitution of various common materials while also offering the same functionality.

The cashew gum, as already mentioned, has physicochemical

characteristics ideal for replacing arabic gum. However, the microcapsules with the gelatin-cashew gum combination showed a different morphology from those obtained with gelatin-arabic gum. As shown in Figure 3.2, the interaction between the gelatin and cashew gums resulted in a complex with an undefined format; however, the oil droplets were completely covered by the polymers, which certainly ensures their protection, although the flow of the powder was reduced compared to powders composed of structures with a rounded shape. Furthermore, complexes formed with gelatin-cashew gum were smaller than those formed with gelatin-arabic gum (Table 3.1), which may be advantageous for some applications since smaller particles have less of an impact on food texture.

Coacervates are dried in order to facilitate their handling and storage as well as to obtain more stable materials. The coacervates were submitted to two different drying methods: freeze drying and spray drying. It was observed that the lyophilized microcapsules presented links by "bridges", a typical characteristic of particles dried by lyophilization. There were no morphological differences between treatments with the same wall material; in other words, different crosslinkers and different ways of incorporating the sinapic acid did not influence the external structure of the microcapsules. However, morphological differences between freeze-dried treatments with different wall materials (gelatin-arabic gum and gelatin-cashew gum) were observed, since treatments with cashew gum showed higher linking by "bridges" than treatments with arabic gum. Furthermore, there was a certain amount of polymer that did not interact to form the microcapsule wall with the gelatin-cashew gum combination (Figure 3.2d).

Figure 3. 2. Optical microscopy of (A) treatment GASb (gelatin-arabic gum as wall materials and sinapic acid added before arabic gum), (B) treatment GCSa (gelatin-cashew gum as wall materials and sinapic acid added after complex coacervation); scanning electron microscopy of (C) treatment GASa (gelatin-arabic gum as wall materials and sinapic acid added after complex coacervation) freeze-dried, (D) treatment GCSa (gelatin-cashew gum as wall materials and sinapic acid added after complex coacervation) freeze-dried, (E) treatment GASa atomized, (F) treatment GCSa atomized; optical microscopy of (G) treatment GASa freeze-dried and rehydrated and (H) treatment GCSa freeze-dried and rehydrated; (I) treatment GASa spray-dried and rehydrated and (J) treatment GCSa spray-dried and rehydrated.



Reference: Own source.

The atomized microcapsules showed a rounded shape, characteristic of dried materials produced by this process. However, it was observed that they were broken within the atomizer, probably due to the pressure exerted by the compressed air and the high temperature. The average particle size of these capsules after the atomizing process was much lower ($9.76 \pm 2.17 \mu\text{m}$ for treatments with arabic gum and $7.55 \pm 1.64 \mu\text{m}$ for treatments with cashew gum) than that obtained before drying, which proves they had broken (Figure 3.2). Furthermore, in both drying processes the particles were agglomerated, which is necessary to facilitate the wettability and dissolution of the powders and also to prevent dust.

The microcapsules dried by both methods were rehydrated and analyzed by optical microscopy. It was observed that drying by lyophilization preserved the original format of the microcapsule after rehydration; however, the same did not happen for the samples from the atomization technique, which formed large agglomerates when dried and lost their initial form when rehydrated (Figure 3.2I and 3.2J). This result can be explained by the conditions of each drying process, such as low temperature and a vacuum for lyophilization, and high temperature and pressure for atomization.

Regarding the application of crosslinkers, the presence of sinapic acid versus transglutaminase, in addition to the different methods of sinapic acid incorporation, did not affect the morphology of the microcapsules.

In a similar study, Alvim & Grosso (2010) studied the influence of the type of crosslinking and drying process on the release of paprika oleoresin encapsulated by complex coacervation using gelatin and arabic gum as wall materials. These authors also obtained microcapsules with a rounded shape

that maintained integrity after the lyophilization process. Samples without the crosslinker were destroyed during the spray drying, however they showed partial resistance with the addition of transglutaminase or glutaraldehyde, which did not happen in this work. These different results can be explained by the different conditions used during the encapsulation procedure, such as concentration of the crosslinker, temperature, time of reaction and spray dryer conditions.

Thus, according to the results presented, lyophilized microcapsules were chosen for the continuation of this research.

3.3. Average particle size and circularity

The particle sizes ranged from 20.53 to 48.72 μm , showing significant differences between treatments (Table 3.1). The use of cashew gum as a wall material, crosslinked with sinapic acid or transglutaminase, gave an average particle size that was twice as small as that obtained for the treatments with only arabic gum. This smaller particle size was considered a positive result, as it will have less of an effect on food texture. The treatment CG showed an undefined complex, which was impossible to measure. The treatments GCSb, GCSa and GCT also showed an undefined format, but they were possible to measure using the arithmetic average of three different diameters. The application of different compounds as crosslinkers and the different methods of sinapic acid incorporation did not influence the mean size. No research using cashew gum as was wall materials for complex coacervation technique was found in the literature. However, this carbohydrate has been used as wall material for ionic gelation technique, obtaining particles size in the range from

600 to 800 μm (DAS; DUTTA; NAYAK; & NANDA, 2014), approximately 20 times larger than the size obtained for the echium oil capsules.

The circularity values are used to represent the roundness of the microcapsule: the closer to 1, the more rounded the capsule. Rounder capsules are better for application in food due to their easy flow. Treatments with arabic gum showed capsules of a rounded shape, within the range of 0.88 to 0.92. Treatments with cashew gum formed a complex with an undefined shape, which made measurement of the circularity impossible. Moreover, the different applications of sinapic acid did not significantly influence the circularity. Comunian et al. (2016) studied the protection of Echium oil by microencapsulation with phenolic compounds using complex coacervation technique and gelatin and gum Arabic as wall materials. These authors also obtained circularity in the range of 0.86-0.92, similar to obtained in this work.

3.4. Accelerated oxidation by Rancimat

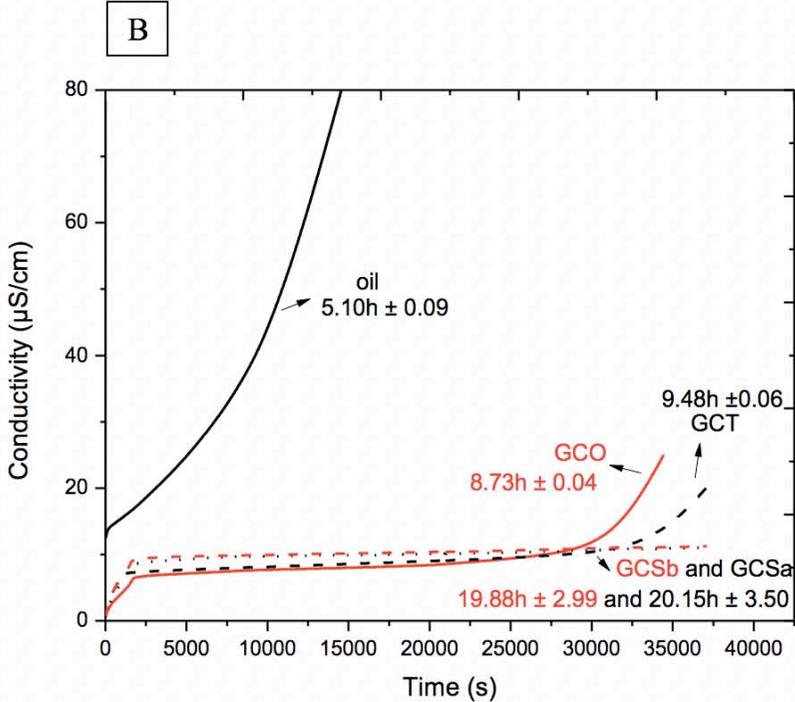
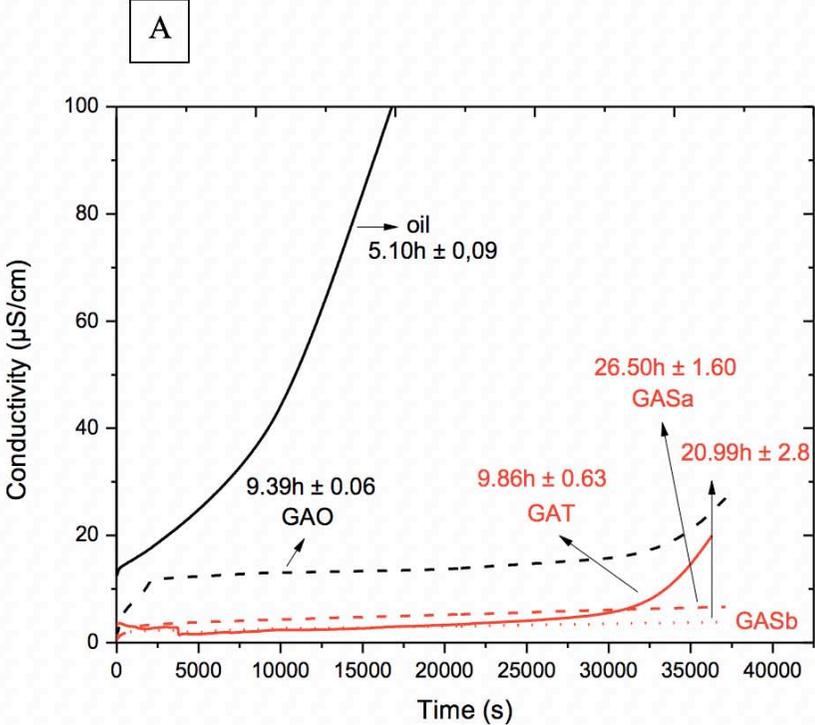
The values for the oxidative stability index (OSI), measured in hours, are presented in Table 3.1, and the oxidation behaviors of encapsulated and free oils are presented in Figure 3.3. The control treatments GAO and GCO presented values of 9.39 ± 0.06 and 8.73 ± 0.04 h, respectively, showing that different wall materials did not have a significant influence on the OSI. The treatments GASb and GASa showed values of 20.99 ± 2.8 h and 26.50 ± 1.60 h, respectively. In other words, the application of sinapic acid increased the oxidative stability of encapsulated oil. The treatment in which sinapic acid was incorporated for 1 min and before the arabic gum (GASb) had double the

stability, whereas the treatment in which the phenolic compound was incorporated for 12 hours (GASa) increased stability by almost three fold.

Treatments GCSb and GCSa presented OSI values of 19.88 ± 2.99 h and 20.15 ± 3.50 h, respectively. Therefore, the addition of this antioxidant doubled the oxidative stability of the encapsulated oil compared to treatment with only the wall material; moreover, the different incorporations of sinapic acid in the microcapsules with gelatin-cashew gum did not influence the OSI of the oil, as seen in Table 3.1. When the transglutaminase enzyme was used as the crosslinker, values of 9.86 ± 0.63 h and 9.48 ± 0.06 h were obtained with GAT and GCT treatments, respectively. As noted, treatment with transglutaminase and control treatments (encapsulated oil with only wall material - GAO and GCO) showed the same OSI; in other words, the addition of transglutaminase as the crosslinker was not more effective than the encapsulation.

Wang, Adhikari, & Barrow (2014) studied the optimization of microencapsulation of tuna oil in gelatin-sodium hexametaphosphate using complex coacervation and observed that the accelerated stability analyzed by Rancimat showed that encapsulated oil stability was more than double that of non-encapsulated oil. In a recent study, Mohammadi et al. evaluated the application of nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil using whey protein concentrate and obtained the maximum value of 12 hours for Rancimat induction period for the samples, while the material un-encapsulated oxidized in 8h. Comparing to the results obtained for echium capsules, the OSI for encapsulated echium oil in treatment GASa was more than five times that of pure echium oil. So, the encapsulation method used for echium oil with sinapic acid adequately protected the oil.

Figure 3. 3. Accelerated oxidation behavior of microcapsules by Rancimat: A) treatments with gelatin-arabic gum as wall material; B) treatments with gelatin-cashew gum as wall material.



Reference: Own source.

3.5. Effect of stress conditions on microcapsules stability

It is known that microcapsules obtained by complex coacervation can be destroyed under certain conditions, which compromises their application in food products. For this reason, the stability of microcapsules produced was studied under certain stress conditions found in food formulations or during food processing, such as different temperatures, pH values, and salt and sucrose concentrations. The rehydrated microcapsules were maintained at different pH values (from 2 to 8), different temperatures (from 2 to 80 °C), different concentrations of salt solutions (from 1 to 5% - w/w) and different concentrations of sucrose solutions (from 1 to 20% - w/w) in order to compare the stability of gelatin-arabic gum and gelatin-cashew gum combinations, in addition to the crosslinking agents and different ways of sinapic acid incorporation.

3.6. Effect of pH on microcapsules stability

As already mentioned, the complex coacervation process involves interactions that depend on specific pH values. These forces of attraction (called ionic bonds, or electrostatic bonds) are very weak, and they can be changed or eliminated by changes in pH.

Figure 3.4 demonstrates that microcapsule resistance was strongly influenced by pH. A low pH value (2.0) was enough to break the microcapsules, except with GASa, GASb and GAT treatments, showing that the wall formed

with the gelatin-cashew gum combination was weaker than that of the gelatin-arabic gum combination. Moreover, treatments with a crosslinking agent and the gelatin-arabic gum combination were more resistant than the GCO and GAO treatments (composed of the wall material and oil), proving that sinapic acid has similar crosslinking action to transglutaminase. The same happened at a high pH (8.0); therefore, sinapic acid and transglutaminase mainly acted on extreme pH values. The other pH values (from 3 to 7) did not cause breakage of the microcapsules, while extreme pH values made the capsule wall more fragile, although it remained intact. At pH 4.0, the value used to promote the complex coacervation, the microcapsules were more isolated for treatment GCSb and more agglomerated for treatment GCO. The difference can be explained by the use of sinapic acid as a crosslinker.

The comparison of the crosslinking action of sinapic acid and transglutaminase shows that the two compounds behaved similarly regarding the stability of the material at different pH values. This showed that transglutaminase can be substituted for sinapic acid. Moreover, different incorporations of sinapic acid did not influence the final result.

There are currently no published studies regarding the influence of pH on stability of coacervate microcapsules. However, particles using biopolymers as wall materials have been evaluated by Chung & McClements (2015) who studied the microstructure and physical properties of biopolymer hydrogel particles through modulation of electrostatic interactions. These authors studied the use of sodium caseinate and pectin in the fabrication of particles and compared the pH values within the 3 to 8 range. They observed that the system was stable at pH values ranging from 3 to 5, while only a few hydrogel particles

were visible from pH 6 to 8. Thus, it is possible to infer that microcapsules produced with gelatin-arabic gum and gelatin-cashew gum crosslinked with sinapic acid are resistant over a wider pH range than the hydrogel particles produced with pectin and sodium caseinate.

3.7. Effect of temperature on microcapsules stability

Heating causes agitation among the molecules, so it can destabilize the complex protein-polysaccharide. For this reason, the treatments were subjected to different temperatures (2, 10, 20, 30, 40, 50, 60, 70 and 80 °C).

The walls of the GCO and GAO treatments were destroyed at a temperature of 40 °C, whereas microcapsules containing sinapic acid and transglutaminase remained intact at higher temperatures (up to 80 °C), demonstrating again the crosslinking action of sinapic acid.

The comparison of different wall material combinations showed that the microcapsules prepared with gelatin-cashew gum presented, as already noted, an undefined format at low temperatures. However, temperatures of 40 °C led to the formation of rounded shape microparticles. Figure 3.5 shows that the wall materials were not surrounding the core as in the treatments with gelatin-arabic gum, but were instead dispersed in the echium oil. This structure was stable up to 80 °C, at which point the release of oil droplets was observed (Figure 3.5). It is important to mention that this rounded structure is more feasible for application in food due to better flow, as already mentioned.

There was no difference between the sinapic acid and transglutaminase treatments, as both kept the capsules intact throughout the temperature range

examined. Therefore, this type of microcapsule can be used in products that undergo heat treatment. Moreover, different methods of sinapic acid incorporation did not influence the final result; in other words, it may be incorporated as the crosslinker without affecting the microcapsule structure.

3.8. Effect of ionic strength on microcapsules stability

It is known that salt concentration is strongly linked to ionic strength, making it a decisive factor in the stability of the microcapsule. Thus, as seen in Figure 3.6, three different concentrations of salt solutions (NaCl) were tested (1, 3 and 5%) (w/w) and then the resistance of the microcapsules was observed.

All treatments prepared with gelatin-arabic gum were resistant at all NaCl concentrations evaluated. Thus, it can be inferred that different salt concentrations do not affect the strength of microcapsules with gelatin-arabic gum as wall materials. This allows for the application of this microcapsule in various food products, despite the fact that 3 and 5% (w/w) salt solutions are high salt concentrations that are hardly found in foods.

However, free oil droplets were observed in treatments with gelatin-cashew gum in the 3 and 5% (w/w) salt solutions. Furthermore, the crosslinking action did not make the particle more resistant in this case.

In addition to pH and temperature influence, there are no studies in the literature regarding the influence of ionic strength on the stability of coacervate microcapsules. Chung & McClements (2015) studied the microstructure and physical properties of biopolymer hydrogel particles through modulation of electrostatic interactions, evaluated the use of sodium caseinate and pectin in

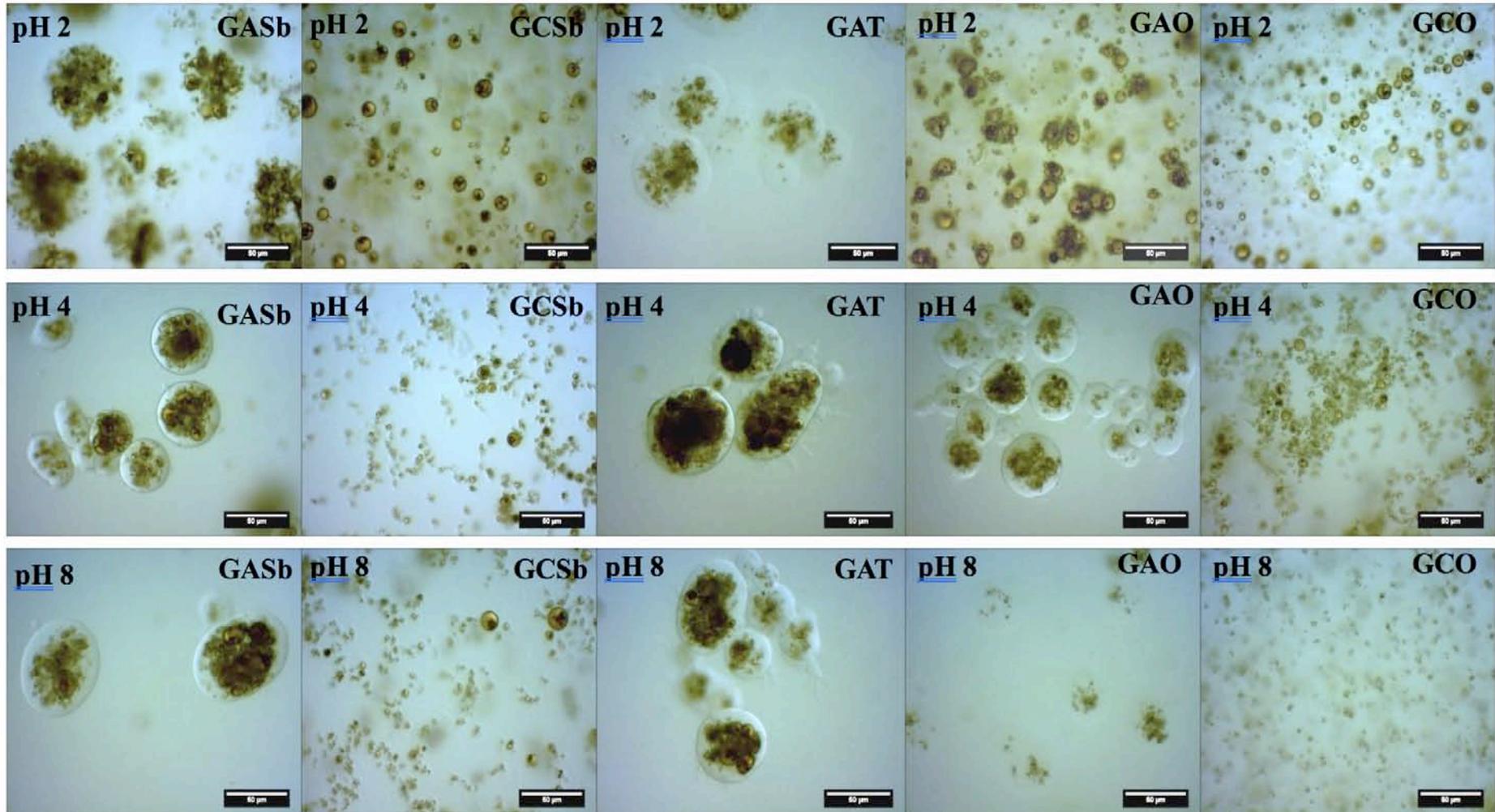
the fabrication of the particles and compared treatments with different sodium chloride concentrations within the range of 0 to 400 mM. They observed that the sodium caseinate-pectin system containing no salt produced small particles, whereas the size of the particles increased with an increase in salt content. The opposite happened to the microcapsules with the gelatin-cashew gum combinations; in other words, the capsules ruptured with an increase in salt concentration, whereas the microcapsules with gelatin-arabic gum remained their original size and format.

3.9. Effect of sucrose on microcapsules stability

Different sucrose concentrations were also analyzed (1, 10 and 20%) (w/w), since small carbohydrate molecules are capable of changing the hydration level of proteins and polysaccharides. Thus, sucrose could possibly influence the structure, and therefore the stability, of the microcapsules produced with this kind of material. However, there were no changes in the structures produced with any of the sucrose concentrations used, showing that sucrose did not influence microcapsule resistance or stability. According to this result, the microcapsules can be applied to products with different sucrose contents while ensuring their integrity.

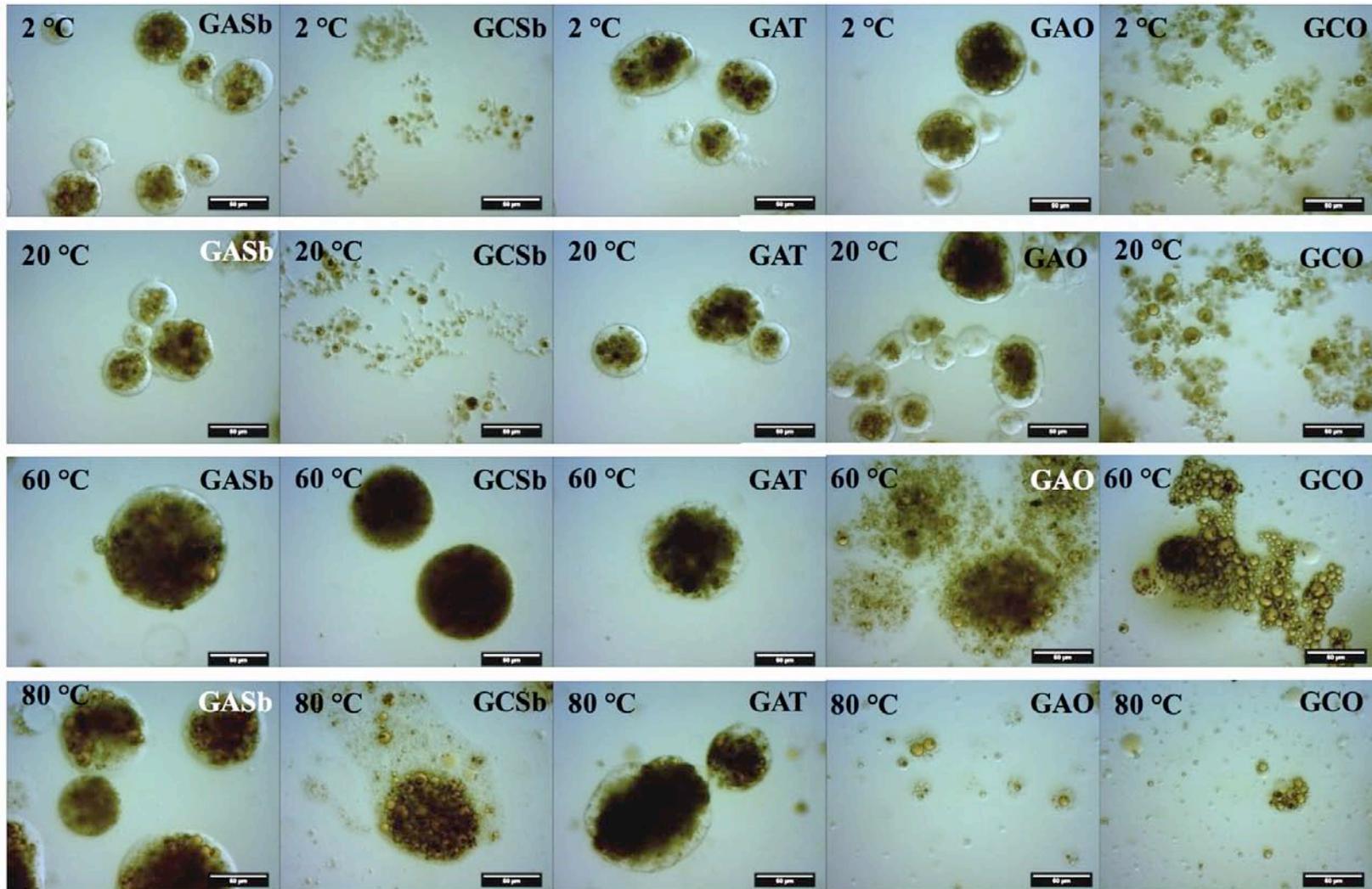
The results obtained regarding microcapsule stability under different stress conditions supports the hypothesis that gelatin-arabic gum microcapsules crosslinked with sinapic acid can be applied in a huge range of foods while maintaining their stability.

Figure 3. 4. Optical microscopy of the treatments maintained in solution of pH 2, 4 and 8.



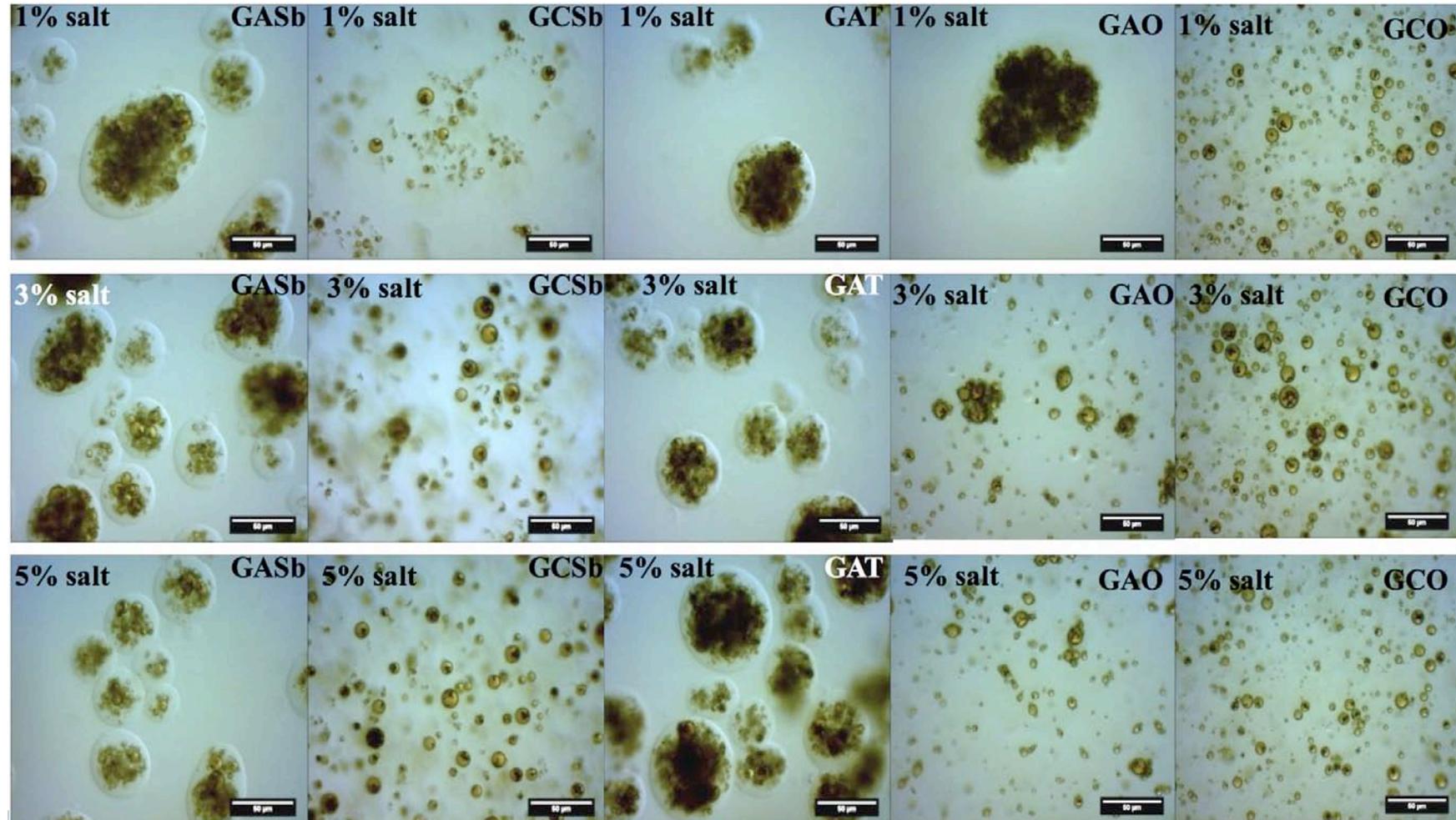
Reference: Own source.

Figure 3. 5. Optical microscopy of the treatments submitted to temperatures of 2, 20, 60 and 80 °C.



Reference: Own source.

Figure 3. 6. Optical microscopy of the treatments in solutions of 1, 3 and 5% (w/w) of salt.



Reference: Own source.

3.10. Crosslinking process of sinapic acid

Sinapic acid was used as a crosslinker in this work for different reasons. First of all, Bunzel et al. (2003) reported that two sinapic acid compounds, (1) 8-8-coupled dehydrodimers, compound 3 (the noncyclic isomer) and (2) lignan thomasidioic acid (the cyclic isomer), were recovered from basic hydrolysis of both soluble and insoluble dietary fibers of wild rice (Figure 3.7a). Chemically, oxidative radical coupling dehydrodimerization of sinapic acid can afford two primary products, 8-O-4- and 8-8-dehydrodimers, only. However, the former was not detected at any significant level in cereal grain dietary fibers evaluated by Bunzel et al. (2003). On the other hand, air oxidation of sinapic acid yields thomasidioic acid as the sole product 100% of the time at pH 8.5 and 30% of the time at neutral pH. Air oxidation of sinapic acid to thomasidioic acid was also described by Charlton & Lee (1997) and Bunzel et al. (2003).

Since sinapic acid 8-8-coupled dehydrodimers are a naturally occurring pathway for crosslinking polysaccharide chains and that sinapic acid itself is promptly oxidized by air to afford medium to high yields of its dehydrodimer thomasidioic acid through an oxidative radical coupling reaction (Figure 3.7b), it can be hypothesized that a similar event happens to some extent when cashew gum and/or arabic gum is exposed to sinapic acid or its dehydrodimer thomasidioic acid.

Therefore, regarding the encapsulation process, it seems likely that the resulting polysaccharide-polysaccharide crosslinkages through a thomasidioic acid moiety would be the major influence for the enhanced mechanical

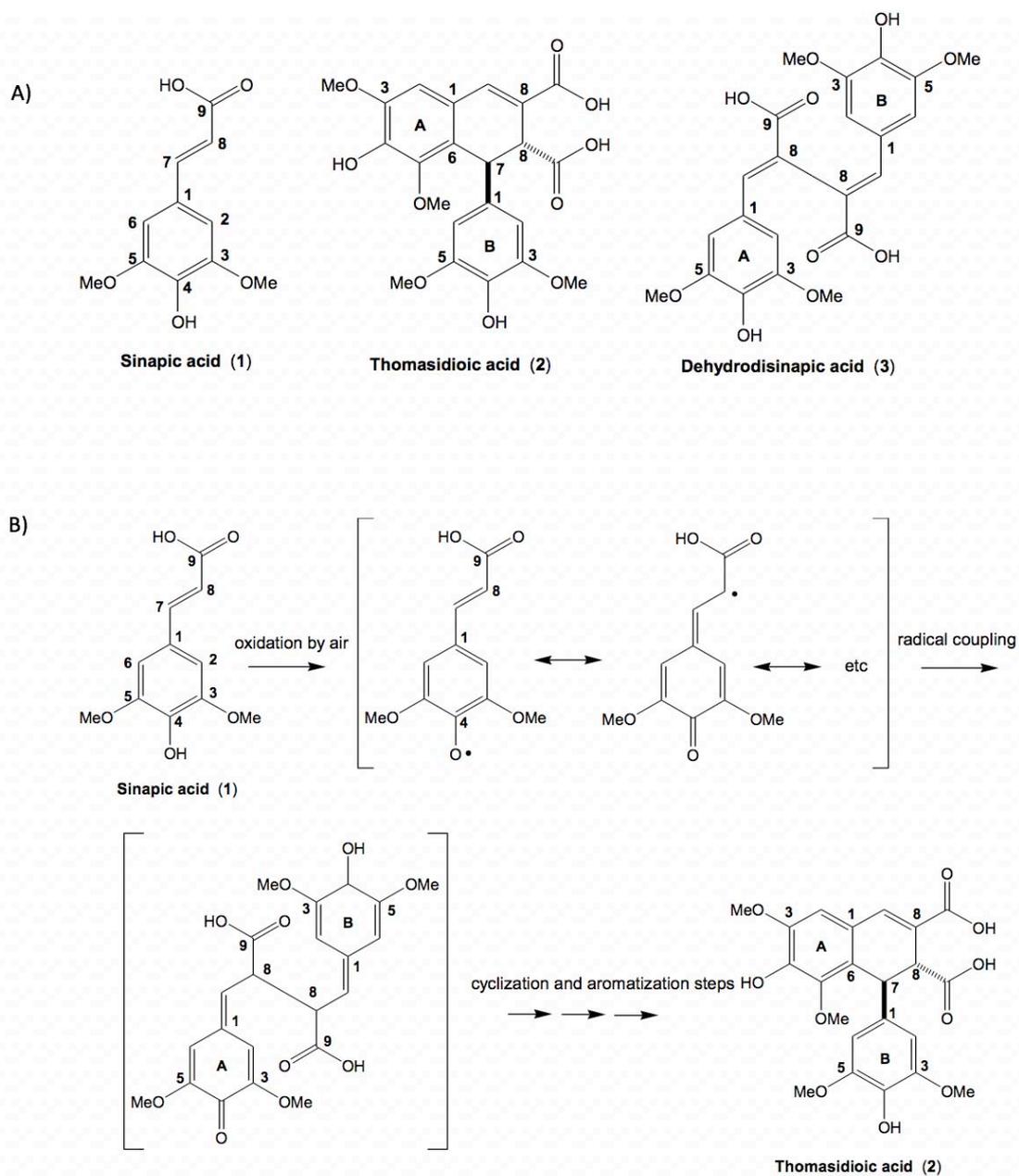
properties of microcapsules formed through the complex coacervation technique with sinapic acid as a crosslinker. The main constraint of this approach is the previously required polysaccharide acylation by free sinapic acid and/or thomasidioic acid without prior acyl group activation by added reagents or catalysts. Even so, given that the whole complex coacervation process was performed under slightly acidic conditions, two possible pathways for crosslinking the cashew gum and/or arabic gum polysaccharides mediated by sinapic and/or thomasidioic acid were envisaged.

The first pathway is based on prior acylation of a polysaccharide chain by free sinapic acid followed by further radical coupling dehydrodimerization and ultimately acylation of another polysaccharide chain (Figure 3.8a) as follows:

- a) Attachment of sinapic acid to a polysaccharide chain through a Fischer esterification-type reaction of the acid's acyl group and an OH group from a suitable sugar residue, under acidic conditions.
- b) Air-induced oxidative radical coupling dehydrodimerization with free sinapic acid.
- c) Attachment of the polysaccharide-bound sinapic acid dehydrodimer to another polysaccharide chain through a Fischer esterification-type reaction of the dehydrodimer's acyl group and an OH group from a suitable sugar residue, under acidic conditions.

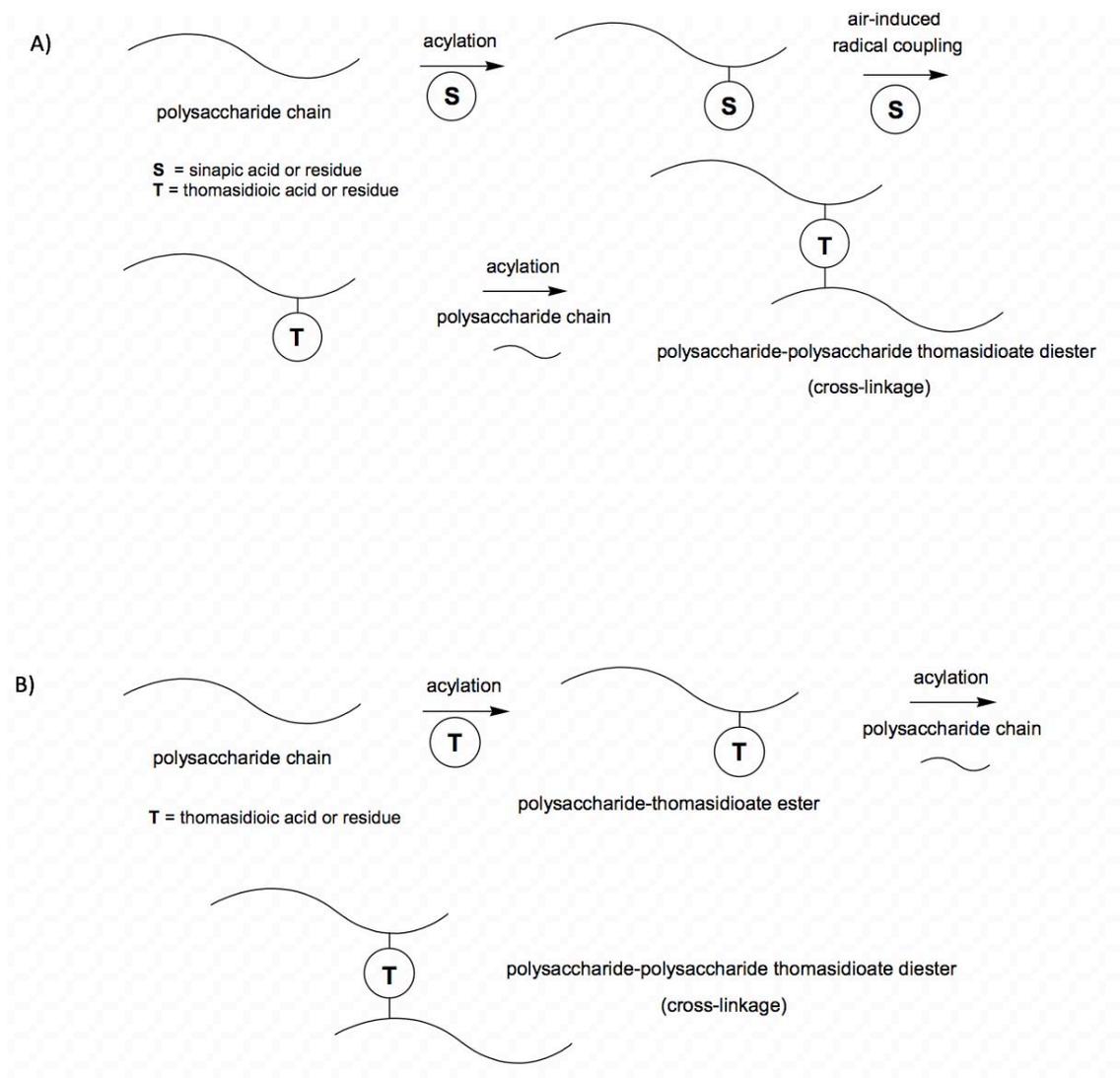
The second proposed pathway for polysaccharide-polysaccharide crosslinkage requires previous formation of thomasidioic acid from air-induced oxidative radical coupling dehydrodimerization of free sinapic acid prior to the later polysaccharide chain acylation (Figure 3.8b).

Figure 3. 7. a) Sinapic acid (1) and dehydrodisinapic acid derivatives thomasidioic acid (2) and compound 3; b) Oxidative radical coupling dehydrodimerization of sinapic acid to form thomasidioic acid. Adapted from Bunzel et al. (2003).



Reference: Own source.

Figure 3. 8. a) Proposed polysaccharide-polysaccharide crosslinkage through a polysaccharide-sinapate ester air-induced oxidative radical coupling dehydrodimerization with a free sinapic acid; b) Proposed polysaccharide-polysaccharide crosslinkage through a polysaccharide-thomasidioate ester.



Reference: Own source.

4. CONCLUSIONS

In this work, microcapsules of echium oil were successfully prepared by a complex coacervation technique using combinations of gelatin-arabic gum and gelatin-cashew gum as wall materials and with the crosslinkers sinapic acid and

transglutaminase. Cashew gum can be used as wall material since it protected the oil (treatments provided oxidative stability four times greater than pure oil). Moreover, sinapic acid, besides being a powerful antioxidant, can also be used as a crosslinker instead of toxic and expensive ones. Gelatin-arabic gum microcapsules crosslinked with sinapic acid for 12 hours showed better oil oxidation stability (almost three times higher than the non-crosslinked gelatin-arabic gum microcapsules), and all microcapsules exhibited optimal morphologic properties and appropriate average particle size. The crosslinked treatments showed greater resistance to different stress conditions than the treatments with just wall material and oil, thereby confirming that these new vehicles of bioactive compounds can be used in food products.

5. ACKNOWLEDGEMENTS

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**Chapter 4. PREPARATION AND CHARACTERIZATION OF ECHIUM OIL
AND SINAPIC ACID MICROPARTICLES BY EMULSION FOLLOWED BY
SPRAY AND FREEZE DRYING**

CHAPTER 4. PREPARATION AND CHARACTERIZATION OF ECHIUM OIL AND SINAPIC ACID MICROPARTICLES BY EMULSION FOLLOWED BY SPRAY AND FREEZE DRYING

ABSTRACT

Echium oil is rich in omega-3, however, is unstable. The objective of this work was the coencapsulation of echium oil and sinapic acid by emulsion followed by spray or freeze drying. Eight treatments were analyzed in relation to process yield, microscopy, A_w , hygroscopicity, moisture, solubility, particle size, x-ray diffraction, thermogravimetry and accelerated oxidation. Particles of rounded shape and undefined format were obtained by spray and freeze-drying, besides ideal physicochemical properties for application (0.091 to 0.365, 3.22 to 4.89%, 57 to 68% and 2.32 to 12.42 μm for A_w , moisture, solubility and particle size, respectively). All treatments protected the oil against oxidation, obtaining induction time of 5.31 h for oil and from 7.88 to 12.94 h for treatments. The better protection to oil was obtained with it emulsified and freeze-dried (L600), concluding the encapsulation increased oxidative stability of oil, besides facilitating its application over the fact the material is in powder form.

Keywords: omega-3 fatty acids, oxidation, microencapsulation, phenolic compound, arabic gum

1. INTRODUCTION

Omega-3 fatty acids (ω -3) are compounds widely used in food, pharmaceuticals and cosmetics products due to its beneficial effects on health, such as reducing the risk of coronary heart disease (KRALOVEC et al., 2012), assisting in the prevention of allergy, diabetes, Alzheimer's disease and neurodegenerative diseases (LAVIE et al., 2009).

The oil extracted from the seed of echium (*Echium plantagineum* L.) contains from 9 to 16% of stearidonic fatty acid, 14% of linoleic acid, 10% of gamma-linolenic acid and 33% of α -linolenic acid and it is being considered an alternative in the substitution of fish oils since it presents a proportion of ω -3 to ω -6 fatty acids that is not found in any other oil (BERTI et al., 2007). However, due to the high degree of unsaturation, this oil is very susceptible to oxidation, and as any oil, it does not solubilize in aqueous media, factors that hamper its storage, handling and application into foods. The microencapsulation technique and the addition of an antioxidant compound are strategies that would facilitate future applications of these compounds.

The phenolic compound sinapic acid has been of great interest in the food industry due to its beneficial effects on human health, acting as an antioxidant agent (LEE et al., 2013). In the case of the encapsulation technique, it has as main objective the protection of the material to be encapsulated, providing the controlled release of the active material in place and time desired, besides facilitating the storage and application (FAVARO-TRINDADE et al., 2008). There are many encapsulation techniques, such as complex coacervation (COMUNIAN et al., 2016a and 2016b – chapters 2 and 3), spray drying (KUCK & NOREÑA, 2016), high pressure homogenization (PARK et al.,

2016), microfluidic devices (COMUNIAN et al., 2014), ionic gelation (BENAVIDES et al., 2016), spray chilling (ORIANI et al., 2016), and others. The method of emulsion followed by spray drying and freeze-drying techniques consists in preparing a mixture of two immiscible liquids (aqueous phase and oil phase), subjected to atomization at high temperature (spray drying) or to freezing followed by sublimation under vacuum conditions and low temperature (lyophilization).

In this context, the objective of this study was to coencapsulate the echium seed oil and the phenolic compound sinapic acid by emulsion using arabic gum as the encapsulating agent/emulsifier, followed by spray drying and freeze drying, resulting in innovative vehicles that will bring health benefits to consumers, greater oxidative stability to the oil and facilitates its application.

2. MATERIAL E METHODS

2.1. Materials

Oil extracted from the seed of echium was used as source of omega-3 (*Echium plantagineum* L.) (NEWmega™ Echium Oil, Ref.15200/ De Wit Speciality Oils, Tescel, Holanda). The phenolic compound sinapic acid and the arabic gum were obtained from Sigma Chemical Co. (St. Louis/MO, United States) and from Nexira (São Paulo/SP, Brazil).

2.2. Study of Emulsions

2.2.1. Preparation of emulsions

For the preparation of single emulsion oil in water (O/W), echium oil was used as oil phase and solution of arabic gum 30% (w/w) as the aqueous phase. Two formulations were prepared, varying the ratio of oil phase to aqueous phase in 1:2 and 1:3.

2.2.2. Study of stability and droplet size

In order to evaluate the stability, the emulsions were visually observed in a measuring cylinder at room temperature and also by optical microscopy during the period of 7 days. In addition, the emulsions were analyzed in relation to stability and droplet size with the aid of the equipment of dispersion and stability analyzer (LumiSizer, Berlin/ Germany) at 4,000 rpm in the temperature of 25 °C.

2.3. Incorporation of sinapic acid

For choosing the best incorporation method of sinapic acid, the process was done in two ways, following what was proposed in a previous work of the group (Comunian et al. 2016b): (1) addition of sinapic acid (200 ppm in relation to the oil) after preparation of the single emulsion oil in water (O/W), which was kept under slow magnetic stirring for 12 hours at 15 °C in BOD TE-391 Tecnal (Piracicaba, São Paulo – Brazil) and (2) with the addition of sinapic acid (200 ppm in relation to the oil) in the aqueous solution of arabic gum, kept under magnetic stirring for 1 minute before preparation of the emulsion. After choosing

the best incorporation method of the phenolic compound, three sinapic acid concentrations were used (200, 600 and 1000 ppm in relation to the oil).

2.4. Drying

The emulsions were frozen in a freezer (-18 °C) and freeze-dried (Terroni, São Carlos, Brazil) for 24 hours. In addition, samples were also dried by atomization, using a spray dryer model LM MSD 1.0 (Labmaq, Ribeirão Preto, Brazil), with heated air flow of 2.50 m/s, inlet air temperature of 150 °C, feed flow rate of 0.6 L/h, nozzle diameter of 1.2 mm, parameters determined after numerous preliminary tests. Thus, eight treatments were obtained, as shown in Table 4.1.

2.5. Characterization of particles

2.5.1. Determination of yield of microencapsulation process

The determination of the yield of microencapsulation by spray dryer and freeze-drying was calculated by Equation 1:

$$Y = W_{is}/W_{fs} \times 100 \quad \text{Eq. 1}$$

Being:

W_{is} = initial amount of solids in solution.

W_{fs} = final amount of solids (powder obtained after the process).

2.5.2. Morphological characterization of microparticles by scanning electron microscopy (SEM)

The characterization of the microparticles was performed by scanning electron microscopy (SEM) using the microscope Tabletop Microscope Hitachi (Tokyo, Japan) TM 3000, with the program TM 3000.

2.5.3. Analysis of particle size and particle size distribution

To obtain the particle size and particle size distribution of the atomized treatments, particle analyzer by laser diffraction SALD- 201V, Shimadzu (Kyoto, Japan) was used. Ethanol was used as sedimentation medium and the average particle size was expressed as D[4,3] by volume distribution.

2.5.4. Water activity

The water activity was determined using the AQUALAB equipment (Decagon Devices, Pullman, WA).

2.5.5. Hygroscopicity

For the determination of hygroscopicity, 0.25 g of samples were stored for one week in a desiccator containing a saturated solution of NaCl (relative humidity of 75%). The hygroscopicity was determined by the mass of water absorbed per 100 g of sample after 7 days of storage, according to the methodology used by Cai & Corke (2000) with some modifications.

2.5.6. Moisture

The determination of the moisture content of the microparticles was performed in the moisture analyzer model MB 35, Ohaus (Ohio, USA).

2.5.7. Solubility

The determination of solubility was done by the gravimetric method according to Eastman & Moore (1984), cited by Cano-Chauca et al. (2005). The method consists of adding 0.40 g of sample into a flask containing 40.0 mL of distilled water. For homogenization, it was used an orbital shaker table, Tecnal (Piracicaba, Brazil) at 100 rpm for 30 minutes, at room temperature. Then, the solution was centrifuged with an Eppendorf Centrifuge, Model 5430 R (Hamburg, Germany) at 1400 g for 5 minutes. An aliquot of 20.0 mL of the supernatant was transferred to a porcelain cup with known mass and kept in an oven at 105 °C until complete evaporation of the water.

2.5.8. Thermogravimetric analysis (TGA)

The TGA curves were obtained using a Thermogravimetric Analyzer, model STA 449 F3 (Netzsh of Brazil, São Paulo, Brazil). Samples of 10 mg were weighted and put in a platinum sample holder. The furnace atmosphere was saturated with super-pure nitrogen with a flow rate of 50 mL/ min. The analysis temperature range was from 25 to 800 °C.

2.5.9. X-ray diffraction

The structure of arabic gum and particles were analyzed by x-ray diffraction (diffractometer Siemens 5100) according to Medeiros et al. (2014) and Xiao et al. (2015), with some modifications. The measurements were obtained by 2θ scanning between 5 and 55° and a scanning rate of $6^\circ\text{C}/\text{min}$, tension of 40 kV and current of 30 mA.

2.5.10. Accelerated oxidation by Rancimat

Accelerated oxidation tests for pure and encapsulated echium oil were carried out with the Rancimat equipment (model 873, Metrohm, Switzerland). The samples were subjected to heating under a purified air flow rate of 20 L/h at 90°C . The induction time of the sample was used as oxidative stability index (OSI). Four g of pure oil and 1.5 g of dried microparticle were used.

2.5.11. Statistical analysis

All experiments were performed in triplicate, with the exception of Rancimat, in duplicate. The data were statistically analyzed using SAS statistical software (Statistic Analysis System), version 9.3, by ANOVA and Tukey test, at 5% of probability.

3. RESULTS AND DISCUSSIONS

3.1. Study of emulsions

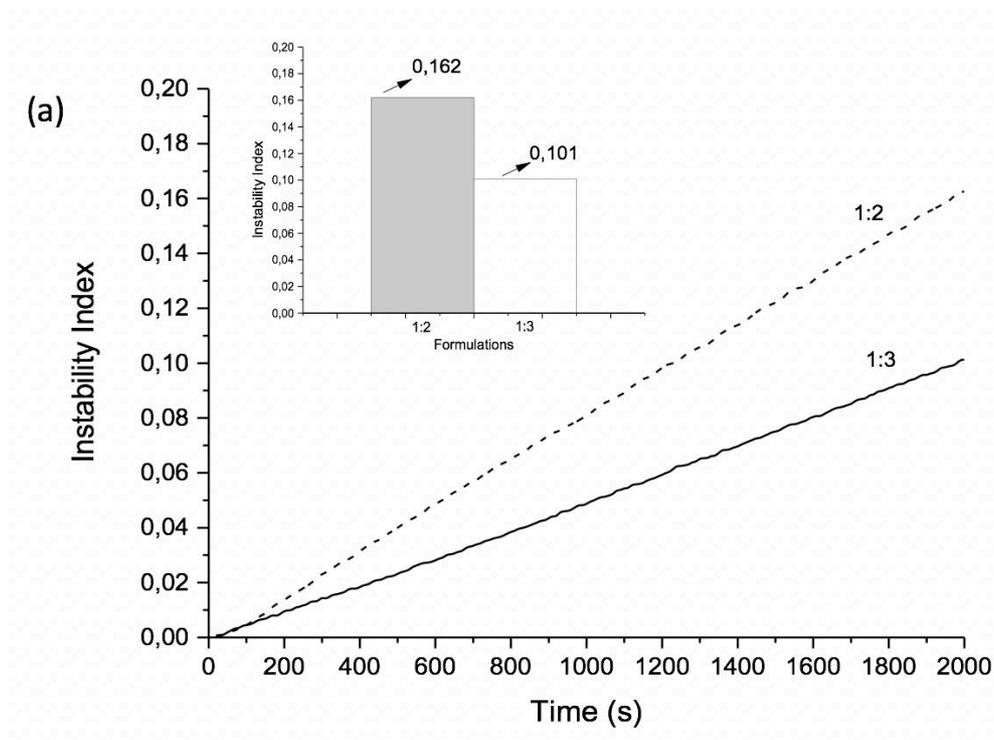
To select the most stable emulsion to be used during the analysis, before application of sinapic acid, two formulations were tested, varying the ratio of aqueous and oil phase, as already mentioned. The two emulsions were stable visually within the period of 7 days, besides not presenting morphological difference between them. The great stability of the two formulations can be explained due to the emulsifying function of arabic gum added as aqueous phase. Thus, to have a non-empirical parameter for emulsion stability determination, they were analyzed with the equipment LumiSizer (Berlin/Germany), using the conditions which estimate the period of 48 days of storage (corresponding to 30 minutes of analysis). The formulation related to the ratio of oil phase: aqueous phase of 1:3 was the most stable, with the lowest instability index (0.101), as shown in Figure 4.1a. It is known that the stability of emulsions is directly linked to the interaction of the interfaces, which can be measured by the hydrophile-lipophile balance (HLB). Thus, it can be said that the best stability presented by formulation with the ratio of 1:3 of oil phase: aqueous phase is intrinsically related to this interphase interaction.

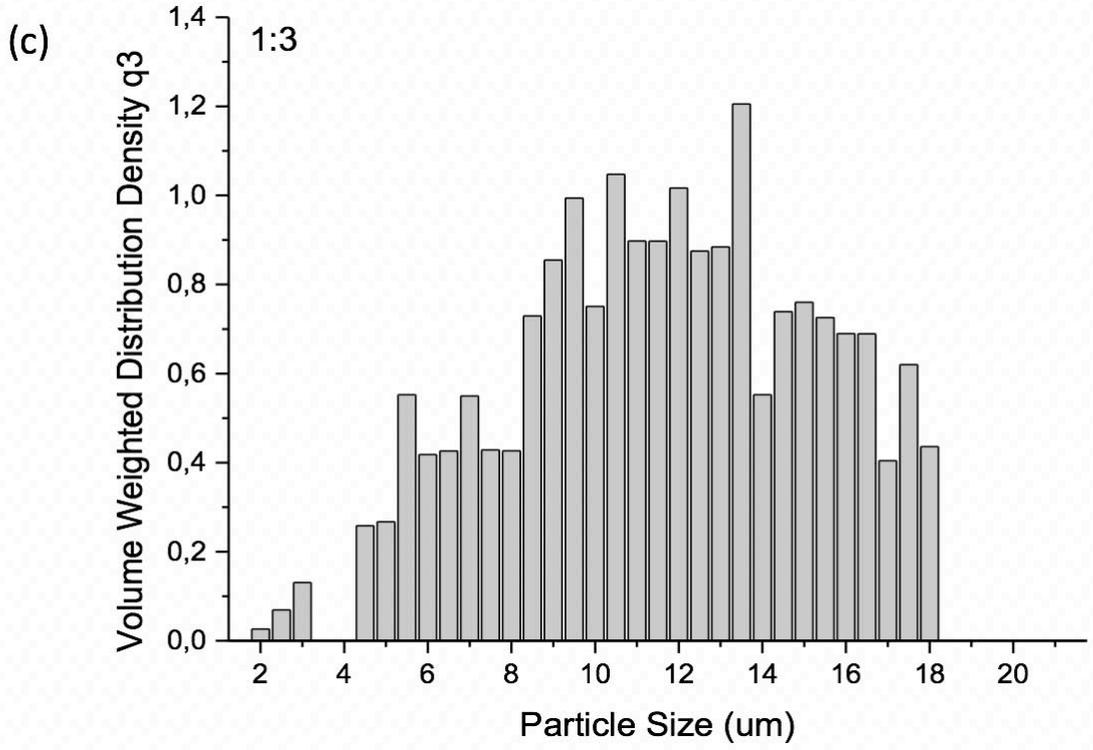
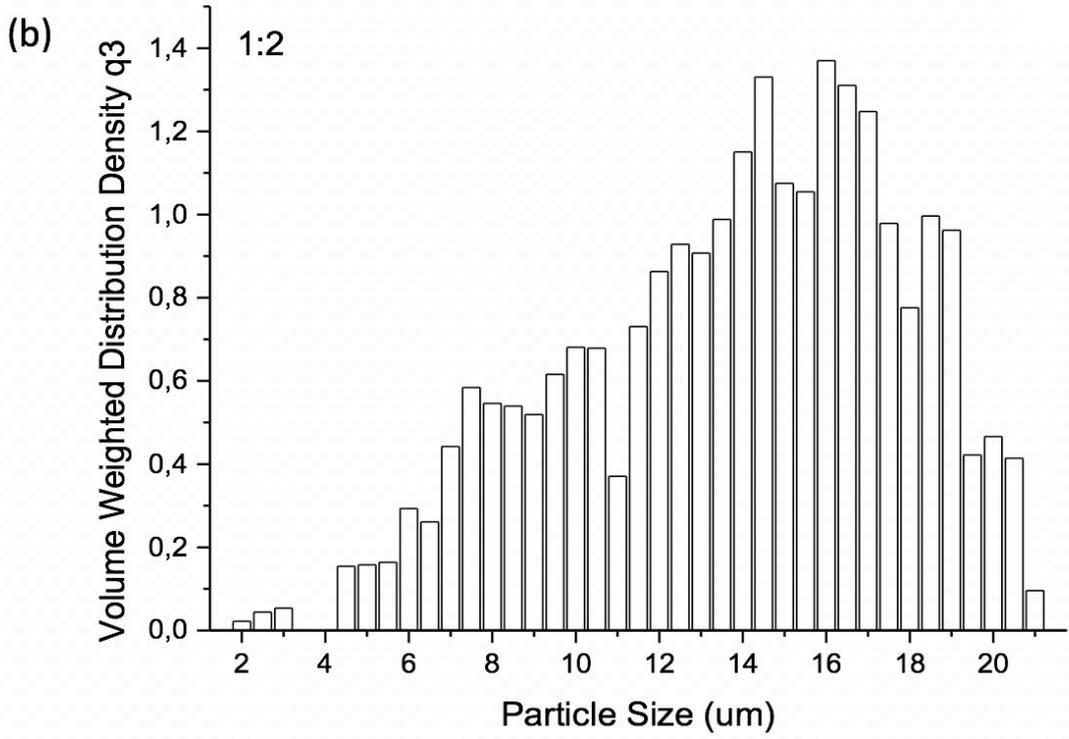
The behavior of each formulation over time can be seen in Figure 4.1a, and it is possible to verify that there was difference between the emulsions from 200 seconds of analysis, time which the best behavior for the emulsion with the proportion of 1:3 of oil phase: aqueous phase was already clear. With the use of the same equipment, it was possible to obtain average droplet size and droplet size distribution for the two tested emulsions. Average size of 14.31 and 10.03

μm for emulsions with the proportion of 1:2 and 1:3 of oil phase: aqueous phase and a unimodal distribution of droplet size were obtained for both formulations, as seen in Figure 4.1b.

According to the above, the emulsion related to the ratio of 1:3 of oil phase: aqueous phase was chosen for the addition of sinapic acid and continuing to research.

Figure 4. 1. (a) Instability index of each emulsion according to the analyzed time and general instability index of the emulsion; (b) Droplet size distribution of the emulsions in the ratio of 1:2 and (c) 1:3 of oil phase: aqueous phase.





Reference: Own source.

3.2. Definition of sinapic acid incorporation way

As already mentioned, to define the best incorporation of the phenolic compound, it was added in two different ways according to Comunian et al. (2016b) (Chapter 3) and subjected to spray drying and freeze drying, resulting in four different treatments: (1) S1 (magnetic stirring for 1 minute and drying by spray dryer), (2) S12 (magnetic stirring for 12 hours and drying by spray dryer), (3) L1 (magnetic stirring for 1 minute and drying by lyophilization) and (4) L12 (magnetic stirring for 12 hours and drying by lyophilization). The treatments were analyzed in relation to accelerated oxidation by Rancimat, obtaining induction time values (or oxidative stability index) (OSI) of 7.51 ± 0.01 , 6.91 ± 0.06 , 11.71 ± 0.74 and 11.02 ± 1.15 hours, respectively.

According to the above, it can be said that there was no significant difference in the incorporation of sinapic acid when stirring continuously for 1 minute and for 12 hours and when subjected to the same drying technique; in other words, different sinapic acid incorporations did not influence in its function as antioxidant. Thus, it was decided that the continuation of the research would be made with the incorporation of sinapic acid in the microparticles kept under stirring for only 1 minute.

3.3 Characterization of microparticles

3.3.1. Yield of drying process

The values obtained for the yield of the drying process are shown in Table 4.1. The values for the atomized treatments are within the range from 49 to 61% and for freeze-dried treatments from 77 to 83%. The yield for drying by

lyophilization was higher, as expected, since the atomization process depends on a number of factors such as production scale, operating parameters, sample volume and there is loss of material during the drying process, mainly due to adherence in the drying chamber and cyclone. It was not possible to establish a relationship between absence/presence of sinapic acid and the yield of drying process.

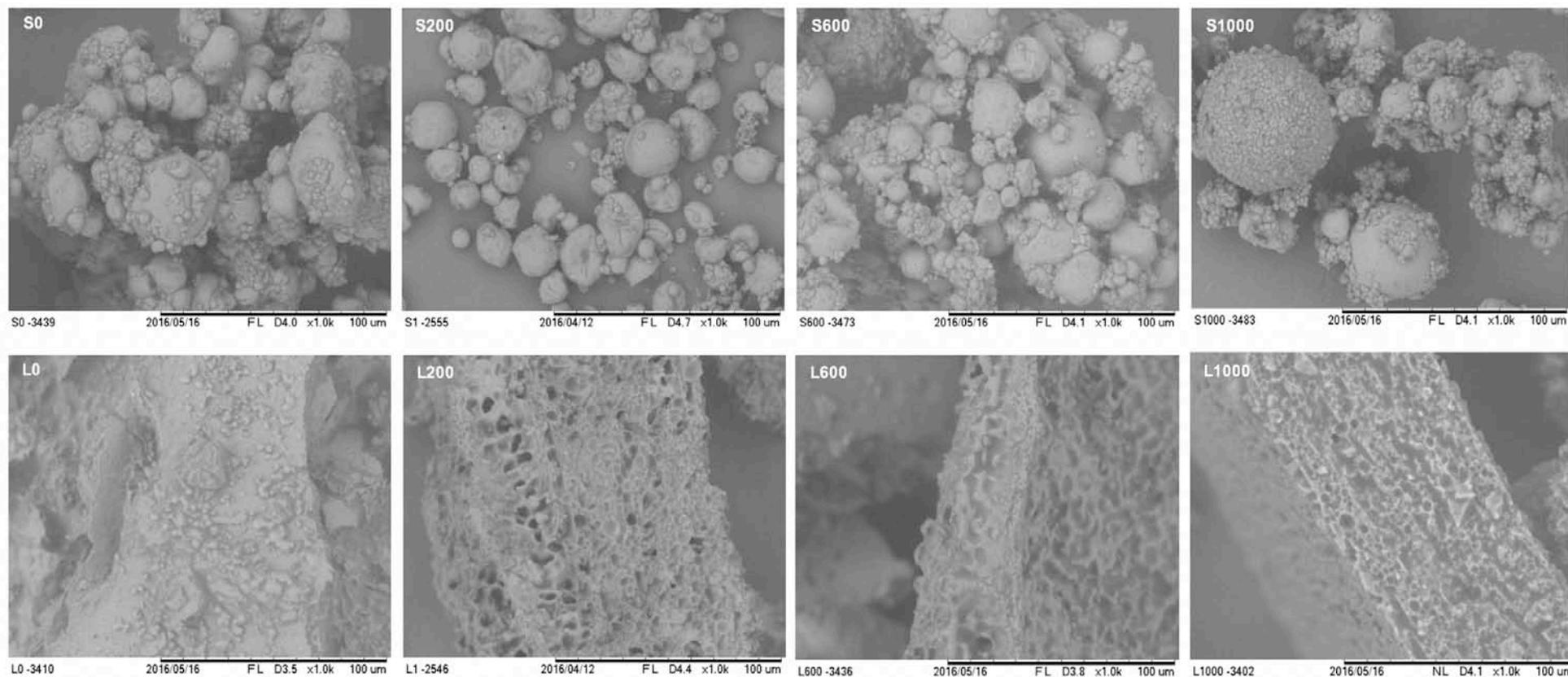
3.3.2. Morphology of microparticles by scanning electron microscopy

The morphology of the microparticles was evaluated by scanning electron microscopy and are presented in Figure 4.2.

Regarding to microparticles obtained by spray drying, it was not possible to observe difference between the ones produced with different concentrations of sinapic acid. In general, the microparticles showed varying size, rounded shape and surface with formation of concavities (Figure 4.2). This formation of concavities may be explained due to shrinkage of the particles due to evaporation of water during drying in the atomizer (COMUNIAN et al., 2011).

Botrel et al. (2014) and Solval et al. (2016) studied the optimization of drying of fish oil by spray drying using inulin as wall material and the physicochemical properties of salmon oil encapsulated by spray drying using powdered egg white, respectively, and obtained morphology similar to that obtained for particles containing echium oil.

Figure 4. 2. Micrographs obtained by scanning electron microscopy of dried treatments by spray dryer: S0, S200, S600 and S1000 (with 0, 200, 600 and 1000 ppm of sinapic acid, respectively); and treatments dried by freeze dryer: L0, L200, L600 and L1000 (with 0, 200, 600 and 1000 ppm of sinapic acid, respectively).



Reference: Own source.

Regarding to the freeze-dried treatments, it was also not observed difference in the morphology of these treatments according to the way of sinapic acid addition, since non-defined shape particles were obtained. The rounded shape of particles obtained by spray drying has the advantage of facilitating the application and the flow this material, properties that are not possible with the material obtained by lyophilization. On the other hand, the drying by lyophilization does not use high temperatures as the atomization process, which decreases the chance of degradation of oil by oxidation.

3.3.3. Water activity, moisture, solubility and hygroscopicity

The values of water activity, moisture, hygroscopicity and solubility of each treatment are shown in Table 4.1.

Regarding to the water activity (A_w), values within the range from 0.091 to 0.365 were obtained, values considered low, ideal for storage of material since it ensures microbiological stability. There were significant differences between the dried treatments by different methods; lyophilized particles presented lower water activity values than atomized ones. This can be explained due to the rapid drying process in the atomizer; it is related to a certain amount of unbounded water still remaining in the sample. Comunian et al. (2016a) (Chapter 2) also coencapsulated echium oil with phenolic compounds, however by complex coacervation technique using gelatin and arabic gum as wall materials and obtained water activity values larger than the values obtained for particles of this work, being within the range from 0.36 to 0.51. Solval et al. (2016) encapsulated salmon oil by spray drying using powder

egg white and obtained water activity values between 0.21 and 0.27, values which, even with different wall material, corroborated with those obtained for the echium oil particles.

In relation to the moisture and hygroscopicity, values within the range from 3.22 to 4.89% and from 1.32 to 2.38 g water/ 100 g sample were obtained, respectively. These values are considered low and facilitate storage and handling. The moisture values were similar for treatments atomized and freeze-dried. Anwar & Kunz (2011) studied the influence of spray drying and freeze drying of fish oil microcapsules and obtained moisture values from 2.23 to 3.01% and 2.7 to 3.23% for treatments dried by atomization and freeze drying, respectively, values which are lower than those obtained for the echium oil particles. Comunian et al. (2016a) (Chapter 2) also evaluated the moisture of echium oil microcapsules with phenolic compounds obtained by complex coacervation using gelatin and arabic gum as wall materials, and obtained moisture values from 6.9 to 9.4%, values nearly twice than obtained for the atomized and freeze dried echium oil particles. In the case of Botrel et al. (2014) and Polavarapu et al. (2011), moisture values in the maximum of 1.89% and within the range from 1.62 to 1.83% were obtained, respectively, for fish oil particles dried by spray dryer using inulin as carrier and for fish oil particles also obtained by atomization, however using beet sugar pectin as wall material. These low values of moisture and water activity are important to ensure the stability of atomized products, because, according to Rosenberg et al. (1990), the increased moisture of the particles can cause the merging of them, reducing the retention of encapsulated material.

According to Nadeau & Puiggali (1995), the hygroscopicity can be defined as the measure of food ability to retain moisture from the environment and it is extremely important for the storage of products, especially for powders. The hygroscopicity values obtained for the echium oil particles were from 5 to 7 times smaller than that obtained by Botrel et al. (2014) (from 9.1 to 11.9 g/100g of sample). This large difference can be explained due to different wall materials and parameters used during drying.

In relation to the solubility, values within the range from 57 to 68% were obtained. This high-water solubility is a positive factor in the case of echium oil particles since it indicates that the presence of arabic gum provides dispersibility of the oil in water, facilitating its use in foods. Botrel et al. (2014) encapsulated fish oil by spray drying using inulin as wall material and obtained solubility values within the range from 71.9 to 84.2%, greater values than those obtained for the echium oil particles, probably due to the different materials used as carrier agents.

Regarding to the incorporation of sinapic acid, it is important to emphasize that its application did not influence on the water activity, moisture, solubility and hygroscopicity of echium oil particles. Even presenting statistical difference, as shown in Table 4.1, this difference can be considered minimal, not affecting the final product.

Table 4. 1. Values of water activity (Aw), moisture, solubility, hygroscopicity, yield of the drying process, average particle size and oxidation index for each treatment.

Treatments	Aw	Moisture (%)	Solubility (%)	Hygroscopicity (g of water/100 g of sample)	Yield of the drying process (%)	Average particle size (µm)	Oxidation index (horas)
S0	0.274±0.06 ^b	4.44±0.50 ^{abc}	64.27±1.75 ^{ab}	1.32±0.09 ^a	60.82 ± 3.89 ^{bc}	5.14 ± 2.81 ^a	8.14 ± 0.03 ^{cd}
S200	0.365±0.11 ^a	4.58±0.09 ^{abc}	68.53±2.70 ^a	1.58±0.21 ^a	55.52 ± 7.73 ^c	2.41 ± 0.53 ^a	7.88 ± 0.51 ^d
S600	0.281±0.03 ^{ab}	4.65±0.60 ^{ab}	62.64±0.98 ^{ab}	1.38±0.09 ^a	52.07 ± 1.63 ^c	3.28 ± 0.98 ^a	8.83± 0.15 ^{cd}
S1000	0.268±0.03 ^b	3.94±0.20 ^{cd}	60.73±2.97 ^{ab}	1.55±0.56 ^a	49.04 ± 3.96 ^c	2.32 ± 1.57 ^a	9.53 ± 0.71 ^{bcd}
L0	0.091±0.01 ^d	3.22±0.36 ^e	60.76±3.79 ^{ab}	2.38±0.72 ^a	79.09 ± 2.68 ^a	--	10.49 ± 0.21 ^{abc}
L200	0.146±0.01 ^{cd}	3.50±0.07 ^{de}	56.42±2.27 ^b	1.85±0.06 ^a	82.93 ± 6.18 ^a	--	11.71 ± 0.74 ^{ab}
L600	0.180±0.01 ^c	4.19±0.30 ^{bc}	59.08±1.96 ^b	1.87±0.08 ^a	83.27 ± 2.09 ^a	--	12.94 ± 1.03 ^a
L1000	0.201±0.01 ^{bc}	4.89±0.39 ^a	57.86±3.00 ^b	2.31±0.19 ^a	77.98 ± 4.11 ^{ab}	--	11.55 ± 1.00 ^{ab}
Pure oil	--	--	--	--	--	--	5.31 ± 0.02 ^e

Reference: Own source.

Equal letters in the same column do not differ at 5% of probability by Tukey test.

- S0, S200, S600 and S1000: Particles obtained by spray drying with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil.
- L0, L200, L600 and L1000: Particles obtained by freeze drying with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil.

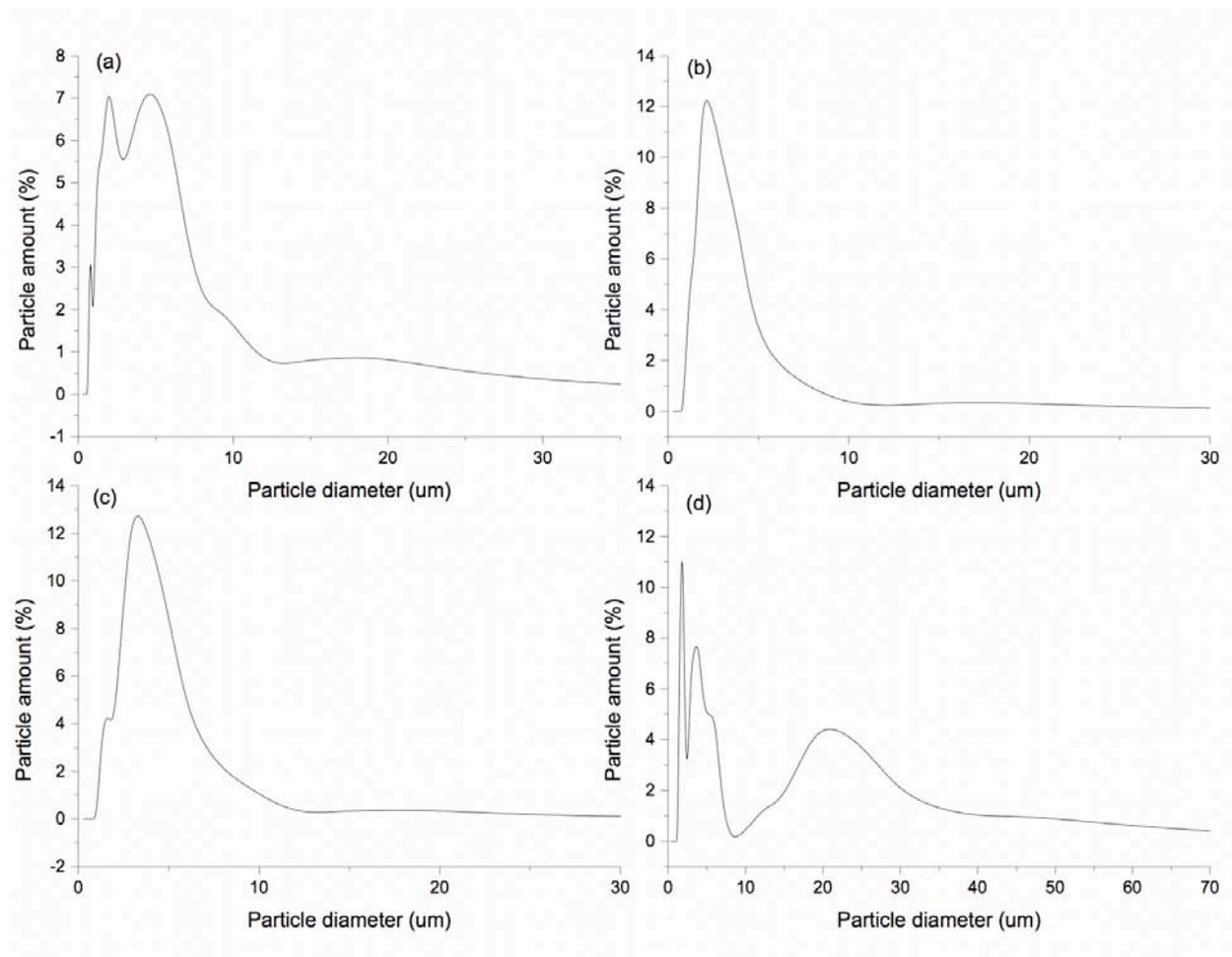
3.3.4. Particle size and particle size distribution for particles obtained by spray drying

The average particle size values and particle size distribution of each treatment dried by atomization are shown in Table 4.1 and Figure 4.3. Values of particle size were within the range from 2.3 to 5.14 μm and presented no significant difference between the treatments, in other words, different sinapic acid concentrations did not affect the average particle size.

The treatments with 200 and 600 ppm of sinapic acid showed particle size distribution with unimodal behavior, while the treatments without sinapic acid and 1000 ppm of this phenolic compound showed multimodal behavior with three populations, i.e., the particles were not homogenous, result from the agglomerating of them. According to Comunian et al. (2011), it can be considered a positive factor since smaller particles occupy the space among the larger, reducing the total volume.

Values of particle sizes from 9.6 to 13.7 μm were obtained by Botrel et al. (2014) when fish oil was atomized using inulin as wall material. Polavarapu et al. (2011) obtained particle sizes from 0.41 to 0.43 μm when they studied the physicochemical characteristics of fish oil encapsulated by atomization using pectin from beet sugar as carrier. This wide range can be explained due to the different compositions of treatments and process parameters used.

Figure 4. 3. Particle size distribution of the atomized treatments: a) S0 (without addition of sinapic acid); b) S200 (200 ppm of sinapic acid); c) S600 (600 ppm of sinapic acid) and d) S1000 (1000 ppm of sinapic acid).



Reference: Own source.

3.3.5. Thermogravimetric analysis (TGA)

According to Yang et al. (2015), thermogravimetric analysis is being widely used to evaluate the thermal stability and the weight loss of different materials according to a wide range of applied temperature.

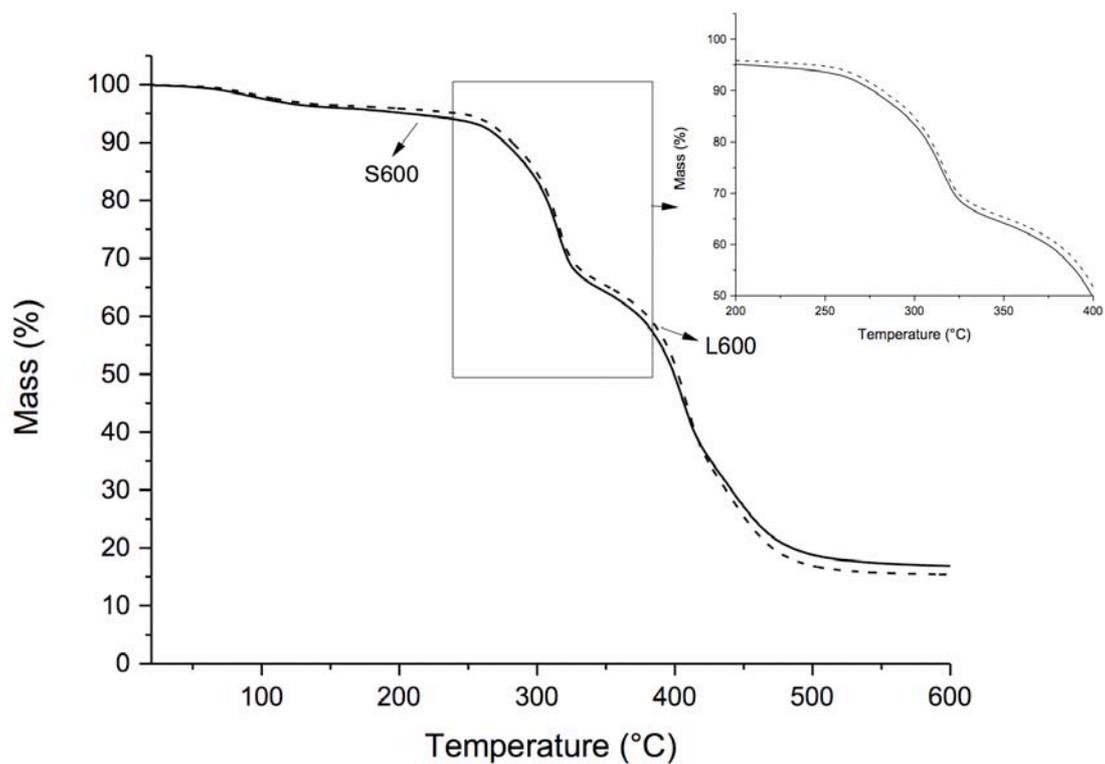
The echium oil particles submitted to the same type of drying showed no difference in thermogravimetric behavior, in other words, different sinapic acid concentrations did not influence on weight loss during the temperature range analyzed, mainly even up to the temperature of 250 °C, since the high temperatures during food processing do not exceed 150-160 °C.

In relation to different drying techniques, it can be stated that the particles obtained by spray drying exhibited behavior slightly weaker than the particles obtained by freeze drying, as shown in Figure 4.4, with treatments S600 and L600; this can be explained due to the conditions used during these drying processes: high temperature and exposure to oxygen in the drying by spray dryer and low temperature and vacuum in the drying by lyophilization. This result corroborates with those obtained by accelerated oxidation by Rancimat (item 3.3.7), which shows that particles obtained by freeze-drying showed longer induction time than particles obtained by spray drying, i.e., greater oxidative protection to the encapsulated oil.

As seen in the curves in Figure 4.4, the treatments showed three weight loss steps. The first step is observed due to the loss of water in the range from 25 to 100 °C. The other two steps are observed within the range from 250 to 400 °C and 400 to 500 °C. According to Xiao et al. (2015) and Yang et al. (2015), these last two steps of weight loss can be attributed to the

decomposition of materials, in this case, arabic gum and echium oil. The same behavior with three weight loss steps was obtained by He et al. (2016) when they studied the stability and controlled release of conjugated linoleic acid in spray-dried particles using OSA-modified starch and xanthan gum as wall materials. Moreover, the fact that the particles were stable up to temperature of 250 °C indicates that the method of emulsion followed by spray drying or freeze drying was feasible for oil protection.

Figure 4. 4. TG curves for treatments S600 and L600 (treatments with 600 ppm of sinapic acid, submitted to spray drying and freeze drying, respectively).



Reference: Own source.

3.3.6. X-ray diffraction

The x-ray diffraction analyzes are widely used in studies of microencapsulation in order to check the state of the material (crystalline or amorphous). The diffractograms obtained for the echium oil and sinapic acid particles produced by spray drying or lyophilization, showed broad peaks, indicating that this material has an amorphous phase. This behavior was expected since the material used as carrier agent is arabic gum (Figure 4.5a and 4.5b).

Different characteristics were obtained by Cerimedo et al. (2014) when they encapsulated fish oil by emulsion using trehalose and sodium caseinate as wall materials followed by freeze drying. In this case, the samples showed crystalline characteristics due to the presence of trehalose. According to Cerimedo et al. (2014) and Buera et al. (2005), the protection function of the matrix which surrounds the encapsulated material is lost when in the crystalline state due to the release of the encapsulated material. In other words, the amorphous characteristic of echium oil particle is a positive factor in the protection of this oil.

3.3.7. Accelerated oxidation by Rancimat

The analysis of accelerated oxidation by Rancimat was performed in order to verify the oxidation of the oil encapsulated by emulsion followed by different drying techniques, besides the different concentrations of sinapic acid. The values obtained for the induction time used as oxidative stability index,

measured in hours, are presented in Table 4.1 and the performance of the oxidative stability of the microparticles is shown in Figures 4.5c and 4.5d.

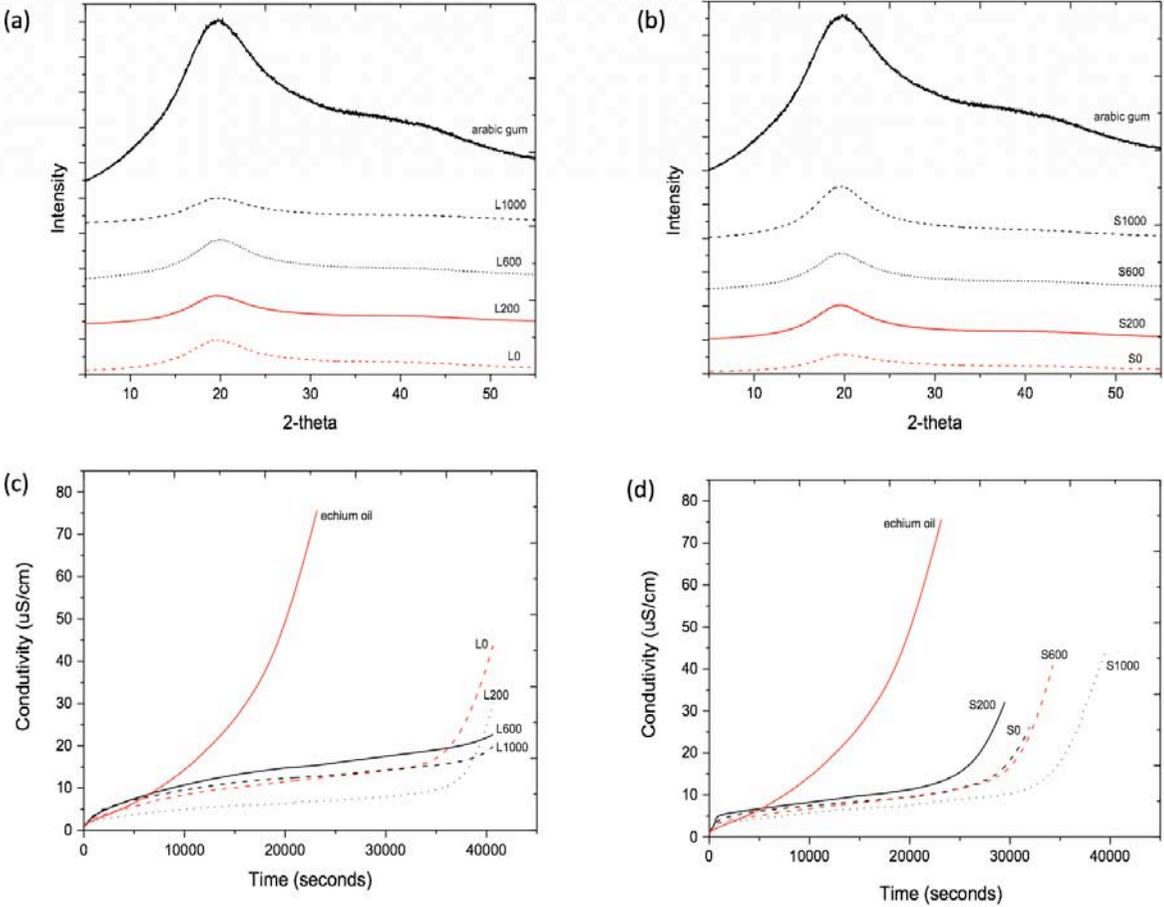
The spray dried treatments showed oxidation index values within the range from 7.8 to 9.5 hours, while the pure oil oxidized in 5.3 hours. In other words, the encapsulation and spray drying technique increased the oxidative stability of oil in 3 hours. The freeze-dried treatments showed oxidation index values between 10.4 and 12.9 hours. Therefore, the encapsulation process by emulsion followed by lyophilization doubled the oxidative stability of the oil. Thus, the freeze-drying process was more effective than spray drying. This can be explained due to high exposure to oxygen at the time of nebulization in the spray dryer, which does not happen with freeze-dried particle. Furthermore, due to the smaller particle size obtained by atomization, they have higher exposure area, resulting in degradation of the oil. It is also important to note that the high temperature used in the spray dryer is crucial to start the oxidation process, mainly to compounds which are sensitive to heat, such as echium oil. In addition, according to Anwar & Kunz (2011), during the spray drying process, there is the breakdown of the large emulsion droplets, which results in a higher remaining oil on the surface of particles, and consequently the oil is exposed to oxygen and therefore subject to oxidation.

Regarding to the application of sinapic acid as antioxidant, there was no statistical difference among the treatments that were submitted to the same drying technique. In other words, different concentrations of the phenolic compound did not significantly differ on oxidative stability index values. Thus, it can be said that only the freeze-drying technique was enough to promote oil protection.

Gallardo et al. (2013) studied linseed oil microencapsulation by spray drying using arabic gum, maltodextrin, methylcellulose and whey protein isolate as carrier agents and evaluated the accelerated oxidation of these particles, resulting in oxidation index values from 3.8 to 9.5 hours, while the pure linseed oil showed value of 2.04 hours. Encapsulated echium oil of this work showed values of oxidation index of 12.9 hours, in other words, the encapsulation by emulsion followed by lyophilization was more effective than spray drying technique used by Gallardo et al. (2013).

Comunian et al. (2016a and 2016b) (Chapters 2 and 3) studied the microencapsulation of echium oil by complex coacervation using gelatin-arabic gum and gelatin-cashew gum as wall materials, respectively, and obtained oxidation index values within the range from 9.97 to 18.77 hours and from 8.7 to 26.5 hours, respectively, values higher than those obtained for the echium oil particles atomized and lyophilized. This difference can be explained due to the higher concentration of sinapic acid used in coacervated capsules and obtainment of a more rigid capsule, since the wall is formed of two polymers linked by ionic interaction and not only arabic gum. However, even so, the values obtained in this work are satisfactory since the encapsulated oil was much more stable than free oil.

Figure 4. 5. X-ray diffraction of particles obtained by (a) lyophilization with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil and (b) spray drying with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil and behavior of accelerated oxidation of pure oil and particles obtained by: (c) lyophilization with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil and (d) spray drying with 0, 200, 600 and 1000 ppm of sinapic acid in relation to the oil.



Reference: Own source.

4.CONCLUSIONS

The coencapsulation of echium oil and sinapic acid by emulsion followed by spray drying or freeze drying were suitable techniques for the oxidative protection of the oil, since all the treatments promoted protection of it; the treatment in which 600 ppm of acid sinapic was added and the particle dried by lyophilization (L600) increased oxidative stability of the encapsulated oil in 2.5 times in relation to the free one. Furthermore, the morphology and values of hygroscopicity, moisture and solubility were optimum for the application, storing and handling of the powder.

5.ACKNOWLEDGEMENTS

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**Chapter 5. COMBINATION OF MICROFLUIDIC DEVICES, IONIC GELATION
AND PHENOLIC COMPOUNDS TO IMPROVE THE OXIDATIVE STABILITY
OF ECHIUM OIL**

(PAPER PUBLISHED IN FOOD CHEMISTRY – ATTACHMENT D)

CHAPTER 5. COMBINATION OF MICROFLUIDIC DEVICES, IONIC GELATION AND PHENOLIC COMPOUNDS TO IMPROVE THE OXIDATIVE STABILITY OF ECHIUM OIL

ABSTRACT

Echium oil is rich in omega-3 fatty acids, which are important because of their benefits to human health; it is, however, unstable. The objective of this work was the coencapsulation of echium oil and quercetin or sinapic acid by microfluidic and ionic gelation techniques. The treatments were analyzed utilizing optical and scanning electron microscopy, encapsulation yield, particle size, thermogravimetry, Fourier transform infrared spectroscopy, stability under stress conditions, and oil oxidative/phenolic compound stability for 30 days at 40 °C. High encapsulation yield values were obtained (91–97% and 77–90% for the phenolic compounds and oil) and the encapsulated oil was almost seven times more stable than the non-encapsulated oil (0.34 vs 2.42 mg MDA/kg oil for encapsulated and non-encapsulated oil, respectively). Encapsulation was shown to promote oxidative stability, allowing new vehicles for the application of these compounds in food without the use of solvents and high temperature.

Keywords: omega-3 fatty acids, sinapic acid, quercetin, microencapsulation, double emulsion, sodium alginate

1. INTRODUCTION

Omega-3 fatty acids (ω -3) are widely used in food and pharmaceutical products due to their benefits to human health (GHORBANZADE, JAFARI, AKHAVAN, & HADAVI, 2017). A source of ω -3 is the echium seed oil (*Echium plantagineum* L.), which contains from 9 to 16% stearidonic fatty acid, an intermediate in the biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), also important for human nutrition (CLOUGH, 1993; BERTI; JOHNSON; DASH; FISCHER, & WILCKENS, 2007; PAYNE; LAD; FOSTER; KHOLA, & GRAY, 2014). Echium oil also has a ratio of omega-3 to omega-6 ideal for health, which is not found in any another type of oil (BERTI; JOHNSON; DASH; FISCHER, & WILCKENS, 2007). The use of this oil in the food industry is hampered due to the instability of its unsaturated fatty acids when they come into contact with light, oxygen and heat. Two strategies that could minimize these limitations, singly or together, are: (1) adding compounds with antioxidant activity—such as phenolic compounds—or (2) microencapsulation.

Phenolic compounds have been of great interest in the food industry due to their activity as antioxidant agents (ABDALLAH et al., 2011; LEE et al., 2013), in addition to their other health benefits, such as the prevention of cancer, inflammation and neurodegenerative diseases (ESFANJANI & JAFARI, 2016). Sinapic acid is the major phenolic acid of canola (RAWEL & ROHN, 2010) and has been studied for its neuro-protective effects against Alzheimer's disease (LEE et al., 2012), cardiac hypertrophy and dyslipidemia (PARI & JALALUDEEN, 2011; ROY & PRINCE, 2013). Quercetin, another phenolic compound, is one of the most common flavonoids present in nature and has

exhibited anti-stress, anti-inflammatory and anti-cancer properties (WATERHOUSE et al., 2014).

Microencapsulation consists of a broad class of techniques in which one or more bioactive materials are contained or immobilized by one or more polymers or lipids, protecting the encapsulated material against environmental conditions to facilitate handling, application and storage of these materials (COMUNIAN & FAVARO-TRINDADE, 2016). Ionic gelation is one such technique, often used to form microparticles whose wall material is composed of a gel-forming polymer, not requiring the use of solvents or high temperatures. Ionic gelation, however, does not afford good control of particle size (which can subsequently range from μm to mm), which can negatively affect resultant food texture. By coupling ionic gelation with microencapsulation using microfluidics, this limitation could be overcome.

Microencapsulation by microfluidics is a promising method for the production of monodisperse droplets—not to mention capsules with multiple compartments— which allows greater control and optimization of the encapsulation efficiency and release of active agents (ZHAO et al, 2011; COMUNIAN; ABBASPOURRAD; FAVARO-TRINDADE; & WEITZ, 2014). A microfluidic device consists of coaxial assemblies of a series of rigid glass capillaries resistant to chemicals; their three-dimensional geometry allows for the controlled production of multiple emulsions. This technique has been little explored for the encapsulation of food ingredients.

The objective of this work was the encapsulation of echium oil by using a combination of ionic gelation and microfluidic techniques. In addition, a phenolic compound (quercetin or sinapic acid) was also tested at various concentrations,

offering benefits to the consumer's health, protection to the encapsulated compounds and the possibility of controlled release. No research related to the encapsulation of these compounds by the combination of these technologies was found in the literature, which suggests the innovative nature of this study.

2. MATERIAL AND METHODS

2.1. Materials

Echium oil (NEWmega™ Echium Oil, Ref.15200, De Wit Specialty Oils, De Waal, Tescel, The Netherlands) was used as core. Sinapic acid and quercetin (Sigma Chemical Co., St. Louis, MO, USA), sodium alginate (Manugel GHB, FMC/ Philadelphia, Pennsylvania), corn oil (Mazola, ACH Food Companies), soy lecithin Ultralect P (ADM/ Decatur, IL, USA) and Tween 80 (Pepsico/ Delaware, USA) were used as antioxidants, wall material, continuous phase, and hydrophobic and hydrophilic surfactant, respectively. Calcium chloride hexahydrate (Fisher Scientific, Waltham, MA, USA) and EDTA disodium salt (Fisher Scientific/ Waltham, MA USA) were used for the formation of a complex that would promote the gelation of the polymer.

2.2. Methods

2.2.1. Microencapsulation process

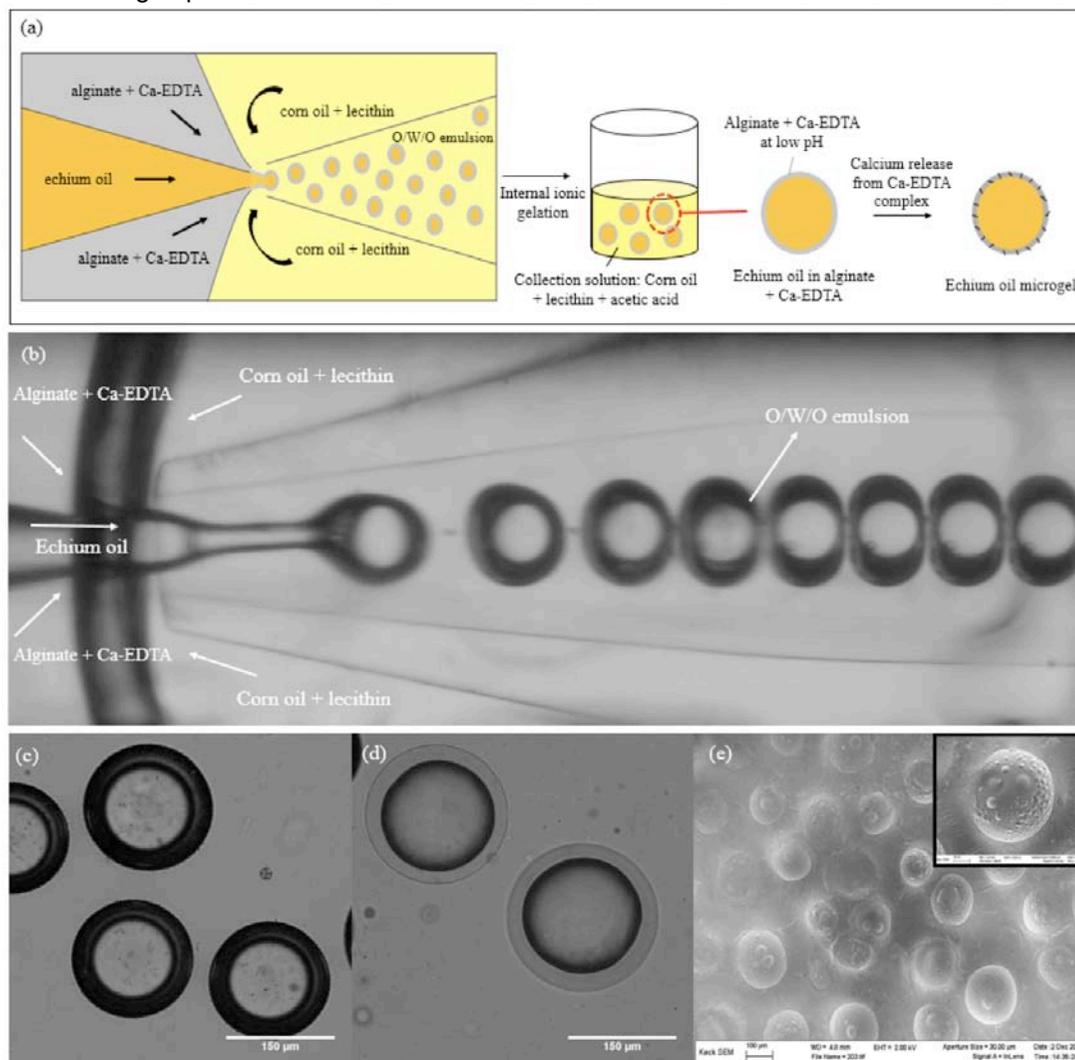
The encapsulation process was performed using a glass microfluidic device (Figures 5.1a and 5.1b) as described by Comunian, Abbaspourrad, Favaro-Trindade, & Weitz, (2014). The cylindrical capillaries used (World Precision Instruments, Inc., Sarasota, Florida, United States) had a diameter and length of 1 mm and 6 in, respectively. These capillaries were inserted into

a square capillary (Harvard Borosilicate Square Tubing), with outer and inner diameters of 1.5 and 1.05 mm, respectively. An oil-in-water-in-oil (O/W/O) double emulsion was produced using this device, composed of echium oil in the internal oil phase, sodium alginate aqueous solution in the middle phase, and corn oil and soy lecithin in the continuous phase.

Five treatments were performed, which either had quercetin added to the inner phase or sinapic acid added to the middle phase, or no phenolic compound added at all (see Table 5.1). The flow rates used were 1300, 870, and 5500 $\mu\text{L/h}$ for the inner, middle and continuous phases for both the control treatment and the S050-Alg treatment; 1300, 870, and 5700 $\mu\text{L/h}$ for the Q500-Alg treatment; 1300, 970, and 5300 $\mu\text{L/h}$ for the S025-Alg treatment; and 1500, 870, and 5000 $\mu\text{L/h}$ for the Q800-Alg treatment. The flow rates were determined in preliminary testing, pumped into the microfluidic device using a syringe pump (New Era Pump Systems, Inc/ Farmingdale, New York, USA).

In combination with the microfluidic technique, the internal ionic gelation technique was used according to Utech et al. (2015). In order to prevent the coalescence of the microcapsules and to obtain a faster gelation process for sodium alginate, a calcium-EDTA complex was prepared by mixing a solution of calcium chloride and a solution of EDTA disodium salt, both at 0.1M. This complex was added to a 2% (w/w) solution of sodium alginate, in equal ratios, resulting in a final alginate concentration of 1% (w/w). The pH of the solution was adjusted to 10. The double emulsion droplets were collected in a plastic beaker containing corn oil, 2% (w/w) soy lecithin and 5% (v/v) glacial acetic acid, with constant stirring, which promoted the gelation of sodium alginate and the formation of microcapsules.

Figure 5. 1. (a) Graphical representation of the encapsulation process: combination of microfluidic device and ionic gelation; optical microscope image of (b) a glass microfluidic device during the production of double emulsions; (c) Q800-Alg capsules before gelation, (d) Q800-Alg capsules after gelation and (e) scanning electron microscope image of S050-Alg capsules.



Reference: Own source.

Table 5. 1. Composition of each treatment, encapsulation yield for the phenolic compounds and for the oil and average particle size before and after gelation of sodium alginate.

Treatments	Inner phase	Middle phase	Continuous phase	Encapsulation yield for the phenolic compounds (%)	Encapsulation yield for the oil (%)	Average particle size before gelation (μm)	Average particle size after gelation (μm)
Control	Echium oil	Sodium alginate	Corn oil + 2% (w/w) soy lecithin	--	85.39 \pm 10.74 ^a	117.22 \pm 12.32 ^e	115.56 \pm 19.27 ^d
S025-Alg	Echium oil	Sodium alginate + 0.025g sinapic acid/ g alginate	Corn oil + 2% (w/w) soy lecithin	91.33 \pm 18.74 ^a	83.29 \pm 10.56 ^a	213.86 \pm 25.84 ^a	227.55 \pm 21.40 ^a
S050-Alg	Echium oil	Sodium alginate + 0.05g sinapic acid/ g alginate	Corn oil + 2% (w/w) soy lecithin	94.04 \pm 23.19 ^a	77.81 \pm 1.80 ^a	148.03 \pm 24.21 ^c	155.56 \pm 26.71 ^c
Q500-Alg	Echium oil + 500 ppm* of quercetin	Sodium alginate	Corn oil + 2% (w/w) soy lecithin	97.90 \pm 4.60 ^a	90.61 \pm 3.99 ^a	135.97 \pm 15.33 ^d	155.71 \pm 17.89 ^c
Q800-Alg	Echium oil + 800 ppm* of quercetin	Sodium alginate	Corn oil + 2% (w/w) soy lecithin	93.19 \pm 13.98 ^a	82.47 \pm 1.41 ^a	185.34 \pm 15.98 ^b	196.77 \pm 22.91 ^b

Reference: Own source.

*500 and 800 ppm in relation to the oil weight

Equal letters in the same column do not differ statistically at a 5% level by the Tukey test.

control: echium oil encapsulated without phenolic compounds;

S025-Alg: echium oil encapsulated with 0.025g of sinapic acid/ g of sodium alginate;

S050-Alg: echium oil encapsulated with 0.055g of sinapic acid/ g of sodium alginate;

Q500-Alg: echium oil encapsulated with 500 ppm of quercetin in relation to the mass of oil;

Q800-Alg: echium oil encapsulated with 800 ppm of quercetin in relation to the mass of oil.

During production of the O/W/O double emulsion (Figure 5.1a and 5.1b), after the sodium alginate gelation, the microcapsules were transferred from an oil medium to a water medium to facilitate handling and control of the material. After sedimentation of the capsules, they were transferred with a pipette into an aqueous solution with 1% (w/w) Tween 80 and centrifuged at 4000 g for 10 minutes to separate the oil and aqueous phases. Due to the difference in density, the microcapsules remained in the aqueous phase. The aqueous solution was washed several times to remove Tween 80 from the media. For specific characterization analysis, the aqueous phase with the microcapsules was frozen in an ultra-freezer (-80 °C) and lyophilized (LabConco freeze-dryer, LabConco Corporation/ Kansas city, MO) for 24 hours to produce a powder.

2.2.2. Encapsulation yield of sinapic acid, quercetin and oil

The yield was determined as a function of the mass difference between the total amount used in the encapsulation process and the amount of compound actually retained in the microcapsules after the process. The capsules were ruptured by adding a 0.1M EDTA disodium salt solution and then it was mixed using a vortex mixer for 1 minute. For the encapsulation yield of the phenolic compounds, methanol was added to the mixture in order to extract them. The samples were stirred with vortex again for 1 minute and centrifuged (Heraeus Multifuge X1R Centrifuge, Thermo Scientific/ Waltham, Massachusetts – USA) at 5000 g for 3 minutes at room temperature. The concentrations of sinapic acid and quercetin were determined in the supernatant using UV-Vis spectrophotometry (UV-Vis Spectrophotometer UV-

2600, Shimadzu Scientific Instruments/ Marlborough, Massachusetts – USA) at wavelengths of 321 and 371 nm for sinapic acid and quercetin, respectively.

For the encapsulation yield of echium oil, after the capsule rupture, hexane was added to the solution in order to extract the oil, then the solution was mixed with a vortex for 1 minute. The sample was centrifuged (Heraeus Multifuge X1R Centrifuge, Thermo Scientific/ Waltham, Massachusetts – USA) at 8000 g for 3 minutes at room temperature, and the supernatant containing hexane and oil was transferred to an eppendorf and maintained at 60 °C until complete evaporation of the solvent. The same extraction process was performed twice to ensure that all the oil was quantified.

2.2.3. Morphology of the microcapsules by optical and scanning electron microscopy

The optical images were obtained using an inverted optical microscope (DMIL LED, Leica) connected to a fast camera (MicroLab 3a10, Vision Research). The scanning electron microscopy images of the microcapsules were obtained using a scanning electron microscope (LEO 1550 FESEM (Keck SEM) (Carl Zeiss, New York/ USA)). Before taking the SEM images, the wet emulsions were placed on the SEM stub and allowed to dry for 24 hours at room temperature.

2.2.4. Particle size and size distribution

The particle size and particle size distribution analyses were performed using ImageJ (NIH, Bethesda, MD), where 100 microcapsules for each

treatment were measured individually. The images were obtained by inverted optical microscopy (DMIL LED, Leica).

2.2.5. Thermogravimetric analysis

TGA curves were obtained using a Q500 Thermogravimetric Analyzer (TA Instruments/ New Castle, DE). The wet samples were conditioned on a platinum support and nitrogen was used in the atmosphere of the furnace, at a flow rate of 60 mL/ min, with a heating rate of 10 °C/ min, starting from 25 to 600°C.

2.2.6. Oxidative stability by FTIR

The spectra of echium seed oil and the microcapsules were obtained in the 4000 to 650 cm^{-1} region, using 16 scans at a resolution of 4 cm^{-1} , using the IRAffinity- 1S Fourier Transform Infrared Spectrophotometer (Shimadzu Scientific Instruments/ Marlborough, Massachusetts - USA). The freeze-dried samples were stored for 0, 7, 15, 21 and 30 days at 40 °C prior to reading. The formation of lipid hydroperoxides, the primary oxidation products, was monitored according to Haider, Majeed, Williams, Safdar and Zhong (2017).

2.2.7. Microcapsule stability under different stress conditions

The wet microcapsules were submitted to various stress conditions, including temperature (10, 20, 30, 40, 50, 60 and 70 °C), pH (2, 4, 6 and 8) and NaCl concentration (1, 3 and 5% w/w) following from work by Comunian et al. (2016b) (Chapter 3). In addition, the samples were also freeze-dried and

resuspended in water. The structures of the microcapsules were evaluated using an inverted optical microscope (DMIL LED, Leica).

2.2.8. Stability of quercetin and sinapic acid

The levels of sinapic acid and quercetin were monitored by UV-Vis spectrophotometry as mentioned in section 2.2.2. Analyses were performed in triplicate, at 0, 7, 15, 21 and 30 days after encapsulation, with the wet material stored in glass vials, protected from light, in the presence of O₂ at 40 °C. In addition to the levels of phenolic compounds inside the microcapsules, the free phenolic compounds were also analyzed in solution, submitted to the same storage conditions.

2.2.9. Oxidative stability by thiobarbituric acid reactive substance (TBARS)

The formation of malonaldehyde, the second oxidation product, was monitored by TBARS analysis, according to Gray, Payne, McClements, Decker, & Lad (2010) and Gomez-Estaca, Comunian, Montero, Ferro-Furtado, & Favaro-Trindade (2016) with the wet samples stored at 0, 7, 15, 21 and 30 days at 40 °C. First, the microcapsules were ruptured as mentioned in section 2.2.2. The samples were then added to an aqueous solution containing 15% (w/w) trichloroacetic acid, 0.375% (w/w) thiobarbituric acid and 2% (w/w) HCl, and kept in a water bath at 90 °C for 15 minutes. An ice bath was used in order to stop the reaction. Butanol was added to the solution, stirred with vortex for 1 minute and then centrifuged at 5000 g for 10 minutes at room temperature. The

supernatant was analyzed by UV-Vis spectrophotometry (UV-Vis Spectrophotometer UV-2600, Shimadzu Scientific Instruments/ Marlborough, Massachusetts – USA) at 532 nm. The same was also done for pure echium oil. The standard curve was made using 1,1,3,3-tetraethoxypropane and the solution used as a blank was submitted to the entire process without the addition of the microcapsules.

2.2.10. Statistical analysis

Data were analyzed using SAS statistical software (Statistical Analysis System, SAS Institute, Cary, NC), version 9.0, using ANOVA and post-hoc Tukey's tests, with statistical significance assumed at $p < 0.05$.

3. RESULTS AND DISCUSSION

Encapsulation by ionic gelation technique occurs because alginate crosslinks with divalent cations. This process can occur in two ways: internal or external gelation. In the case of internal gelation, the method used in this work, a dispersion of insoluble calcium particles is added into the alginate solution and the release of these ions is induced by the reduction of pH via the addition of acid, resulting in the gelation of alginate (LEONG et al., 2016).

For the production of echium oil microcapsules, a Ca-EDTA complex was added into the sodium alginate solution. The gelation of alginate occurred when the droplets were collected in corn oil (the same material used as continuous phase for the O/W/O emulsion) containing acetic acid. According to Utech et al. (2015), the acid diffuses into the droplets, causing the pH reduction and

consequently the dissociation of calcium ions from the Ca-EDTA complex. The released calcium ions react with the alginate chains, promoting the gelation of the polymer and then the formation of the microcapsules containing echium oil as core and sodium alginate as the wall material (Figure 5.1a).

3.1. Morphology of microcapsules by optical and scanning electron microscopy

Optical microscope images for the Q800-Alg treatment (addition of 800 ppm quercetin) before and after gelation, and a SEM image for the S050-Alg treatment (0.05 g of sinapic acid/ g alginate) are shown in Figures 5.1c, 5.1d and 5.1e, respectively. The treatments did not show any morphological differences, that is, the addition of the phenolic compounds in the inner oil phase or in the middle aqueous phase did not influence the formation of the spherical and mononucleate microcapsule. The SEM image shows the presence of particles adhering to the surface of the microcapsule. These particles can be explained by the presence of soy lecithin, which was used as an emulsifier in the continuous oil phase during the formation of the O/W/O emulsion. The emulsifiers have a hydrophilic and a hydrophobic part, which enables them to act at the water-oil interface in order to stabilize the emulsion. Thus, soy lecithin, used in the continuous oil phase, acts at the alginate-corn oil interface and—after the gelation of the polymer and the transfer of the microcapsules from an oil medium to a water medium—the lecithin remains in the wall material. The lecithin did not affect the functionality of the microcapsule, as demonstrated in later results.

Ren, Ju, Xie, & Chu (2010) produced similar microcapsules when they studied the formation of monodisperse alginate microcapsules with an oil core generated with a microfluidic device; however, because they used a combination of three devices, our control over, and stability of emulsions would be more precise.

3.2. Encapsulation yield of sinapic acid, quercetin and oil

The values of encapsulation yield for the phenolic compounds and oil are presented in Table 5.1. Values from 91 to 97% were obtained for sinapic acid and quercetin and from 77 to 90% for oil. For the two phenolic compounds, we hypothesized that the yield for sinapic acid would be lower than that for quercetin because sinapic acid was added to the aqueous phase —middle wall— and thus its release would be more likely, however this was not the case. There were no significant differences among treatments, showing that addition of a phenolic compound to either the oil phase or the aqueous phase does not influence their encapsulation yield. Considering the oil encapsulation yield, no significant differences among the treatments were observed. The presence of phenolic compounds did not interfere with the amount of encapsulated oil, which shows that the entire encapsulation process, used in combination with the addition of phenolic compounds, was efficient and suitable for the task.

So far, no research has reported the encapsulation of sinapic acid and quercetin using a combination of the microfluidic and ionic gelation techniques. A study conducted by Comunian et al. (2016a) (Chapter 2) has reported the encapsulation of echium oil and sinapic acid or rutin by complex coacervation using gelatin and arabic gum as wall materials and obtained an encapsulation

yield for sinapic acid of 39 to 50%, values almost half of those obtained using the microfluidic and ionic gelation combination employed in this study. In the case of quercetin, Vidal et al. (2016) studied the synthesis and characterization of nanoparticles for the encapsulation of quercetin using polyhydroxybutyrate-co-hydroxyvalerate as wall material by the double emulsion technique and obtained an encapsulation yield of 51% for quercetin, again around half of that obtained in this work.

Several authors have studied the encapsulation of oil; however, only two papers report the encapsulation of oil by microfluidic devices using alginate as the wall material; moreover, these studies did not examine encapsulation yield or stability of the capsules (LIU et al., 2013; REN; JU; XIE; & CHU, 2010). Comunian et al. (2016b) (Chapter 3) obtained an encapsulation yield for echium oil of 73 to 99% when it was encapsulated by the complex coacervation technique using gelatin and arabic gum as wall materials, values similar to those obtained in this work. Moreover, Ghorbanzade, Jafari, Akhavan, & Hadavi (2017) were able to encapsulate fish oil in nano-liposomes, obtaining a yield of 92.22%, which corroborates that obtained in this study for the Q500-Alg treatment.

3.3. Average particle size and particle size distribution

Values for average particle size were in the range of 117 to 213 μm before alginate gelation and 115 to 227 μm after gelation (Table 5.1). There were significant differences among treatments, suggesting the amount of added phenolic compounds had an influence on the average particle size. The control treatment produced a smaller average particle size compared to the other

treatments, which was expected, as the presence of phenolic compounds in the inner or middle phases causes an increase in solution viscosity, which may influence particle size. For the sinapic acid treatment, a higher concentration was expected to lead to a larger average particle size, because it would trigger the destabilization of the structure (section 3.5), however this did not occur. On the other hand, an increase in quercetin concentration did lead to a parallel increase in the particle size. It is important to note that even with a significant difference among the treatments, the particle size distribution for all treatments was unimodal, which demonstrates the homogeneity of the microcapsules produced.

The addition of sinapic acid to the microcapsule wall (S025-Alg treatment) resulted in a bigger particle size, as compared to the control and the treatments with quercetin. This can be explained by the influence of surface charges. Alginate has a negative charge, and both sinapic acid and calcium ions have a positive charge. For this reason, sinapic acid and the calcium ions compete for alginate, thus affecting the gelation of this polysaccharide, making the microcapsules less stable and, consequently, bigger. Particle size values within the range of 256 to 337 μm were obtained by Ren, Ju, Xie, & Chu (2010) when they studied the formation of monodisperse alginate microcapsules with an oil core using a microfluidic device with a combination of three capillaries, obtaining particle sizes bigger than those for echium oil.

3.4. Thermogravimetry analysis (TGA)

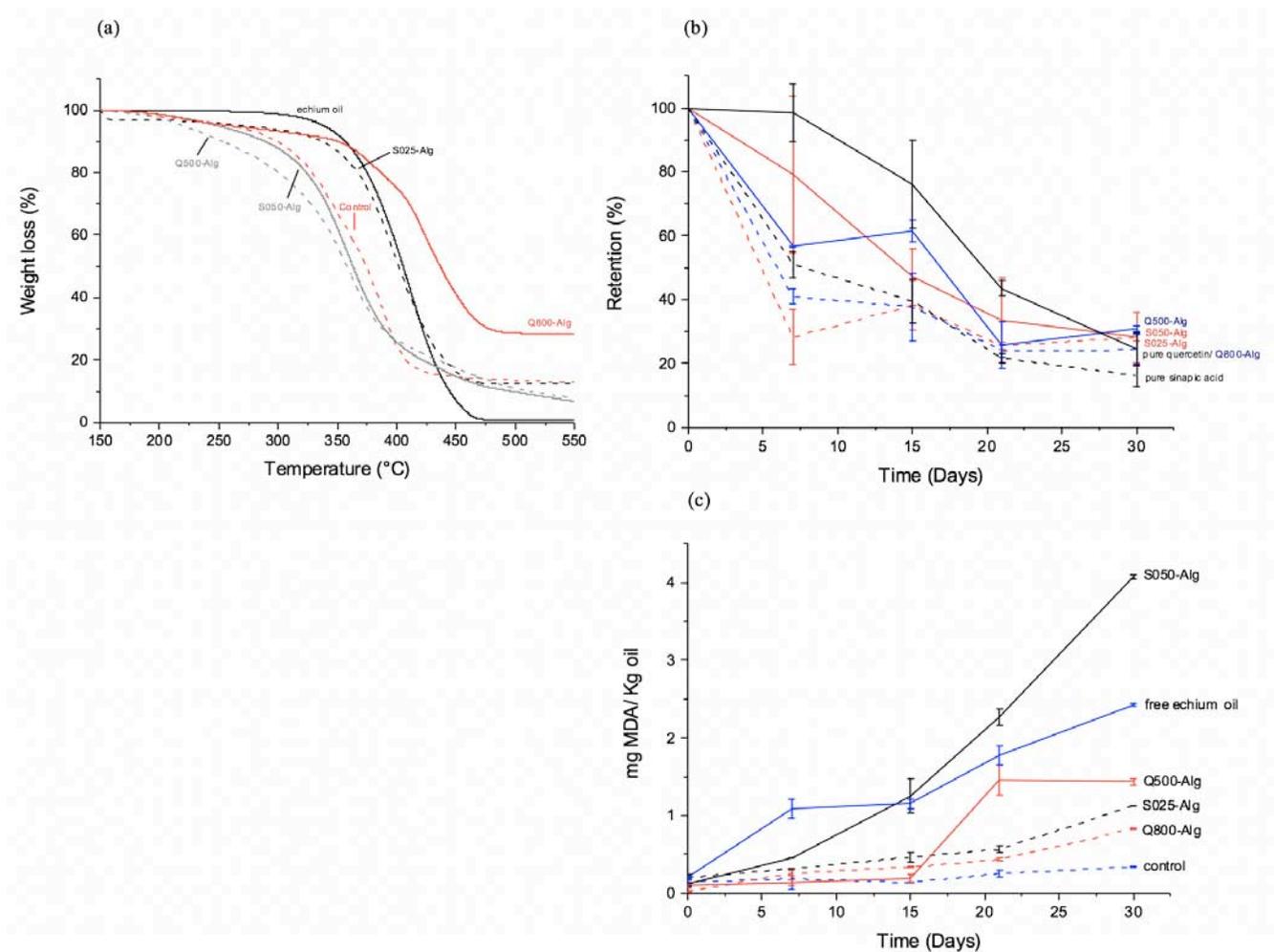
The thermogravimetric behavior of the treatments and the non-encapsulated echium oil is shown in Figure 5.2a. All treatments displayed mass

loss in three steps. The first step occurred in the 25 to 120°C range, which corresponds to the loss of water. This step was not shown in Figure 5.2a since the wet microcapsules were analyzed. Thus, for the purpose of comparison, the mass after this first step was considered 100%. The other two steps were observed from 200 to 350°C and from 350 to 450°C, with variation for some treatments. They can be attributed to the decomposition of the alginate and oil, respectively. This result is in accordance with Yang et al. (2015), who reported the encapsulation of poppy-seed oil by complex coacervation using gelatin and arabic gum as wall materials.

Comparing the control (echium oil encapsulated without phenolic compound) with the S025-Alg and S050-Alg treatments (oil encapsulated with 0.025 and 0.05 g of sinapic acid/ g of alginate), it appears that the treatment with a higher sinapic acid concentration was less thermally stable than both the control and the treatment with lower sinapic acid concentration. Sinapic acid, a proton donor, competes with calcium, a cation, in its interaction with alginate, a negatively charged carbohydrate. Thus, a higher concentration of sinapic acid in the capsule wall would lead to the capsule's destabilization. This higher concentration will then provide less oil protection, as will be discussed further in sections 3.7 and 3.8. When sinapic acid was added at a lower concentration, however, it did not influence the microcapsule structure. As for the Q500-Alg and Q800-Alg treatments (oil encapsulated with 500 and 800 ppm of quercetin in relation to the total mass of oil), it was shown that the treatment with a higher concentration of quercetin was more thermally stable than both the control and the treatment with a lower concentration.

Between the sinapic acid and quercetin treatments, it appears that the Q800-Alg treatment was the most thermally stable, followed by the S025-Alg treatment. This behavior can be related to the concentration of quercetin and its function as an antioxidant in comparison to sinapic acid. The Q500-Alg and S050-Alg treatments showed similar behavior. Thus, how much and in which phase a phenolic compound is added are important factors to consider when designing a thermally stable microcapsule.

Figure 5. 2. a) TG curves for each treatment and pure echium oil; (b) Retention (%) of encapsulated and non-encapsulated quercetin and sinapic acid for the samples stored for 30 days at 40 °C and (c) oxidative stability of the encapsulated and non-encapsulated echium oil by thiobarbituric acid reactive substance (TBARS).



Reference: Own source.

3.5. Stability of quercetin and sinapic acid

Retention of the encapsulated phenolic compounds was 25–30% after a 30-day storage, while for the non-encapsulated compounds, the retention values were 16 and 24% for sinapic acid and quercetin, respectively (Figure 5.2b).

There were significant differences in retention among all the treatments; it was not possible, however, to obtain a relationship between a phenolic compound's concentration and its retention. However, non-encapsulated sinapic acid and quercetin were less stable than their encapsulated counterparts, that is, the encapsulation of these compounds protected them against degradation. Comparing the different concentrations of sinapic acid, it can be affirmed that the higher the concentration of this compound, the greater the retention within 30 days of storage. The same was not observed for quercetin: the higher the quercetin concentration, the lower its retention during storage, probably because it acted better as an antioxidant than sinapic acid did.

Wang, Waterhouse, & Sun-Waterhouse (2013) studied the encapsulation of canola oil and the effect of quercetin addition to the oil core by co-extrusion. After 30 days of storage at 20 °C, no quercetin was found in the encapsulated beads with alginate and pectin as wall materials. That is, even with quercetin acting as antioxidant (and thus decreasing its concentration) in the microcapsules in the present study with echium oil, its retention was still greater than in the system used by Wang, Waterhouse, & Sun-Waterhouse (2013). As for sinapic acid, no research was found about its stability when encapsulated,

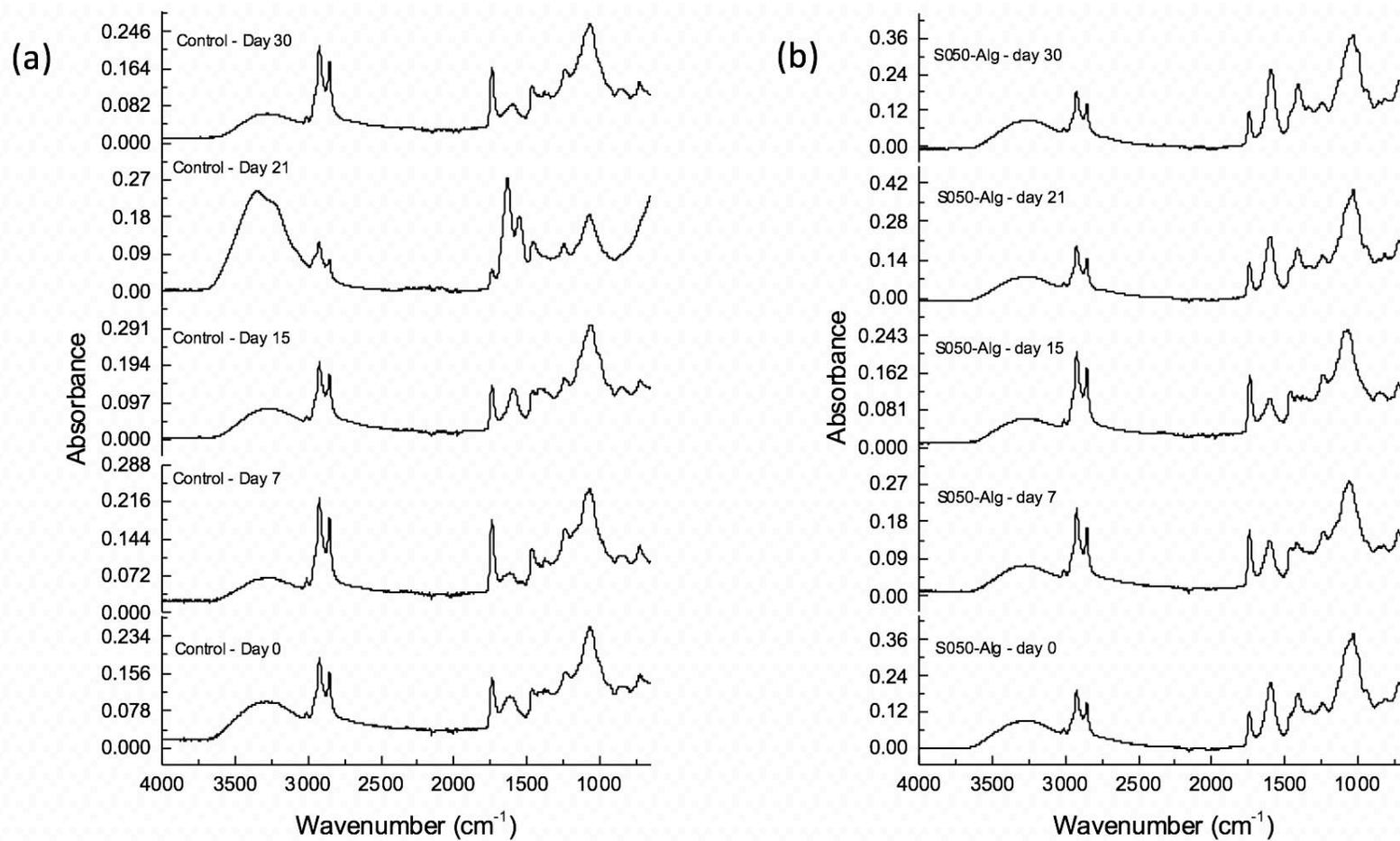
so we can state that the encapsulation of echium oil and sinapic acid by using a combination of the microfluidic and ionic gelation techniques provided good protection for this phenolic compound, because the retention for the non-encapsulated sample was lower after the 30-day storage period.

3.6. Oxidative stability by FTIR

Oxidative stability can be analyzed by Fourier transform infrared spectroscopy according to hydroperoxide formation in the ranges 3200–3600 cm^{-1} and 1730–1750 cm^{-1} . According to Haider, Majeed, Williams, Safdar, & Zhong (2017), peaks in these wavelength ranges are related to the formation of hydroperoxides (first oxidation product) and the formation of ketones and aldehydes, respectively.

Hydroperoxide formation was analyzed in the encapsulated echium oil of the lyophilized samples stored for 30 days at 40 °C. In Figure 5.3a, the control (microcapsule without phenolic compound) showed a significant increase in absorbance for these wavelength ranges (3200–3600 cm^{-1} and 1730–1750 cm^{-1}) at 21 days of storage, which then decreased until the 30th day. It is known that hydroperoxides are the first products of oxidation and are unstable compounds; thus, they decompose rapidly after they are produced, forming aromatic compounds. This suggests that the formation of the second product of oxidation begins after 21 days of storage. This result corroborates those obtained in section 3.8 (Oxidative stability by thiobarbituric acid reactive substance), which shows that the formation of malonaldehyde (the second product of oxidation) for the control was significant after 21 days.

Figure 5. 3. Fourier Transform Infrared Spectrophotometry spectra for the following treatments: (a) control and (b) S050-Alg stored for 30 days at 40 °C.



Reference: Own source.

In contrast, for the non-encapsulated echium oil and all other treatments with added phenolic compounds, no significant increase in absorbance for these wavelength ranges was found, because the oxidation and formation of malonaldehyde occurred more rapidly. In other words, the formation and decomposition of hydroperoxide occurred so rapidly that it could not be detected. The same behavior was observed by Haider, Majeed, Williams, Safdar, & Zhong (2017) when they studied the encapsulation of krill oil by emulsion and later electrostatic interaction of chitosan with tripolyphosphate.

3.7. Oxidative stability by thiobarbituric acid reactive substance (TBARS)

Oxidative stability by thiobarbituric acid reactive substance was measured with wet samples stored for 30 days at 40 °C. The formation of malonaldehyde (MDA) (second product of oxidation) provides a more comprehensive idea about the oxidation of the encapsulated oil compared to the non-encapsulated sample (Figure 5.2c). A significant difference in MDA formation was observed between treatments during 30 days of storage, the non-encapsulated echium oil having a higher formation of malonaldehyde (2.4 mg MDA/ kg of oil) compared to the other treatments (from 0.3 to 4 mg MDA/ kg of oil), with the exception of the S050-Alg treatment (0.05 g sinapic acid/ g alginate). This behavior for the S050-Alg treatment was expected since the presence of a higher concentration of sinapic acid in the capsule wall destabilized its structure, affecting the protection of the oil.

The oxidative stability of echium oil has also been studied by Gray, Payne, McClements, Decker, & Lad (2010) in emulsions using SDS or Tween 20 as emulsifiers. Authors found a higher formation of malonaldehyde for non-encapsulated echium oil (15 mmol/ kg of oil) compared to emulsions (from 1 to 3 mmol/ kg of oil), with samples stored for 7 days at 40 °C. Comparing the results, echium oil encapsulated by a combination of microfluidic devices and ionic gelation techniques promoted greater oxidative stability since the formation of malonaldehyde for the control (oil encapsulated without the presence of phenolic compounds) started to be significant only at 21 days of storage (0.25 mg MDA/ kg of oil), and not at 7 days of storage, as in the results obtained by Gray, Payne, McClements, Decker, & Lad (2010). Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas (2014) encapsulated fish oil using monolayered and multilayered emulsions by spray drying with maltodextrin and chitosan as wall materials. They analyzed the oxidative stability by TBARS at three different temperatures, but only for 12 days. Mohammadi, Jafari, Esfanjani, & Akhavan (2016) studied the application of a nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil, evaluating the TBARS values within a span of 20 days, demonstrating a significant increase in oxidation by the first week of storage. In comparison, the technique used in the present work for the encapsulation of echium oil prevented a significant formation of malonaldehyde in the first 21 days (especially for the control), which argues for its effectiveness. In addition, Pourashouri et al. (2014) also studied the oxidative stability of spray-dried microencapsulated fish oils with different wall materials—storing their samples for 60 days at 20 °C and evaluating TBARS. Comparing their approach to our

own, the combination of the microfluidic device and ionic gelation used in our study provided more protection against oxidation, since our samples (stored at 40 °C) showed the same TBARS values as the samples stored at 20 °C from this study.

Comparison of the control with treatments where phenolic compounds were added (Q500-Alg, Q800-Alg and S025-Alg) revealed that the control had lower malonaldehyde formation, which was not expected. This could be explained by the possible action of quercetin as pro-oxidant along with destabilization of the microcapsule structure by the presence of sinapic acid in the microcapsule wall. According to Behling, Sendão, Francescato, Antunes, & Bianchi (2004), flavonoids can act as pro-oxidants, depending on the concentration and total number of hydroxyls. In addition, between quercetin and sinapic acid, it was evident that the treatment with higher quercetin concentration (Q800-Alg) protected the oil better than the treatments with sinapic acid (S025-Alg and S050-Alg).

It follows that the combination of microfluidic devices and ionic gelation techniques was enough to protect the oil, as the control (without quercetin or sinapic acid) showed better oxidative stability. However, even with the addition of these interfering compounds, oil protection was still guaranteed because they underwent a lower rate of malonaldehyde formation compared to non-encapsulated echium oil.

3.8. Microcapsule stability under different stress conditions

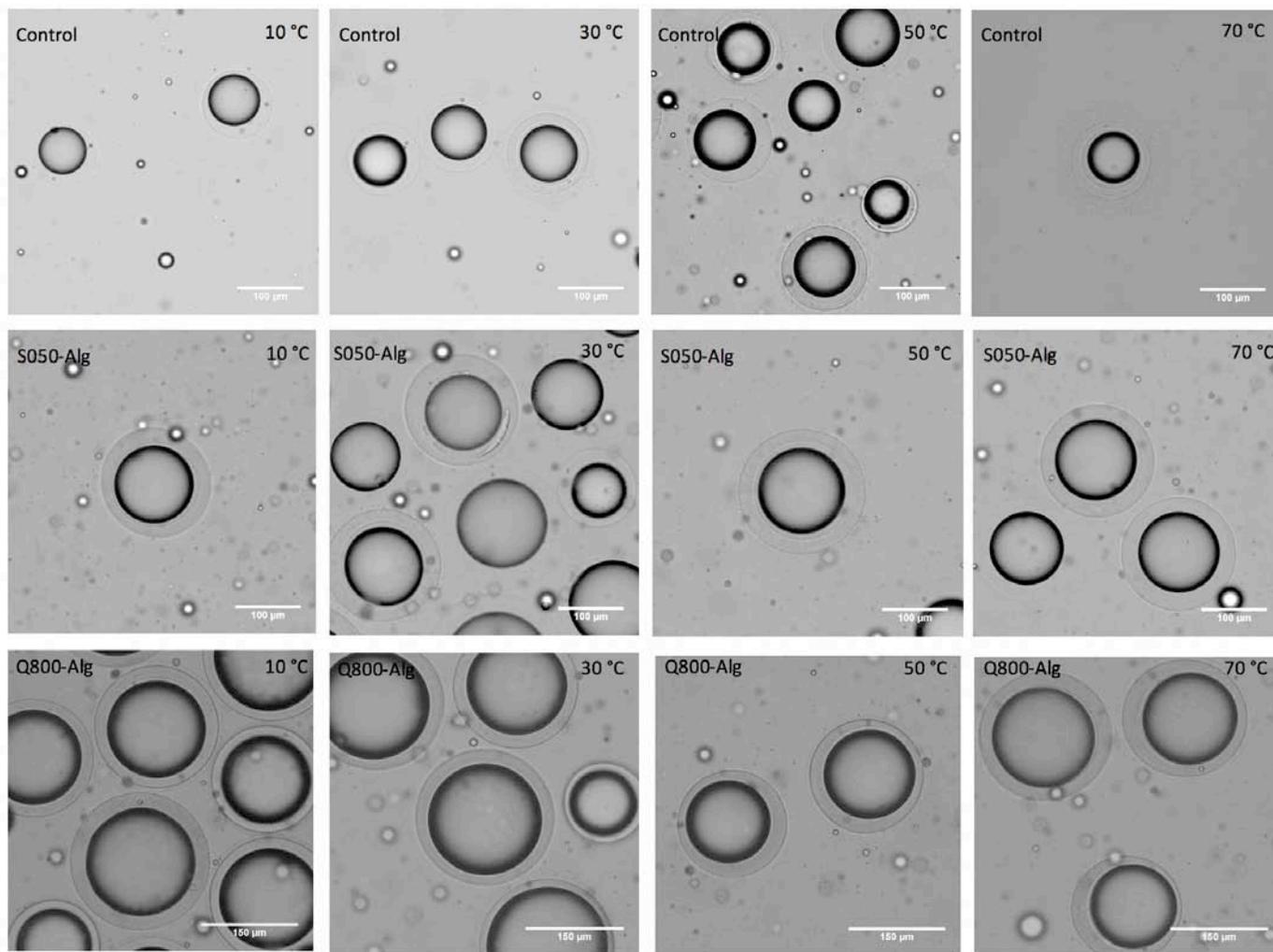
In the temperature range tested, all treatments retained their structure. With increasing temperature however, there was an increase in shell thickness for the treatment involving sinapic acid (Figure 5.4). This can be explained by the presence of this compound in the sodium alginate solution. As already mentioned, sinapic acid affects the ionic gelation process and, consequently, the structure of the capsule. Comunian et al (2016b) (Chapter 3) studied the effect of sinapic acid as a crosslinking agent on echium oil microcapsules obtained by complex coacervation using the combinations gelatin-arabic gum and gelatin- cashew gum as wall materials, and observed a high potential of this phenolic compound for binding to polysaccharides and proteins. The same was expected to occur with sodium alginate; however, due to the nature of the ionic gelation process, the sinapic acid in this case did not produce a robust effect on the formation of the microcapsule and consequently on the protection of echium oil. Regardless, the control and treatment with quercetin both showed good stability, with intact microcapsules within the temperature range tested, showing that treatments obtained by combining microfluidic devices and ionic gelation techniques can be applied to products over a wide temperature range, ensuring the protection of the encapsulated oil.

In relation to different pH values and salt concentrations, a similar pattern of behavior was observed. In this case, the control and treatment with sinapic acid showed an increase in shell thickness at pH 8 and in all concentrations of salt analyzed (1, 3 and 5% w/w), whereas the treatments with quercetin retained the same structure. The structure of the microcapsules, however, was

not affected. Thus, submitting the treatments to different stress conditions did not affect oil protection.

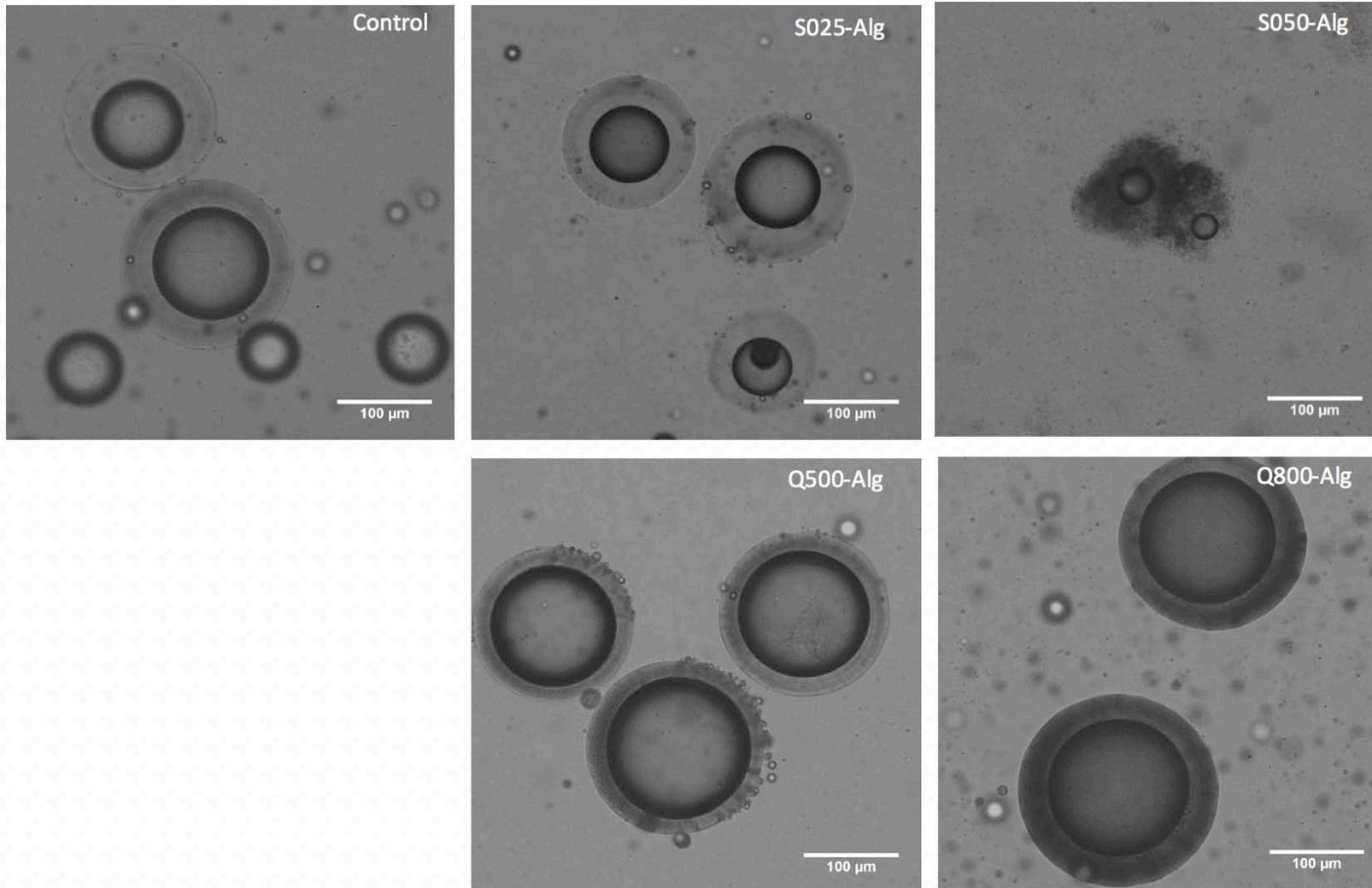
After freezing, lyophilization and resuspension of the microcapsules in water, it was possible to confirm that the treatments with quercetin were the most stable, since they presented an intact structure even after all these steps (Figure 5.5). The S050-Alg treatment (with the higher concentration of sinapic acid) produced an undefined shape after resuspension in water, showing that the presence of sinapic acid, as already mentioned, can destabilize the structure of the microcapsule, causing the loss of its initial form. This result corroborates those obtained for thermogravimetric analysis and oxidative stability by thiobarbituric acid reactive substance in sections 3.4 and 3.7.

Figure 5. 4. Optical microscope images of the following treatments: (a) Control, (b) S050-Alg and (c) Q800-Alg submitted to different temperatures.



Reference: Own source.

Figure 5. 5. Optical microscope image of each treatment freeze-dried and resuspended in water.



Reference: Own source.

4. CONCLUSIONS

Microencapsulation of echium oil by a combination of microfluidic and ionic gelation techniques is feasible, since high values of encapsulation yield and oxidative stability of echium oil during storage at 40 °C were obtained. The control (microcapsules without addition of phenolic compounds) provided high oil oxidative stability; and the control, all treatment with quercetin, and the treatment with a low concentration of sinapic acid were stable under different conditions of stress. Moreover, the Q800-Alg treatment (microcapsule with higher quercetin concentration) showed the best thermal stability, followed by the control, suggesting that this type of microcapsule can be applied to different products. Thus, the combination of the microfluidic and ionic gelation techniques was sufficient to protect the oil. In the future, this combination of techniques can be further explored for the encapsulation of food ingredients, since no solvent or high temperature was used during the process.

5. ACKNOWLEDGMENTS

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**Chapter 6. ENHANCING STABILITY OF OMEGA-3 FATTY ACIDS AND
BETA-SITOSTEROL BY THEIR COENCAPSULATION USING DIFFERENT
COMBINATIONS OF WALL MATERIALS AND CROSSLINKERS**

CHAPTER 6. ENHANCING STABILITY OF OMEGA-3 FATTY ACIDS AND BETA-SITOSTEROL BY THEIR COENCAPSULATION USING DIFFERENT COMBINATIONS OF WALL MATERIALS AND CROSSLINKERS

ABSTRACT

Intake of omega-3 fatty acids and phytosterols aids in the reduction of cholesterol and serum triglycerides. However, both fatty acids and phytosterols are susceptible to oxidation. This work coencapsulated echium oil (source of stearidonic and alpha-linolenic fatty acids) and beta-sitosterol (phytosterol) by complex coacervation using different combinations of wall materials, and sinapic acid (SA) and transglutaminase as crosslinkers. High encapsulation yields were obtained (29–93% for SA; 68–100% for the mixture of oil and phytosterols) and retention of 49–99% and 16% for encapsulated and free SA, at 30 days-storage. Treatment with gelatin-arabic gum and 0.075 g SA/g gelatin showed the best results: 0.07 mg MDA/g capsule, and retention of 96, 90 and 74% for alpha-linolenic, stearidonic acid and beta-sitosterol at 30 days of storage. Thus, coencapsulation of echium oil and phytosterol using SA as the crosslinker was possible, obtaining effective vehicles for protection and application of these compounds in foods.

Keywords: encapsulation, complex coacervation, sinapic acid, phytosterol, echium oil, cashew gum

1. INTRODUCTION

Omega-3 and omega-6 fatty acids have been extensively studied due to their beneficial effects on health (KRALOVEC; ZHANG S.; ZHANG W; & BARROW, 2012). Stearidonic acid (SDA) and alpha-linolenic acid (ALA) are important omega-3 fatty acids. SDA, a long chain fatty acid produced by the desaturation of ALA, is found in small amounts in plants, fish and algae, and it plays a valuable role in human nutrition because it is an intermediate in the biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (COUPLAND & HEBARD, 2002; BERTI et al., 2007; LEMKE et al., 2013). The echium seed oil is of great interest to researchers, as it contains 9–16% SDA and 33% ALA, in addition to 14% linoleic acid and 10% gamma-linolenic acid (omega-6 fatty acids), and has been an alternative to the use of fish oil, due to its unique ratio of omega-3 to omega-6 fatty acids (BERTI et al., 2007). Similarly, phytosterols, compounds obtained from refined oils of plants, seeds, whole grains and legumes, also have many health benefits (SANCLEMENT et al., 2012).

According to Espinosa et al. (2015), the consumption of an omega-3 fatty acid and phytosterol mixture leads to a reduction in triglycerides and cholesterol serum levels. However, these compounds are very susceptible to oxidation and are insoluble in water. Consequently, their application in food products is particularly challenging. An alternative to minimize such problems would be the microencapsulation of these compounds.

Microencapsulation is intended to protect compounds against adverse environmental conditions, to mask unpleasant odors and flavors, and to avoid the evaporation of volatile compounds through the coating or dispersibility of

compounds (core) by one or more materials (wall materials), facilitating their application in food products (SANTOS et al., 2015). The complex coacervation technique consists of the electrostatic interaction between two polymers with opposite charges. According to Comunian and Favaro-Trindade (2016), it is an ideal process for the encapsulation of hydrophobic compounds, considering the first step in the process is the preparation of an oil-in-water (O/W) emulsion.

The crosslinking process has been widely used together with the complex coacervation technique, to enhance the capsule strength. Previous work from our research group showed that in addition to being an antioxidant, the phenolic compound sinapic acid (SA) is an effective crosslinker and can enhance the stability of microcapsules obtained by coacervation (COMUNIAN et al., 2016a; COMUNIAN et al., 2016b). Besides having several beneficial effects on health, such as a neuroprotective effect against Alzheimer's disease, and ability to ameliorate cardiac hypertrophy and dyslipidemia (LEE et al., 2012; ROY & PRINCE, 2013), SA represents a suitable alternative to traditional crosslinkers, such as transglutaminase, which is expensive and does not provide health benefits, and glutaraldehyde, which is toxic. In a recent study from our group, echium oil was coencapsulated with SA and/or rutin, by complex coacervation (COMUNIAN et al., 2016a). However, for the inclusion of these compounds in the microcapsule, a water-in-oil-in-water (W/O/W) double emulsion was produced with the addition of SA or rutin in the internal aqueous phase, as they are water-soluble compounds. The results showed that protection of the echium oil was promoted, but it was not better than when SA was applied as a crosslinker (COMUNIAN et al., 2016a; COMUNIAN et al., 2016b). Therefore, we conducted further work on echium oil encapsulation by

complex coacervation, with the specific purpose of studying the application of SA as a crosslinker. In this instance, the best parameters for the inclusion of this compound in the microcapsule were investigated (COMUNIAN et al., 2016b). However, the inclusion of phytosterol and the oxidative stability of the oil and of the encapsulated compounds were not studied, and are important factors to guarantee the viability of the microcapsule and their application in food products. In addition, the encapsulation of phytosterols and the coencapsulation of this type of mixture (two lipophilic bioactive compounds with different physicochemical properties) have been little explored in the literature.

In this context, the current work aimed to coencapsulate echium oil—rich in SDA and ALA—and phytosterol, by the complex coacervation technique, using SA as a crosslinking agent (and as an antioxidant). Also, to evaluate the stability of these compounds under pre-determined conditions, with the intention to protect and apply these compounds in foods.

2. MATERIAL AND METHODS

2.1. Material

As source of ω -3, oil extracted from echium seed (*Echium plantagineum* L.) (NEWmega™ Echium Oil, De Wit Speciality Oils, Tescel, Netherlands) was used. Sinapic acid, gelatin, arabic gum, and cashew gum were obtained from Sigma Chemical Co. (St. Louis, MO, USA), Gelnex (Santa Catarina, Brazil), Nexira (São Paulo, Brazil) and EMBRAPA Tropical Agroindustry (Fortaleza). The mixture of phytosterols, composed by beta-sitosterol (70-80%), beta-sitostanol (0-15%), campesterol (0-15%), stigmasterol (0-2%) and campesterol (0-5%) was obtained from DuPont-Danisco (Barueri, Brazil).

2.2. Methods

2.2.1. Microencapsulation

For the preparation of microcapsules, the method adopted by Comunian et al. (2016b), with some modifications, was used. The ratios of 1:1 of gelatin: arabic gum and 1: 2.5 of gelatin: cashew gum were fixed, with 50% of core (echium oil + phytosterol mixture) in relation to the total polymer mass (gelatin, arabic gum and cashew gum solution 5% - w/w). Treatments with different concentrations of sinapic acid as a crosslinking agent were prepared, which were compared to transglutaminase, totalizing in 10 treatments as shown in Table 6.1.

The oil and the phytosterol mixture (0.132g phytosterols/ g oil) were emulsified with gelatin solution (5% - w/w) in order to obtain a simple oil-in-water (O/W) emulsion at 10,000 rpm/ 3 minutes with Ultraturrax (Ika, Germany). The solution of arabic or cashew gum (5% - w/w) was added to these emulsions by magnetic stirring at 40 °C. To promote complex coacervation, the pH was adjusted to 4.0 at 40 °C under constant magnetic stirring, and thereafter the temperature was gradually reduced to 10 °C in an ice bath. The coacervate material was stored for 24 h at 7 °C to promote decantation. The removal of the supernatant (water) was done with the use of a dispenser. The coacervates were then frozen in a freezer (-18 °C) and freeze-dried (Terroni/ São Carlos - SP).

Table 6. 1. Composition of each treatment and yield values of sinapic acid, oil and phytosterol mixture.

Treatments	Polysaccharide in combination with gelatin	Concentration of sinapic acid (g/g of gelatin)	Concentration of transglutaminase (U/ g of gelatin)	Sinapic acid encapsulation yield (%)	Encapsulation yield of the oil + phytosterols (%)
GA00	Arabic gum	0.000	--	--	98.77 ± 7.55 ^a
GA025	Arabic gum	0.025	--	29.19 ± 1.68 ^d	94.82 ± 5.00 ^a
GA05	Arabic gum	0.050	--	84.09 ± 9.04 ^{ab}	98.53 ± 12.43 ^a
GA075	Arabic gum	0.075	--	87.53 ± 2.72 ^a	97.83 ± 16.11 ^a
GC00	Cashew gum	0.000	--	--	100.81 ± 5.25 ^a
GC025	Cashew gum	0.025	--	51.18 ± 2.99 ^c	100.73 ± 1.02 ^a
GC05	Cashew gum	0.050	--	67.46 ± 0.85 ^{bc}	102.95 ± 2.70 ^a
GC075	Cashew gum	0.075	--	93.07 ± 8.89 ^a	102.91 ± 3.86 ^a
GAT15	Arabic gum	--	15.00	--	68.91 ± 1.46 ^b
GCT15	Cashew gum	--	15.00	--	95.02 ± 0.62 ^a

Reference: Own source.

Equal letters in the same column do not differ statistically at 5% level by the Tukey test.

- GA00, GA015, GA05 and GA075: Treatment with a ratio of 1:1:0.5 gelatin, arabic gum and echium oil containing sinapic acid as crosslinker at concentrations of 0, 0.025, 0.05 and 0.075 g/g of gelatin and 5% (w/w) gelatin solution.
- GC00, GC025, GC05 e GC075: Treatment with a ratio of 1:1:0.5 gelatin, cashew gum and echium oil containing sinapic acid as crosslinker at concentrations of 0, 0.025, 0.05 and 0.075 g/g of gelatin and 5% (w/w) gelatin solution.
- GAT15: Treatment with a ratio of 1:1:0.5 gelatin, arabic gum and echium oil containing transglutaminase as crosslinker at concentration of 15 U/g of gelatin and 5% (w/w) gelatin solution.
- GCT15: Treatment with a ratio of 1:1:0.5 gelatin, cashew gum and echium oil containing transglutaminase as crosslinker at concentration of 15 U/g of gelatin and 5% (w/w) gelatin solution.

2.2.2. Crosslinking process

2.2.2.1. Crosslinking with sinapic acid (SA)

Based on a previous method (COMUNIAN et al., 2016b), the SA crosslinking agent was incorporated, as follows: (a) gelatin-arabic gum treatments as wall material: aqueous SA solution (at 0 to 0.075 g SA/g gelatin) was added to microcapsules after the complex coacervation process and maintained under low magnetic stirring at 15 °C for 12 h in a BOD TE-391 Tecnal (Piracicaba, Brazil); (b) treatments with gelatin-cashew gum as wall material: SA was added after the preparation of the simple O/W emulsion, kept under magnetic stirring at 40 °C for 1 min, followed by the addition of cashew gum solution and the complex coacervation process.

2.2.2.2. Crosslinking with transglutaminase

An aqueous solution containing 15 U transglutaminase/g gelatin at pH 6.0 was prepared and added to the solution of the microcapsules after coacervation. The mixture was maintained under low magnetic stirring at 15 °C for 12 h in a BOD TE-391 Tecnal (Piracicaba, Brazil).

2.2.2. Characterization of microcapsules

(a) Sinapic acid encapsulation yield

The SA encapsulation yield was determined as a function of the mass difference of SA contained in the powders and the total amount used in the encapsulation process. For the capsule rupture, 0.1 g of the sample was diluted in a solution containing 5 mL methanol, 5 mL ZnSO₄ (5% w/w) and 0.8 g KCl, sonicated for 10 min and centrifuged at 4000 rpm, at 25 °C for 10 min. The

supernatant was filtered and analyzed by high-performance liquid chromatography (HPLC) (Shimadzu; Kyoto, Japan). An amide C18 column was used, with a 45:55 (v/v) water:methanol mobile phase at pH 3.5, a flow rate of 1 mL/min and detection and volume of injection of 325 nm and 10 μ L, respectively.

(b) Oil and phytosterols encapsulation yield

The oil and phytosterols encapsulation yields were determined as a function of the mass difference of oil and phytosterols contained in the powders and the total amount used in the encapsulation process. For the rupture of the capsules, the same method mentioned in section 2.2.2a was used. After the capsules rupture, the oil and phytosterols were extracted using the Bligh and Dyer (1959), with some modifications. For Bligh and Dyer method, the capsules (0.5 g of the powders), 5 mL chloroform, 10 mL methanol and 4 mL water were added and the mixture gently vortexed for 1 min. Next, 5 mL chloroform and 5 mL of 1.5% (w/w) sodium sulfate solution were added. The mixture was again gently vortexed for 1 min and kept standing for 30 min, for phase separation. A known volume of chloroform, the phase in which oil and phytosterols are present, was oven-dried at 60 °C until the solvent evaporated, followed by quantification of the retained oil and phytosterols.

(c) Fourier Transform Infrared Spectroscopy (FTIR)

The spectra of sinapic acid, echium seed oil, phytosterols, gelatin, arabic gum, cashew gum and microcapsules were obtained in the region of 4000-600 cm^{-1} using the Perkin Elmer FT-IR Spectrometer equipment (Massachusetts,

USA) with the aid of Spectrum One software version 5.3.1.

(d) X-ray diffraction

The structure of gelatin, arabic gum, cashew gum and microcapsules were analyzed by x-ray diffraction (Siemens 5100 diffractometer) according to Xiao, Li, & Zhu (2015), with some modifications. The measurements were obtained by 2θ scanning between 5 and 55° and a scanning rate of $6^\circ\text{C}/\text{min}$, tension of 40 kV and current of 30 mA.

(e) Stability of sinapic acid

The SA levels were monitored by HPLC (Shimadzu; Kyoto, Japan), as mentioned in section 2.2.2a. The analyzes were performed on the powders, in triplicate, at 0, 7, 15 and 30 days after encapsulation. The powders were stored protected from light, in the presence of oxygen and at 37°C . In addition to the SA levels in the powder microcapsules, standard solutions of SA (non-encapsulated SA), were stored under the same conditions.

(f) Stability of omega-3 fatty acids

The levels of omega-3 fatty acids were monitored by gas chromatography/mass spectrometry (GC/MS) (Agilent 7890 A GC system). The analyzes were performed in powders, in triplicate, stored as described in section 2.2.2e. For the rupture of the microcapsules, 1 g sample was added to 20 mL of 0.5M NaOH solution in methanol and 0.2 g ZnSO_4 . The mixture was vortexed and ultrasonicated for 1 and 10 min, respectively, and then centrifuged at 9000 rpm for 6 min. A 1 mL aliquot of the ruptured capsules was collected,

vortexed for 15 s and kept in a water bath at 100 °C for 5 min. After cooling the samples, 2 mL of 14% BF₃-methanol was added, vortexed for 15 s and maintained in a water bath at 100 °C for 5 min. Then, 1 mL of isooctane was added and stirred vigorously for 30 s, followed by the addition of 5 mL of saturated NaCl solution and brief homogenization. After phase separation, the upper isooctane layer was transferred to a microtube and completely evaporated with nitrogen gas. The samples were then resuspended in 500 µL isooctane and transferred to a vial for chromatographic analysis. A J&W DB-23 60 m × 250 µm × 0.15 µm Agilent 122-2361 column was used, with the following chromatographic conditions: split 50:1; inlet temperature: 250 °C; injector temperature: 250 °C; injection volume: 1 µL, carrier gas: helium, and heating ramps: 80 to 175 °C at 5 °C/min, 175 to 230 °C at 3 °C/min and 230 °C for 5 min.

(g) Quantification of malonaldehyde

The MDA levels were monitored by HPLC (Agilent Technologies 1200 series; Santa Clara, CA, USA). The analyzes were performed in powders, in triplicate, stored as described in section 2.2.2e. For the rupture of the microcapsules, 1 g sample was added to 20 mL of 0.5 M NaOH solution in methanol and 0.2 g ZnSO₄. The mixture was vortexed and ultrasonicated for 1 and 10 min, respectively and then centrifuged at 9000 rpm for 6 min. Then, 100 µL of the ruptured microcapsules was added to 12.5 µL of 0.2% 2,6-butylated hydroxytoluene and 6.25 µL of 10 N NaOH. After 30 min at 60 °C, 750 µL of 7.2% trichloroacetic acid containing 1% KI was added. The samples were cooled on ice for 10 min and centrifuged at 12,000 rpm for 10 min. Five hundred

μL of the supernatant was added to 250 μL of 0.6% 2-thiobarbituric acid and then heated in a water bath at 95 °C for 30 min. After cooling, the oil was extracted by vortexing the mixture with 750 μL butanol for 15 s and centrifugation at 12,000 rpm for 10 min. The supernatant was recovered and analyzed by HPLC, using 60% potassium phosphate buffer/40% methanol as the mobile phase, at a flow rate of 1 mL/min. A Phenomenex reverse-phase C18 column, 50 μL injection volume, 4.9–5.3 min retention time and wavelengths of 515 and 553 nm for excitation and emission, respectively, were used.

(h) Stability of phytosterol

Phytosterol levels were monitored by GC/MS (Agilent 7890 A GC system). The analyzes were performed in powders, in triplicate, stored as described in section 2.2.2e. For the rupture of the microcapsules, 250 mg sample was added to 4 mL of 2 M KOH in methanol and 0.05 g ZnSO_4 . The mixture was vortexed and ultrasonicated for 1 and 10 min, respectively and then centrifuged at 9,000 rpm for 6 min. Next, 750 μL of this sample was vortexed for 15 s and kept in a water bath at 60 °C for 1 h. After cooling, the first extraction of the phytosterols was done by adding 600 μL hexane and 400 μL ultrapure water (Direct-Q, Millipore, USA). The mixture was vortexed for 15 s and centrifuged at 5,000 rpm for 4 min. The organic phase was separated and transferred to a microtube. The second extraction was then done with the remainder of the sample, by adding 600 μL hexane, followed by vortex and centrifugation under the same conditions. The upper phase was removed and transferred to the same microtube containing the organic phase of the first

extraction. The hexane was evaporated. Then, derivatization was performed with the addition of 100 μL *N,O*-bis-(trimethylsilyl)acetamide and 50 μL pyridine, the solution mixed by vortex and heated in a water bath at 70 $^{\circ}\text{C}$ for 15 min. Finally, 350 μL of hexane was added, the solution then vortexed for 15 s and transferred to a vial for chromatographic analysis. An Agilent Technologies J&W HP-5 column (30 m \times 320 μm \times 0.25 μm) was used, with 1:50 split, inlet and injector set at 250 $^{\circ}\text{C}$, 1 μL injection volume, detector at 300 $^{\circ}\text{C}$, and helium as carrier gas. After an initial 150 $^{\circ}\text{C}$ for 3 min, the temperature was ramped at 5 $^{\circ}\text{C}/\text{min}$ up to 310 $^{\circ}\text{C}$ and held for 10 min. A post run of 320 $^{\circ}\text{C}/5$ min was performed.

(i) Statistical analysis

The data were analyzed using SAS (version 9.2; SAS Institute, Inc., Cary, NC, USA), by analysis of variance (ANOVA) and Tukey's test, at the 5% significance level.

3. RESULTS AND DISCUSSION

(a) Encapsulation yield of sinapic acid, echium oil and phytosterols

Sinapic acid encapsulation yield values for all treatments are shown in Table 6.1. These values ranged from 29–87% and 51–93% for the treatments with arabic and cashew gum, respectively. It is observed that with increasing SA concentration, the encapsulation yield increases, both for arabic and cashew gum treatments. Namely, the higher the concentration of SA added, the lower its loss during the encapsulation process. It is also noted that the treatments with cashew gum showed higher SA yields than with arabic gum. This can be explained by the stronger bonds between SA and cashew gum than between this phenolic and arabic gum.

In a previous work of this research group, the microencapsulation of echium oil with SA or rutin was studied and encapsulation yields values of 39–50% and 58–80% for SA and rutin, respectively, were obtained (Comunian et al., 2016a). This lower value of encapsulation yield compared to the current study can be explained due to the manner of incorporation of the phenolic compound into the microcapsule, given, in the previous research, the SA was inside the microcapsule. In this earlier work, SA and rutin were incorporated into the internal aqueous phase of a W/O/W double emulsion and did not undergo any interaction with other compounds, facilitating their release into the second aqueous phase (COMUNIAN et al., 2016a) (Chapter 2). The results obtained in this work indicate that the incorporation of SA as a crosslinker provided more

interactions of this with the wall materials, making it difficult to release and consequently increasing the value of encapsulation yield.

Regarding the echium oil and phytosterol encapsulation yields, there was a significant difference between the treatments, obtaining values ranging from 68.91–100%, as shown in Table 6.1. A comparison of the different wall materials compositions, reveals that the gelatin-cashew gum combination promoted less oil loss during the encapsulation process. This result corroborates with the SA encapsulation yield, considering that among the treatments, those that used this combination of wall materials also presented higher yields for the phenolic. Namely, the crosslinking reaction between SA and cashew gum promoted a greater retention of oil and phytosterols, than SA and arabic gum, as expected. Based on previous results obtained by the research group, we hypothesized that greater crosslinking of SA with cashew gum (presence of mannose, glucose and xylose as terminal residues) would be due to a small difference in its composition, compared to arabic gum composition. Still, according to the previous results, one of the ways that crosslinking occurs is by the Fischer esterification reaction with a hydroxyl (-OH) group from a sugar residue, which is present in cashew gum structure (COMUNIAN et al., 2017; COMUNIAN et al., 2016b).

A comparison of the different crosslinking agents, affirms that the SA was more efficient than transglutaminase for the microcapsules prepared with the gelatin-arabic gum combination. The treatments with transglutaminase presented oil and phytosterol encapsulation yields of 68.91 and 95.02% for the treatments with the gelatin-arabic gum and gelatin-cashew gum combinations, respectively. However, in the treatment with the gelatin-cashew gum

combination, the phenolic compound was as good as the enzyme, which is a traditional crosslinker and does not provide any health benefits.

No work on the coencapsulation of echium oil and phytosterols was found in the literature; however, in a previous work of the group, encapsulation of echium oil and sinapic acid or rutin by complex coacervation was studied by incorporating such phenolic compounds into the internal aqueous phase of the double water-in oil-in water (W/O/W) emulsion, since they are water-soluble (COMUNIAN et al., 2016a) (Chapter 2). In this work, they obtained oil encapsulation yield values within the range of 73.16-99.80%, which corroborate those obtained for the mixture of echium oil + phytosterols. Timilsena, Adhikari, Barrow, & Adhikari (2016) studied the microencapsulation of chia oil using protein and the gum extracted from the chia seed by complex coacervation and obtained oil encapsulation yield values of 95-98.2% and of 91,7-96.6% for treatments dried by atomization and lyophilization, respectively. Thus, it can be stated that the presence of phytosterols in the core did not influence the loss of the encapsulated materials during the process, which was expected, since phytosterol is a non-polar compound, which facilitates the formation of the oil-in-water emulsion (O/W), which is the first step of the coacervation process.

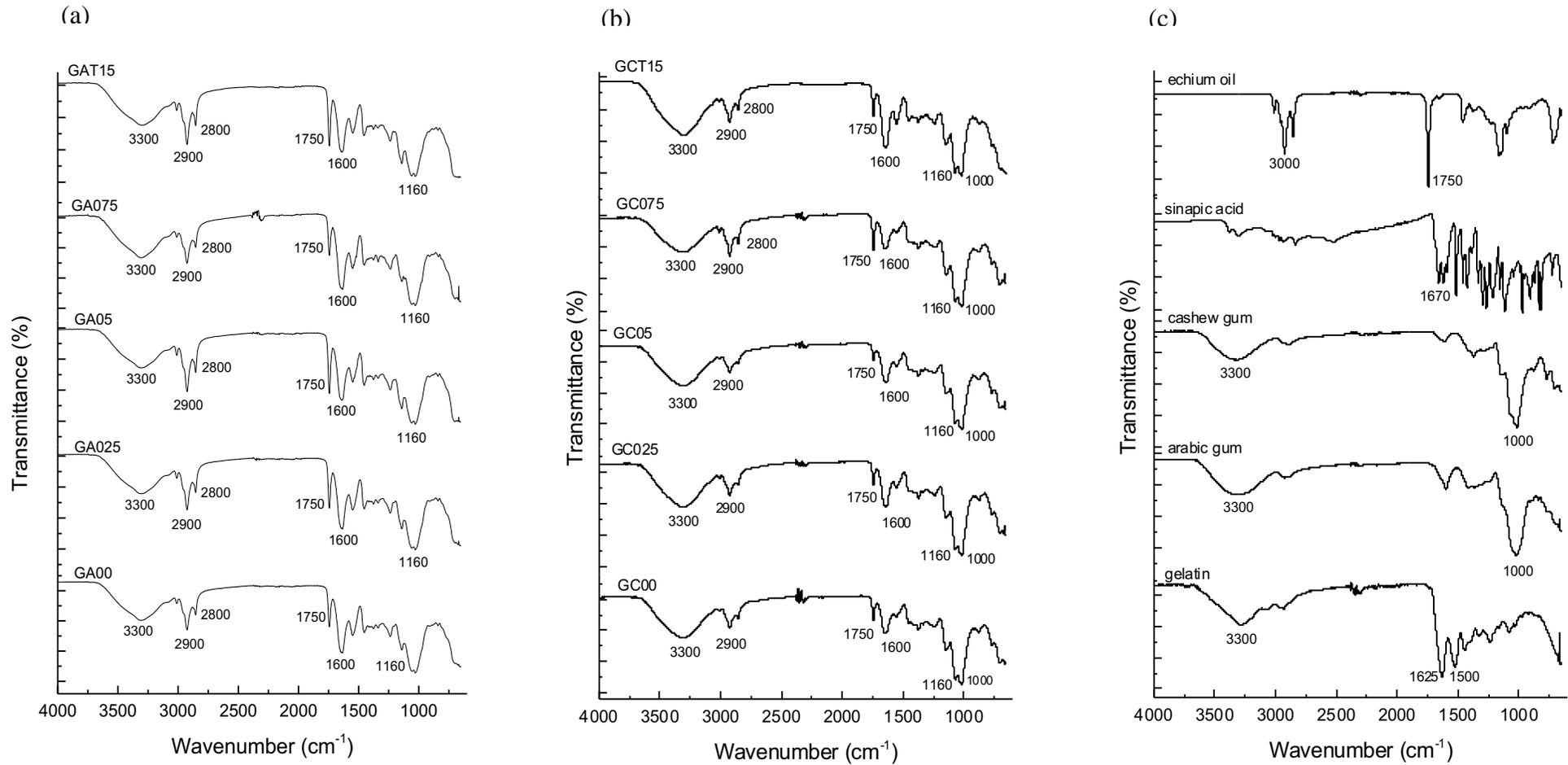
(b) Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectra obtained for free and encapsulated materials and wall materials are shown in Figure 6.1. According to Santos et al. (2015), the positively-charged protein has the amino group in its composition, whereas the negatively-charged polysaccharide has carboxyl groups. These groups can be

observed at peaks around 3300 and 2900 cm^{-1} , respectively, and also in all treatments. The arabic and the cashew gums showed very similar behavior, as expected. As we previously obtained, when encapsulating the echium oil with the incorporation of SA or rutin in the internal aqueous phase of a W/O/W double emulsion (Comunian et al., 2016a), the spectrum of SA showed intense vibrations between 3650 and 2500 cm^{-1} and between 1750 and 600 cm^{-1} , representing -OH and carbonyl groups (C=O), respectively.

The spectra obtained for the echium oil and for the mixture of phytosterols presented the same behavior. The spectra of the echium oil showed peaks around 1750 and 3000 cm^{-1} . According to Oriani et al. (2016) and Matos-Jr et al. (2017), these peaks represent vibration of the -CO bond of the ester functional group and carboxylic acids, respectively. In addition, the peaks present in all spectra of the free materials are also present in the spectra of the treatments. In other words, the SA, the echium oil and the phytosterol mixture are intact and encapsulated.

Figure 6. 1. Spectra obtained by FTIR for (a) treatments with gelatin-arabic gum combination, (b) treatments with gelatin-cashew gum combination and (c) wall and encapsulated materials.



Reference: Own source.

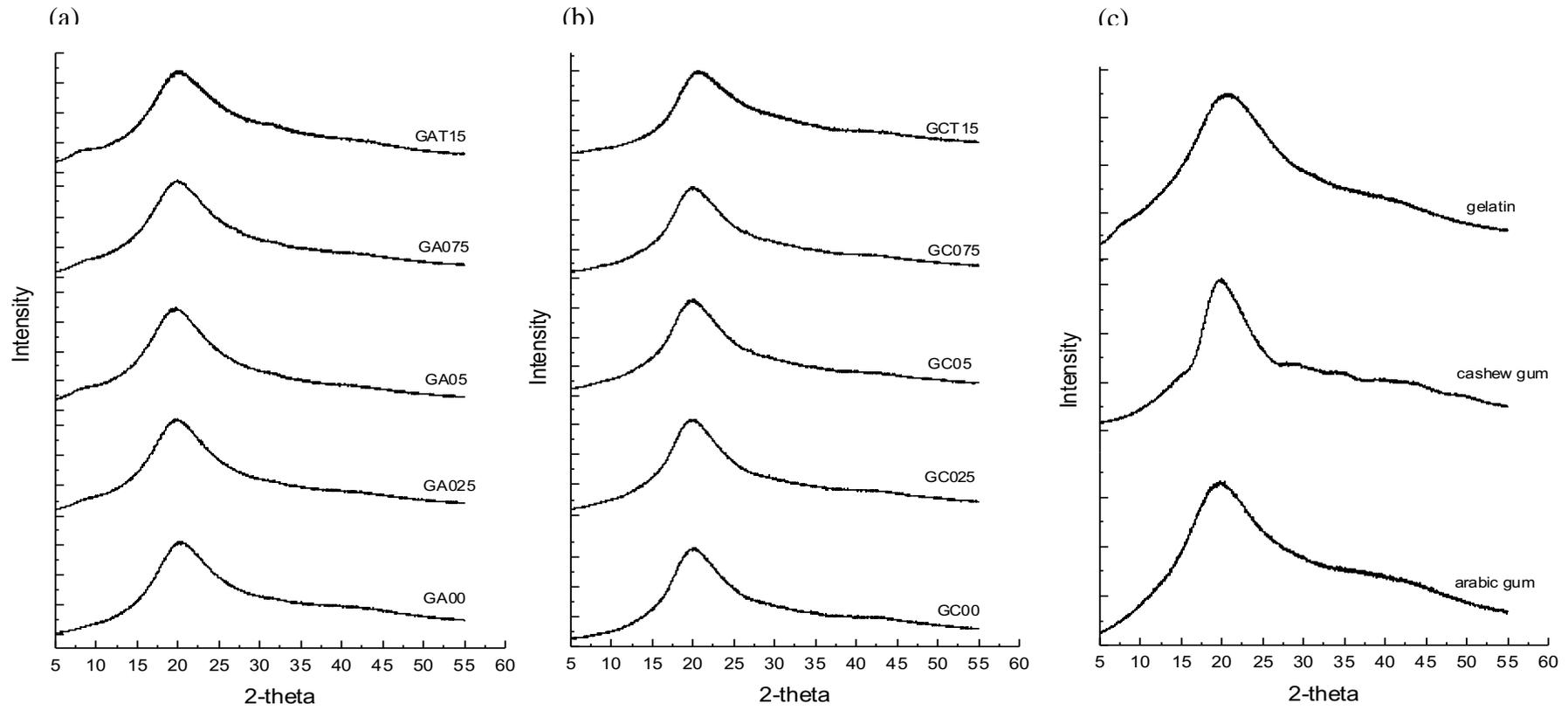
(c) X-ray diffraction

The diffractograms of the wall materials and of each treatment are shown in Figure 6.2. The analysis of the diffractograms allows inferring that all the materials used in the preparation of the microcapsules, as well as the powders obtained from the microcapsules, were in the amorphous state. For microcapsules, the amorphous state is advantageous because it provides greater physical stability to the structure, therefore greater retention and protection for the bioactive than the crystalline form.

The gelatin diffractogram showed two peaks, one small peak around 10° and a sharper peak around 22° . According to Xiao et al. (2015), the first peak may be related to the helical structure of the collagen, which is where the gelatin is obtained. When comparing the treatments, this peak disappears, except for the treatment GAT15 (composition in Table 1). That is, it is an indication that the complex is formed as a function of the disruption of the helix.

In a recent paper, Huang et al. (2016) studied the formation of complexes using chitosan and arabic gum as wall materials and genipin as the crosslinking agent and obtained diffractograms with similar behaviors to echium oil and phytosterol microcapsules. The similar behaviors are justified given that the materials are all in the amorphous state and because the same encapsulation method has been used.

Figure 6. 2. X-ray diffraction (a) of the treatments with gelatin-arabic gum combination, (b) of the treatments with the gelatin-cashew gum combination and (c) of the wall materials.



Reference: Own source.

(d) Stability of sinapic acid

The treatments were stored at 37 °C in order to evaluate the stability of the sinapic acid in the microcapsule compared to the one non-encapsulated. The values obtained are shown in Table 6.2.

It is observed that the microencapsulated phenolic compound presented greater stability in the conditions submitted when compared to the one not encapsulated. Comparing the treatments with 30 days of storage, the microcapsules presented retention values within the range of 45-96% and of 49-99% for the treatments with arabic and cashew gum, respectively. On the other hand, the retention of non-encapsulated sinapic acid under the same conditions was only 16.3%. In other words, the encapsulation by complex coacervation with both wall material combinations provided the protection of the phenolic compound even with it being used as a crosslinking agent.

The gelatin-cashew gum combination as wall materials promoted greater sinapic acid protection than the gelatin-arabic gum combination. One hypothesis for this result would be a stronger crosslinking reaction with cashew gum, which corroborates the results obtained for encapsulation yield for sinapic acid and for the mixture of echium oil and phytosterols (item 3a). However, it was not possible to establish a relationship between the sinapic acid retention values for the same combination of wall material and different concentrations of the phenolic compound.

Table 6. 2. Stability (%) of encapsulated and free sinapic acid during 30 days-storage at 37 °C.

Treatments/ Days	0	7	15	30
GA00	--	--	--	--
GA025	100.00 ±0.01 ^{a,A}	96.60± 6.07 ^{a,A}	96.74±3.92 ^{a,A}	96.89±6.39 ^{aA}
GA05	100.00±0.01 ^{a,A}	78.52±14.04 ^{bc,A}	42.68±12.28 ^{b,B}	49.07±11.78 ^{c,B}
GA075	100.00±0.01 ^{a,A}	101.81±4.76 ^{a,A}	94,85±16,48 ^{a,A}	57.29±11.90 ^{bc,B}
GC00	--	--	--	--
GC025	100.00±0.01 ^{a,A}	103.54±4.15 ^{a,A}	101.81±2.94 ^{a,A}	99.51±5.53 ^{a,A}
GC05	100.00±0.01 ^{a,AB}	105.61±7.07 ^{a,A}	87.12±8.80 ^{a,BC}	73.87±8.13 ^{b,C}
GC075	100.00±0.01 ^{a,A}	100.01±5.06 ^{a,A}	98.92±10.75 ^{a,A}	49.88±4.69 ^{c,B}
GAT15	--	--	--	--
GCT15	--	--	--	--
Free SA	100.00±0.01 ^{a,A}	59.84±1.17 ^{c,B}	32.91±0.76 ^{b,C}	16.27±0.06 ^{d,D}

Reference: Own source.

Equal lowercase letters in the same column and equal capital letters in the same line do not differ by Tukey test at 5% probability level.

- GA00, GA015, GA05 and GA075: Treatment with a ratio of 1:1:0.5 gelatin, arabic gum and echium oil containing sinapic acid as crosslinker at concentrations of 0, 0.025, 0.05 and 0.075 g/g of gelatin and 5% (w/w) gelatin solution.
- GC00, GC025, GC05 e GC075: Treatment with a ratio of 1:1:0.5 gelatin, cashew gum and echium oil containing sinapic acid as crosslinker at concentrations of 0, 0.025, 0.05 and 0.075 g/g of gelatin and 5% (w/w) gelatin solution.
- GAT15: Treatment with a ratio of 1:1:0.5 gelatin, arabic gum and echium oil containing transglutaminase as crosslinker at concentration of 15 U/g of gelatin and 5% (w/w) gelatin solution.
- GCT15: Treatment with a ratio of 1:1:0.5 gelatin, cashew gum and echium oil containing transglutaminase as crosslinker at concentration of 15 U/g of gelatin and 5% (w/w) gelatin solution.

As previously obtained by the research group, the echium oil was encapsulated with sinapic acid or rutin by complex coacervation, incorporating these phenolic compounds into the internal aqueous phase of the water-in oil-in water (W/O/W) double emulsion, however its stability over time has not been studied (COMUNIAN et al., 2016a) (Chapter 2). For comparison purposes, no work on encapsulation of sinapic acid and the study of its stability has been found in the literature. In other words, the coencapsulation of echium oil, phytosterol and sinapic acid may be considered a pioneer in the protection of this phenolic compound.

(e) Stability of encapsulated omega-3 fatty acids

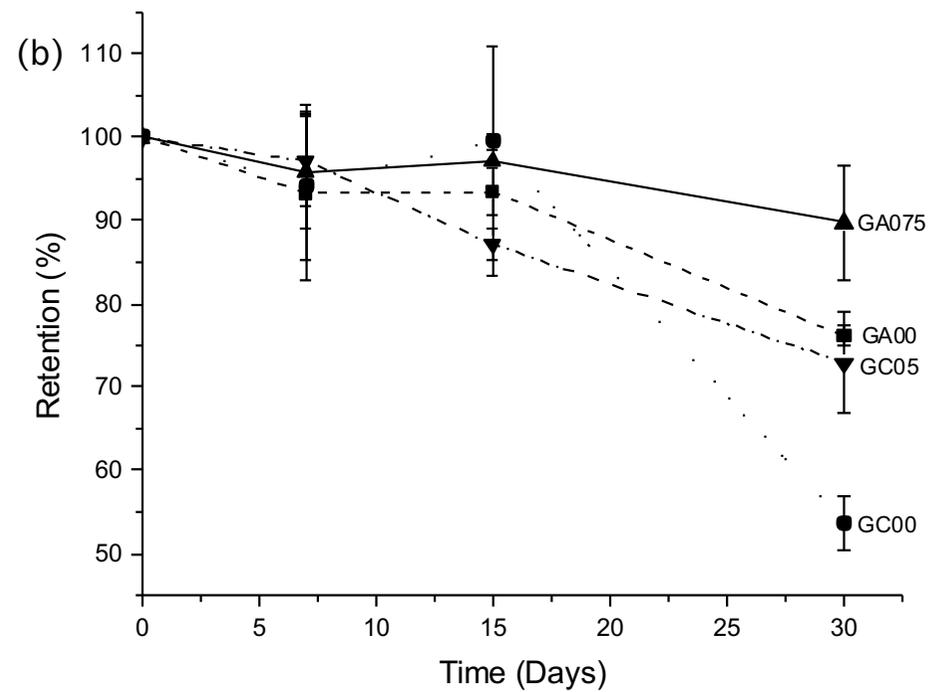
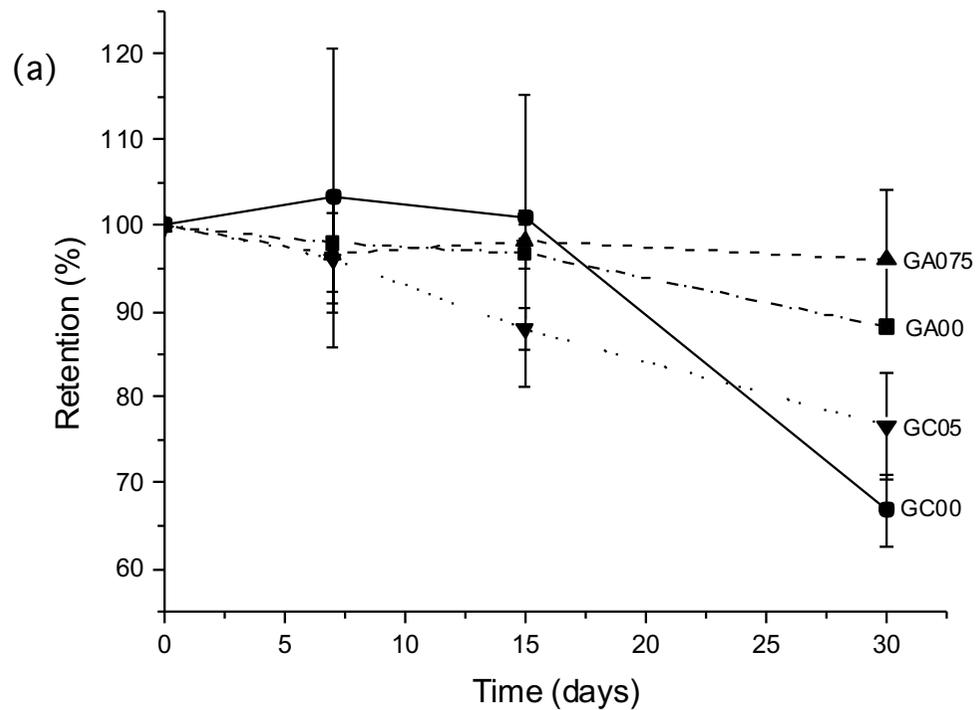
The study of the stability of omega-3 fatty acids present in the encapsulated echium oil was done in four treatments chosen based on preliminary results obtained by our group: two treatments with the gelatin-arabic gum combination (GA075 and GA00) and two treatments with the gelatin-cashew gum combination (GC05 and GC00) (compositions in Table 1). This choice was made, to compare the effect of the presence/absence of the crosslinking agent on core protection. Similar to SA, the levels of omega-3 fatty acids, ALA and SDA were monitored during the 30-day period with samples stored at 37 °C.

The results obtained for the stability of these fatty acids are shown in Figures 6.3a and 6.3b. Treatments with gelatin-arabic gum combination promoted greater protection for both fatty acids than treatments with the gelatin-cashew gum combination. Regarding the incorporation of SA, it can be affirmed that its presence in the wall of the microcapsule increased the protection of both

omega-3 fatty acids compared to the treatment with the same combination of wall material, but without SA addition. Hence, the treatment GA075 provided the greatest protection to both fatty acids. This result concurs with those obtained for MDA quantification (section 3f) and phytosterol stability (section 3g). However, they do not corroborate those obtained for SA stability, as expected. It can be explained due to the action of SA as an antioxidant, reducing its concentration over time for treatment GA075.

Umesha et al. (2015) studied the encapsulation of ALA-rich oil by emulsion followed by spray drying, using whey protein as the wall material and, also, the application of the particles in biscuits. These authors evaluated the stability of ALA under various storage conditions, reaching up to 94% oil retention. Rasti, Erfanian and Selamat (2017) studied the encapsulation of fish oil in nanoliposomes and their application in bread and milk. After 7-day storage of these products containing the nanoliposomes, the authors detected 86–96% EPA and 87–96% DHA, values that confirm those obtained for the treatment GA075 (microcapsules of echium oil and phytosterols with 0.075 g SA/g gelatin), in the current study.

Figure 6. 3. Stability of (a) alpha linolenic acid and (b) stearidonic acid present in the encapsulated oil stored for 30 days at 37 °C.



Reference: Own source.

(f) Monitoring of malonaldehyde levels

The formation of MDA, one of the main low molecular weight compounds, formed by the decomposition of lipid peroxidation products, was monitored in the treatments GA00, GA075, GC00 and GC05, during storage at 37 °C for 30 days (Figure 6.4a). Values of 0.07, 0.32, 1.13 and 1.44 mg MDA/g capsule were obtained for treatments GA075, GC05, GA00 and GC00, respectively. As MDA is generated during lipid oxidation, the higher its concentration, the more oxidized the material is, namely, among the treatments, the GA075 promoted greater protection to the encapsulated materials.

According to the results presented in Figure 6.4a, the encapsulation of echium oil and phytosterols by complex coacervation with the gelatin-arabic gum combination as wall materials and the incorporation of SA as crosslinker (0.075 g SA/g gelatin – treatment GA075) promoted the oxidative stability of the materials at 37 °C over time. In contrast, treatment GC05 (microcapsule with gelatin-cashew gum combination and the addition of 0.05 g SA/g gelatin as a crosslinker) showed an increase in the MDA concentration over time, albeit, at a lower rate than the treatments without SA present. Therefore, the different wall material combinations and addition of SA have a significant influence on the oxidative stability of the encapsulated materials.

Gray et al. (2010) studied the oxidative stability of echium seed oil during storage for 7 days at 40 °C, by analysis of thiobarbituric acid reactive substances (TBARS), a method similar to that used for the MDA quantification. These authors observed that from 2 days of storage, the TBARS concentration increased significantly over time, reaching approximately 15 mmol/kg oil. In

comparison, the current procedure that used gelatin-arabic gum and gelatin-cashew gum provided greater protection to the echium oil and phytosterols.

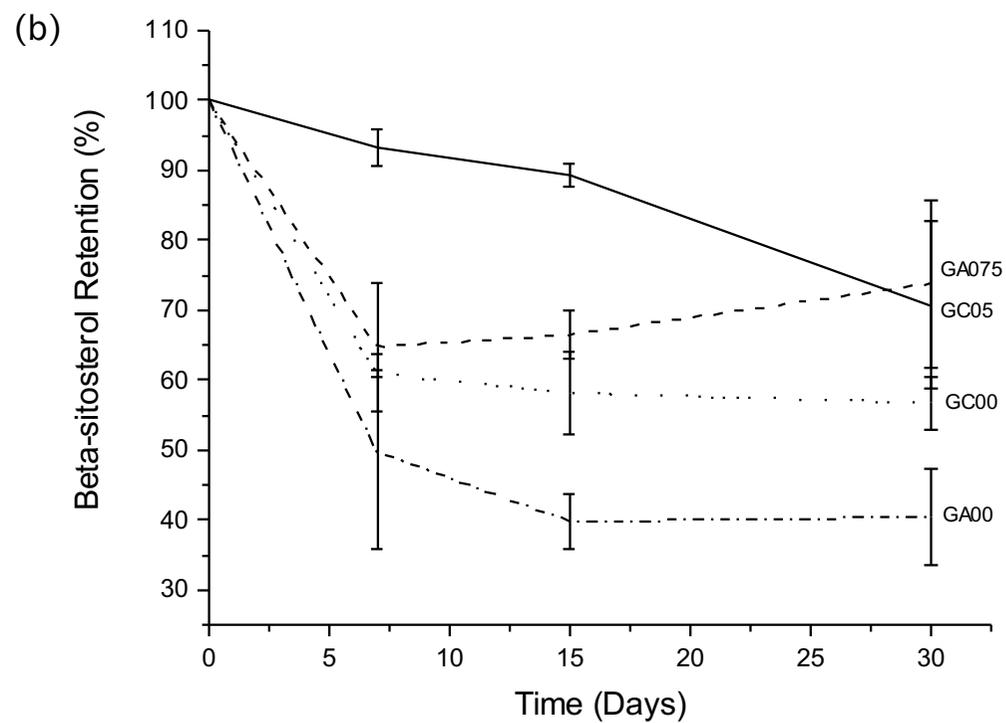
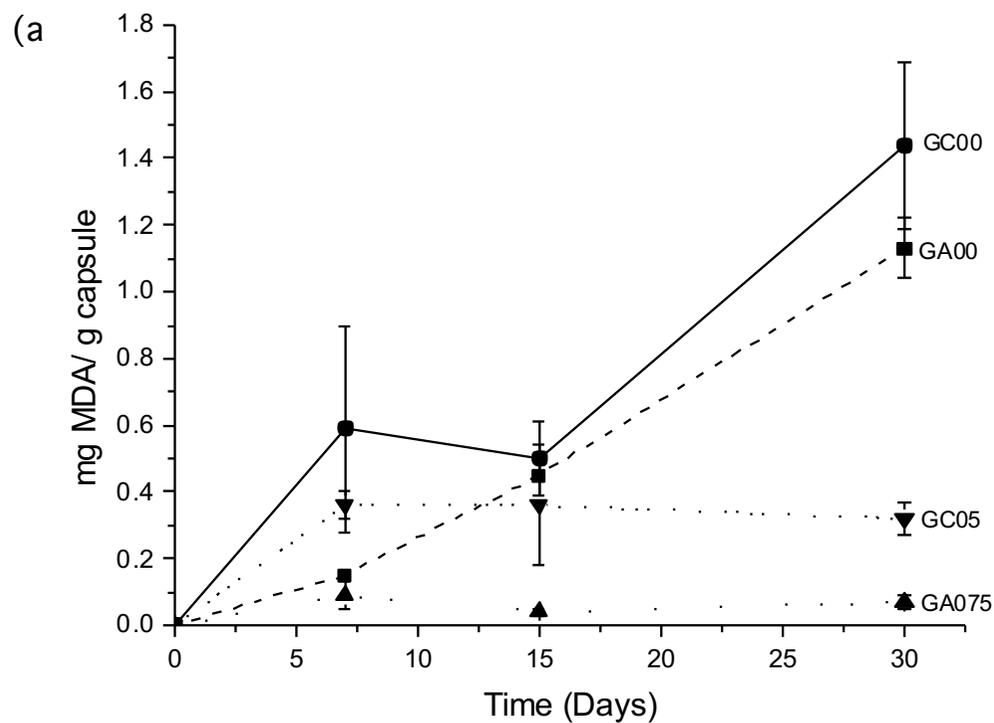
(g) Stability of encapsulated phytosterols

Similar to the omega-3 fatty acids, the study of the stability of encapsulated beta-sitosterol (the phytosterol with the highest concentration present in the used mixture) was done in the treatments GA00, GA075, GC00 and GC05 during the 30-day storage at 37 °C and are shown in Figure 6.4b. There was a significant difference among the treatments and over time, obtaining retention values of 40.42, 56.73, 73.77 and 70.74% for treatments GA00, GC00, GA075 and GC05, respectively, at 30 days of storage.

Comparing treatments with different wall materials without the addition of SA as crosslinker, it can be observed that the combination gelatin-cashew gum promoted greater protection to phytosterol than the gelatin-arabic gum combination, which was not expected given the results of fatty acid stability and MDA formation presented different behaviors. When the same combination of wall material is compared, microcapsules with the crosslinking agent promoted greater protection of beta-sitosterol, as was already expected, considering the addition of SA probably makes the capsule wall more resistant. Finally, comparing different wall material combinations with the addition of SA, the treatment with gelatin and arabic gum and 0.075g SA/g gelatin presented a higher retention value of phytosterol when stored for 30 days (73.78%). This result is in agreement with the fatty acid stability (section 3e) and MDA formation (section 3f) results, which showed that among the treatments, GA075 presented better conditions for the protection of the encapsulated compounds.

Few studies on encapsulation of phytosterols are found in the literature. Khalid et al. (2017) studied the encapsulation of beta-sitosterol in an O/W emulsion obtained by emulsification by microchannels. These authors obtained 80% core retention, when the samples were stored at 4 and 25 °C. In this instance, the retention values were higher than those achieved in the current work, for beta-sitosterol encapsulated by complex coacervation using the gelatin-arabic gum and gelatin-cashew gum combinations with the addition of SA as a crosslinker. However, it can be explained due to the higher storage temperature (37 °C) used in the present study. Moreover, even with the retention values of 73.78 and 70.74%, it can be stated that it was possible to protect phytosterol at high temperature.

Figure 6. 4. (a) Monitoring of the formation of malonaldehyde in encapsulated materials and stored for 30 days at 37 °C; (b) stability of encapsulated beta-sitosterol during the 30-day period with samples stored at 37 °C.



Reference: Own source.

4. CONCLUSIONS

The production of microcapsules of echium oil, phytosterols and the crosslinker SA, using gelatin-arabic gum and gelatin-cashew gum wall material combinations was feasible, considering the process promoted protection to the encapsulated materials. The treatment with gelatin-arabic gum combination and 0.075 g SA/g gelatin (GA075) was the best among those studied. Hence, this study obtained innovative vehicles that will bring benefits to consumers' health, protection of the compounds under consideration and the possibility of controlled release.

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**Chapter 7. DEVELOPMENT OF FUNCTIONAL YOGURT CONTAINING FREE
AND ENCAPSULATED ECHIUM OIL, PHYTOSTEROL AND SINAPIC ACID**

(PAPER PUBLISHED IN FOOD CHEMISTRY – ATTACHMENT E)

CHAPTER 7. DEVELOPMENT OF FUNCTIONAL YOGURT CONTAINING FREE AND ENCAPSULATED ECHIUM OIL, PHYTOSTEROL AND SINAPIC ACID

ABSTRACT

The consumption of omega-3 fatty acids and phytosterol promotes the reduction of cholesterol and triacylglycerol levels. However, such compounds are susceptible to oxidation, which hampers their application. The objective of this work was to coencapsulate echium oil, phytosterols and sinapic acid (crosslinker/antioxidant), and incorporate the obtained microcapsules into yogurt. The microcapsules were evaluated for particle size, accelerated oxidation by Rancimat, and simulation of gastric/intestinal release. The yogurts were assessed for morphology, pH, titratable acidity, color, rheology and sensory analysis. The microcapsules (13–42 μm) promoted protection against oil oxidation (induction time of 54.96 h). The yogurt containing microcapsules, presented a pH range from 3.89–4.17 and titratable acidity range from 0.798–0.826%, with good sensorial acceptance. It was possible to apply the microcapsules in yogurt, without compromising the rheological properties and physicochemical stability of the product.

Keywords: omega-3 fatty acids, encapsulation, complex coacervation, functional food, rheology

1. INTRODUCTION

Omega-3 fatty acids (ω -3) are compounds widely used in food, pharmaceutical and cosmetic products, due to their beneficial effects on health (KRALOVEC; ZHANG; ZHANG; & BARROW, 2012). The oil extracted from the echium seed (*Echium plantagineum* L.) contains 9–16% stearidonic acid (18:4 ω -3), which is a long-chain polyunsaturated ω -3 fatty acid. Stearidonic acid is rare in plants and very important in human nutrition because it is an intermediate in the biosynthesis of eicosapentaenoic acid (EPA, C20:5 ω -3) and docosahexaenoic acid (DHA, C22:6 ω -3), which are also associated with valuable human benefits (BERTI; JOHNSON; DASH; FISCHER; & WILCKENS, 2007; ZANETTI et al. 2013). Sterols, also known as steroid alcohols, are an important class of organic molecules that occur naturally in fungi (e.g. ergosterol), animals (e.g. cholesterol) and plants (phytosterols), and play essential roles in the physiology of eukaryotic organisms (DAMODARAN; PARKIN; & FENNEMA, 2010).

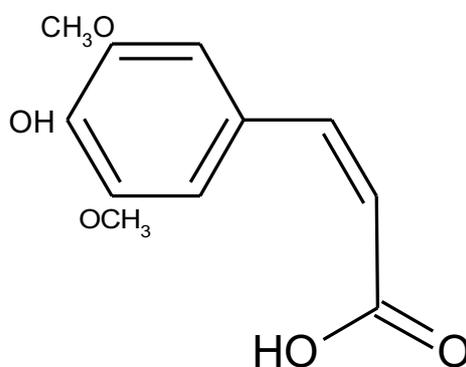
The consumption of both, ω -3 fatty acids and phytosterol, is effective in lowering the blood cholesterol and triacylglycerol levels (ESPINOSA; INCHINGOLO; ALENCAR; RODRIGUEZ-ESTRADA; & CASTRO, 2015). However, these compounds are highly susceptible to oxidation, which hampers their use and application. Two strategies used to minimize this problem include the addition of a compound with antioxidant function and microencapsulation.

Microencapsulation by complex coacervation, is caused by electrostatic interactions between two or more oppositely charged colloids (ROCHA-SELMÍ et al., 2013). Besides the formation of resistant microcapsules by complex

coacervation, the use of a crosslinking agent is being explored, in order to stabilize the structure of the complexes (CHEN et al., 2012).

Sinapic acid (SA) is both an antioxidant (ESPINOSA; INCHINGOLO; ALENCAR; RODRIGUEZ-ESTRADA; & CASTRO, 2015) and, as demonstrated in a previous work by our research group, displays a crosslinking effect on microcapsules produced by complex coacervation, using gelatin-arabic gum and gelatin-cashew gum combinations, as wall materials (COMUNIAN et al., 2016b). SA is the main phenolic acid of canola (RAWEL & ROHN, 2010) and has been studied in relation to its neuroprotective effects against Alzheimer's disease (LEE et al., 2012), cardiac hypertrophy and dyslipidemia (ROY & PRINCE, 2013). Its structural formula (Figure 7.1), possesses only one phenolic group.

Figure 7. 1. Structure of sinapic acid.



Reference: Own source.

It is known that functional foods are those containing bioactive compounds which help in protecting the body against disease and maintaining health. Microencapsulation is of extreme importance for the development of functional foods because several bioactive compounds are unstable when in

contact with light, oxygen and heat (FAVARO-TRINDADE; COMUNIAN; SOUZA; SANTOS; & OLIVEIRA, 2016). Coencapsulation of the bioactive compounds echium oil and phytosterols, has not been previously described in the literature and publications regarding the application of this type of microcapsule in food are scarce. Therefore, in this work, echium oil and phytosterols were coencapsulated, using SA, as a crosslinking and antioxidant agent. The obtained microcapsules were evaluated for their gastrointestinal release properties and their application in yogurt, to produce a functional product, by physicochemical, sensorial and rheological analyses. In order to produce a functional product, the proportion of echium oil and/or microcapsules added to yogurt, followed the standards of Brazil's National Health Surveillance Agency (ANVISA) Directors' Collegiate Resolution (RDC) No 54 of 12 November 2012, ANVISA Technical Report No 56 (2014), and the minimum daily recommendation of EPA and DHA (200 mg/day), as well as equivalent ratio of EPA and stearidonic acid, according to Decker, Akoh and Wilkes (2011).

2. MATERIALS AND METHODS

2.1. Material

Oil extracted from the *E. plantagineum* L. seed, was used as the ω -3 source (NEWmega™ Echium Oil, Ref. 15200, De Wit Speciality Oils, De Waal, Tescel, The Netherlands). Gelatin, arabic gum, cashew gum and SA, were obtained from Gelnex (Santa Catarina, Brazil), Nexira (São Paulo, SP, Brazil), The Brazilian Agricultural Research Corporation (EMBRAPA) Tropical Agribusiness (Fortaleza, Ceará, Brazil) and Sigma Chemical Co. (St. Louis,

MO, USA), respectively. The mixture of phytosterols, comprised of β -sitosterol (70–80%), β -sitostanol (0–15%), campesterol (0–15%), stigmasterol (0–2%) and campesterol (0–5%), was obtained from DuPont Danisco (Barueri, SP, Brazil). The pulp, strawberry essence and cochineal carmine dye, used in the production of yogurt, were obtained from Borsato Industrial Ltda (Farroupilha, RS, Brazil), Frutarom do Brasil (São Paulo, SP, Brazil) and Fuchs Gewurze do Brasil Ltda (Itupeva, SP, Brazil), respectively. The yogurt starter culture Granoferm BY220 (20 U), was obtained from Granolab do Brasil SA Tecnologia Para a Industria Alimentícia (Curitiba, Paraná, Brazil). Whole milk was donated by the prefecture of "Fernando Costa" campus of the University of São Paulo (Pirassununga, SP, Brazil).

2.2. Microencapsulation

The microcapsules were prepared, as described by Comunian et al (2016b) (Chapter 3). The echium oil and phytosterol (0.132 g phytosterols/g oil) mixture was used as the core and added at 50% (w/w) in relation to the total mass of polymers (gelatin-arabic gum and gelatin-cashew gum, respectively), to 5% (w/w) gelatin solution and homogenized (Ultra-Turrax T25, IKA, Germany), at 10,000 rpm for 3 min, obtaining an oil-in-water (O/W) emulsion. Solutions of 5% (w/w) arabic gum or cashew gum were, respectively, added to the O/W emulsion, under magnetic stirring at 40 °C. For complex coacervation, the respective emulsions were adjusted to pH 4.0 and the temperature reduced to 10 °C, in an ice bath.

Ratios of 1:1 gelatin-to-arabic gum and 1:2.5 gelatin-to-cashew gum, were used. The final percentages obtained were 32, 32, 32 and 4% (w/w, dry basis) of gelatin, arabic gum, echium oil and phytosterols, respectively, for the treatments with the combination of gelatin and arabic gum as wall materials. For the gelatin-cashew gum combination as the wall materials, 18.24, 45.62, 31.93 and 4.21% (w/w, dry basis) of gelatin, cashew gum, echium oil and phytosterols, respectively, were present in the final microcapsules. Treatments were prepared at four concentrations of SA as crosslinker, totaling eight treatments, as shown in Table 7.1.

Table 7. 1. Composition of treatments, values of average particle diameter and oxidative stability index for each treatment.

Treatments	Wall materials combination	Proportion of polymers	Concentration of phenolic (g/g gelatin)	Average particle size (μm)	Oxidative stability index (hours)
GA00	Gelatina: GA	1:1	0.000	32.61 \pm 14.09 ^c	10.13 \pm 0.26 ^f
GA025	Gelatina:GA	1:1	0.025	42.96 \pm 10.86 ^a	13.27 \pm 0.17 ^{de}
GA050	Gelatina:GA	1:1	0.050	37.16 \pm 10.07 ^b	28.06 \pm 0.35 ^b
GA075	Gelatina: GA	1:1	0.075	40.79 \pm 13.06 ^{ab}	54.96 \pm 1.52 ^a
GC00	Gelatina: GC	1:2.5	0.000	13.38 \pm 2.58 ^e	9.63 \pm 0.17 ^f
GC025	Gelatina: GC	1:2.5	0.025	15.28 \pm 3.06 ^{ed}	14.11 \pm 0.65 ^{cd}
GC050	Gelatina: GC	1:2.5	0.050	14.88 \pm 3.06 ^{ed}	16.57 \pm 0.16 ^c
GC075	Gelatina: GC	1:2.5	0.075	17.88 \pm 4.75 ^d	17.02 \pm 0.25 ^c
Echium oil	--	--	--	--	5.10 \pm 0.09 ^g

Reference: Own source.

Equal letters in the same column do not differ statistically at level of 5% by the Tukey test.

Based on Comunian et al. (2016b), for the crosslinking process, SA (concentrations presented in Table 7.1) was added to the solution containing the microcapsules (after the complex coacervation process, pH adjustment and temperature reduction) and maintained under low magnetic stirring at 15 °C for 12 h, in a BOD TE-391 incubator (Tecnal, Piracicaba, São Paulo, Brazil).

The coacervate material was stored at 7 °C for 24 h, to promote decantation. Then, the samples were frozen (-18 °C) and lyophilized in a Terroni freeze-dryer (São Carlos, SP, Brazil) for 24 h, at a pressure of 1 to 0.1 kPa, and initial and final temperature of -20 and 30 °C, respectively.

2.3. Characterization of microcapsules

2.3.1. Average particle size

The mean particle size of 100 microcapsules from each treatment was measured, using ImageJ, and the geometric mean of each treatment was calculated. Images of the microcapsules were obtained, using a light microscope (Bel Photonics BIO3, Italy).

2.3.2. Accelerated oxidation by Rancimat

Accelerated oxidation tests for pure echium oil and the microcapsules, were performed with a Rancimat apparatus (model 873, Metrohm, Switzerland). Samples (4 mL of pure echium oil and/or 1.5 g of microcapsules) were heated under a purified air flow rate of 20 L/h at 90 °C. The sample induction time, measured in hours, was used as the oxidative stability index.

2.3.3. Simulation of the release of SA in simulated gastric and intestinal fluids

The release of the SA present in the microcapsules was monitored for 0, 15, 30, 60, 90, 120 and 180 min in simulated gastric and intestinal fluids, under constant magnetic stirring at 37 °C. The aqueous gastric fluid solution contained 0.9% (w/w) NaCl and 0.3% (w/w) pepsin, at pH 1.8. The aqueous intestinal fluid contained 0.9% (w/w) NaCl, 1% (w/w) pancreatin, 1% (w/w) trypsin, and 0.3% (w/w) bile salts, at pH 7.8 (Gbassi, Vandamme, Ennahar, & Marchioni, 2009). A 0.2-g aliquot of microcapsule was added to 100 mL of each fluid and 1-mL aliquots were removed at each time interval, then centrifuged (Centrifuge 5430R, Hamburg, Germany) at 7500 rpm for 5 min at 4 °C. The supernatant was filtered and analyzed by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). A C18 column (Amide) was used, with a 45:55 water:methanol (pH 3.5) mobile phase, at a flow rate of 1 mL/min, and detection at 325 nm. The sample injection volume was 10 µL.

2.3.4. Simulation of the release of the oil and the mixture of phytosterols in the gastric and intestinal systems

The release of the oil and phytosterol present in the microcapsules was monitored for 0, 15, 30, 60, 90, 120 and 180 min, in simulated gastric and intestinal fluids, under constant magnetic stirring, at 37 °C, as described in section 2.3.3. Nile Red dye (Sigma-Aldrich) (1.5 mg) was added to the oil and phytosterols mixture, in order to stain the material, allowing detection by spectrophotometry at 547 nm. A 1.5-g aliquot of microcapsule was added to

100 mL of each fluid and a 5-mL aliquot was removed at each time interval, followed by addition of 2 mL butanol. The mixture was vortexed for 1 min and centrifuged at 7500 rpm for 3 min (Centrifuge 5430R, Hamburg, Germany). The butanol phase was used for spectrophotometric (Thermo Scientific, Genesys 10S UV-Vis, Shanghai, China) quantification, using butanol as the blank.

2.4.Preparation of yogurt

The microcapsule corresponding to the best treatment (GA075) was chosen for incorporation into yogurt. The yogurt was made in the dairy plant of the Campus "Fernando Costa", at the University of São Paulo (Pirassununga, SP). The following steps were involved in the yogurt manufacture: (1) heat treatment of milk (90 °C for 30 min); (2) addition of sugar; (3) cooling to 45 °C; (4) addition of yogurt starter culture; (5) incubation at 45 °C for 3 h; (6) cooling and maturation for 24 h, and (7) mixing of ingredients (strawberry pulp, strawberry essence and carmine dye) and of microcapsules and/or non-encapsulated echium oil, phytosterols and SA. The yogurts were packed in 200-mL plastic bottles and stored at 4 °C, for 0, 7, 15, 21 and 30 days. The basic yogurt formulation contained 10% (w/w) sugar, 2% (v/v) strawberry pulp, 0.075% (v/v) strawberry essence, and 0.025% (v/v) carmine dye. The three different treatments were differentiated in relation to the presence or absence of microcapsules and free bioactive compounds, as follows: T1: control yogurt, composed of the basic yogurt formulation; T2: yogurt containing the non-encapsulated bioactive compounds: 6% (w/w) echium oil, 0.1% (w/w) phytosterol mixture and 0.05% (w/w) SA; T3: yogurt containing 2% (w/w) microcapsules. The treatments T2 and T3, presented an echium oil

concentration equivalent to 15% of the minimum daily intake of EPA (100 g portion).

2.5. Characterization of yogurt

2.5.1. Morphological characterization

The yogurt was morphologically characterized, using an optical microscope (Bel Photonics, Italy) and by scanning electron microscopy (SEM), using a Hitachi SEM TM-3000 Tabletop Microscope (Hitachi High-Technologies Corp., Tokyo, Japan), equipped with the TM-3000 program. For the optical microscopy, an aliquot of yogurt was placed on a glass slide, without any preparation. For the SEM, the yogurt was frozen (-25 °C) and then lyophilized, as described in section 2.2.

2.5.2. Determination of pH and titratable acidity

The pH of the samples was analyzed using a pH meter (Marte MB-10, São Paulo, Brazil). Titratable acidity was determined by titrating up to pH 8.2, with 0.1 N NaOH and expressed as a percentage of lactic acid (AOAC, 1995; BRAZIL, 1986), for the yogurt samples stored at 4 °C, for 0, 7, 15, 21 and 30 days, respectively.

2.5.3. Determination of instrumental color

The color of the stored yogurt samples (4 °C, for 0, 7, 15, 21 and 30 days) was analyzed, using a HunterLab Mini Scan XE Plus colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA). The CIE L* a* and b* color

parameters obtained may vary from white (100) to black (0), from green (–) to red (+), and from blue (–) to yellow (+), respectively. The total color difference (ΔE), at 30 days of storage, was obtained by equation 1.

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \text{ (Eq. 1)}$$

2.5.4. Rheological characterization

The viscosity of the formulations was determined using a rotational rheometer AR 2000, TA Instruments (New Castle, Delaware, USA), with parallel plate geometry (60 mm, gap 500 μm) and the software Rheology Advantage Instrument control AR and Rheology Advantage Data Analysis. The rheological measurements were obtained at 6 °C, according to the method of Sah, Vasiljevic, McKechnie and Donkor (2016). A 3-mL aliquot of yogurt was pre-sheared for 30 s, at a shear rate of 500 s^{-1} , maintained in equilibrium for 300 s, and then analyzed for 4 min. For the frequency sweep analysis, an oscillatory stress of 0.2 Pa was set (this value was in the region of linear viscoelasticity) and a frequency ramp from 0.1 to 10 Hz, was used. The analyzed results of this test, were storage modulus (G'), loss modulus (G''), loss tangent (G''/G') and complex modulus ($((G')^2 + (G'')^2)^{1/2}$). Also, the flow curve was analyzed at a shear rate from 0.1 to 100 s^{-1} and the results modeled, according to the Herschel-Bulkley equation (Eq. 2):

$$\sigma = \sigma_0 + k \cdot \gamma^n \text{ (Eq. 2)}$$

where, σ_0 , k and γ represent the yield stress (Pa), consistency index (Pa s) and shear rate (s^{-1}), respectively. Values of n are necessary to define the type of fluid: $n = 1$: Newtonian fluid; $n < 1$: pseudoplastic fluid and $n > 1$: dilating fluid (Sah, Vasiljevic, McKechnie & Donkor, 2016).

2.5.5. Consumer acceptance test

Sensory evaluation is a valuable tool for the development and quality evaluation of products with added polyunsaturated oils. The sensory acceptance analysis was performed in individual cabins, according to the method described by Meilgaard et al. (1999). The samples were evaluated using a 9-point hedonic scale (1 - "I highly disagree" and 9 - "I liked it very much"), for the attributes of taste, texture, color, flavor and overall acceptance. A total of 126 untrained tasters, of both genders and of various age, participated in the test. The tasters were selected on the basis of being regular consumers of yogurt and for their willingness to participate in the trials. The sample was presented monadically, in coded cups and in a random manner. This study was approved by the Research Ethics Committee of the College of Animal Science and Food Engineering, of the University of São Paulo (number of the Certificate of Presentation for Ethical Assessment (CAAE): 52954916.6.0000.5422).

2.6. Statistical analysis

The data were statistically analyzed using the statistical program SAS (Statistic Analysis System), version 9.0, by analysis of variance (ANOVA) and Tukey's test, at the significance level of 5%.

3. RESULTS AND DISCUSSION

3.1. Characterization of microcapsules

3.1.1. Average particle size

The average particle sizes obtained for the treatments ranged from 13 to 42 μm (Table 7.1). There was a significant difference among all the treatments. The treatments with gelatin-arabic gum presented particle size values almost three times higher than the treatments with gelatin-cashew gum. Ifeduba et al. (2015) also studied the microencapsulation of stearidonic acid by complex coacervation, using gelatin and arabic gum as wall materials and reported average particle sizes ranging from 286 to 296 μm for microcapsules produced using a low homogenization speed (600 rpm) and 45 to 56 μm for microcapsules produced at high homogenization speed (19,400 rpm). This broad range of average particle sizes obtained, can be attributed to several factors, such as differences in the wall materials, the concentration of polymers and encapsulated material, temperature and homogenization speed of the process. According to Comunian and Favaro-Trindade (2016), the average particle size is a critical factor to be considered when incorporating the microcapsules into a food product, because they may negatively interfere with the texture of the final product. The microcapsules obtained in this work, can be applied in food because they presented average diameters below 100 μm .

3.1.2. Accelerated oxidation by Rancimat

Table 7.1 presents the induction time (hours), which was used to represent the oxidative stability index of the encapsulated and pure oil. The

oxidative stability index ranged from 9.95–54.96 h for treatments and 5.10 h for pure echium oil. A comparison of the pure echium oil with the same encapsulated oil, without the crosslinking agent (treatments GA00 and GC00), shows that it was possible to increase the oxidative stability of the oil two-fold. Namely, encapsulation by complex coacervation, using gelatin-arabic gum (GA) and gelatin-cashew gum (GC) as wall materials, respectively, promoted greater oxidative stability to echium oil.

For treatments with addition of various SA concentrations, the oxidative stability index increased with increasing phenolic compound concentration, obtaining up to 10-fold more oxidative stability for the oil when encapsulated with the treatment GA075 and 3.5-fold more in the case of treatments GC050 and GC075. Thus, the encapsulation of echium oil by complex coacervation, using SA as a crosslinking and antioxidant agent, is an effective technique to promote increased stability to the encapsulated material.

Wang et al. (2015) studied the microencapsulation of tuna oil fortified with multiple lipophilic ingredients, such as vitamins A, D3, E, and K2, as well as curcumin and coenzyme Q10, using gelatin and sodium hexametaphosphate as wall materials. These authors obtained oxidative stability index values ranging from 5.59–6.23 h, which are similar to pure echium oil. In comparison, the technique used in this work, provided considerably greater oxidative stability to the oil. However, a direct comparison with the current study is not practical given that a different oil was studied by Wang et al. (2015).

Previously, Comunian et al. (2016a, 2016b) studied the coencapsulation of echium oil and phenolic compounds by complex coacervation using gelatin-arabic gum and gelatin-cashew gum combinations as wall materials,

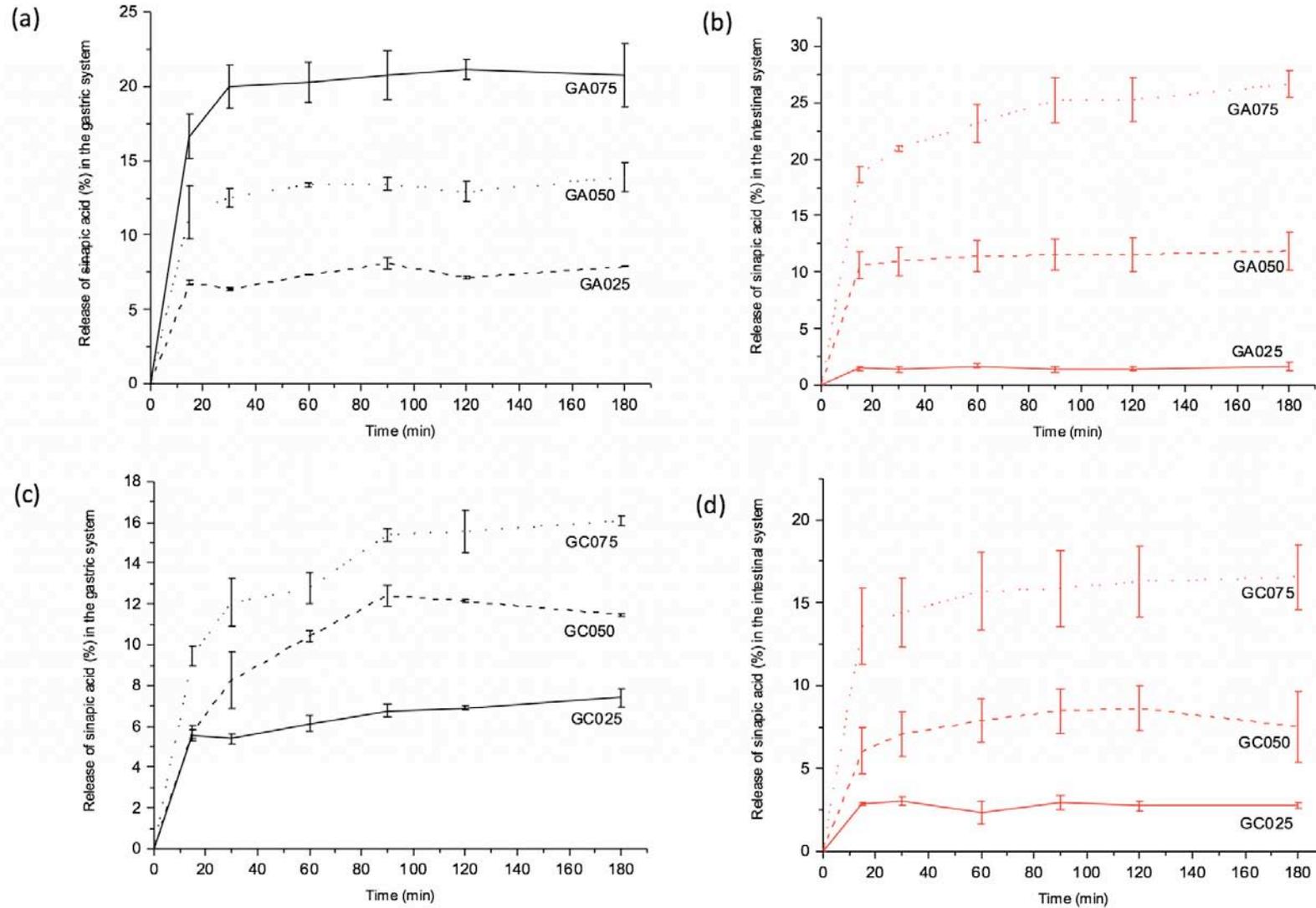
respectively, and obtained oxidation index values ranging from 9.97–18.77 and 8.73–26.50 h, respectively. The difference between these values and that obtained for the microcapsules with echium oil, SA and phytosterols, can be explained by the different concentrations of SA applied, which were 200 ppm SA relative to the mass of oil (Comunian et al., 2016a, 2016b) and 0.05 g SA/g gelatin for the microcapsules, respectively.

3.1.3. Simulation of the release of SA

The behavior of the release of SA from each treatment, under conditions simulating the gastric and intestinal system, is presented in Figure 7.2. It can be stated that the higher the concentration of SA, the greater its release in the system, both for the gelatin-arabic gum and the gelatin-cashew gum combinations as wall materials, ranging from 6–20 and 2.5–25% for gelatin-arabic gum treatments in the gastric and intestinal system, respectively, and from 7–16 and 2.5–17%, for treatments with gelatin-cashew gum in the gastric and intestinal system, respectively, for 180 min.

Initially, the release rates were higher in both fluids, due to diffusion through the wall of the rehydrated microcapsule. Over the analyzed time, the rate of release of the phenolic compound decreased, until reaching a plateau. Considering that SA has beneficial health properties, it would be better to have a higher release of SA with echium oil and phytosterols, however, this did not occur. Furthermore, these low release values can be explained by the crosslinking reaction of the SA with the protein and the polysaccharide, according to Comunian et al. (2016b) (Chapter 3).

Figure 7. 2. Simulation of the gastric (a and c) and intestinal (b and d) release of sinapic acid over 180 min, from the treatments prepared with gelatin-arabic gum (a and b) and gelatin-cashew gum (c and d).



Reference: Own source.

By comparing the same treatments in the different fluids, it can be affirmed that there was greater release in the intestinal system. However, by comparing different combinations of wall materials, with the same SA concentrations and in the same fluid, it can be stated that the microcapsules with the gelatin-arabic gum combination showed a higher phenolic compound release than the treatments with the gelatin-cashew gum combination. Again, this higher release rate for treatments with the gelatin-arabic gum combination can be attributed to the stronger crosslinking bonds between SA and cashew gum. Thus, it could be hypothesized that the composition of the polysaccharides affects the extent of crosslinking by SA. Both arabic gum and cashew gum are polysaccharides composed of D-galactose, L-arabinose, and L-rhamnose, in addition to D-glucuronic acid and 4-O-methyl-D-glucuronic acid (MOTHÉ & CORREIA, 2002), differing only in the presence of mannose, glucose and xylose, as terminal residues in cashew gum. According to Comunian et al. (2016b) (Chapter 3), one of the ways for the crosslinking reaction to occur between SA and a polysaccharide is by the Fischer esterification reaction, with an OH group from a sugar residue, which was present mainly in cashew gum. This suggests that the presence of mannose, glucose and xylose, in the composition of cashew gum, promoted a greater crosslinking reaction with SA.

3.1.4. Simulation of release of oil and phytosterols mixture

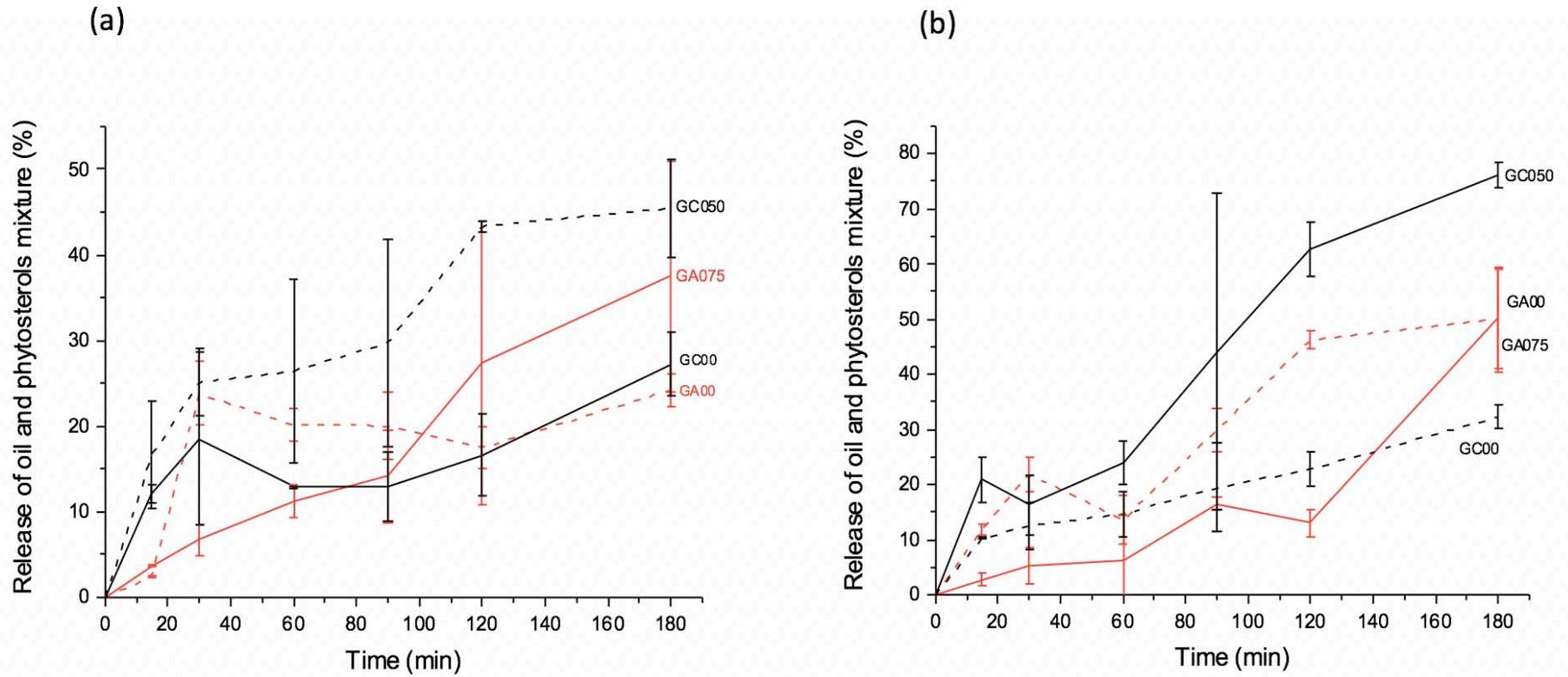
The study of the release behavior of echium oil and the phytosterols mixture, was done with four treatments that were chosen based on the results of accelerated oxidation by Rancimat. Thus, two treatments with the gelatin-

arabic gum combination (GA075 and GA00) and two treatments with the gelatin-cashew gum combination (GC050 and GC00) were investigated, to compare the effect of the presence and absence of the crosslinking agent on the release of the core. The treatments GA075 and GC050 were chosen due to the better oxidative stability afforded to echium oil.

The behavior of the release of the oil and phytosterols from these treatments, under conditions simulating the gastric and intestinal system, is shown in Figure 7.3. The core release rates ranged from 24–45 and 32–76% for the gastric and intestinal fluids, in 180 min. Treatments with the presence of SA, presented a higher rate of release of the core in the gastric fluid than the treatments without the phenolic compound, which was not expected and a reasonable explanation cannot be provided for this observation. In the case of the intestinal fluid, no clear trend was detected in the association between the presence or absence of the phenolic compound, and the release of the core.

Tello et al. (2016) studied the controlled release of soybean oil containing paprika oleoresin, in microcapsules obtained by complex coacervation, using gelatin and arabic gum as wall materials, and transglutaminase and glutaraldehyde as crosslinking agents. These authors documented oil release values in the gastric system (pH 1.2, at 37 °C for 2 h) that ranged from 17–32.3% for microcapsules with transglutaminase and from 9.1–10.3% for microcapsules with glutaraldehyde. These authors obtained lower release rates for paprika oleoresin compared to the values obtained in the release of echium oil and phytosterols, besides a shorter analysis time (2 h). Furthermore, glutaraldehyde is toxic and not allowed for food applications (COMUNIAN & FAVARO-TRINDADE, 2016).

Figure 7. 3. Simulation of the release of the mixture of oil and phytosterols in (a) gastric fluid and (b) intestinal fluid, during 180 minutes.



Reference: Own source.

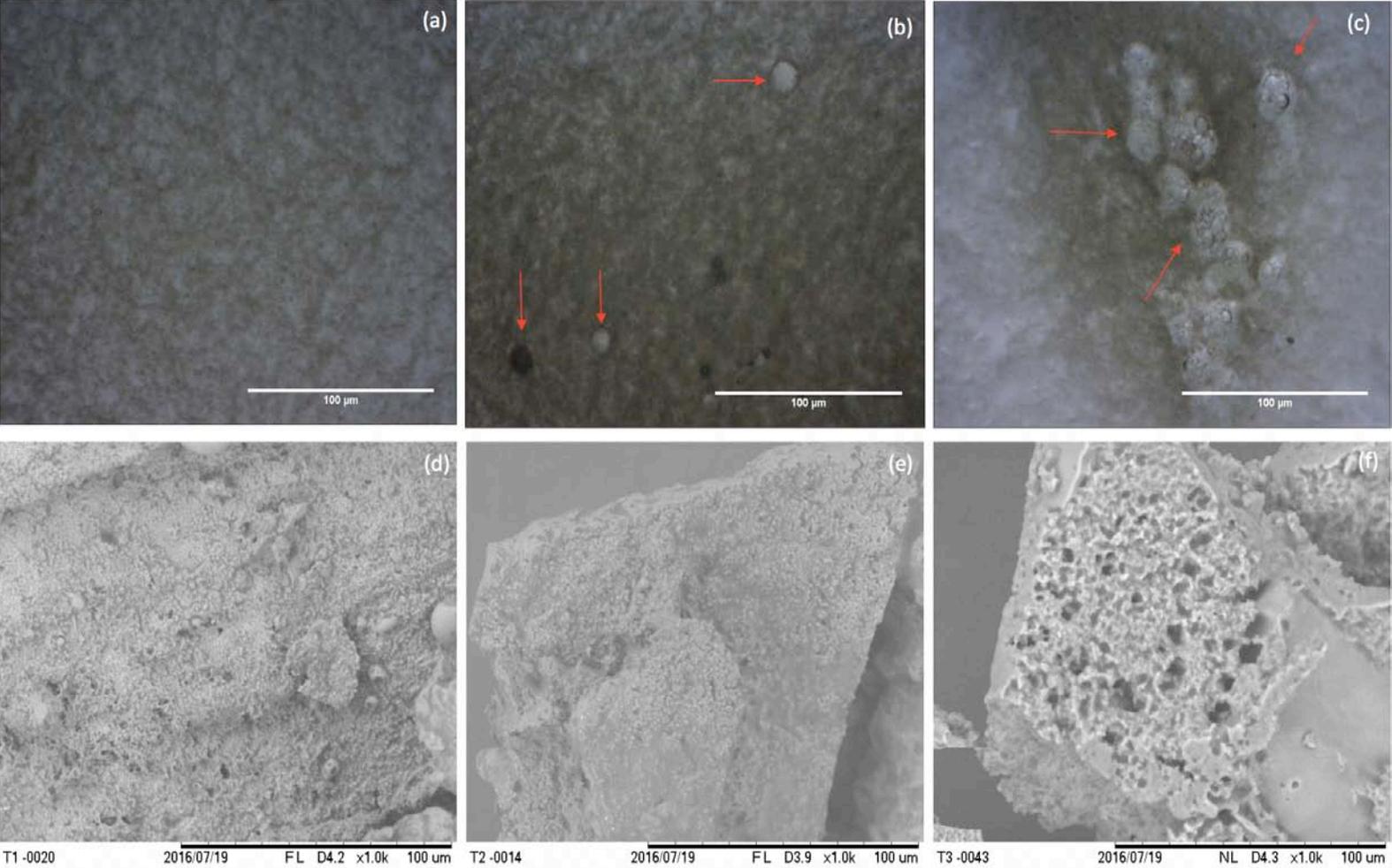
3.2. Yogurt characterization

Based on the results obtained for the different microcapsules, the treatment GA075 was chosen for the application in strawberry flavored yogurt. The yogurt control and yogurt with the added non-encapsulated echium oil, phytosterols and SA, were also produced, totaling three treatments, as mentioned in section 2.4.

3.2.1. Morphological characterization

Figure 7.4 shows the micrographs obtained by optical microscopy and SEM of the three yogurt formulations. It is possible to observe the presence of the droplets of oil and phytosterols, in the treatment with the non-encapsulated bioactive compounds (Figure 7.4b). The mixture of phytosterols at room temperature, shows a viscous behavior, however, when at lower temperatures, such as in yogurt (cooling temperature), the phytosterols solidify, which makes the homogenization of the final product difficult. In the case of treatment T3, in which microcapsules were added to the yogurt, it is possible to observe the structure of the microcapsules in the final product (Figure 7.4c). It is inferred that the encapsulation of phytosterol helps, even in its application in food, because it allows a homogeneous dispersion of the ingredient. These characteristics were not observed in Figure 7.4a, corresponding to the treatment T1 (yogurt control) because it is composed only of the basic formulation (refer to section 2.4.).

Figure 7. 4. Optical microscopy of the yogurts (a) T1 (yogurt control - without bioactive compounds added), (b) T2 (yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added), and (c) T3 (yogurt with added microcapsules). Scanning electron microscopy of the yogurts (d) T1 (yogurt control - without bioactive compounds added), (e) T2 (yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added), and (f) T3 (yogurt with added microcapsules).



Reference: Own source.

SEM was performed to visualize changes in the microstructure of the yogurt gel, due to the variation in the composition of the three treatments. Morphological differences were observed in the yogurt gel structures (Figure 7.4d, 7.4e and 7.4f), revealing a more open and larger pore sized network was present in treatment T3, corresponding to the yogurt with added microcapsules (Figure 7.4f) than the remaining treatments. According to Sah et al. (2016), this can be explained due to the thermodynamic incompatibility between the polysaccharide present in the wall of the microcapsule and the milk proteins. However, this incompatibility did not damage the structure and the physicochemical and sensory characteristics of the final product were retained.

3.2.2. Determination of pH and titratable acidity

The values of pH and titratable acidity, measured as a percentage of lactic acid, for each treatment stored at 4 °C for 30 days, are shown in Table 7.2. The pH values ranged from 4.14 to 4.05 and from 4.10 to 4.02, for samples stored for 0 and 30 days, respectively. Titratable acidity ranged from 0.789–0.826 and 0.782–0.802%, for samples stored for 0 and 30 days, respectively.

There was a significant difference ($p < 0.05$) in the values of pH and titratable acidity among treatments stored for the same duration and, also, for the same treatment throughout the 30 days of storage. However, it was not possible to establish an association for pH and titratable acidity as a function of the presence and/or absence of the microcapsules and the non-encapsulated bioactive compounds in the yogurt. These results show that the incorporation of the microcapsules of echium oil, phytosterols and SA affected the pH and the

titratable acidity of the yogurt, however, they did not influence the quality of the final product. Thus, pH around 4.0 is ideal for maintaining the structure of the microcapsules intact, preventing the release of the encapsulated materials during the storage of the yogurt.

Sah et al. (2016) obtained pH 4.48–4.50 at 1 day of storage and pH 4.23–4.29 at 28 days of storage at 4 °C, for probiotic yogurt enriched with pineapple peel rich in fiber. These values corroborate with those obtained in the current study, for yogurt with the added microcapsules of echium oil, phytosterols and SA.

For yogurt enriched with fish oil microcapsules and stored for 21 days, Tamjidi, Nasirpour and Shahedi (2011) obtained titratable acidity values ranging from 0.77–0.97%. Similar values in the current study were recorded for yogurt with added microcapsules of echium oil, phytosterols and SA but only early on in the storage. According to Tamjidi et al. (2011) and Serra et al. (2009), the decrease in pH and the increase in titratable acidity of the yogurt samples during storage, can be explained by the metabolic activity of the dairy culture used in yogurt production, which implies the fermentation of lactose with the production of lactic acid. These changes in pH and titratable acidity were not observed in yogurts with added microcapsules of echium oil, phytosterols and SA, ensuring that the incorporation of these capsules into yogurt does not interfere with the stability of the product during the evaluated storage duration.

3.2.3. Color

The color parameters L^* (brightness), a^* (green (-) to red (+)), and b^* (blue (-) to yellow (+)), for each treatment stored at 4 °C for 30 days, are presented in Table 7.2. The total color difference (ΔE) values were obtained, by comparing the values recorded at 0 and 30 days of storage.

The L^* values ranged from 62.43–62.59 and 62.64–62.75, for yogurts at 0 and 30 days of storage, respectively. There was a statistical difference among treatments for the same day and among different days for the same treatment, however, this difference may be considered small. Due to the slight variation in these values, it can be affirmed that all the samples were bright during the analyzed period. However, there was no evident trend of behavior among the samples. Namely, no relation was apparent between the presence and/or absence of microcapsules and non-encapsulated bioactive compounds and brightness values. Sah et al. (2016) obtained L^* values ranging from 84.53–90.43 and 84.84–91.54 at 1 and 28 days storage at 4 °C, for probiotic yogurt enriched with pineapple peel rich in fiber. The values obtained by these authors were higher than those obtained for the treatments of this work, due to the different compositions, mainly in relation to the presence of fibers.

The a^* values ranged from 8.63–8.99 and 8.93–9.11, for the yogurts stored for 0 and 30 days, respectively. These values originated from the pink/red strawberries used to produce the yogurt. With the small variation presented among the treatments and over the 30 days, it can be reaffirmed that the incorporation of the microcapsules did not interfere with the yogurt coloration compared to the yogurt control.

Likewise, the b^* values ranged from -0.89 to -0.62 and from -0.87 to -0.47 for yogurts stored for 0 and 30 days, respectively. There were statistical differences among the treatments for the same time evaluated and also for the same treatment over the 30 days. This difference can be attributed to the compositions of each treatment because the microcapsules are also composed of gelatin and arabic gum as wall materials. However, even though there was a statistical difference in the instrumental color of the final product, this difference did not influence the quality of the yogurt.

Tamjidi, Nasirpour and Shahedi (2011) obtained a^* values from -2.94 to -1.69 and b^* values from 11.12 to 16.87 over the 21-day storage period for yogurt enriched with microcapsules of fish oil. These values are considerably different to those obtained in the current study, for yogurt with the microcapsules of echium oil, phytosterols and SA because the latter yogurt was made with strawberry pulp and carmine dye.

Regarding the total color difference (ΔE), values of 0.32 ± 0.11 , 0.26 ± 0.05 and 0.47 ± 0.11 were obtained, for the treatments T1 (yogurt control), T2 (yogurt with non-encapsulated bioactive compounds added) and T3 (yogurt with added microcapsules), respectively. Treatments T1 and T2 were statistically different ($p < 0.5$) to treatment T3. Thus, the addition of microcapsules promoted a greater color change during storage than the yogurt control, probably due to the presence of gelatin and arabic gum as wall materials. However, this change did not interfere with the acceptance of the product, as observed in section 3.2.5.

Table 7. 2. Values of L*, a*, b*, pH and titratable acidity for each treatment in the period of 30 days.

Treatments/ days	0	7	15	21	30
L*					
T1	62.59 ± 0.01 c, A	62.77 ± 0.08 a, A	62.69 ± 0.03 b, A	62.83 ± 0.01 a, A	62.65 ± 0.04 bc, B
T2	62.43 ± 0.03 d, B	62.71 ± 0.03 b, A	62.72 ± 0.02 b, A	62.79 ± 0.01 a, B	62.64 ± 0.02 c, B
T3	62.43 ± 0.10 b, B	62.81 ± 0.23 a, A	62.69 ± 0.04 a, A	62.74 ± 0.02 a, C	62.75 ± 0.04 a, A
a*					
T1	8.99 ± 0.08 c, A	9.18 ± 0.03 ab, A	9.03 ± 0.12 c, A	9.26 ± 0.04 a, A	9.11 ± 0.08 bc, A
T2	8.99 ± 0.04 cd, A	9.14 ± 0.07 ab, A	9.07 ± 0.09 bc, A	9.25 ± 0.04 a, A	8.93 ± 0.10 d, B
T3	8.63 ± 0.14 c, B	9.04 ± 0.08 a, B	8.83 ± 0.09 b, B	8.92 ± 0.07 ab, B	8.94 ± 0.09 ab, B
b*					
T1	-0.89 ± 0.05 c, B	-0.76 ± 0.09 bc, B	-0.79 ± 0.07 bc, B	-0.73 ± 0.06 ab, B	-0.62 ± 0.12 a, A
T2	-0.89 ± 0.08 ab, B	-0.86 ± 0.09 a, B	-1.02 ± 0.08 b, C	-0.93 ± 0.05 ab, C	-0.87 ± 0.09 a, B
T3	-0.62 ± 0.04 b, A	-0.45 ± 0.03 a, A	-0.61 ± 0.07 b, A	-0.53 ± 0.03 ab, A	-0.47 ± 0.08 a, A
pH					
T1	4.14 ± 0.03 a, A	4.13 ± 0.03 a, A	4.16 ± 0.02 a, AB	3.93 ± 0.07 c, A	4.05 ± 0.02 b, A
T2	4.11 ± 0.01 b, B	4.09 ± 0.01 b, B	4.15 ± 0.01 a, B	3.88 ± 0.01 d, A	4.02 ± 0.01 c, B
T3	4.10 ± 0.01 b, B	4.07 ± 0.01 c, B	4.17 ± 0.01 a, A	3.89 ± 0.01 e, A	4.05 ± 0.01 d, A
Titratable acidity (% of lactic acid)					
T1	0.789 ± 0.03 b, A	0.776 ± 0.01 b, B	0.800 ± 0.03 ab, B	0.825 ± 0.01 a, A	0.795 ± 0.01 ab, A
T2	0.799 ± 0.01 bc, A	0.803 ± 0.01 b, A	0.805 ± 0.01 b, B	0.828 ± 0.01 a, A	0.782 ± 0.01 c, A
T3	0.826 ± 0.03 ab, A	0.798 ± 0.01 b, A	0.835 ± 0.00 a, A	0.825 ± 0.01 ab, A	0.802 ± 0.01 b, A

Reference: Own source.

Equal lowercase letters in the same row and capital letters in the same column do not differ statistically at the level of 5% by the Tukey test.

T1: yogurt control (without bioactive compounds addition);

T2: yogurt added of non-encapsulated echium oil, phytosterols and sinapic acid;

T3: yogurt added of microcapsules.

3.2.4. Rheological characterization

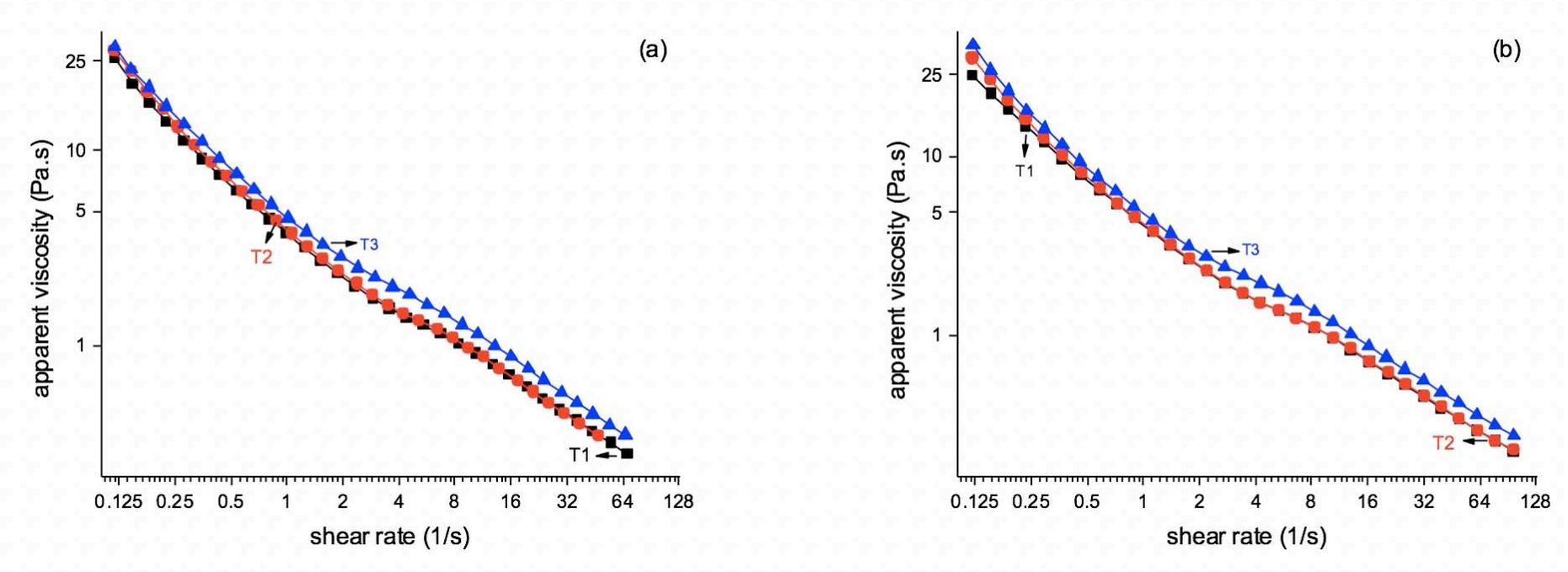
Rheological characterization analyzes allow studying the deformations and flows of matter, when submitted to a certain stress rate. In the current study, the apparent viscosity of the yogurts decreased with increased rate of deformation during the shear (Figure 7.5a and 7.5b) and remained stable, without significant difference throughout the storage (Table 7.3). However, there was a significant difference among the treatments from the first day of analysis, with values ranging from 0.526–0.689 and 0.539–0.663 Pa.s, for the samples stored for 0 and 30 days, respectively, and a deformation rate of 20 s^{-1} . The treatment T3, presented higher values than the treatments T1 and T2, as expected, due to the addition of the microcapsules, which increases the solids content of the final product. This higher viscosity influenced the evaluation of the attribute "texture", in the sensorial analysis (refer to section 3.2.5). However, despite this significant difference, the addition of the microcapsules did not influence the quality of the final product.

In the case of the yield stress (Table 7.3), only treatment T1 (yogurt control) showed a significant difference during the 30 days. When comparing treatments on the same day, there was a statistical difference, as expected, considering the varied compositions presented visually different firmness. Also, according to Sah et al. (2016), yield stress is positively correlated to firmness. In the case of consistency index values (Table 7.3), there was a significant difference for treatment T2 over time and among treatments stored for the same duration. It was not possible to establish an association regarding the increase in the consistency index values for treatment T2, however, the difference

among the treatments was expected, due to the variation in the compositions and apparent viscosities, as above-mentioned.

The G' values (elastic modulus or storage modulus), which measures the ability of the material to store energy, and G'' (viscous or loss modulus), which measures the ability of the material to dissipate energy, were also obtained, in order to calculate the complex modulus (G^*) for each treatment stored at 4 °C for 30 days, as presented in Table 7.3. The G^* values obtained, ranged from 65.20–83.45 and 57.99–99.18 Pa at 0 and 30 days of storage, respectively. There was no significant difference for treatments T1 (yogurt control) and T3 (yogurt added of microcapsules) during the time analyzed. However, there was a significant difference between treatments on the same day, with higher values for treatment T3. That is, the addition of the microcapsules promoted the formation of a weak gel, as per the other treatments, however, with increased elastic behavior compared to the other yogurt samples.

Figure 7. 5. Effect of storage time on apparent viscosity as a function of deformation rate, for treatments T1 (black curve), T2 (red curve), and T3 (blue curve), at (a) 7 and (b) 30 days of storage. T1 refers to the yogurt control (without bioactive compounds added); T2 refers to yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added, and T3 refers to the yogurt with microcapsules added.



Reference: Own source.

Table 7. 3. Yield stress, consistency index (K), Complex modulus (G*) (at frequency of 1 Hz) and apparent viscosity at shear rate of 20 s-1 ($\eta_a, 20$) for each treatment stored at 4 °C for 30 days.

Yield stress (Pa)					
Treatments	Day 0	Day 7	Day 15	Day 21	Day 30
T1	1.30 ^{a,A}	0.98 ^{b,A}	1.00 ^{b,A}	0.92 ^{b,AB}	0.90 ^{b,B}
T2	1.05 ^{a,A}	1.05 ^{a,A}	0.98 ^{a,A}	0.77 ^{a,B}	0.89 ^{a,B}
T3	1.10 ^{a,A}	1.10 ^{a,A}	1.40 ^{a,A}	1.35 ^{a,A}	1.58 ^{a,A}
K (Pa.s)					
Treatments	Day 0	Day 7	Day 15	Day 21	Day 30
T1	2.95 ^{a,B}	2.93 ^{a,B}	3.63 ^{a,A}	3.54 ^{a,A}	3.57 ^{a,C}
T2	3.43 ^{b,AB}	3.42 ^{b,AB}	3.78 ^{ab,A}	3.87 ^{ab,A}	4.10 ^{a,B}
T3	4.30 ^{a,A}	4.25 ^{a,A}	4.59 ^{a,A}	5.14 ^{a,A}	4.19 ^{a,A}
G*(Pa)					
Treatments	Day 0	Day 7	Day 15	Day 21	Day 30
T1	65.96±11.40 ^{a,A}	56.74±2.65 ^{a,B}	61.46±4.66 ^{a,C}	54.33±1.91 ^{a,B}	57.99±1.36 ^{a,C}
T2	65.20±0.00 ^{b,A}	65.63±3.37 ^{b,A}	77.62±2.67 ^{a,B}	60.68±1.41 ^{b,B}	74.81±0.00 ^{a,B}
T3	83.45±5.71 ^{a,A}	87.28±7.73 ^{a,A}	93.90±0.00 ^{a,A}	90.89±6.13 ^{a,A}	99.18±5.51 ^{a,A}
η (Pa.s)					
Treatments	Day 0	Day 7	Day 15	Day 21	Day 30
T1	0.526±0.00 ^{a,B}	0.529±0.03 ^{a,B}	0.552±0.02 ^{a,B}	0.538±0.00 ^{a,B}	0.539±0.01 ^{a,C}
T2	0.540±0.00 ^{a,B}	0.55±0.00 ^{a,B}	0.541±0.01 ^{a,B}	0.539±0.02 ^{a,B}	0.567±0.00 ^{a,B}
T3	0.689±0.01 ^{a,A}	0.673±0.00 ^{a,A}	0.691±0.03 ^{a,A}	0.758±0.08 ^{a,A}	0.663±0.00 ^{a,A}

Reference: Own source.

Equal lowercase letters in the same row and capital letters in the same column do not differ statistically at the level of 5% by the Tukey test.

T1: yogurt control (without bioactive compounds addition);

T2: yogurt added of non-encapsulated echium oil, phytosterols and sinapic acid;

T3: yogurt added of microcapsules.

3.2.5. Consumer acceptance test

The scores obtained for the flavor, color, texture, taste and overall acceptability attributes are presented in Table 7.4. There was a significant difference among the treatments for all attributes, except for color.

For the flavor attribute, the treatment with the added microcapsules (T3) presented similar acceptance to treatment T1 (yogurt control), with average scores of 6.73 and 6.65, respectively, followed by treatment T2 (yogurt with non-encapsulated bioactive compounds added), with a mean score of 6.21. Thus, the incorporation of the encapsulated bioactive compounds into the product improved the flavor in comparison to the addition of their non-encapsulated counterparts because the echium oil may present an unpleasant flavor, after a certain time of exposure to oxygen.

Regarding the color attribute, there was no significant difference in the average scores attributed to the samples. Therefore, the addition of the microcapsules did not influence the visual color of the final product.

In the case of the texture attribute, treatments T1 (yogurt control) and T2 (yogurt with the non-encapsulated bioactive compounds added) presented better acceptance than treatment T3 because the yogurt viscosity increased with the addition of microcapsules, as observed in the rheological characterization (refer to section 3.2.4). Nevertheless, the yogurt with added microcapsules, obtained a score corresponding to "I liked it lightly", while the other treatments obtained scores corresponding to "I liked it regularly", proving that the incorporation of the capsules did not have a negative influence on the acceptance by the tasters.

The texture had a marked influence on the scores obtained for the taste and overall acceptability attributes. In this case, the treatments T1 and T2 showed better acceptance than treatment 3 (yogurt with added microcapsules). However, treatment T3 maintained the score corresponding to "I liked slightly", and treatments T2 and T3 received scores corresponding to "I liked it regularly". Notably, treatment T2 showed droplets of oil and particles of the phytosterol mixture on the surface, promoting the rejection by several tasters.

Table 7. 4. Scores obtained in sensory analysis for the attributes flavor, color, texture, taste and general acceptance.

Treatments	Flavor	Color	Texture	Taste	General acceptance
T1	6.65 ± 1.57 ^{ab}	7.14 ± 1.26 ^a	7.19 ± 1.35 ^a	6.97 ± 1.49 ^a	7.07 ± 1.20 ^a
T2	6.21 ± 1.72 ^b	6.96 ± 1.36 ^a	7.13 ± 1.49 ^a	6.81 ± 1.61 ^a	7.78 ± 1.50 ^{ab}
T3	6.73 ± 1.46 ^a	7.04 ± 1.29 ^a	6.55 ± 1.70 ^b	6.22 ± 1.83 ^b	6.47 ± 1.46 ^b

Reference: Own source.

Equal letters in the same column do not differ statistically at the level of 5% by the Tukey test.

T1: yogurt control (without bioactive compounds addition);

T2: yogurt added of non-encapsulated echium oil, phytosterols and sinapic acid;

T3: yogurt added of microcapsules.

4. CONCLUSIONS

Efficient coencapsulation of echium oil and phytosterols was achieved, using gelatin-arabic gum and gelatin-cashew gum combinations, respectively, as wall materials and the phenolic compound SA, as the crosslinking and antioxidant agent. Specifically, the microcapsules provided oxidative stability to the encapsulated bioactive compounds, and the average particle size and

release of the bioactive compounds into the simulated gastric and intestinal fluids, indicate their suitability for food applications. Additionally, the process of encapsulation by complex coacervation was effective for the application of these bioactive compounds into yogurt, considering that the physicochemical, rheological and sensorial properties of the yogurt with added microcapsules were similar to the yogurt control (without bioactive compounds addition) and superior to the yogurt with the non-encapsulated bioactives added. Furthermore, the improved oxidative stability of the oil, induced by microencapsulation, guarantees the quality of the final product.

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Chapter 8. GERAL CONCLUSION

CHAPTER 8. GERAL CONCLUSION

It was possible to conclude that the microencapsulation was a great alternative to improve the stability of echium oil, phytosterols and phenolic compounds. First, when echium oil and phenolic compounds were encapsulated by complex coacervation, the combinations of gelatin-arabic gum and gelatin-cashew gum and the incorporation of sinapic acid as crosslinker were ideal for the protection of them. Moreover, the coencapsulation of echium oil and sinapic acid by emulsion followed by spray drying or freeze drying were also suitable for the oxidative protection of the oil and the morphology and values of hygroscopicity, moisture and solubility were optimum for the application, storing and handling of the powder. In addition, the microencapsulation of echium oil by a combination of microfluidic and ionic gelation techniques was also feasible, since high values of encapsulation yield and oxidative stability of echium oil during storage at 40 °C were obtained.

In this way, the production of microcapsules of echium oil, phytosterols and sinapic acid-crosslinked using gelatin-arabic gum and gelatin-cashew gum combinations was feasible since the process promoted protection to the encapsulated materials; the treatment with the gelatin-arabic gum combination and 0.075 g of sinapic acid/ g of gelatin (GA075) was the best among those presented. So, the application of treatment GA075 into yogurt was effective, considering that the physicochemical, rheological and sensorial properties of the yogurt with added microcapsules were similar to the yogurt control and superior to the yogurt with the non-encapsulated bioactives, thereby confirming that these new vehicles of bioactive compounds can be used in food products.

ATTACHMENTS

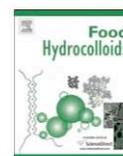
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Review

Microencapsulation using biopolymers as an alternative to produce food enhanced with phytosterols and omega-3 fatty acids: A review



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ABSTRACT

Omega 3 fatty acids and phytosterols are prominent bioactive compounds in food industry due to their health benefits such as the reduction in cholesterol and triglycerides levels, and therefore reducing the risk of cardiovascular diseases. However, they are very susceptible to oxidation when exposed to high temperature, high concentration of oxygen and incidence of light. Microencapsulation is being one of the most used alternatives for the purpose of protection and controlled release of these bioactive compounds. Encapsulation techniques which utilize biopolymers are considered featured since they allow the formation of edible, nontoxic and easy handling materials. This review will address the methods of spray drying, ionic gelation, complexation and complex coacervation, various polymers used as wall material, the cross-linking process used together with the complex coacervation and the characterization analyzes commonly used, especially in the encapsulation of omega-3 fatty acids and phytosterols, unstable bioactive compounds and of importance in maintaining of health. Finally, concluding remarks on future applications of these techniques and these bioactive compounds will be considered.

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Contents

1. Introduction	443
1.1. Omega-3 fatty acids	444
1.2. Phytosterols	444
2. Microencapsulation	444
2.1. Biopolymers used by the encapsulation of phytosterols and omega-3 fatty acids	446
2.1.1. Gelatin	446
2.1.2. Gum Arabic	447
2.1.3. Pea protein	448
2.1.4. Pectin	448
2.1.5. Casein	448
2.1.6. Soy protein isolate	448
2.1.7. Chitosan	448
2.1.8. Alginate	448
2.1.9. Whey protein	449
2.2. Mechanisms of release	449
3. Microparticles characterization	449
3.1. Particle size	449
3.2. Encapsulation efficiency	450
3.3. Optical, confocal and scanning electron microscopy	450
3.4. Water activity, moisture, hygroscopicity and solubility	450
3.5. Fourier transform infrared spectroscopy (FTIR)	450

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3.6.	Thermogravimetric analysis (TG)	451
3.7.	Controlled release and stability of the encapsulated material	451
3.8.	Differential scanning calorimetry (DSC)	451
3.9.	Oxidation analyzes by thiobarbituric acid reactive substances (TBARS) and peroxide value	451
3.10.	Accelerated oxidation by rancimat	451
4.	Techniques applied for encapsulation of omega 3 and phytosterols using biopolymers and cyclodextrins	451
4.1.	Spray drying	451
4.2.	Ionic gelation or ionotropic gelation by extrusion processes	452
4.3.	Complex coacervation	452
4.4.	Complexation	453
5.	Conclusions	454
	Acknowledgements	455
	References	455

1. Introduction

Fatty acids (FAs), compounds formed by an aliphatic chain and a carboxylic group, are the main constituents of lipids. The FAs are classified into saturated and unsaturated, and the last ones are unstable due to the presence of double bonds in their structure. There are several types of unsaturated fatty acids, and those belonging to omega-3 family are considered prominent because of their benefits to human health. These fatty acids have double bonds separated by a methylene from the third carbon atom from the methyl-terminus.

Omega-3 fatty acids (ω -3) are compounds widely used in the food, cosmetic and pharmaceutical fields due to their beneficial health effects such as reduction of the risk of coronary heart disease (Kralovec, Zhang, Zhang, & Barrow, 2012), are precursors to anti-inflammatory mediators, prevention of inflammatory mediated disorders including allergy, diabetes, Alzheimer's disease and related neurodegenerative diseases (Lavie, Milani, Mehra, & Ventura, 2009). There are several sources of omega-3, both from animal (marine fish oil) and from vegetable (echium seed oil, chia oil) source. In addition to these natural sources of ω -3, there are many choices of food enriched with these fatty acids, such as milk and dairy products, juices, eggs, baby food, meat products, margarine and baked products, as well as supplements, representing a large percentage within the functional food market. In the case of baked products, the oxidative stability of omega-3 fatty acids can be ensured by the encapsulation process. Gökmen, Mogol, Lumaga, Fogliano, Kaplun, & Shimoni (2011) studied the development of functional bread containing nanoencapsulated omega-3 fatty acids. According to these authors, the encapsulation decreased lipid oxidation as measured by the formation of hexanal and nonanal in breads during baking. However, further research still might be necessary for this kind of application. According to Serna-Saldivar, Zorrilla, Parra, Stagnitti, and Abril (2006), the carrier ingredients used to protect the oil, rich in unsaturated fatty acids, can affect the physical properties of the bread, causing changes in texture, handling and sensory properties of the final product. Moreover, the wall materials cannot release the encapsulated omega-3 fatty acids at the processing temperature.

There is also a class of lipids known as sterol. The sterols are lipids composed of three six-carbon rings and one five-carbon ring also linked to an aliphatic chain and a hydroxy at the carbon 3 of the ring (Damodaran, Parkin, & Fennema, 2010). In the same way as fatty acids, sterols can be from animal sources (zoosterol, as cholesterol or coprostanol) and plant origin (phytosterols, as β -sitosterol, campesterol and stigmasterol - the most commonly occurring in the human diet). The phytosterols have beneficial properties to human health such as reduction in the intestinal

cholesterol absorption (Ostlund, Racette, Okeke, & Stenson, 2002), as well as helping to maintain the membrane integrity (Watson & Preedy, 2004). There are some options of food products enriched with phytosterols, such as bakery products and margarines. However, this number is not as significant as with omega-3, corresponding to a smaller percentage within the functional food market.

Despite of high consumer demand and consequently high industrial interest in food enhanced with these both bioactive compounds, for the food processors, preparing food added of omega-3 fatty acids and/or phytosterol is still nowadays not a easy task, since there are many challenges to be overcome; moreover, these compounds are both very unstable, water insoluble and omega-3 fatty acids from fish source – the most common one – have also very unpleasant aroma. However, the technology of microencapsulation has been used to overcome all of these drawbacks.

Microencapsulation has been widely used in the food field. This technique consists in the formation of small "packaging", called microcapsules, microspheres or microparticles, which consist of structures that have one or more bioactive materials involved or immobilized by one or more polymer or a lipid. According to Favaro-Trindade, Pinho, & Rocha (2008), these structures are applied with several objectives, including: reducing the reactivity of the material that is being encapsulated, protection of the encapsulated material against evaporation or loss into other medium, to facilitate handling, application and storage of the encapsulated material, to promote controlled release and mask unpleasant taste and flavor.

In the case of omega-3 and phytosterols, this technique has been very useful, since it can: avoid their interaction with other compounds in foodstuffs; offer protection against light, oxygen and metals; it can enable their dispersion in water for aqueous formulations, as beverages; to turn them, that are both liquids, in powder, that facilitates their transport, application and manipulation; and to mask or to reduce the aroma of oils from fish source.

There are several techniques that have been used or at least studied for microencapsulation of omega-3 and phytosterols which resulted in these compounds more protected, such as complex coacervation (Barrow, Nolan, & Holub, 2009; Comunian, Boillon, Thomazini, Nogueira, Castro, & Favaro-Trindade, 2016; Conto, Grosso, & Gonçalves, 2013), spray drying (Chen, McGillivray, Wen, Zhong, & Quek, 2013), high-pressure homogenization (Salminen, Aulbach, Leuenberger, Tedeschi, & Weiss, 2014), emulsion followed by spray granulation, spray drying and freeze drying (Anwar & Kunz, 2011), emulsion followed by high-pressure homogenization (Chen, McGillivray, Wen, Zhong, & Quek, 2013), ultrasonic atomizer (Klaypradit & Huang, 2008); all these techniques show important impact on the functionality of omega-3 and phytosterols,

depending on the desired final application. However, this review will give more attention for the techniques that used biopolymers as encapsulation agents, as spray drying, complex coacervation and ionic gelation; besides of the method of complexation, using cyclodextrins.

Chen, McGillivray, Wen, Zhong, & Quek, (2013) coencapsulated fish oil, phytosterols and limonene by spray drying and used whey protein isolate and sodium caseinate as wall materials. These authors obtained good quality microcapsules, with higher retention of EPA and DHA and better flavor/odor profile. In another study, Klaypradit and Huang (2008) encapsulated tuna oil by emulsion followed by ultrasonic atomization and freeze drying using chitosan, maltodextrin and whey protein isolate as wall materials and Tween 80 as emulsifier. The combination of chitosan and maltodextrin presented the highest emulsion stability showing that this encapsulation method could improve the stability of tuna and other oils. Several researchers showed that chitosan is unable to produce stable emulsion when it is alone (Klaypradit & Huang, 2008); however, when this polymer is mixed in appropriate concentrations, with for example, maltodextrin, it is possible to obtain stable emulsion. According to Rodriguez, Albertengo, and Agullo (2002) and Klaypradit and Huang (2008), it can be explained due to the chitosan emulsification capacity combined to electrosteric and viscosifying stabilization mechanisms, besides the chitosan cationic nature with hydrophilic zones. More recently, Esquerdo, Dotto, and Pinto (2015) encapsulated fish oil by nanoemulsions using chitosan as wall material and Tween 80 as emulsifier. These authors showed that the microstructure was able to protect the material regarding to oxidation and the chitosan could be used as encapsulating agent for unsaturated fatty acids.

Besides the many studies in the literature, the number of patents related to omega-3 and phytosterols is very large, mainly related to the application of these compounds in food products and in pharmaceutical field. Lima (2013) applied microencapsulated omega-3 fatty acid with natural polymers into chocolate, which increased the bioavailability of ω -3 fatty acids and maintained its original shelf life. Malaki et al. (2015) obtained a patent related to the application of an encapsulated composition composed by whey protein isolate, low weight carbohydrates, antioxidant and polyunsaturated fatty acids in different food products such as infant formula, cereals, powdered drink mixes, instant coffees and teas, flavors, fragrances, colorants and other dry food products. They obtained materials with superior encapsulation performance, cost-effective and that protect the polyunsaturated fatty acids against oxidation, besides a good sensory impression.

So, this review focuses on the processes that use biopolymers and cyclodextrins as wall materials for encapsulation of omega-3 and phytosterols and the application of these microcapsules into food, discussing relevant advances and different approaches.

1.1. Omega-3 fatty acids

As mentioned, the fatty acids are the main constituents of lipids. They can be classified as saturated or unsaturated, having only single bonds and containing at least one double bond, respectively. The fatty acids containing double bond (or unsaturated) in the third carbon counted from the methyl-terminus are known as omega-3 fatty acids (ω -3). The main members of this family are alpha-linolenic acid (ALA) (18:3 n-3), eicosapentaenoic acid (EPA) (20:5 n-3), docosahexaenoic acid (DHA) (22:6 n-3) and stearidonic acid (C18:4 n-3), as shown in Fig. 1.

There are some sources of ω -3 viable to be added into food products; they are from animal origin (marine oils) and vegetable origin (as echium, chia, camelina, perilla and flax seed oil). The omega-3 fatty acids from animal origin are derived primarily from

certain fish oils and eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are the most important; moreover, they are precursors of anti-inflammatory mediators and have been shown advantages in preventing of diseases inflammatory, allergy, diabetes and Alzheimer's disease (Kralovec, Zhang, Zhang, & Barrow, 2012; Lavie et al., 2009). The ω -3 fatty acids from vegetable origin extracted from echium seed oil (*Echium plantagineum* L.) contains 33% of α -linolenic acid and from 9 to 16% of stearidonic fatty acid, which belongs to the ω -3 family and is an intermediate in the biosynthesis of EPA and DHA (Berti, Johnson, Dash, Fischer, Wilckens Hevia, 2007; Zanetti, Monti, & Berti, 2013). The chia (*Salvia hispanica* L.) seeds contain the highest known percentage of α -linolenic fatty acid of any plant source, ranged from 64.8 to 56.9% (Ayerza & Coates, 2011). Camelina (*Camelina sativa* L.), perilla (*Perilla frutescens* L.) and flax (*Linum usitatissimum* L.) seeds containing around of 36, 53, and 57%, respectively, of α -linolenic fatty acid (Sultana, 1996).

Omega-3 fatty acids are being widely studied due to triglyceride reduction capability, promoting the reduction of risk of cardiovascular disease (Poole et al., 2013), reducing the risk of coronary heart disease in adults (Khandelwal et al., 2013) and in the treatment of people with depression and bipolar disorder (Mischoulon & Freeman, 2013). However, due to the presence of double bonds in their long-chain, these molecules are susceptible to changes as oxidation, besides being very hydrophobic, what hamper its application in some foods and beverages.

1.2. Phytosterols

In relation to phytosterols, plant sterols, they have structural similarity with cholesterol, differing only in the presence of side chains in the C-24 carbon; the main phytosterols are: beta-sitosterol, beta-sitostanol, campesterol, campestanol and stigmasterol (Carr, Ash, & Brown, 2010), as observed in Fig. 2. They are obtained from refined oils of plants, seeds, whole grains and legumes (Sanclément et al., 2012).

In the same way as the omega-3 fatty acids, phytosterols have many benefits, such as the improvement of the profile of serum lipids, reduction of the risk of cardiovascular disease and anticancer effect (Awad, Fink, Williams, & Kim, 2001; Bradford & Awad, 2010; Ito, Ohtsubo, Kusu, & Hakamata, 2012; Ju, Clausen, Allred, Almada, & Helferich, 2004; Kendall & Jenkins, 2004; Liu & Ruan, 2013; Ostlund, 2002; Woyengo, Ramprasath, & Jones, 2009). However, phytosterols are also unstable when subjected to certain environmental conditions, very hydrophobic and viscous.

Next, this review will briefly present the mechanisms that use polymers as wall material to form structures or vehicles for protecting omega-3 fatty acids and phytosterols; finally, a consideration of the future scope for encapsulation of these compounds in different applications will be presented.

2. Microencapsulation

Microencapsulation is a technique in which one or more compounds (core or internal phase) are surrounded or immobilized by one or more materials (shell, carrier or wall material) in order to be protected from external factors such as light, high concentration of oxygen, heat, moisture, preventing evaporation of volatiles compounds, masking unpleasant tastes and odors, and for development of value-added products. So, this technology has been used in the food industry since it facilitates the application of unstable ingredients, in addition to improving the processing and texture of ingredients due to lower hygroscopicity, increased solubility and dispersibility in different kind of materials. Besides, there is the possibility of release control in the site of action of the compounds

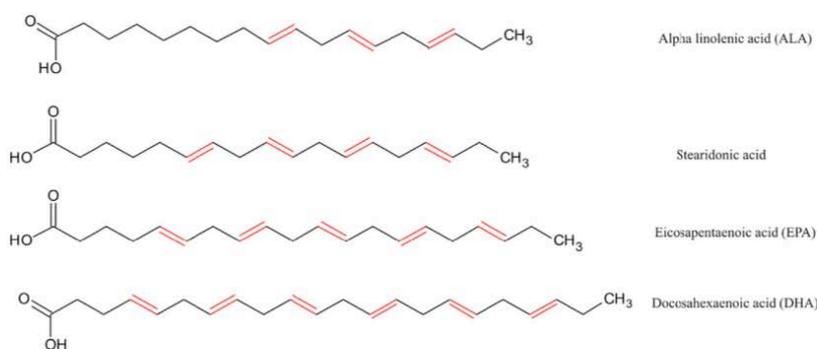


Fig. 1. Chemical structures of the main omega-3 fatty acids.

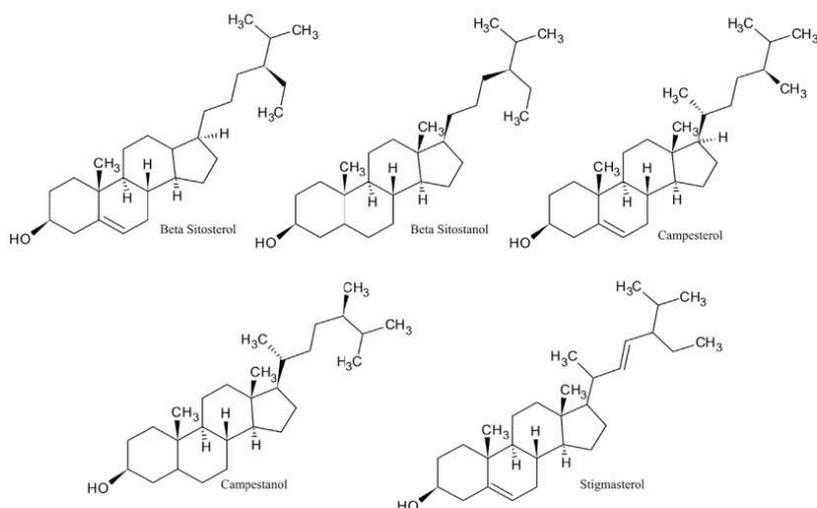


Fig. 2. Chemical structures of major phytosterols.

of interest. The cited advantages can be seen in various applications, such as the cited one by Gallardo et al. (2013), who microencapsulated linseed oil by spray drying and applied it into bread manufacturing. In a recent research, Marsanasco, Piotrkowski, Calabró, Alonso, and Chiaramoni (2015) encapsulated omega-3, omega-6 and tocopherol by liposomes and incorporated it orange juice as new functional food. Marsanasco, Márquez, Wagner, Chiaramoni, and Alonso (2015) encapsulated omega-3, omega-6 and vitamin E by liposomes and applied it into chocolate milk, obtaining a functional food product.

There are several techniques that have been used or at least studied for microencapsulation of different food ingredients. Moreover, these techniques are classified into three different methods: (1) Physical methods, such as, spray drying, spray chilling/cooling/congealing, supercritical fluids encapsulation processes, process with microfluidic devices and spray coating; (2) Chemical methods, as polymerization; and (3) Physico-chemical methods, such as, complex coacervation, liposomes, micelles, emulsions, process with nanostructured lipid matrices, solvent evaporation, and molecular inclusion. All of these techniques can

be adapted for the encapsulation of omega 3 fatty acids (Cao, He, Zhang, & Wang, 2011; Conto et al., 2013; Eratte, Mcknight, Gengenbach, Dowling, Barrow, Adhikari, 2015; Gómez-Mascaraque & López-Rubio, 2016; Marsanasco, Márquez, Wagner, Chiaramoni, & Alonso, 2015; Umesha, Manohar, Indiramma, Akshitha, & Naidu, 2015) and phytosterols (Chen, Zhong, Wen, McGillivray, & Quek, 2013; Meng, Pan, & Liu, 2012), although, as already mentioned, this review focuses on the methods that use biopolymers and cyclodextrins.

For the choice of technique to be used, it is necessary to analyze the final product in which it will be applied, the desired particle size, release mechanisms and the physicochemical properties of the material used as the encapsulating agent and core (Favaro-Trindade, Pinho, & Rocha, 2008). Besides, in the case of a food ingredient, the cost and safety of the method and wall material also must be considered in the selection.

According to Coupland and Hayes (2014), there are different formulation challenges for food compounds encapsulation. One of them is the choice of materials to be used in the encapsulation process. There are many kinds of polymers that cannot be applied

in food products, limiting the use of various compounds. In addition, the economics and marketing concerns of food need to be taken into consideration. However, even with several challenges mentioned, the encapsulation technique has been increasingly explored, achieving excellent results in obtaining ingredients with functional appeal.

The particles produced by encapsulation methods are known as microcapsules or microspheres, as can be seen in Fig. 3. Both structures (microcapsules and microspheres) are very important for practical applications and can have similar and different utilities or functionalities; the controlled release of the active material, for example, depends on the location of this compound inside the microcapsule and/or microsphere. The obtention of microcapsules or microspheres depends mainly on the encapsulation technique used, as mentioned following. Microcapsules are reservoir type particles, where the core is surrounded by a shell (Suave, Dallagnol, Pezzin, Silva, Meier, Soldi, 2006); moreover, it can have one or multiple nucleus. Liposomes, complex coacervation, microfluidic devices, spray coating and molecular inclusion are examples of techniques of encapsulation that produce microcapsules (Comunian, Abbaspourrad, Favaro-Trindade, & Weitz, 2014; Xiao, Liu, Zhu, Zhou, & Niu, 2014). Microspheres are matrix type particles, i.e., structures where the bioactive compound is spread by all the volume of the particle, sometimes including in its surface (Suave et al., 2006). Spray drying and spray chilling are examples of methods of encapsulation that produce microspheres (Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, & Rojas, 2015; Okuro, Thomazini, Balieiro, Liberal, & Favaro-Trindade, 2013).

In relation of the biopolymers that have been used, studied or could be useful for the encapsulation of omega 3 fatty acids and phytosterols, the next section will illustrate some of them.

2.1. Biopolymers used by the encapsulation of phytosterols and omega-3 fatty acids

2.1.1. Gelatin

Gelatin, one of the most used hydrocolloids in food industry, may be obtained from pig skin and bones (46% and 23.1%, respectively), from cowhide (29.4%) and from fish (1.5%). Moreover, it is produced from the partial hydrolysis of collagen (acidic or basic hydrolysis). In relation to the gelatin charge, it has a negative charge above its isoelectric point (IEP) and positive charge below it. According to Mishra (2015), Chap. 12, IEP is the pH at which the charge of gelatin is zero. Furthermore, its isoelectric point (IEP) varies according to its obtainment; due to minor changes in the amide groups, the gelatin obtained by acid hydrolysis (gelatin type A) has IEP value in alkaline medium (it ranges from pH 7 to 9). In the case of gelatin obtained by alkali hydrolysis, due to the large amount of free carboxylic acid, it has IEP value in acid medium (it ranges from 4 to 5). Knowing the isoelectric point of the gelatin is essential to understand the complex coacervation process (item 4.3). Different IEP result in different ranges of coacervation pH values, which can vary from 3.2 to 5.50 for the combination of gelatin and gum Arabic, for example (Mishra, 2015, Chap. 12). In addition to IEP, the encapsulation of omega-3 fatty acids and phytosterols can be affected by other factors such as pH and ionic strength, mainly in

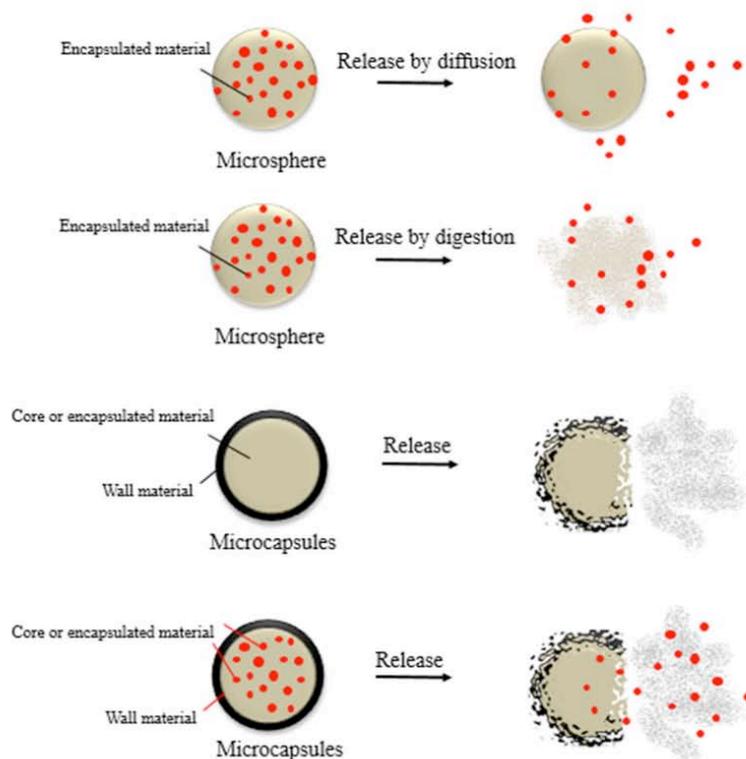


Fig. 3. Scheme of microsphere and microcapsule structure.

the complex coacervation, which is a technique that uses gelatin as wall material very often (Burgess, 1990; Kaushik, Dowling, Barrow, & Adhikari, 2014; Mishra, 2015, Chap. 12). Furthermore, gelatin has tensoactive properties, acting as a surfactant in food applications (Diaz-Calderón, Caballero, Melo, & Enrione, 2014; Duconseille, Astruc, Quintana, Meersman, & Sante-Lhoutellier, 2015).

Gelatin is one of the most used biopolymers as wall material, as it is shown in Table 1. Besides its low cost, gelatin gel melts at temperatures around 30–40 °C, what helps in the controlled release of the encapsulated compounds. This temperature range is similar to body temperature; however, this kind of material may take up to 30 min to melt, depending on the concentration of the solution and also on the interactions to its neighbors. Thus, gelatin is an important biopolymer in the microencapsulation of omega-3 fatty acids and phytosterol, since it has suitable properties for its use in various encapsulation techniques such as spray drying and complex coacervation.

2.1.2. Gum Arabic

Gum Arabic, highly water-soluble polymer, is an acacia tree secretion, and it is found in Arab countries such as Sudan and also African countries (Islam, Phillips, Slijivo, Snowden, & Williams, 1997). According to Damodaran et al. (2010), the gum Arabic is a material composed of two fractions: (1) polysaccharides chain, corresponding to greater part of its structure (D-galactose, L-arabinose, L-rhamnose, D-glucuronic acid), and (2) protein content, which corresponds to about 2% of the total weight. As well as all protein, the protein fraction of gum Arabic also changes the charge, however, due to its small proportion, this charge change has slight influence on the total charge of gum Arabic. Also according to Damodaran et al. (2010), the polysaccharide structures are highly ramified and the uronic acid units occur more often as non-reducing ends, having acid character and being responsible for the hydrophilic part of the polymer, whereas the protein structure

is responsible for the hydrophobic part of the gum Arabic. These amphiphilic character allows gum Arabic to act as emulsifier.

In the food industry, gum Arabic is widely used as a stabilizer, emulsifier and thickener, and also acts as flavor fixative (Ribeiro & Seravalli, 2004). As mentioned in this review, the process of complex coacervation makes use of different wall material combinations (item 4.3), however the pair gelatin-gum Arabic is the most traditional one due to several factors such as: (1) the hydrophobic material is totally coated by polymers, ensuring the protection of omega-3 fatty acids and phytosterols against adverse environmental conditions; (2) producing microcapsules of rounded shape (characteristic not observed in the most other wall material combinations) and this morphology helps in the application of microcapsules in a food product due to the easy flow. Moreover, according to Eastoe and Leach (1977), Duce, Richard, Saulnier, Popineau, & Boury (2004), Schmitt, Sanchez, Banon, and Hardy (2010) and Kaushik et al. (2014), due to the appropriate amino acid profile and charge density of gelatin, and a polysaccharide configuration of gum Arabic, which preserves the charges and allows the water to be blocked between the chains, the combination gelatin and gum Arabic helps the formation of the complex coacervate due to the increased interaction between these two polymers.

Additionally, gum Arabic is negatively charged, whereas gelatin has a negative charge above its isoelectric point (IEP) and positive charge below it. When pH of the combination of gelatin and gum Arabic is below the IEP of the gelatin, the coacervate is formed due to the interaction of these polymers in suitable temperature, ionic strength, concentrations and polymer ratios, becoming therefore, a feasible combination with gelatin to form microcapsules by complex coacervation, as noted in Table 1.

Gum Arabic is also very useful for encapsulation of hydrophobic compounds by spray drying since it has emulsifying ability. Besides, it forms low viscosity solutions in high concentration, what is very

Table 1

Some examples of polymers combinations used as wall materials by complex coacervation and their compounds encapsulated.

Polymers combination	Encapsulated material	Source
Gelatin and chitosan	Limonene oil	Prata & Grosso (2015)
Gelatin and OSA	—	Wu & McClements (2015)
Gelatin and gum Arabic	Xylitol	Santos, Bozza, Thomazini, & Favaro-Trindade (2015)
Lactoferrin and gum arabic	—	Gulão, Souza, Silva, Coimbra, & Garcia-Rojas (2014)
Whey protein and pectin	<i>Lactobacillus acidophilus</i>	Ribeiro et al. (2014)
Gelatin and gum arabic	Vitamin C	Comunian et al. (2013)
Gelatin and gum arabic	Aspartame	Rocha-Selmi, Theodoro, Thomazini, Bolini, & Favaro-Trindade (2013a)
Gelatin and gum arabic	Sucralose	Rocha-Selmi, Bozza, Thomazini, Bolini, & Favaro-Trindade (2013b)
Pea protein isolate and alginate	—	Klemmer, Waldner, Stone, Low, & Nickerson (2012)
Casein and pectin	Acetaminophen	Barakat et al. (2012)
Soy protein isolate and pectin	—	Chen, Li, Ding, & Duo (2012)
Hydrolyzed collagen and chitosan	Lavender oil	Ocak (2012)
Soybean protein isolate and pectin	propolis	Nori et al. (2011)
Soy globulins and chitosan	—	Liu et al. (2011)
Gelatin and gum Arabic	Peppermint oil	Dong et al. (2011)
Gelatin and gum arabic	Paprika oleoresin	Alvim & Grosso (2010)
Gelatin and sodium carboxymethylcellulose	Polyisobutylene	Dai et al. (2010)
Soybean protein isolate and pectin	Succinimide	Mendanha et al. (2009)
Casein and pectin	probiotics	Oliveira, Moretti, Boschini, Baliero, Freitas, & Favaro-Trindade (2007), Oliveira, Moretti, Boschini, Baliero, Freitas, & Freitas (2007)
Gelatin and polyphosphate	Fish oil (source of Omega-3 fatty acids)	Barrow et al. (2009)
Soy protein isolate and gum arabic	Omega-3 ethyl ester	Conto et al. (2013)
Whey protein isolate and gum arabic	Tuna oil (source of omega-3 fatty acids) and probiotic bacteria	Eratte et al. (2015)
Gelatin and acacia	Eicosapentaenoic acid ethyl ester	Lamprecht et al. (2001)

important to facilitating pumping and to improving the productivity of the process.

Thus, according to the above, in the same way as gelatin, gum Arabic is considered a viable biopolymer for the encapsulation of omega-3 and phytosterols, especially by spray drying and complex coacervation techniques.

2.1.3. Pea protein

According to Kent & Doherty (2014), the peas are used worldwide as sources of starch, fiber and protein because of its economic viability, including easy obtainment and peeling. Pea protein has several characteristics such as high temperature stability and the ability to act as an emulsifier.

The pea protein has some limitations to its application as wall material in complex coacervation due to a relatively broad range of the isoelectric point from pH 4 to 6, depending on raw material and process history. Therefore, the source has to be chosen well to avoid solubility problems at certain pH-depending applications. However, even with these limitations, researchers have been able to use this protein as encapsulating agent (Table 1).

Thus, due to the mentioned limitations, pea protein may be considered a biopolymer little explored in the encapsulation field; however, not less important than gelatin and gum Arabic.

2.1.4. Pectin

Pectin, a linear polysaccharide obtained from the extraction of the albedo of citrus fruits and apple pulp, is considered a good material to be used as encapsulating agent since it is degradable by the microbiota, what facilitates the controlled release of the encapsulated compounds. It is classified according to the degree of methoxylation (methyl groups esterified to the carboxylic group) and it is widely used by the food industry due to its gelling power (Fathi, Martin, & McClements, 2014; Ribeiro & Seravalli, 2004). As pectin is an anionic polysaccharide, an inexpensive and abundant material, it has been used to encapsulate lots of compounds by complex coacervation (Table 1).

Pectin can be considered a good alternative for the encapsulation of omega-3 and phytosterols, since it is easy to obtain and can be applied as wall material for various encapsulation techniques (spray drying, complex coacervation and ionic gelation).

2.1.5. Casein

Casein, protein derived from milk (representing around 80% of milk proteins), has been of interest to the food industry due to its composition (it contains all the common amino acids and it is also rich in essential amino acids) and its interactions with ions like calcium phosphate, providing an additional role for nutritional value. Besides its ability to form micelles, it is into three subgroups, including α -casein, β -casein and κ -casein (Damodaran et al., 2010; Koury, Scheed-Bergdahl, & Bergdahl, 2014). According to Sgarbieri (2005), the α -caseins are divided in α_{S1} and α_{S2} , depending on the presence of fast migration proteins designated α_S -caseins. The α_{S1} -casein is the main and consists of a polypeptide chain with 199 amino acid residues. In the case of β -caseins, they represent from 30 to 35% of total caseins. As opposed to α_{S1} -casein, β -casein forms colloidal suspensions in the presence of Ca^{++} , besides presenting temperature, concentrations and pH that equilibrium (association - dissociation) occurs. In the case of κ -casein, it is the only glycosylated casein and consists of three monosaccharides (galactose, *N*-acetyl-galactosamine or *N*-acetyl neuraminic acid). In the encapsulation process, the functionality of caseins depends on their process history, that means in which constitutional form are used: as salt (e.g. sodium caseinate), as micelles (native casein entities like in milk) or as protein particle suspension (e.g. like in acidified milk drinks).

Although the mean charge of a casein micelle at pH 6.8 is negative, in its surface spots with different partial charges from negative to positive values are found depending on the casein type and its role in the micellar structure. Therefore it depends on the processing or selecting of casein type if casein presents a charge interaction functionality like gum Arabic for example, and thus, might be expected to be a fitting combination to encapsulate with gelatin due to the electrostatic interactions resulted from the opposite charges. If casein is applied in an acidified system in non-micellar form, some types of pectin might be the adequate interaction partner like shown in the literature and seen in Table 1.

2.1.6. Soy protein isolate

Soybean is a commonly cultivated product in the world of significant economic value. As mean value, it contains about 40% of protein and 20% of oil on a dry basis. According to Damodaran and Arora (2013), the soy protein isolate used in food for humans is obtained from defatted soy flour, which contains about 50% of protein on a dry basis. It is an abundant, inexpensive and renewable raw material (Ortiz, Mauri, Monterrey-Quintero, Trindade, Santana, & Favaro-Trindade, 2009).

The soy protein isolate, because it is a protein of low cost and wide availability, is being very explored as encapsulating material when the complex coacervation technique is used, however not as much as gelatin and gum Arabic (Table 1). Soy protein isolate has been also used to encapsulate by spray drying (Ortiz et al. 2009).

2.1.7. Chitosan

Chitosan, polysaccharide derived from chitin, is a cationic and biodegradable polymer, found mainly in crustacean shell and considered highly renewable and economically viable material. It has been used by food industries mainly in health area, presenting antimicrobial and antioxidant activities (Canella & Garcia, 2001; Fathi et al., 2014; Janegitz, Lourenço, Lupetti, & Fatibello-Filho, 2007). In addition, studies have shown the relation of chitosan to body weight reduction (Trivedi et al., 2015).

In relation to its use as encapsulating material, its solubility only at low pH values may limit its application, however, due to its mucoadhesive nature, the controlled release of the encapsulated material in the gastrointestinal medium can be facilitated. To control the release by mucoadhesive nature of an encapsulating partner is possible to use an insoluble coating together or modify the material into suitable viscosity and chemistry according to the desired place to release the active compound.

For complex coacervation process, it is necessary that the used polymers have opposite charges. Therefore, if one polymer is protein, which can change the charge due to pH, the way how to do the encapsulation depends on the sign of the charge of the polysaccharide. In the case of chitosan, the process would begin with the protein in an acid medium, followed by raising the pH in order to obtain a negative charged protein binding to chitosan. Thus, chitosan, as well as having its beneficial properties to health, it can be considered a feasible biopolymer to be applied in various encapsulation techniques, especially in coacervation techniques.

2.1.8. Alginate

The alginate, an anionic and hydrophilic polysaccharide of natural origin, has been used in the pharmaceutical and food field, acting in microencapsulation processes as well as building membranes with regulated permeability. This biopolymer can be obtained from marine plants (Zia, Zia, Zuber, Rehman, & Ahmad, 2015).

The alginate is composed by carboxylic acid, hydroxyl functional groups, glycosidic and glycolic bonds. Due to the presence of charges, this polymer has been used as wall materials for the

encapsulation technique of complex coacervation. This polysaccharide is also very useful for the immobilization of thermal unstable materials, as omega 3 and phytosterol, by ionic gelation, because it is able to jellyfy at room temperature. The gelling capacity of a polymer is related to its composition. The difference between alginate and other polymers is that alginate consists of α -L-guluronics and β -D-manuronics acids, varying in relation to manuronics and guluronics residues and its level of polymerization. In this case, alginate gels are easily formed when in contact to cations Ca^{++} or Mg^{++} .

Moreover, alginate, besides being a good choice as wall material for complex coacervation technique, it has similar properties to pectin, being also applied in the ionic gelation method.

2.1.9. Whey protein

The whey is considered a byproduct of cheese production, it is rich in proteins (β -lactoglobulin, α -lactoglobulin, immunoglobulin and serum albumin), and it is being widely used by the food industry due to their nutritional and functional properties.

The whey proteins (β -lactoglobulin and α -lactoglobulin) have negative charge at a pH of 6.8 like found in fresh milk, making its use as wall materials possible in complex coacervation technique, since it can participate in the electrostatic interactions in the same way as the other biopolymers cited in this review. In addition, due to their globular structure if native in a solution, these proteins provide only low viscosities even at high concentrations. They present also properties of emulsifiers and therefore provide this functionality process where encapsulation is done by emulsification. In other words, the whey protein is a polymer suitable to provide needed functionality when applied as wall material.

It is important to highlight that heat-induced changes the whey protein conformation due to disulfide bonds, being an essential information to control the functionality of the material. According to Sikorski (2002), thermal changes are responsible by loss of solubility due to aggregation of the proteins, β -lactoglobulin and immunoglobulin for example, besides the increase in solubility as a resulting of the breaking down of structures, as collagen for example. In the case of proteins that present low number of S–S-bonds, the heat-induced leads to an increased solubility. However, even with these changes in the properties, these changes probably will not influence on the activity as wall materials of these proteins; in the case of whey protein for example, several researches on encapsulation with this type of material are found in the literature (Van Leusden, Den Hartog, Bast, Postema, Van der Linden, & Sagis, 2016; Wang, Liu, Chen, & Selomulya, 2016), showing that encapsulated materials were protected.

2.2. Mechanisms of release

Besides the protection of the encapsulated material, the microencapsulation techniques also aimed optimal release conditions of the active material in appropriate locations and times in order to obtain better effectiveness and availability of the compounds of interest. It is important to note that the release of the omega-3 and phytosterol should be avoided during processing and storage of food products, occurring in the consumer's body.

According to Xu et al. (2013), Zhang, Decker, and McClements (2014) and Moomand and Lim (2015), it is possible to analyze how these cited conditions are applied in the controlled release study due to the microcapsules and/or complex subjected to in vitro gastrointestinal conditions using simulated gastric and intestinal fluids. These release mechanisms depend on various conditions, such as diffusion, solvents, pH and temperature.

The release by diffusion is a mechanism that varies according to the biopolymer used as wall material and the encapsulation

technique. The active material diffusion rate also depends on conditions of microcapsule wall (integrity) or distribution of the material in the microspheres, besides the presence of pores, which facilitates the diffusion and, consequently, the release of the compounds of interest. Moreover, the state in which the wall materials are, also determines the diffusion of active material. According to Re (1998), compounds in glassy state are more impermeable than the gummy state, then showing the importance of studying the temperatures and heat flow associated with thermal transition of the compounds. In this case, due to the presence of pores formed in the wall material, this type of release is most noticeable when the spray chilling technique is used (method not shown in this review).

In relation to the solvent, it is a potential factor in the release of active materials since it assists in the disruption or dissolution of the wall and consequently in the desired release of the compound of interest. Any kind of molecular interaction that occurs during the encapsulation process (spray drying, complex coacervation, ionic gelation or complexation) can be undone with the addition of solvents. At the same time, the pH values determine the release of the encapsulated compounds since the variations in these values change the solubility of the wall materials. This method is widely used for microcapsules obtained by complex coacervation.

In relation to release by temperature, these release method is one of the methods most commonly used due to a change of the physical state of the wall materials and/or fusion of these materials when applied temperature changes. This method is not recommended when sensitive compounds to high temperature are encapsulated, such as omega-3 fatty acids and phytosterols, however can be applied to capsules formed with gelatin as wall material, for example. Moreover, the combination of these factors can also be done aiming the best conditions for the desired controlled release.

3. Microparticles characterization

There are several types of analyzes used for the characterization of these kind of materials. These analyzes can be classified in (1) characterization methods and quality control for the microcapsules, (2) characterization methods and quality control for the fatty acids and phytosterols encapsulated, (3) methods for the right combination or interaction capability of biopolymers. For the first classification, particle size, optical, confocal and scanning electron microscopy, water activity, moisture, hygroscopicity and solubility analyzes can be cited. For the second classification, the methods are controlled release, stability of the encapsulated material, oxidation analyzes by thiobarbituric acid reactive substances (TBARS), peroxide value and accelerated oxidation by rancimat. In relation to the third classification, encapsulation efficiency, Fourier transform infrared spectroscopy (FTIR), thermogravimetric analysis (TG) and differential scanning calorimetry (DSC) can be mentioned. All these methods are cited below to give an overview about their importance in the omega-3 fatty acids and phytosterols encapsulation.

3.1. Particle size

The particle size is one of the most decisive factors in the application of the microcapsules obtained by complex coacervation into food products, since certain sizes may interfere on the texture and final flavor. The particles can be measured by scattering and refractive index, or even by optical microscopy, aided by software available online ImageJ (ImageJ, Image Processing and Analysis in Java), depending on the structure available for each researcher (Comunian et al., 2011, 2014). Which one of these two working ways will be performed in an application depends on system's microstructure and target defined needed accuracy.

If the analysis is performed by scattering and refractive index, it is possible to measure the particle size prior to drying step (in the case of complex coacervation technique for example), but several characteristics of the solvent like ionic strength have to be chosen well to avoid dilution related changes of the microstructure. For some systems, alternatively a dispersion of the sample in a solution or solvent in which the capsules and/or spheres do not solubilize (in the case of spray drying technique, for example) is used. In this case, the type of encapsulation technique and the wall material used is decisive in the choice of solvent (Chen, Zhong, Wen, McGillivray, & Quek, 2013).

In the case of complexes formed by the complexation technique, this analysis may be difficult due to obtainment of samples with undefined format.

3.2. Encapsulation efficiency

The amount of encapsulated material depends on several factors, including the encapsulation technique employed, the compounds used as encapsulating agents and as the core and the process conditions (time, temperature and pH). The encapsulation efficiency is necessary to know how much of the active material is inside and outside of the microcapsule and/or complex and thus know how much sample is necessary to add in the final food product. The lower the amount of capsule (and higher the encapsulation efficiency), the better the texture and flavor of the food. In other words, the less capsule applied to the food, the more difficult is to be perceived by the consumer, not affecting the sensory characteristics of the product. It is not possible affirm a limit of microcapsule to be applied in the final product, since it depends on the concentration of active material encapsulated and regulations of each country for each type of compound.

The encapsulation efficiency can be measured in several ways, depending on the encapsulated material (High-performance liquid chromatography, gas chromatography coupled to mass spectrometry, spectroscopy or titration) (Carneiro, Tonon, Grosso, & Hubinger, 2013; Comunian, Thomazini, Alves, De Matos Junior, Balieiro, & Favaro-Trindade, 2013; Conto et al., 2013; Lamprecht, Schäfer, & Lehr, 2001; Santos et al., 2014). In the case of oil encapsulation, it is often made only the measurement of oil by the technique of Bligh & Dyer (Bligh & Dyer, 1959).

3.3. Optical, confocal and scanning electron microscopy

The study of the morphology of the microcapsules and/or complexes is important to define the format, location of compounds within the structure and physical characterization. Depending on the encapsulation and drying techniques used, it can be obtained microcapsules with rounded shape or with no defined format, an important feature for the application of the material and which can be analyzed by these various microscopy techniques.

According to Joye and McClements (2014), optical microscopy is usually used to study the microstructure in general, while the confocal, with the help of dyes, it is possible to view certain compounds and study their behavior depending on the location that they are within the microcapsule and/or complex. Comunian et al. (2016) studied the morphology of microcapsules prepared with echium oil (source of omega-3) and phenolic compounds by complex coacervation using gelatin and gum Arabic as wall materials and applied Nile red as fluorophore to dye the oil and verify its location inside the capsule. The micrographs were analyzed by confocal microscopy. Moreover, with confocal microscopy is also possible to determine 3D images. When it comes to nano-sized samples or there is a need to study the surface characteristics, the use of scanning electron microscopy is more recommended. A

confocal microscopy of microcapsule obtained by complex coacervation is presented in Fig. 4.

3.4. Water activity, moisture, hygroscopicity and solubility

Besides the size and morphology of the microcapsules, physicochemical characteristics are also important in the characterization of the components and their ability to form a biopolymer complex. Future applications depend directly on the hygroscopicity, solubility, moisture and water activity of the capsule and the complex formed.

Analysis of water activity for omega-3 and phytosterols microcapsules is important to assess the microbiological stability of the material. According to Damodaran et al. (2010), water activity values below 0.6 ensure microbiological safety. In the case of hygroscopicity and solubility values, they help to determine the storage conditions and the type of product which the capsules would be applied, respectively, since the hygroscopicity corresponds to the amount of water that the material can absorb from the environment and the solubility corresponds to the material capacity of dissolving in a specific media. The obtaining of the material moisture values is also essential, since this value assists in determining the capsule composition on a dry basis, a decisive factor for the encapsulation efficiency calculations, for example. Thus, these properties can be controlled according to the desired application.

3.5. Fourier transform infrared spectroscopy (FTIR)

During the encapsulation process, reactions or interactions can occur, affecting the structure of the encapsulated material, wall materials and/or the microcapsule and complex. The Fourier Transform Infrared Spectroscopy (FTIR) can be used for the determination of possible compounds or radicals formed by these reactions or interactions and consequently to analyze if the encapsulated material suffered any influence during the encapsulation process and/or if the wall of the capsule (or complex) was actually formed.

One example for this analysis is shown by Torres-Giner, Martinez-Abad, Ocio, and Lagaron (2010), when they studied the

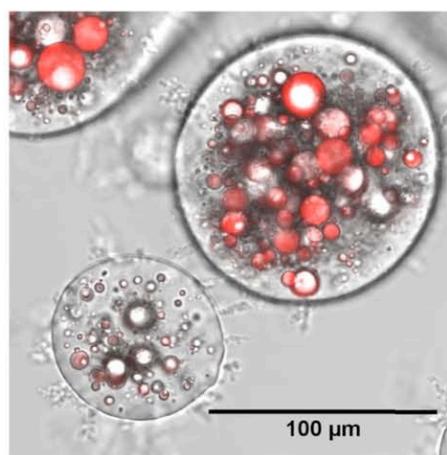


Fig. 4. Confocal microscopy of microcapsules obtained by complex coacervation using gelatin and gum Arabic as wall materials.

stabilization of a nutraceutical omega-3 fatty acid by encapsulation in ultrathin electrosprayed zein prolamine. According to these authors, by ATR-FTIR was possible to verify that the encapsulation was more efficient against degradation under ambient conditions.

3.6. Thermogravimetric analysis (TG)

Thermogravimetric analysis (TG) is important when it is necessary to evaluate the behavior of the material in relation to weight loss according to the applied temperature which can vary from 0 to 800 °C. For example, the cross-linking process is widely used for the purpose of forming more rigid capsules. In this case, the TG analysis is important to evaluate the range of temperature that the capsule is stable and/or hard enough to protect the encapsulated material. Peng et al. (2014) studied the chemical composition, antimicrobial property and microencapsulation of Mustard (*Sinapis alba*) seed essential oil with genipin by complex coacervation and carried out thermogravimetric analysis of the microcapsules at a heating rate of 20 °C/min for 25–550 °C. With the aid of this analysis, they observed that low concentration of genipin (compound used as crosslinker) resulted in greater weight loss, in other words, less stable capsules. Recently, Comunian et al. (2016) studied the encapsulation of echium oil by complex coacervation using gelatin and gum Arabic as wall material and performed the thermogravimetric analysis of the microcapsules at a heating rate of 10 °C/min in the temperature range of 25–800 °C. These authors observed that the higher the concentration of the phenolic compound (sinapic acid) added in the microcapsule, the lower the weight loss in the range from 250 to 430 °C.

3.7. Controlled release and stability of the encapsulated material

The main objectives of microencapsulation are to promote the controlled release and protect the compounds against adverse environmental conditions (light, oxygen, moisture), increasing their stability. According to Quirós-Sauceda, Ayala-Zavala, Olivas, and Gonzalez-Aguilar (2014), for the controlled release analysis, it is necessary to study parameters such as moisture, temperature, pH variations and mechanical disruption (Anderson, Hansen, Lukianova-Hleb, Hafner, & Lapotko, 2010; Kiser, Wilson, & Needham, 2000) to obtain the best conditions for the release of drugs or active material in predetermined place and time in the organism or in the food in which the microcapsules and/or complexes were applied. With the same principle, the study of stability is extremely important to obtain a material resistant to environmental conditions and therefore feasible for applications.

Thus, it can be inferred that the characterization of the microcapsules and/or complex obtained by the technologies cited in this review has to be chosen in a way that allows differentiating several independent aspects regarding all functionalities needed to obtain a product of quality and with functional appeal.

3.8. Differential scanning calorimetry (DSC)

The differential scanning calorimetry is an essential method for evaluating the physical state of the wall material and core, associating the behavior of heat flow and different temperatures, obtaining information such as glass transition temperature (T_g), melting and crystallization temperature. According to Nollet (2004), this is a technique often used when information related to conformational changes are needed. One example of this application is shown by Awad, Helgason, Weiss, Decker, & McClements (2009) when they studied the effect of omega-3 fatty acids on crystallization, polymorphic transformation and stability of tri-palmitin solid lipid nanoparticle suspension.

3.9. Oxidation analyzes by thiobarbituric acid reactive substances (TBARS) and peroxide value

In the case of microencapsulation of omega-3 fatty acids and phytosterol, analysis of oxidation of these compounds is essential, since one of the goals of encapsulation of these materials is their protection against oxidation. The evaluation of the oxidation by Thiobarbituric acid reactive substances (TBARS) and peroxide value are the main methods for measurement of oxidation. Usually, to carry out these analyzes, the microcapsules (or microspheres) are submitted to different storage conditions for a certain period. However, according to Kaushik et al. (2014), these methods present some disadvantages such as unstable measurements and intermediate products (peroxide value) and is not a good indicator at lower oxidation levels (TBARS).

3.10. Accelerated oxidation by rancimat

In addition to the analyzes mentioned in item 3.9, the oxidation can also be assessed by rancimat. In this case, the samples are subjected to conditions that accelerate the oxidation process, thus obtaining the results in function of oxidation time. According to Kaushik et al. (2014), the advantage of this method is the assessment of a large number of samples with good reproducibility. However, it needs high-cost equipment, making the analysis expensive.

4. Techniques applied for encapsulation of omega 3 and phytosterols using biopolymers and cyclodextrins

4.1. Spray drying

Spray drying is the technique most commonly used for the encapsulation of various food ingredients and consists in the atomization of a solution, suspension or emulsion, formed between the active ingredient and an encapsulating material (carrier), in a drying chamber where it comes into contact with a heater medium (usually hot air) and dry almost instantaneously (Fig. 5a). Generally this technique produces matrix type microparticles, or microspheres, that are hollow structures (Subtil et al. 2014), with size ranged from 10 to 120 μm.

It is a low cost technology, fast, continuous process, that produces free-flowing powders and despite of the fact that is necessary to use high temperatures in this process, the time of contact is too short (few seconds) meaning the energy is used for evaporation only and not for rising the temperature of the powder particles, enabling the application of this technology to encapsulate heat sensitive materials with low thermal degradation. So, this is interesting for the encapsulation of bioactive compounds such as omega 3 and phytosterols, which are often heat-sensitive.

Among the carriers used in this technique, can be cited the polysaccharides maltodextrin (Rubilar et al., 2012), gum Arabic (Rubilar et al., 2012), chitosan (Klinkesorn, Sophanodora, Chinachoti, McClements, & Decker, 2005) and some types of modified starches and proteins, as gelatin (Castro-Muñoz, Barragán-Huerta, & Yáñez-Fernández, 2015), whey protein (Umesha et al., 2015) and soy protein (Augustin et al., 2014). These materials have relatively low costs and do have no taste, aroma or color which facilitates the application of the product obtained in different systems.

Umesha et al. (2015) encapsulated garden cress seed oil, which is rich in α-linolenic acid (ALA), using whey protein concentrate as wall material by spray drying method. The encapsulated oil was applied to biscuits and stored in different conditions. Moreover, in all the conditions, the oxidation rate of ALA was high when it was

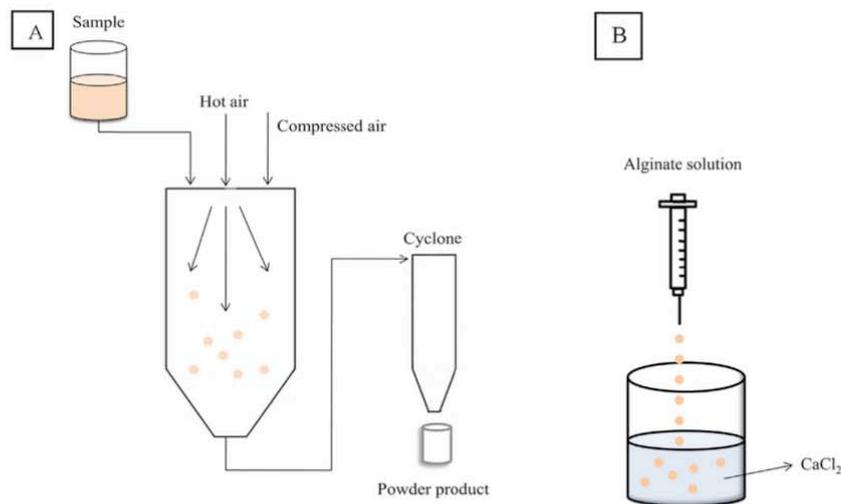


Fig. 5. Illustration of spray drying and ionic gelation methods, respectively.

free compared to when it was encapsulated, indicating that this encapsulation method protected the fatty acid in biscuits. In other study, [Chen, McGillivray, Wen, Zhong, & Quek, \(2013\)](#) coencapsulated fish oil with phytosterol ester and limonene by spray drying and freeze drying using whey protein and soluble corn fiber as wall materials. According to these authors, the oxidation of the oil was similar for both methods during the accelerated storage, so either freeze-drying or spray drying provided protection to the core materials. Recently, [Jiménez-Martín et al. \(2015\)](#) encapsulated omega-3 fatty acids by spray drying using lecithin and lecithin-chitosan fish oil emulsions with maltodextrin as wall material. These authors observed that microcapsules with lecithin-chitosan protected the material against lipid oxidation during storage at 30 and 60 °C, showing that this method is suitable to encapsulate omega-3 fatty acids.

Thus, according to the results reported in many studies, it can be said that the spray drying technique is suitable and feasible for encapsulating of phytosterols and oils containing omega-3 fatty acids, providing protection to these compounds of interest and possible application in food products.

4.2. Ionic gelation or ionotropic gelation by extrusion processes

Ionic gelation or ionotropic gelation by extrusion processes produce matrix or core-shell (reservoir) particles, depending on nozzle design ([Fig. 5b](#)). It produces narrow size distribution of particles with sizes from 1 to 10000 µm. These processes are continuous and have high production capacity. The most common extrusion process uses the biopolymer alginate as wall material and is based on the gelification capacity of this polymer in the presence of ionic calcium, without the need of heating. Due to the mild process conditions needed, it is suitable for the encapsulation of omega 3 and phytosterols.

However, few studies addressing the encapsulation of omega-3 fatty acids by ionic gelation were found in the literature, which makes it an innovative idea for the encapsulation and, therefore, protection of these compounds. [Mwangi, Ho, Ooi, Tey, & Chan \(2016\)](#) studied a method for forming ionically cross-linked chitosan microcapsules from Pickering emulsion templates using the

same idea of ionic gelation technique. These authors obtained microcapsules suited for the rapid controlled release of lipophilic compounds. In the case of phytosterols, stigmastanol was encapsulated by [Fujiwara et al. \(2013\)](#) by the external ionic gelation technique using sodium alginate, corn starch and chitosan as wall materials. According to these authors, the method was feasible due to the high yield and encapsulation efficiency values.

According to the above, it is observed that extrusion or ionic gelation processes, methods which guarantee the protection of the encapsulated compounds, are rarely explored in relation to the encapsulation of omega-3 fatty acids and phytosterols. It can be explained because the polymers used as wall material for ionic gelation process, as alginate and carrageen, have no surface property and are hydrophilic compounds, so, for encapsulation of hydrophobic materials, it is necessary to include an emulsifier and to prepare an emulsion before the gelation process. So, it is an innovative idea, which gives a quite promising field for future research and applications of interest in the food industry.

4.3. Complex coacervation

Complex coacervation is an encapsulation technique based on the electrostatic interaction between two polymers with opposite charges ([Fig. 6](#)). It is a relatively simple methodology and useful to elaborate water and heat resistant microcapsules, with size about 0.1–500 µm. One disadvantage of this method is that it typically yields particle dispersions that the most of the time need a post-process, such as spray drying or freeze drying, in order to obtain a more stable material and of simple application. This method is feasible for the encapsulation of hydrophobic compounds, since that the first step is an oil in water (O/W) emulsion, so it is perfect for the encapsulation of omega-3 fatty acids and phytosterols.

There are several combinations used as wall material for complex coacervation encapsulation, as it is shown in [Table 1](#); however, as already mentioned, the protein gelatin and the polysaccharide gum Arabic are the most widely used biopolymers due to the perfect combination of their charges and low cost ([Alvim & Grosso, 2010](#)). However, this perfect combination is not achieved with all kinds of combination of the oppositely charged polymers. A

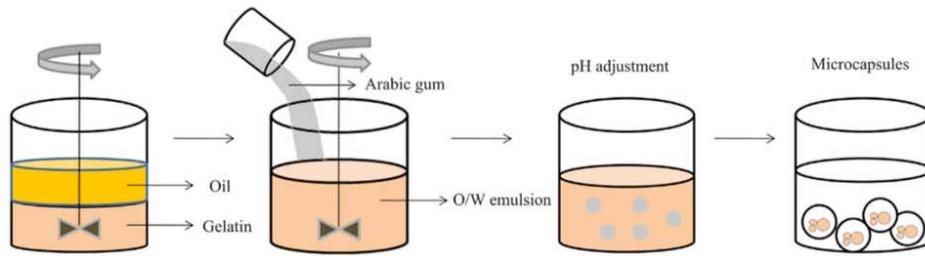


Fig. 6. Complex coacervation technique procedure.

preliminary study analyzing the zeta potential of the biopolymer is important in order to obtain the best relationship between the charge and concentration of these materials.

Omega-3 fatty acids are some of the compounds more encapsulated by complex coacervation because of their importance and instability, as already mentioned in the course of this review and also because this technique is widely used to encapsulate lipophilic compounds. It is not possible to encapsulate hydrophilic materials by complex coacervation without any prior step due to the process occur in an aqueous medium, hindering the aggregation of the material by polymers. Furthermore, the multilayer coating obtained by complex coacervation provides better protection to omega-3 fatty acids and phytosterols against oxidation. This technique also produces more stable and heat resistant microcapsules, of high encapsulation efficiency and of rounded shape; this last characteristic helps in the future application due to the easy flow (Barrow, Nolan, & Jin, 2007; Kaushik et al., 2014; Taneja & Singh, 2012). Eratte, Wang, Dowling, Barrow, and Adhikari (2014) studied the encapsulation of omega-3 rich tuna oil by complex coacervation using whey protein isolate and gum Arabic as wall materials and obtained capsules stable against oxidation with potential application into food products.

Recently, the idea of coencapsulation was used by Eratte et al. (2015) when they encapsulated omega-3 fatty acids and probiotic bacteria by complex coacervation followed by spray and freeze drying. Protein whey isolate and gum Arabic were used as wall materials, tuna oil as a source of fatty acids and *Lactobacillus casei* 431 as probiotics. According to these authors, the materials were successfully encapsulated and the oxidative stability of the tuna oil was improved both in the presence and absence of bacterial cells, showing that these capsules may be useful in case of release of these two important ingredients in functional foods and nutraceutical applications. Comunian et al. (2016) studied the coencapsulation of echium seed oil, vegetable source of omega-3 fatty acids, with phenolic compounds sinapic acid or rutin by complex coacervation using gelatin and gum Arabic as wall materials, obtaining rounded microcapsules, with good values for encapsulation process yield for the oil and for the phenolic compounds.

Tamjidi, Nasirpour, and Shahedi (2014) studied the rheological characteristics of yogurt enriched with fish oil microcapsules obtained by complex coacervation using gelatin and acacia as wall materials. The yogurt with microcapsules presented higher apparent viscosity than the control and a non-Newtonian shear-thinning flow behavior; moreover, it was possible to obtain a yogurt which provides the fish oil health effect.

Differently of omega-3 fatty acids, no work on encapsulation of phytosterols by complex coacervation was found in the literature. Many studies on the encapsulation of this material are being done, however with other encapsulation methods, mainly with the formation of liposomes (not mentioned in this review) and spray

drying. This shows that there are still many possibilities for encapsulation of phytosterols, since the study of their stability and controlled release in the organism are extremely important for obtaining quality products, and hence the maintenance of health. In this way, it can be said that the microencapsulation by complex coacervation technique, besides being the most widely used for encapsulation of omega-3 fatty acids, still needs to be explored in relation to encapsulation of phytosterols. As already mentioned, it is a technique that uses inexpensive materials, besides it ensures the oxidative protection of compounds of interest as observed in the studies cited.

Furthermore, along with the complex coacervation technique, the cross-linking process has been widely used for the purpose of forming more resistant capsules. This process consists in the addition of a compound (anionic or cationic) in the wall material in order to bind themselves and then, stabilize and modify the structure of the complex (Chen, Li, Ding, & Duo, 2012). The traditional cross-linking agents used in this process are formaldehyde and glutaraldehyde, which are toxic and prohibited in food applications. For this reason, in order to replace them, the cross-linking function of specific natural compounds are of interest. Ferulic acid (Cao, Fu, & He, 2007) and genipin (Li et al., 2015), are being studied for this reason; transglutaminase, an enzyme very applied in cheese, meat products and edible films, and tannic acid are the compounds most used with this function (Dong et al., 2008; Peng et al., 2014). To briefly summarize, the major difference between the process of complex coacervation and cross-linking is that the first one is the encapsulation technique itself, while the second one consists of the addition of a compound in the coacervate microcapsule in order to stabilize its structure.

The cross-linking technique has been widely used for the encapsulation of omega-3 fatty acids by complex coacervation. Conto et al. (2013) evaluated the encapsulation of omega-3 fatty acids by complex coacervation using soy protein isolate and gum Arabic as wall materials, varying the polymer concentration, the core and cross-linker agent (transglutaminase). According to these authors, it was possible to obtain the microcapsules and, in addition to that, to obtain a final functional food, it would be necessary to add 0.4 g of microcapsules in 100 g of final product portion. In other study, Wang, Adhikari, and Barrow (2014) microencapsulated tuna oil by complex coacervation using the combination gelatin-sodium hexametaphosphate as wall materials and transglutaminase as crosslinking agent. These authors obtained high values of encapsulation efficiency and samples more stable than non-encapsulated oil.

4.4. Complexation

Complexation is a technique used for different purposes; however, it is considered one of the simplest methods of encapsulation

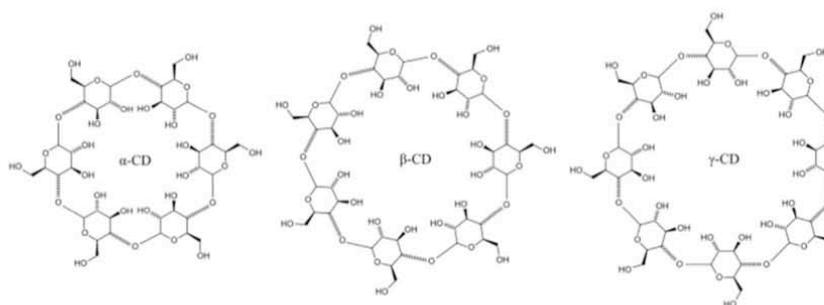


Fig. 7. Structures of α , β and γ -cyclodextrin.

Table 2
Characterization of the three cyclodextrins.

Types of cyclodextrins	Glucopyranose units	Solubility in water	Rigidity of the structure	Size of the largest internal diameter (nm)
Alfa-cyclodextrin	6	medium	medium	0.57
Beta-cyclodextrin	7	low	strong	0.78
Gama-cyclodextrin	8	high	weak	0.95

Reference: Astray et al. (2009).

which consists of only a mixture of cyclodextrin (α -cyclodextrin (α -CD), β -cyclodextrin (β -CD) and γ -cyclodextrin (γ -CD) (Fig. 7 and Table 2) with the hydrophobic material that will be encapsulated. The β -cyclodextrin is the most used of the three mentioned because of the purification cost.

Cyclodextrins (CDs) are oligomers in the form of truncated cone (Fig. 7); the outer region of the structure is composed by hydrogen atoms and glycosidic oxygen bridge, while the interior by free electrons. The CDs are formed by glycosidic bonds and produced from the processing of starch by bacteria, which help in the protection of flavors, vitamins and natural dyes (Fathi et al., 2014). According to Astray, Gonzalez-Barreiro, Mejuto, Rial-Otero, and Simal-Gandara (2009), the obtaining of the cyclodextrin is composed by four steps, including: (1) the culture of the microorganism which produces the cyclodextrin glucosyl transferase enzyme (CGT-ase); (2) the separation, concentration and purification of the enzyme; (3) enzymatic conversion and (4) separation, purification and crystallization of the cyclodextrins.

The CDs have been very used for encapsulation, because its shape allows to form complex, involving hydrophobic compounds inside. In this case, there is no formation of round microcapsules as in the case of complex coacervation technique; however it is also a method that allows the protection and controlled release of lipophilic and sensitive compounds when exposed to various environmental conditions. According to Astray et al. (2009), the complexes formed by CDs are also known as inclusion complexes, since CDs can involve the encapsulated compound partially or completely.

Omega-3 fatty acids and phytosterols are materials often encapsulated by this technique. Yoshii, Furuta, Yasunishi, Linko, & Linko (1996) studied the complexation of omega-3 polyunsaturated fatty acids eicosapentaenoic (EPA) and docosahexaenoic acids (DHA) using CD. The obtained samples were stable to oxidation and most EPA remained unoxidized. Recently, Vestland, Jacobsen, Sande, Myrset, and Klaveness (2015) produced compactible powders of omega-3 and β -cyclodextrin. These authors analyzed the powders produced by vacuum drying, freeze drying or spray granulation of aqueous mixtures of omega-3 oil and β -cyclodextrin, confirming the true complexation.

The encapsulation of phytosterols with the use of CDs by the complexation technique was studied by Meng et al. (2012) when they evaluated the preparation and properties of phytosterols in their inclusion in complexes of hydroxypropyl- β -cyclodextrin. The inclusion efficiency values (it is also known as encapsulation efficiency for other authors) were in the range from 92 to 98% and, according to these authors, the formed complexes presented high solubility in water, which facilitates the application of the final product.

Besides the CDs, spring dextrans are also used for the complexation technique for encapsulation of lipophilic compounds. Xu et al. (2013) studied the stability and controlled release of polyunsaturated fatty acids ω 3/ ω 6 encapsulated by spring dextrin. According to these authors, the complex improved the stability of α -linolenic fatty acid (ALA) and linoleic (LA) and enabled the controlled release of 21.7 and 18.5% of ALA and LA of its complex when the gastrointestinal tract was simulated.

No application of complex of omega-3 fatty acids or phytosterols with CDs into food products was found in the literature. Thus, according to the above, it can be said that complexation technique, besides being a simple method of encapsulation, has potential in protecting omega-3 fatty acids and phytosterols and is also a technique which can provide a promising field for the future since the application of this material has been little explored.

5. Conclusions

The potential of the microencapsulation technique has greatly increased, since this process has been one of the best alternatives to improve the stability of phytosterols and omega-3 fatty acids and also control the release of these compounds to the maintenance of health. These materials have often been encapsulated by techniques which use biopolymers such as complex coacervation, complexation, spray drying and ionic gelation, as they allow the formation of microcapsules and/or microparticles and/or complex of low cost and easy handling. Moreover, the encapsulated phytosterols and/or omega-3 fatty acids are applied to food products in order to provide better nutritional attributes with functional appeal, and thereby help in preventing of cardiovascular diseases.

Encapsulation of omega-3 fatty acids has been more explored than of phytosterols. Many studies on the encapsulation of phytosterols are found, however there is no study using complex coacervation and only one using complexation in the literature, showing an area that needs to be explored in the future. Moreover, microcapsules and/or complex composed by biopolymers have wide application, not just in the food field, but also in the pharmaceutical and cosmetics industries.

Acknowledgements

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Protection of echium oil by microencapsulation with phenolic compounds



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ABSTRACT

The consumption of omega-3 enables the reduction of cardiovascular disease risk; however they are unstable. The aim of this work was to encapsulate echium oil (*Echium plantagineum* L.), a rich source of omega-3 fatty acids, with phenolic compounds (sinapic acid and rutin) by double emulsion followed by complex coacervation or by complex coacervation with sinapic acid in the capsule wall. Analyses of morphology, particle size, circularity, water activity, moisture, Fourier transform infrared spectroscopy, thermogravimetry, process yield, accelerated oxidation and identification and quantification of fatty acids present in the encapsulated oil were performed. Samples presented values of encapsulation process yield of phenolics and oil in the range of 39–80% and 73–99%, respectively. Moreover, all samples protected the oil against oxidation, obtaining induction time (accelerated oxidation) of 5 h for pure oil and values in the range from 10 to 18 h for samples. Thus, better protection to the oil was possible with sinapic acid applied in the capsule wall, which enhances its protection against lipid oxidation.

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1. Introduction

There are several sources of omega-3 fatty acids (ω -3 FA) that can be added to food products. These sources can be from animal origin (marine oils extracted from cold water fish) or vegetable origin, such as echium oil (Whelan, 2009). *Echium plantagineum* L. has a Mediterranean and Macaronesian origin (Berti, Johnson, Dash, Fischer, & Wilckens, 2007) and contains 33% of α -linolenic acid and from 9 to 16% stearidonic acid, an intermediate in the biosynthesis of EPA and DHA, (Clough, 1993; Payne, Lad, Foster, Kholá, & Gray, 2014), which are important omega-3 FA. Besides, the echium oil is being used in replacement of fish derived oils, since it presents unique ratio of omega-3 to omega-6 FA of 1.8:1, suitable for nutraceutical applications (Berti et al., 2007).

Omega-3 FA have been assessed for reducing the risk of cardiovascular disease (Poole et al., 2013); however they are very susceptible to oxidation, which hinders their application in food. Lipid oxidation could be delayed by different factors such as packaging and modified atmosphere. Moreover, other two strategies that could minimize these limitations are: (1) adding a compound with antioxidant function and (2) microencapsulation.

Phenolic compounds, such as sinapic acid and rutin, have been of great interest in the food industry, as they act as antioxidant agents (Lee et al., 2013). Sinapic acid, the main phenolic acid of canola, has been studied regarding its neuroprotective effect against Alzheimer's disease (Lee et al., 2012), cardiac hypertrophy and dyslipidemia (Roy & Prince, 2013). In relation to rutin, phenolic compound found in plants and food sources such as onions, grape, bean, apple and tomato, it has several pharmacological activities, including anti-allergenic, anti-inflammatory and vasoactive properties (Jantrawut, Assifaoui, & Chambin, 2013; Kim, Kwon, & Jang, 2011). Espinosa, Inchingolo, Alencar, Rodriguez-Estrada, and Castro (2015) studied the effect of eleven compounds on oxidative stability of emulsions prepared with echium oil and it was observed that sinapic acid and rutin were the most efficient to delay lipid oxidation, so they were distinguished from the compounds analyzed, which make them potential alternative to be applied as antioxidant in products, specially with echium oil.

The encapsulation is a process which retains a bioactive (solid, liquid or gas) inside another (wall material) in order to protect the material against adverse environmental conditions, thereby increasing the shelf-life and promoting the controlled release of the active compound in the microcapsule (Nedovic, Kalusevic, Manojlovic, Levic, & Bugarski, 2011; Shahidi & Han, 1993).

In this context, the aim of this work was to protect echium oil. In order to achieve this aim, the echium oil was encapsulated in the presence of the phenolic compounds rutin and sinapic acid.

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2. Material & methods

2.1. Materials

As core were used echium oil (NEWmega™ Echium Oil, Ref.15200, from De Wit Specialty Oils (De Waal, Tescel, The Netherlands), sinapic acid and rutin from Sigma Chemical Co. (St. Louis, MO, USA). As wall material were used gelatin and Arabic gum from Gelita South America (Mococa/SP, Brazil) and Nexira (São Paulo/SP, Brazil), respectively. Polyglycerol ricinoleic acid (PGPR 90) (Danisco, Denmark) was used as an emulsifier.

2.2. Methods

2.2.1. Microencapsulation

For the production of microcapsules, a primary water-in-oil emulsion (W/O) consisting of sinapic acid or rutin solution in the internal aqueous phase (0.50 mg of phenolic compound/mL of water) and echium seed oil in the external oil phase was prepared at a ratio of 2:1 (v:v oil phase:aqueous phase). Polyglycerol ricinoleic acid (PGPR 90) was used as the emulsifier in the concentration of 0.5% (w/w) and added in the oil phase.

The primary emulsions (W/O) were emulsified in the gelatin solution (7.5%-w/w) with pH 6.0 to obtain a double emulsion water-in-oil-in-water (W/O/W) with different concentrations of phenolic compounds as showed in Table 1. The homogenization steps were performed at 12,000 rpm for 4 min (primary emulsion – W/O) and 10,000 rpm for 3 min (double emulsion – W/O/W) with an Ultra-Turrax apparatus (Ika, Germany). The Arabic gum solution (7.5%-w/w) was added to these emulsions by magnetic stirring at 40 °C for 3 min.

Besides the application of sinapic acid in the internal phase of the primary emulsion, a sample was also obtained where it was added directly to the primary emulsion, before the addition of Arabic gum solution (SIN-GEL). In this case, the double emulsion was not prepared, but only the primary emulsion composed of echium oil and gelatin solution. Moreover, the control sample composed of only echium oil and the wall materials (gelatin and Arabic gum) was also prepared.

Samples with different concentrations of the primary emulsion (50, 75 and 100% – SIN50, SIN75 and SIN100 and RUT50, RUT75 and RUT100, respectively) in relation to the total mass of the polymer (7.5%-w/w) were prepared, in addition to samples in which sinapic acid was used in the capsule wall and the oil was encapsulated without a phenolic compound (SIN-GEL and Control, respectively), resulting in eight samples (Table 1). The ratio of polymers (gelatin and Arabic gum) was fixed at 1:1.

Table 1
Concentrations and proportions used for each sample in the process of encapsulation.

Sample	Concentration of phenolic (µg/g of capsule)	Proportion of phenolic in relation to the oil (ppm)	Proportion of core in relation to total amount of polymer (%)	Concentration of oil (g/g of capsule)
SIN50	65.1	200	50	0.24
SIN75	87.0	200	75	0.32
SIN100	104.8	200	100	0.39
SIN-GEL	88.9	200	50	0.33
RUT50	65.1	100	50	0.24
RUT75	87.0	100	75	0.32
RUT100	104.8	100	100	0.39
Control	–	–	50	0.33

SIN50 and RUT50: sample with a 1:1:0.5 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN75 and RUT75: sample with a 1:1:0.75 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN100 and RUT100: sample with a 1:1:1 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN-GEL: sample with a 1:1:0.5 proportion of gelatin, Arabic gum and echium oil with sinapic acid as a cross-linker. Control: sample with a proportion of 1:1:0.5 of gelatin, Arabic gum and echium oil.

To promote complex coacervation, the pH was adjusted to 4.0 at 40 °C under magnetic stirring and the temperature was gradually lowered to 10 °C in an ice bath. The material was stored for 24 h at 7 °C to promote decantation. The coacervates were frozen (–18 °C) and freeze-dried (Terroni/São Carlos – SP, Brazil) for 24 h at a pressure of 1 to 0.1 kPa pressure, at a temperature of –20 °C and a final temperature of 30 °C. All the procedure of capsules fabrication is presented in a schematic representation in Fig. 1.

2.2.2. Characterization of coacervate samples

2.2.2.1. Yield of encapsulation process of phenolic compounds. The yield was determined by the mass difference of the phenolic compound contained in the microcapsules and the total quantity used in the encapsulation process. For the rupture of the capsule, 0.2 g of sample was diluted in 5 mL of methanol, 5 mL of ZnSO₄ solution (5%-w/w) and 0.8 g of KCl, subjected to ultrasound for 10 min and centrifuged at 4000 rpm at 25 °C for 10 min. The supernatant was filtered and analyzed by High Performance Liquid Chromatography (HPLC) (Shimadzu, Kyoto, Japan). A C18 column was used with water:methanol as the mobile phase at a ratio of 45:55 (water at pH 3.5), flow of 1 mL/min. The detection wavelength was 325 and 356 nm and the injection volume was 10 and 20 µL for sinapic acid and rutin, respectively. The yield of the encapsulation process was calculated from Eq. (1).

$$\text{Yield} = \frac{(\text{Phenolic added} - \text{phenolic in the capsule}) \times 100}{\text{Phenolic added}} \quad (1)$$

2.2.2.2. Yield of encapsulation process of echium oil. The yield was determined by the difference in the mass of the oil contained in the microcapsules and the total quantity used in the encapsulation process. For the rupture of the capsule, the same method mentioned in Section 2.2.2.1 was used. After the rupture of the capsule, the procedure continued with the Bligh Dyer method with some modifications for oil extraction (Bligh & Dyer, 1959). In the broken capsules, it was added 5 mL of chloroform, 10 mL of methanol and 4 mL of distilled water. The mixture was lightly stirred for 1 min and then 5 mL of chloroform and 5 mL of sodium sulfate solution (1.5% w/w) were added to it, stirring gently in the vortex for 1 min more. The samples were allowed to stand for 30 min and a known volume of the chloroform phase was withdrawn and kept in oven at 60 °C to evaporate the solvent. The quantification of the oil was made from the remaining mass after evaporation of the chloroform. Thus, knowing the mass of oil added to the microcapsule and the volume of evaporated chloroform, it was possible to obtain the yield of encapsulated oil.

2.2.2.3. Morphological characterization of the microcapsules by optical microscopy, scanning electron microscopy (SEM) and confocal microscopy. Wet microcapsules were analyzed by optical microscopy using Bio3 equipment (Bel Photonics, Italy) and by confocal microscopy, using an Axio Observer Z.1 and an LSM 780-NLO Zeiss microscope, with a 40× objective. A laser with a wavelength of 561 nm was used for excitation along with a 569–691 nm emission filter for the fluorophore Nile Red. The pinhole was set to 1 Airy unit in each channel and the image size was 1024 × 1024, with an optical zoom of 1.7×. Lyophilized microcapsules were analyzed by SEM using a TM 3000 table-top microscope (Hitachi, Tokyo, Japan), and TM 3000 software.

2.2.2.4. Particle size analysis. To obtain the particle size, 200 microcapsules of each sample were individually measured using ImageJ software. A BIO3 microscope (Bel Photonics, Italy) was used to obtain the images.

2.2.2.5. Circularity. To determine the circularity the ratios between the smallest and the largest diameter of the capsules were used. These measurements were obtained from 100 capsules for each sample using

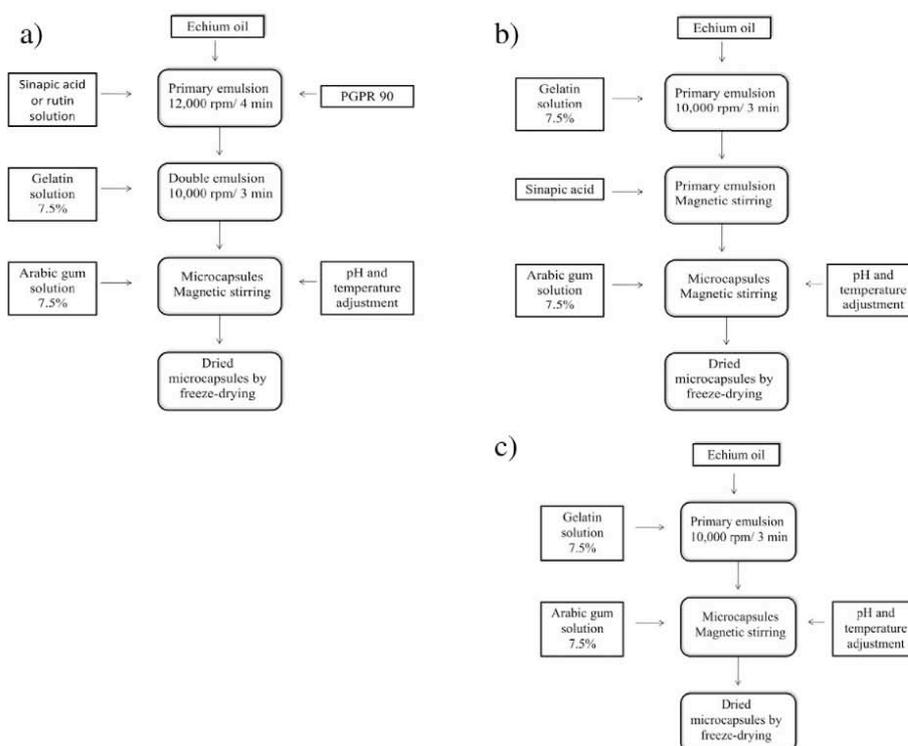


Fig. 1. Schematic representations of the procedure of the capsules fabrication: a) samples SIN50, SIN75, SIN100, RUT50, RUT75 and RUT100; b) sample SIN-GEL; c) sample control.

ImageJ software. A BIO3 microscope (Bel Photonics, Italy) was used to obtain the images.

2.2.2.6. Water activity and moisture. Water activity and moisture content were determined using AQUALAB equipment (Decagon Devices, Pullman, WA) and moisture analyzer (Ohaus model MB 35, Ohio, USA).

2.2.2.7. Fourier transform infrared spectroscopy. The spectra of the ingredients and the microcapsules were obtained in the region of 4000 to 600 cm^{-1} using a Perkin Elmer FTIR spectrometer (Massachusetts, USA) with Spectrum One software version 5.3.1.

2.2.2.8. Thermogravimetric analysis. The thermogravimetric curves (TG) were obtained using Shimadzu TGA-50 equipment, which had been calibrated at a heating rate of 10 $^{\circ}\text{C min}^{-1}$ with calcium oxalate monohydrate. Sample masses of 8 mg were used, which were placed in a platinum sample holder. The furnace atmosphere was saturated with super-pure nitrogen at a flow rate of 50 mL/min. The analysis temperature range was 25 to 800 $^{\circ}\text{C}$.

2.2.2.9. Identification and quantification of fatty acids (FA) in encapsulated oil. FA concentrations were monitored by gas chromatography (Agilent 7890 A GC System, Agilent Technologies Inc., Santa Clara, United States), with a J&W DB-23 column (60 m \times 250 $\mu\text{m} \times$ 0.15 μm ; Agilent 122–2361). The analyses were performed in triplicate. After the rupture of the capsules, 150 mg of oil extracted from the microcapsules was weighed; 5 mL of hexane was added to this sample and mixed on a vortexer. For the esterification of FA, 50 μL of this diluted oil

(corresponding to 1.5 mg of oil) was added to 15 mL of a standard solution (C23:0) in dichloromethane and 1 mL of 0.5 M NaOH in methanol was added. This mixture was mixed on a vortexer for 15 s and placed in a water bath at 100 $^{\circ}\text{C}$ for 5 min. After cooling, 2 mL of BF₃-methanol 14% was added, then the sample was shaken and placed in a water bath at 100 $^{\circ}\text{C}$ for 5 min. One milliliter of iso-octane was added, shaken vigorously for 30 s and added of saturated NaCl solution. Then, the sample was homogenized. After phase separation, the iso-octane layer was transferred to a 1.5 mL microtube and dried completely with nitrogen gas. The residue was resuspended in 500 μL of iso-octane and 200 μL of this mixture was transferred to a vial with an insert for chromatographic analysis.

2.2.2.10. Accelerated oxidation. Accelerated oxidation tests for the pure and for echium oil encapsulated were performed with the aid of Rancimat equipment (model 873, Metrohm, Switzerland). Samples were subjected to heating under a purified air flow rate of 20 L/h at 90 $^{\circ}\text{C}$. The induction time of sample was used as oxidative stability index. Besides, it was used 5 g of pure oil and 1.5 g of microcapsule, and this analysis was performed in duplicate.

2.2.2.11. Statistical analysis. All experiments were done three times, except Rancimat, which was done twice. Data were statistically analyzed using SAS statistical software (Statistic Analysis System), version 9.2, by ANOVA and Tukey's test at 5%.

3. Results and discussion

3.1. Yield of encapsulation process of phenolics and oil

The values obtained for the process yield are shown in Table 2. In relation to the yield of the process for rutin and sinapic acid, the obtained values were within the range from 39 to 81%. There was a significant difference for the samples RUT75 and RUT100 (75 and 100% core in relation to the total amount of polymer). The yield values of these samples were considered good and were approximately double compared to those obtained for samples with sinapic acid (SIN75 and SIN100) with the same concentration of core. The sample in which sinapic acid was applied in the capsule wall (SIN-GEL) showed a yield of 50%. The expected result would be that the higher the concentration of phenolic compound in the microcapsule, the greater its effective action as antioxidant, in other words, RUT75 and RUT100 samples would present better oxidative protection to the oil than the other samples. However, that is not what happened, as shown in the accelerated oxidation test (item 3.7). This can be explained due to the polarity of the sinapic acid – a compound more polar than rutin. According to Shahidi and Zhong (2011), polar antioxidants are more effective in less polar media, what happened to sinapic acid in this oil system.

In the case for the yield of the process for oil, values between 73 and 99% were obtained, with no significant difference among the samples. So, different concentrations of the phenolics and oil, and different position of sinapic acid, did not affect the mass of oil that was encapsulated. Oil losses are related to the amounts that were in the beaker or in the Ultra-Turrax during the process.

Rocha-Selmi, Theodoro, Thomazini, Bolini, and Favaro-Trindade (2013) and Rocha-Selmi, Bozza, Thomazini, Bolini, and Favaro-Trindade (2013) studied the encapsulation of aspartame and sucralose, hydrophilic compounds, by double emulsion followed by complex coacervation using gelatin and Arabic gum as wall materials; they obtained yields ranging from 43 to 89% and from 45 to 71%, respectively; these values corroborate those obtained for sinapic acid and rutin.

3.2. Morphological characterization of the microcapsules by optical microscopy, SEM and confocal microscopy

The micrographs are shown in Figs. 2 and 3. For optical microscopy, there were no morphological differences among all samples, showing round and multinucleated microcapsules. It was also noted that the use of sinapic acid in the microcapsule wall did not affect the morphology, showing that it can be incorporated in different positions without damaging the structure.

In the same way, Santos, Bozza, Thomazini, and Favaro-Trindade (2015); Rocha-Selmi, Theodoro et al. (2013) and Rocha-Selmi, Bozza et al. (2013) obtained round, multinucleated microcapsules when

xylitol, sucralose and aspartame were encapsulated, respectively, using the double emulsion technique followed by complex coacervation. Regarding to the SEM results (Fig. 3), it was observed that there was increased surface roughness with an increasing concentration of the core in relation to the total mass of the polymer; in other words, samples SIN100 and RUT100 were rougher than SIN75 and RUT75. However, samples SIN50, RUT50 and control showed smooth surfaces. Regarding the sample SIN-GEL, it was observed that the presence of sinapic acid in the capsule wall made the surface rougher than when it was located within the microcapsule. However, this feature did not affect the functionality of the material. The micrographs showed that the microcapsules were linked by “bridges” due to the freeze-drying method. This structure complicates applications in food products where good flow is necessary; however, it becomes a positive factor regarding wettability due to the presence of canals in these agglomerates, through which water infiltrates. Besides, the dehydration process facilitates the microcapsule preservation, its packaging and transport; and the microcapsule circularity can be recovered putting or applying the microcapsules in wet products.

The micrographs obtained from confocal microscopy are also presented in Fig. 2. The red dye Nile Red was applied to the oil in order to verify the location of the oil within the microcapsule. In these micrographs, it is possible to observe that the sample in which the double emulsion was used as one of the steps presented a light red coloration due to the lower amount of oil compared to samples in which the core was only oil.

3.3. Fourier transform infrared spectroscopy

For analysis by FTIR, spectra were obtained for the wall materials, the cores and for the samples (Fig. 4).

In relation to the wall material, according to Santos et al. (2015), the peaks around 3300 cm^{-1} present in the spectrum of gelatin represent amino groups (when the protein is positively charged), while peaks about 2900 cm^{-1} present in the spectrum of Arabic gum represent carbonyl groups (negatively charged); these peaks were also present in the samples.

The spectrum of sinapic acid showed intense vibrations between 3650 and 2500 cm^{-1} and between 1750 and 600 cm^{-1} . A similar spectrum was obtained by Tan and Shahidi (2013). According to these authors, peaks around 1700 cm^{-1} represent carbonyl groups ($\text{C}=\text{O}$) and between 3300 and 3500 cm^{-1} represent hydroxyl groups in the phenolic portion. These peaks present in the spectrum of sinapic acid were also present in the spectra of the samples, showing that the structure of this compound remained intact. For rutin, very intense vibrations between 1700 and 1000 cm^{-1} were obtained. According to Yan, Li, Zhao, and Yi (2011), peaks around 1450 cm^{-1} represent CH_2 or CH_3 bonds. It is observed that all samples with rutin showed similar

Table 2
Values of water activity, moisture, particle size, circularity and accelerated oxidation index.

Samples	Yield for phenolics (%)	Yield for oil (%)	Water activity	Moisture (%)	Particle size (um)	Circularity	Accelerated oxidation index (hours)
SIN50	39.48 ± 5.99 ^b	99.80 ± 0.34 ^a	0.51 ± 0.08 ^{ab}	9.12 ± 1.30 ^a	45.14 ± 13.94 ^{bc}	0.89 ± 0.12 ^{ab}	10.18 ± 0.00 ^b
SIN75	43.37 ± 10.18 ^b	95.69 ± 7.46 ^a	0.49 ± 0.03 ^{ab}	9.43 ± 0.36 ^a	42.39 ± 12.96 ^c	0.90 ± 0.11 ^{ab}	9.97 ± 0.00 ^b
SIN100	46.35 ± 4.21 ^b	94.37 ± 6.37 ^a	0.53 ± 0.02 ^a	8.89 ± 0.62 ^{ab}	45.01 ± 14.71 ^{bc}	0.87 ± 0.12 ^{ab}	10.06 ± 0.28 ^b
SIN-GEL	50.55 ± 5.98 ^b	87.08 ± 12.95 ^a	0.32 ± 0.07 ^c	7.43 ± 0.23 ^{ab}	42.22 ± 13.13 ^c	0.90 ± 0.14 ^{ab}	18.77 ± 2.42 ^a
RUT50	58.16 ± 7.88 ^a	73.16 ± 4.89 ^a	0.43 ± 0.13 ^{abc}	8.98 ± 1.92 ^{ab}	47.31 ± 14.86 ^{ab}	0.92 ± 0.08 ^a	10.04 ± 0.05 ^b
RUT75	78.70 ± 18.03 ^a	91.31 ± 10.78 ^a	0.49 ± 0.17 ^{abc}	8.71 ± 1.85 ^{ab}	51.75 ± 17.97 ^a	0.90 ± 0.12 ^{ab}	10.26 ± 0.19 ^b
RUT100	80.97 ± 20.17 ^a	85.61 ± 15.01 ^a	0.43 ± 0.07 ^{abc}	8.14 ± 1.07 ^{ab}	42.55 ± 18.11 ^c	0.87 ± 0.12 ^{ab}	10.06 ± 0.05 ^b
Control	–	79.49 ± 18.22 ^a	0.36 ± 0.04 ^{bc}	6.93 ± 0.52 ^b	44.86 ± 13.75 ^{bc}	0.86 ± 0.12 ^b	10.05 ± 0.05 ^b
Pure oil	–	–	–	–	–	–	5.15 ± 0.04 ^c

Equal letters in the same column do not differ significantly by the Tukey test at 5% probability.

SIN50 and RUT50: sample with a 1:1:0.5 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN75 and RUT75: sample with a 1:1:0.75 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN100 and RUT100: sample with a 1:1:1 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN-GEL: sample with a 1:1:0.5 proportion of gelatin, Arabic gum and echium oil with sinapic acid as a cross-linker. Control: sample with a proportion of 1:1:0.5 of gelatin, Arabic gum and echium oil.

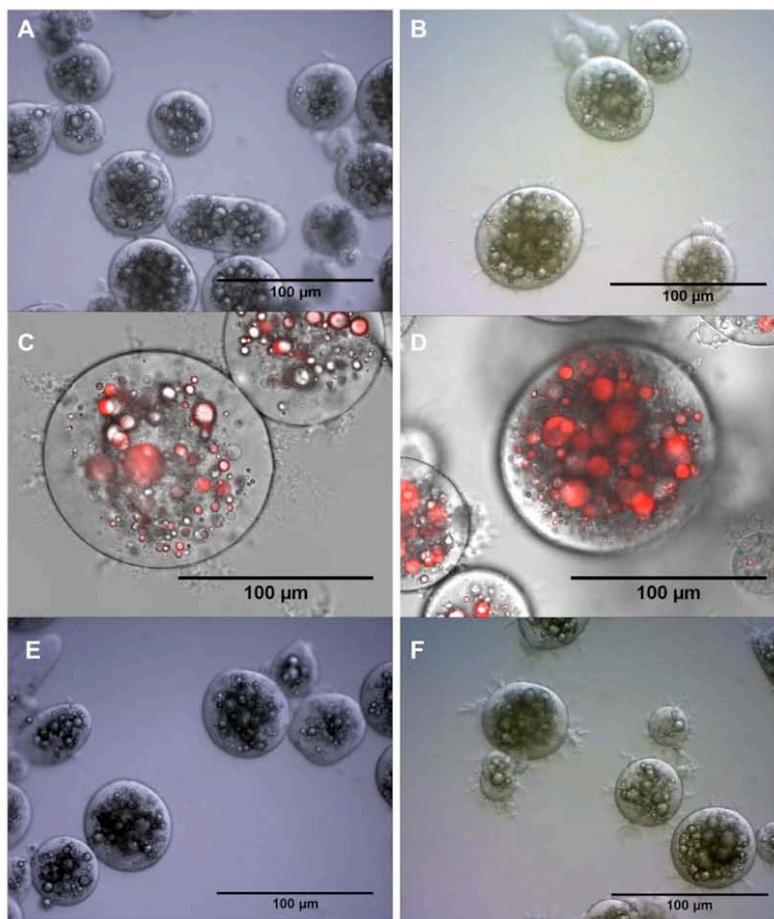


Fig. 2. Micrographs obtained by optical microscopy for the samples (A) SIN50 and (B) SIN-GEL; confocal microscopy for the samples (C) SIN50 and (D) SIN-GEL; optical microscopy for the freeze-dried and rehydrated samples (E) SIN50 and (F) SIN-GEL.

behavior, differing only in the intensity of the peaks, since the difference is related to the concentration of the compounds.

3.4. Analysis of particle size, circularity, water activity and moisture

The values obtained for water activity, moisture, average particle size and circularity are shown in Table 2. The analyses of water activity and moisture were performed on freeze-dried capsules and particle size and circularity were performed in the wet ones.

The values of water activity were between 0.36 and 0.51, which can be considered low and, since these values are below 0.6, they ensure microbiological stability (Fennema, Damodaran, & Parkin, 2010). Regarding the moisture values, they were within the range from 6.9 to 9.4% and are also considered suitable for materials dried by freeze-drying. These values facilitate the storage, handling and application of the powders.

Regarding the average particle size values for wet microcapsules, the mean diameter ranged from 42 to 51 μm , showing significant differences among the samples, although it was not verified a clear trend. According to Favaro-Trindade, Pinho, and Rocha (2008), capsules obtained by complex coacervation can present particle sizes in the range from 1

to 500 μm , which is within the range obtained in this work. Furthermore, they were smaller than 100 μm , which can facilitate their application in food products due to the low sensory perception in the mouth of consumer (Thies, 1995).

Regarding the circularity, values were obtained in the range from 0.86 to 0.92. As already mentioned, the value closer to 1, the more rounded the capsule is, facilitates application in food due to easy flow. The values obtained in this work, even with significant differences, can be considered close to 1 and, consequently, indicate a rounded shape. This result was confirmed by optical and confocal microscopy. It is important to highlight that this analysis was performed with wet microcapsules. If they are applied in a dried food, maybe there is no advantage, since the lyophilized microcapsules lose the circularity, as seen in SEM micrographs (Figs. 2 and 3).

3.5. Thermogravimetric analysis

To carry out the thermogravimetric analysis, the behavior in relation to the mass loss according to the temperature applied for each sample was analyzed (Fig. 5). It was observed small weight loss with the increase of temperature up to 100 $^{\circ}\text{C}$; this first thermal degradation of

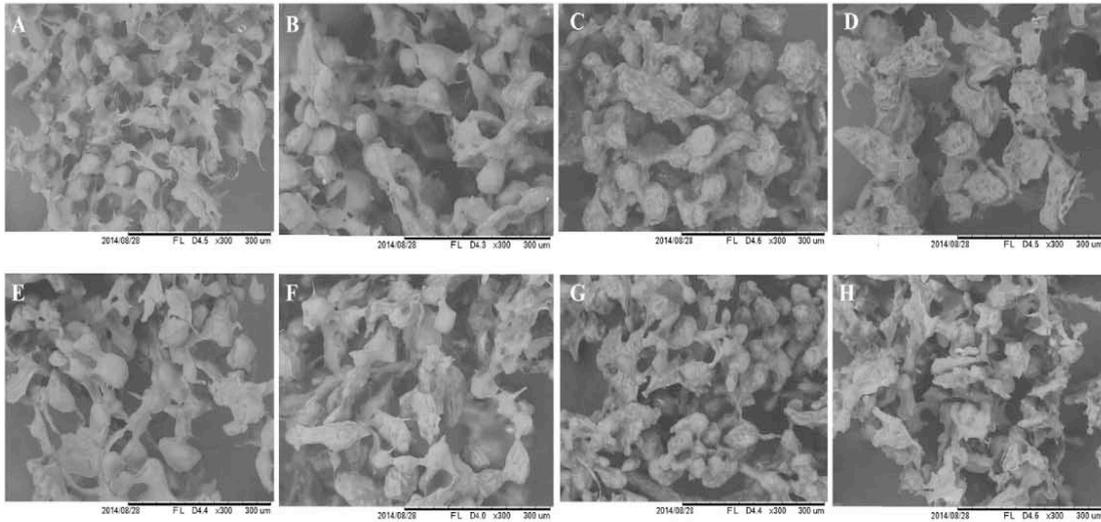


Fig. 3. Micrographs obtained by scanning electron microscopy for the samples (A) SIN50; (B) SIN75; (C) SIN100; (D) SIN-GEL; (E) RUT50; (F) RUT75; (G) RUT100 and (H) control.

the dried microcapsules is owed to the weight loss of remaining water. After this first thermal degradation, the sample weight kept constant in the temperature range from 100 to 250 °C. However, this was followed by the second thermal degradation in the range from 250 to 430 °C; according to Xiao, Li, and Zhu (2015), degradation in this range is related

to the evaporation of oil and partial decomposition of the wall materials. In this case, approximately 80% of the total mass was decomposed.

In Fig. 5, it can be seen that SIN100 was more stable than SIN75, which was more thermally stable than SIN50, in other words, the higher the concentration of sinapic acid (μg of sinapic acid/g of capsule)

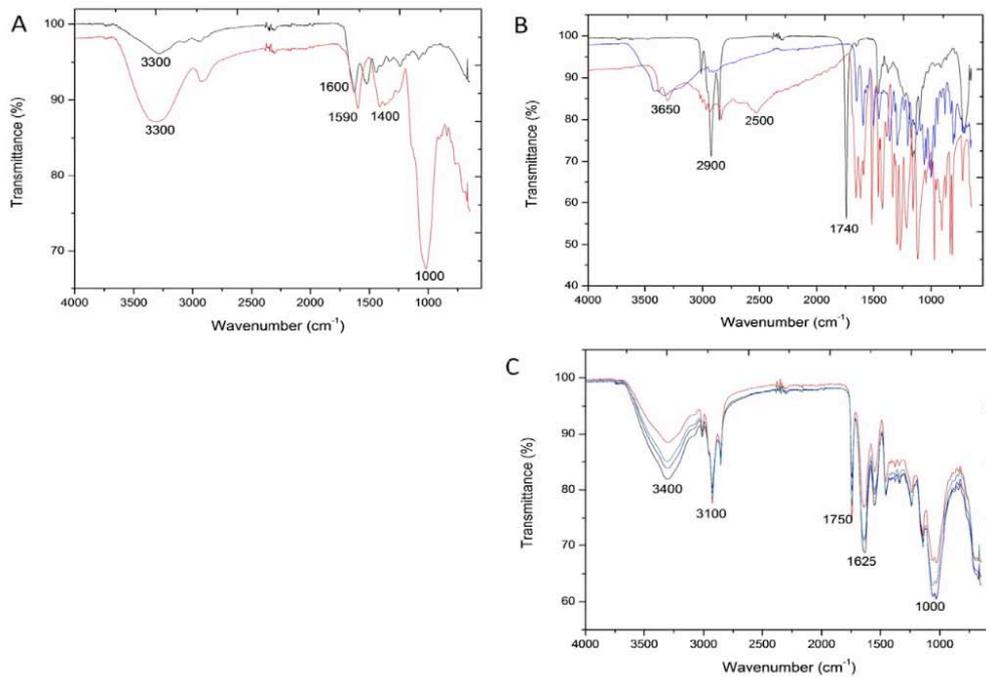


Fig. 4. Spectra obtained by FTIR for (A) gelatin (black curve) and Arabic gum (red curve); (B) echium oil (black curve), rutin (blue curve) and sinapic acid (red curve); (C) dried samples SIN50 (black curve), RUT50 (red curve), SIN-GEL (blue curve) and control (green curve).

(Table 1), the lower the weight loss in the range from 250 to 430 °C. This small difference can be attributed to the low concentration of sinapic acid in relation to the content of gelatin (0.001 g/g of gelatin). According to Peng et al. (2014), who studied the encapsulation of mustard essential oil by complex coacervation with genipin, low concentrations of genipin such as 0.025 g of genipin/g of gelatin resulted in less stable microcapsules, which decomposed at temperatures below 300 °C.

The same relation cannot be applied to the samples with rutin. It is observed in Fig. 5 that the sample RUT50 was more stable than the other samples (RUT75 and RUT100), which showed similar behaviors, including the control sample (encapsulated oil without phenolic compound); moreover, in this case, sinapic acid was more effective than rutin.

3.6. Identification and quantification of fatty acids (FA) present in encapsulated echium oil

Nine FA were identified in the microcapsules, including palmitic (C16:0), stearic (C18:0), oleic (C18:1 n9 cis), elaidic (C18:1 n9 trans), linoleic (C18:2 n6 cis), alpha (C18:3 n3) and gamma linolenic (C18:3 n6), stearidonic (C18:4 n3) and gondoic acid (C20:1 n9), where alpha linolenic and stearidonic are the omega-3 FA.

The FA compositions for each sample are presented in Table 3. It was observed that with an increasing of oil concentration, the FA content also increased, which was expected. The SIN-GEL and control samples, i.e. microcapsules in which the core was composed only of oil, presented oil concentrations similar to the SIN100 sample. For this reason, the concentrations of FA were also similar.

As already mentioned, the echium oil has a ratio of 1:1.8 of omega-6 to omega-3 FA. This ratio is considered suitable for nutraceutical applications and it is not found in other vegetable oil. The encapsulation process used in this study has maintained this ratio, since the concentrations of omega-3 and omega-6 FA presented in Table 3 for each sample are in agreement with that expected.

3.7. Accelerated oxidation by Rancimat

The samples and pure oil were submitted to accelerated oxidation by Rancimat in order to compare the effect of encapsulation, the presence of phenolic compounds in the microcapsules and the position of sinapic in the microcapsules to protect echium oil against oxidation. Table 2 shows the induction time values, values used as oxidative stability index, measured in hours, for all samples and for the pure echium oil.

In comparison to control sample (oil encapsulated without phenolic compound), it was observed that the pure echium oil took about 5 h to oxidize, while the encapsulated oil without phenolic compounds took

approximately 10 h, in other words, the encapsulation of echium oil by complex coacervation offered twice more stability to the oil.

In relation to the oxidative stability of pure echium oil compared to samples in which the oil was encapsulated in the presence of phenolic compound within the microcapsule, it can be stated that the encapsulation process together with the addition of an antioxidant compound (rutin or acid sinapic) also provided twice more stability for the echium oil (Table 2). However, as noted, control sample and the samples with phenolic compounds within the microcapsule had the same induction time, in other words, the addition of phenolic compounds was not more effective than just encapsulation. This can be explained due to the position of the phenolic compounds in the microcapsule, protected and with little contact to the oil, which hindered its action as antioxidant.

However, for the sample in which sinapic acid was applied to the microcapsule wall, it took about 18 h to oxidize, so, the encapsulation of echium oil by complex coacervation with the addition of the phenolic compound in the microcapsule wall offered almost four times more stability for the oil when compared to the pure echium oil and almost twice when compared to the encapsulated oil without antioxidants or with them within the microcapsule. This can be explained as due to sinapic acid position in the microcapsule wall, enhancing its contact with the oil and thus its antioxidant function.

Wang, Adhikari, and Barrow (2014) studied the microencapsulation of tuna oil by complex coacervation using gelatin-sodium hexametaphosphate as wall material and genipin as crosslinking agent and compared the action of nine different antioxidants, added in the tuna oil, by accelerated oxidation (Rancimat). According to these authors, the addition of antioxidant also increased the oxidative stability of tuna oil, obtaining great values of induction time (29.27 ± 1.04 h).

4. Conclusions

With these results, it can be concluded that encapsulation of echium oil by complex coacervation improves its chemical stability. In this case, the use of double emulsion before the coacervation procedure was not as effective as the complex coacervation process with the addition of the phenolic compound in the capsule wall. In other words, the encapsulation of this oil with sinapic acid added directly in the gelatin solution is the most effective sample and offers almost four times more stability for the oil.

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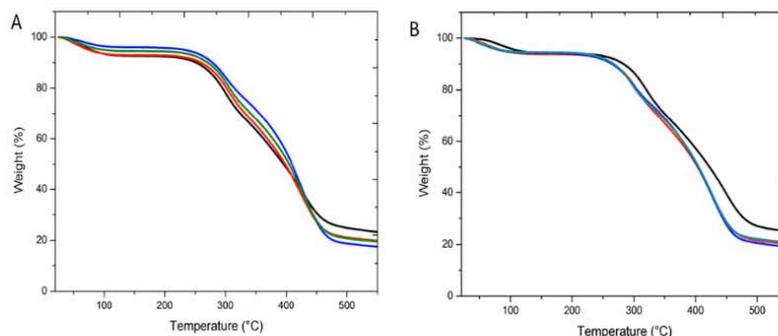


Fig. 5. Thermogravimetric behavior for each dried sample with (A) sinapic acid: samples SIN50 (Black curve), SIN75 (red curve), SIN100 (blue curve) and SIN-GEL (green curve); (B) with rutin: samples RUT50 (black curve), RUT75 (red curve), RUT100 (blue curve) and control (green curve).

Table 3
Concentration and composition of oil and major acids for each sample.

Sample	Oil (g oil/g of capsule)	Palmitic acid C16:0 (mg/g of capsule)	Stearic acid C18:0 (mg/g of capsule)	Oleic acid C18:1 n9 cis (mg/g of capsule)	Elaidic acid C18:1 n9 trans (mg/g of capsule)	Linoleic acid C18:2 n6 (mg/g of capsule)	α -linolenic acid C18:3 n3 (mg/g of capsule)	γ -linolenic acid C18:3 n6 (mg/g of capsule)	Stearidonic acid C18:4 n3 (mg/g of capsule)	Condoic acid C20:1 n9 (mg/g of capsule)
SIN50	0.24	19.71 ± 1.39 ^{bc}	16.89 ± 7.06 ^a	26.44 ± 2.46 ^{cd}	2.48 ± 1.19 ^a	25.80 ± 2.40 ^c	50.13 ± 7.12 ^{cd}	25.63 ± 11.70 ^a	31.05 ± 11.68 ^{ab}	1.90 ± 0.94 ^a
SIN75	0.32	28.22 ± 1.70 ^{ab}	17.55 ± 0.86 ^a	39.63 ± 3.50 ^{ab}	1.54 ± 0.26 ^b	38.75 ± 3.11 ^{ab}	74.23 ± 2.01 ^b	24.64 ± 1.07 ^a	33.09 ± 0.30 ^{ab}	1.78 ± 0.12 ^a
SIN100	0.39	32.43 ± 0.53 ^{ab}	19.05 ± 0.60 ^a	49.83 ± 3.08 ^a	2.05 ± 0.65 ^a	48.27 ± 2.91 ^a	102.50 ± 7.78 ^a	34.55 ± 2.56 ^a	44.61 ± 3.34 ^a	2.39 ± 0.25 ^a
SIN-GEL	0.33	24.67 ± 2.27 ^{abc}	18.72 ± 5.42 ^a	32.54 ± 0.13 ^{abcd}	2.20 ± 1.51 ^a	31.17 ± 0.09 ^{bc}	65.93 ± 0.89 ^{bc}	28.96 ± 9.58 ^a	37.19 ± 12.40 ^{ab}	1.64 ± 1.40 ^a
RUT50	0.24	13.33 ± 2.63 ^c	7.93 ± 1.82 ^a	19.97 ± 3.54 ^d	0.75 ± 0.16 ^b	19.39 ± 4.37 ^c	43.72 ± 1.77 ^d	13.42 ± 2.54 ^a	18.06 ± 3.18 ^b	0.95 ± 0.06 ^a
RUT75	0.32	20.54 ± 2.24 ^{bc}	12.23 ± 1.58 ^a	28.21 ± 1.76 ^{bcd}	0.90 ± 0.03 ^a	28.16 ± 0.84 ^{bc}	59.97 ± 2.01 ^{bcd}	20.03 ± 0.52 ^a	24.61 ± 2.77 ^{ab}	1.21 ± 0.08 ^a
RUT100	0.39	28.82 ± 2.12 ^{ab}	14.12 ± 4.12 ^a	49.60 ± 6.05 ^a	1.50 ± 0.11 ^a	46.52 ± 4.81 ^a	93.09 ± 10.39 ^a	25.29 ± 7.60 ^a	31.15 ± 9.32 ^{ab}	2.03 ± 0.01 ^a
Control	0.33	36.92 ± 9.54 ^a	20.21 ± 2.15 ^a	37.46 ± 6.24 ^{abc}	2.44 ± 1.48 ^a	40.11 ± 6.54 ^{ab}	95.96 ± 0.76 ^a	28.12 ± 4.35 ^a	36.67 ± 5.46 ^{ab}	2.13 ± 0.46 ^a

The analyzes were performed in triplicate. Equal letters in the same column do not differ significantly by the Tukey test at 5% probability. SIN50 and RUT150: sample with a 1:1:0.5 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN100 and RUT100: sample with a 1:1:1 proportion of gelatin, Arabic gum and primary emulsion containing sinapic acid and rutin, respectively. SIN-GEL: sample with a 1:1:0.5 proportion of gelatin, Arabic gum and echium oil with sinapic acid as a cross-linker. Control: sample with a proportion of 1:1:0.5 of gelatin, Arabic gum and echium oil.

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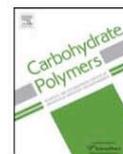
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Effect of different polysaccharides and crosslinkers on echium oil microcapsules



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ABSTRACT

Microencapsulation by complex coacervation using gelatin and arabic gum (AG) as wall materials and transglutaminase for crosslinking is commonly used. However, AG is only produced in a few countries and transglutaminase is expensive. This work aimed to evaluate the encapsulation of echium oil by complex coacervation using gelatin and cashew gum (CG) as wall materials and sinapic acid (S) as crosslinker. Treatments were analyzed in relation to morphology, particle size, circularity, accelerated oxidation and submitted to different stress conditions. Rounded microcapsules were obtained for treatments with AG (45.45 μm) and microcapsules of undefined format were obtained for treatments with CG (22.06 μm). The S incorporation for 12 h improved the oil stability by three fold compared to oil encapsulated without crosslinkers. Treatments with CG and S were resistant to different stress conditions similar to treatments with AG and transglutaminase, making this an alternative for delivery/application of compounds in food products.

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1. Introduction

Echium oil is a plant-based oil which contains 9–16% stearidonic acid, 14% linoleic acid, 10% gamma-linolenic acid and 33% alpha-linolenic acid. It is being considered as an alternative to fish oils, as it presents a unique ratio of omega-3 to omega-6 fatty acids that is not found in any other plant (Berti et al., 2007). However, this oil is very unstable, and is also a hydrophobic material which hampers its application in beverages, for instance. Thus, one alternative to minimize the problems related to echium oil is the microencapsulation process.

The microencapsulation technique can be defined as a process in which one or more materials are surrounded by a membrane, in order to protect against environmental conditions, and also to promote the controlled release of the materials in specific locations and conditions. The complex coacervation encapsulation technique consists of the electrostatic interaction between oppositely charged

macromolecules. Microcapsules are obtained by this interaction, where the formation of a shell around the encapsulated material is possible under specific conditions of pH and temperature (Xiao, Liu, Zhu, Zhou, & Niu, 2014). Many wall material combinations are used in the complex coacervation technique, including casein and pectin (Baracat et al., 2012), gelatin and arabic gum (Comunian et al., 2013; Santos, Bozza, Thomazini, & Favaro-Trindade, 2015), alginate and pea protein isolate (Klemmer, Waldner, Stone, Low, & Nickerson, 2012), pectin and soy protein isolate (Mendanha et al., 2009) and gelatin and chitosan (Prata and Grosso, 2015). The combination of gelatin and arabic gum is the most common and effective one.

Gelatin, a hydrocolloid majorly obtained from the bones and skins of mammals and fish, has amphoteric character and cationic properties at pH below its isoelectric point (IEP) and anionic characteristics at pH values above its IEP, which makes it a great polymer to be used as wall material in the complex coacervation technique (Xiao et al., 2014). On the other hand, arabic gum has some limitations. It is produced in just a few countries (such as Senegal and Sudan), and variations in its quality and composition make its obtainment and standardization difficult. The use of new polysaccharides in the complex coacervation process is important

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to increase the range of choices and, consequently, to decrease the cost of the final product, in order to obtain innovative and excellent products. A new option is the cashew gum, a macromolecule similar to arabic gum due to its thermal and rheological behavior (Mothé and Rao, 2000). It is an exudate from the *Anacardium occidentale* tree and is composed of galactose, arabinose, glucose and rhamnose (Abreu, Oliveira, Paula, & Paula, 2012). Additionally, some studies have shown health benefits such as antitumor and antihypertensive activities (Carestiato, Aguila, & Mothé, 2009). The cashew gum has a negative charge, which makes it a feasible and effective alternative as a wall material in the complex coacervation technique.

Microcapsules obtained by complex coacervation are also known to be fragile under certain conditions. For this reason, specific compounds are used as crosslinkers in order to obtain more resistant structures. The traditional crosslinkers are formaldehyde and glutaraldehyde, which are toxic and prohibited in the food industry. Transglutaminase has also been widely used, however it is an expensive material which limits its application (Peng, Zhao, Huang, Chen, & Zhao, 2014). Furthermore, countries such as Spain do not allow the use of transglutaminase in food products.

The sinapic acid compound, extracted from fruits (lemons, oranges, tangerines, strawberries, blueberries), vegetables (onions, garlic, broccoli), cereal grains (rye, rice and oat) and herbs and spices (borage, thyme, nutmeg) (Niciforovic and Abramovic, 2014), is a phenolic compound with antioxidant functions and has been studied regarding its neuroprotective effect against Alzheimer's disease (Lee et al., 2012) and cardiac hypertrophy and dyslipidemia (Roy and Prince, 2013). Sinapic acid also has possible reactions with proteins and enzymes, which lead to covalent bonds and thereby crosslinking reactions (Rawel and Rohn, 2010). Moreover, the chemistry of hydroxycinnamic acid derivatives concerning polysaccharide–polysaccharide and lignin–polysaccharide crosslinking in grass cell walls (Ralph, Quideau, Grabber, & Hatfield, 1994) and in soluble and insoluble dietary fibers of many cereal grains (Bunzel et al., 2003) seems to be a clue towards understanding the enhanced mechanical properties of microcapsules formed through a coacervation with sinapic acid as a crosslinker. In grasses, it is well-known that ferulic acid is esterified to grass cell wall polysaccharides (arabinoxylans) at the C-5 position of α -L-arabinofuranoside moieties (Hatfield, Ralph, & Grabber, 1999). Dimerization of such polysaccharide-ferulate esters provides a pathway for crosslinking polysaccharide chains (Ralph et al., 1994). Similarly, another hydroxycinnamic acid derivative, sinapates, were also claimed to have a similar role to ferulates in crosslinking polysaccharides in cereal grains and presumably in grass cell walls in general (Bunzel et al., 2003). Thus, the use of sinapic acid as a crosslinker instead of the traditional crosslinkers can be an alternative for achieving more rigid and stable microcapsules.

The current research therefore aimed to compare the use of cashew gum and sinapic acid, instead of arabic gum and transglutaminase, as the polysaccharide-encapsulant and crosslinker, respectively, to encapsulate echium oil by complex coacervation and submit the capsules to different temperatures, pH levels, and salt and sucrose concentrations. To the authors' best knowledge, there are no published studies regarding the use of cashew gum as a wall material and sinapic acid as a crosslinker, making this an innovative idea.

2. Material and methods

2.1. Materials

Echium oil (NEWMega™ Echium Oil, Ref.15200/De Wit Speciality Oils, Tescel, Netherlands) was used as the core. The

encapsulants were gelatin, purchased from Gelnex (Santa Catarina, Brazil), arabic gum (composed by protein (0.99% – w/w), rhamnose (4% – w/w), arabinose (46% – w/w), galactose (38% – w/w) and glucuronic acids (6.5% – w/w), purchased from Nexira (São Paulo/SP, Brazil), and cashew gum (composed by β -D-galactose (72–73% – w/w), α -D-glucose (11–14% – w/w), arabinose (4–6.5% – w/w), rhamnose (3.2–4% – w/w) and glucuronic acids (4.7–6.3% – w/w)) (De Paula, Heatley, & Budd, 1998) which was obtained from EMBRAPA Tropical Agribusiness (Fortaleza/Ceará, Brazil). The crosslinkers used were sinapic acid (Sigma, St. Louis, MO, USA) and transglutaminase (Ajinomoto, São Paulo, Brazil) with an activity of 100 U/g. Lycopene (Lycovit Dispersion 20%) from BASF (Ludwigshafen, Germany) was used to dye the oil.

2.2. Methods

2.2.1. Purification and characterization of cashew gum

For purification of cashew gum, it was dispersed in 96% ethanol and the precipitate was dried at 60 °C for 24 h. An aqueous solution of 6% (w/w) of the obtained powder was prepared and centrifuged at 5000 rpm for 15 min at 20 °C with the equipment Centrifuge 5430R, Eppendorf AG (Hamburg, Germany). The supernatant was filtered to obtain the purified cashew gum solution. This solution was stored in Petri dishes and maintained at 65 °C for 24 h in a kiln (Fanem, Model 315 SE, São Paulo/Brazil) for complete drying and obtaining powder material. The purified cashew gum is referred to throughout the text as cashew gum. The study was adapted from Torquato et al. (2004).

For the characterization of cashew gum, analysis of protein content (Baethgen and Alley, 1989), ash content (AOAC, 2005) and solubility (Cano-Chauca, Stringheta, Ramos, & Cal-Vidal, 2005) were carried out.

2.2.2. Microencapsulation process

2.2.2.1. Complex coacervation.

The microcapsules were produced according to Nori et al. (2011), with some modifications. A concentration of 50% (w/w) of oil in relation to the polymer mass, with 8% (m/m) of lycopene in relation to the oil, was added to a 5% (w/w) gelatin solution and homogenized at 10,000 rpm for 3 min with an Ultraturrax T25 (IKA, Germany), obtaining an oil in water emulsion. Then, in order to perform the complex coacervation, a 5% (w/w) arabic or cashew gum solution was added to the oil in water emulsion under magnetic stirring at 40 °C. The pH was adjusted to 4.0 and the temperature reduced to 10 °C via an ice bath. After preliminary tests, the proportions of gelatin:arabic gum and gelatin:cashew gum were fixed at 1:1 and 1:2.5, respectively.

2.2.2.2. Crosslinking.

Sinapic acid (0.05 g/g of gelatin) was added in two different ways: (1) after preparing the simple oil in water emulsion (oil in gelatin solution) and before the addition of the arabic gum solution, sinapic acid was added while using a magnetic stirrer over 1 min at 40 °C; (2) after the complex coacervation process, following the pH adjustment and temperature reduction, sinapic acid was added to the solution with the microcapsules and maintained under low magnetic stirring for 12 h at 15 °C in the BOD TE-391 incubator (Tecnal/Piracicaba, São Paulo – Brazil).

For the crosslinking with transglutaminase, a 100 mL aqueous solution was prepared containing the enzyme at a concentration of 15 U/g of gelatin and a pH of 6.0. The solution was added to the microcapsules after the coacervation and maintained under low magnetic stirring for 12 h at 15 °C in the BOD incubator.

In addition, the control treatments were prepared, making up ten treatments in total (Table 1 and Fig. 1).

2.2.2.3. Drying processes of microcapsules.

The coacervates were stored for 24 h at 7 °C to allow the microcapsule precipitate to set-

Table 1

Composition of each treatment, particle size and circularity values for each treatment in the wet form and accelerated oxidation index for the freeze-dried ones.

Treatments	Wall material	Crosslinking agent	Incorporation of crosslinkers	Average particle size (μm)	Circularity	Accelerated oxidation index (hours)
GASb	Gelatin-arabic gum	Sinapic acid	Before arabic gum*	48.72 ± 16.09^a	0.88 ± 0.14^a	20.99 ± 2.87^b
GASa	Gelatin-arabic gum	Sinapic acid	After complex coacervation**	46.18 ± 13.41^a	0.92 ± 0.11^a	26.50 ± 1.60^a
GCSb	Gelatin-cashew gum	Sinapic acid	Before arabic gum*	24.21 ± 9.53^b	–	19.88 ± 2.99^b
GCSa	Gelatin-cashew gum	Sinapic acid	After complex coacervation**	21.45 ± 5.43^b	–	20.15 ± 3.50^b
GAT	Gelatin-arabic gum	Transglutaminase	After complex coacervation**	44.87 ± 14.37^a	0.88 ± 0.14^a	9.86 ± 0.63^c
GCT	Gelatin-cashew gum	Transglutaminase	After complex coacervation**	20.53 ± 4.83^b	–	9.48 ± 0.06^c
GAO***	Gelatin-arabic gum	–	–	45.49 ± 19.18^a	0.87 ± 0.15^a	9.39 ± 0.06^c
GCO****	Gelatin-cashew gum	–	–	21.89 ± 4.77^b	–	8.73 ± 0.04^d
GC****	Gelatin-cashew gum	–	–	–	–	–
GA****	Gelatin-arabic gum	–	–	42.06 ± 13.19^a	0.90 ± 0.14^a	–
Echium oil	–	–	–	–	–	5.10 ± 0.09^e

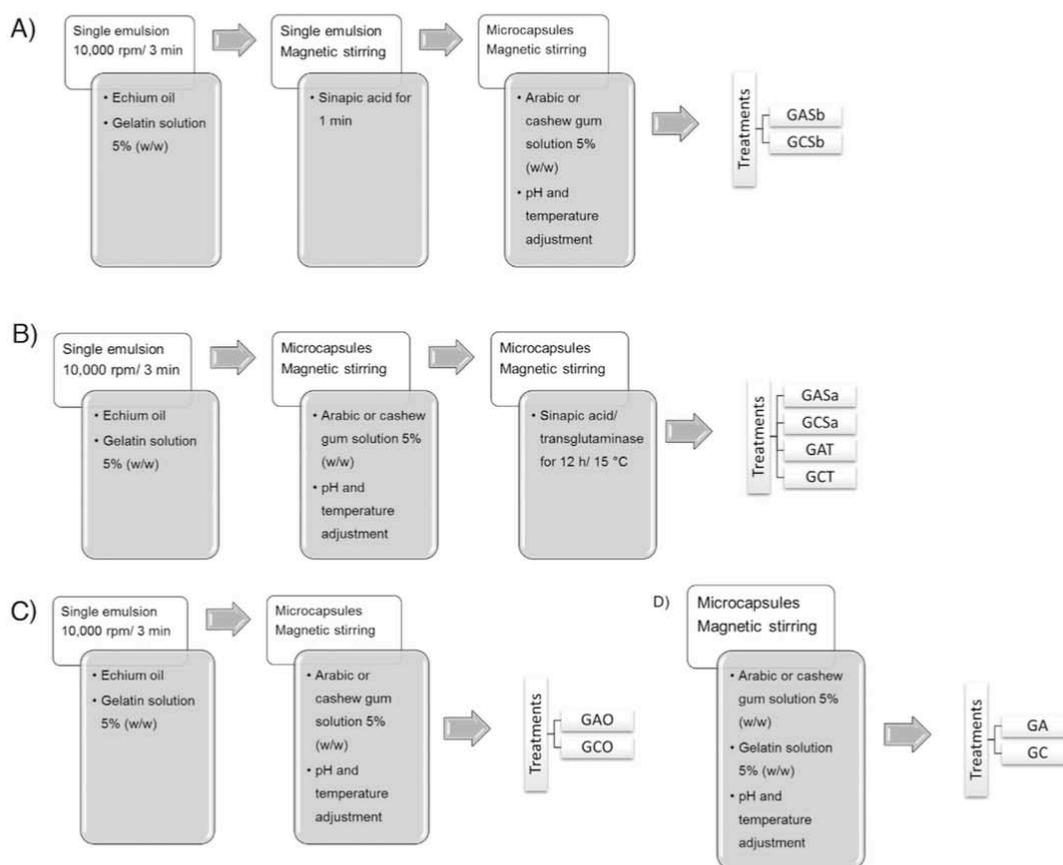
Equal letters in the same column do not differ at 5% of significance by Tukey test.

* Stirring for 1 min.

** Stirring for 12 h.

*** Control treatments: with wall materials and echium oil without crosslinkers.

**** With wall materials only.

**Fig. 1.** Diagram about the process of complex coacervation and crosslinking for treatments (A) GASb and GCSb; (B) GASa, GCSa, GAT and GCT; (C) GAO and GCO; and (D) GA and GC.

tle. The supernatant (water) was removed with a dispenser. Then, the samples were frozen and lyophilized (Freeze drier Terroni, São Carlos, SP – Brazil). For comparison, the coacervates were also atomized using a spray dryer, model LM MSD 1.0 (Labmaq, Ribeirão Preto/SP, Brazil), with a heating air flow of 2.50 m/s, inlet air temperature of 150 °C, feed flow rate of 0.6 L/h and a nozzle diameter

of 1.2 mm. In this case, the capsules were suspended in water and atomized right after the encapsulation and crosslinking process.

2.2.3. Microcapsule characterization

2.2.3.1. Morphology. The morphology of wet microcapsules was analyzed by optical microscopy (BIO3 equipment – Bel Photonics,

Italy), and dehydrated microcapsules were analyzed by scanning electron microscopy (SEM) using the Tabletop Microscope TM 3000 (Hitachi, Tokyo, Japan).

2.2.3.2. Particle size and circularity. To obtain the particle size, 100 microcapsules per treatment were individually measured using the ImageJ software. The BIO3 microscope (Bel Photonics, Italy) was used to obtain the images. To calculate the circularity of the microcapsule, the smallest and the biggest diameters of 100 capsules were measured individually for each treatment. These measurements were made with wet microcapsules.

2.2.3.3. Accelerated oxidation by Rancimat. Accelerated oxidation analysis for the encapsulated oil was performed with the Rancimat equipment (model 873, Metrohm, Switzerland). Dried capsule samples (1.5 g) were submitted to heating under a purified air flow rate of 20 L/h at 90 °C. The induction times (hours) of the samples were used as the oxidative stability index (Comunian et al., 2016).

2.2.4. Stress conditions submitted

The analyses of stress conditions were performed according to Chung and McClements (2015), with modifications. The wet coacervates were submitted to different stress conditions, such as different temperatures (2, 10, 20, 30, 40, 50, 60, 70 and 80 °C) during 5 min, pH values (2, 3, 4, 5, 6, 7 and 8), NaCl solutions (1, 3 and 5%) (w/w) and sucrose solutions (1, 10 and 20%) (w/w). To evaluate different temperatures, the wet coacervates were dispersed in a beaker containing 50 mL of an aqueous solution at 2 °C which was maintained on a heater plate under constant stirring, while controlling the increase of temperature up to 80 °C with a thermometer. To test different pH values, the wet coacervates were dispersed in an aqueous solution, the pH was adjusted with a solution of NaOH 0.1 M or H₃PO₄ 0.1 M and the mixtures were stirred for 2 min at room temperature. To test different concentrations of NaCl and sucrose, the coacervates were again dispersed in aqueous solutions with the respective concentrations, and the mixtures were stirred for 2 min at room temperature. After being submitted to each one of these conditions, the microcapsules structures were evaluated using a BIO3 optical microscope (Bel Photonics, Italy).

2.2.5. Statistical analysis

Data were statistically analyzed using SAS statistical software (Statistic Analysis System), version 9.2, by ANOVA and the Tukey's test at 5%.

3. Results and discussion

3.1. Characterization of cashew gum

In order to characterize the cashew gum, analyzes of protein, ash and solubility were performed, obtaining values of $0.26 \pm 0.03\%$, $0.6 \pm 0.09\%$ and $71.11 \pm 0.08\%$, respectively. Even the cashew gum presenting similar composition to gum arabic, its content of protein, ash and the solubility are different, obtaining approximated values of 2%, 3.39% and 50%, respectively (BeMiller and Huber, 2010; Weinbreck, Vries, Schrooyen, & Kruijff, 2003). This can be considered a small difference, however enough to influence on the structure of the capsules, as it will be shown following.

3.2. Morphology by optical and scanning electron microscopy

The complex coacervation process using gelatin and arabic gum as wall materials is known for producing multinucleated microcapsules with a rounded shape (Comunian et al., 2016). Furthermore, as these wall materials are commonly used, researchers have sought

new polymers for application in the food, cosmetic and pharmaceutical industries, ensuring the substitution of various common materials while also offering the same functionality.

The cashew gum, as already mentioned, has physicochemical characteristics ideal for replacing arabic gum. However, the microcapsules with the gelatin-cashew gum combination showed a different morphology from those obtained with gelatin-arabic gum. As shown in Fig. 2, the interaction between the gelatin and cashew gums resulted in a complex with an undefined format; however, the oil droplets were completely covered by the polymers, which certainly ensures their protection, although the flow of the powder was reduced compared to powders composed of structures with a rounded shape. Furthermore, complexes formed with gelatin-cashew gum were smaller than those formed with gelatin-arabic gum (Table 1), which may be advantageous for some applications since smaller particles have less of an impact on food texture.

Coacervates are dried in order to facilitate their handling and storage as well as to obtain more stable materials. The coacervates were submitted to two different drying methods: freeze drying and spray drying. It was observed that the lyophilized microcapsules presented links by "bridges", a typical characteristic of particles dried by lyophilization. There were no morphological differences between treatments with the same wall material; in other words, different crosslinkers and different ways of incorporating the sinapic acid did not influence the external structure of the microcapsules. However, morphological differences between freeze-dried treatments with different wall materials (gelatin-arabic gum and gelatin-cashew gum) were observed, since treatments with cashew gum showed higher linking by "bridges" than treatments with arabic gum. Furthermore, there was a certain amount of polymer that did not interact to form the microcapsule wall with the gelatin-cashew gum combination (Fig. 2d).

The atomized microcapsules showed a rounded shape, characteristic of dried materials produced by this process. However, it was observed that they were broken within the atomizer, probably due to the pressure exerted by the compressed air and the high temperature. The average particle size of these capsules after the atomizing process was much lower ($9.76 \pm 2.17 \mu\text{m}$ for treatments with arabic gum and $7.55 \pm 1.64 \mu\text{m}$ for treatments with cashew gum) than that obtained before drying, which proves they had broken (Fig. 2). Furthermore, in both drying processes the particles were agglomerated, which is necessary to facilitate the wettability and dissolution of the powders and also to prevent dust.

The microcapsules dried by both methods were rehydrated and analyzed by optical microscopy. It was observed that drying by lyophilization preserved the original format of the microcapsule after rehydration; however, the same did not happen for the samples from the atomization technique, which formed large agglomerates when dried and lost their initial form when rehydrated (Fig. 2i and j). This result can be explained by the conditions of each drying process, such as low temperature and a vacuum for lyophilization, and high temperature and pressure for atomization.

Regarding the application of crosslinkers, the presence of sinapic acid versus transglutaminase, in addition to the different methods of sinapic acid incorporation, did not affect the morphology of the microcapsules.

In a similar study, Alvim and Grosso (2010) studied the influence of the type of crosslinking and drying process on the release of paprika oleoresin encapsulated by complex coacervation using gelatin and arabic gum as wall materials. These authors also obtained microcapsules with a rounded shape that maintained integrity after the lyophilization process. Samples without the crosslinker were destroyed during the spray drying, however they showed partial resistance with the addition of transglutaminase or glutaraldehyde, which did not happen in this work. These different results can be explained by the different conditions used during the

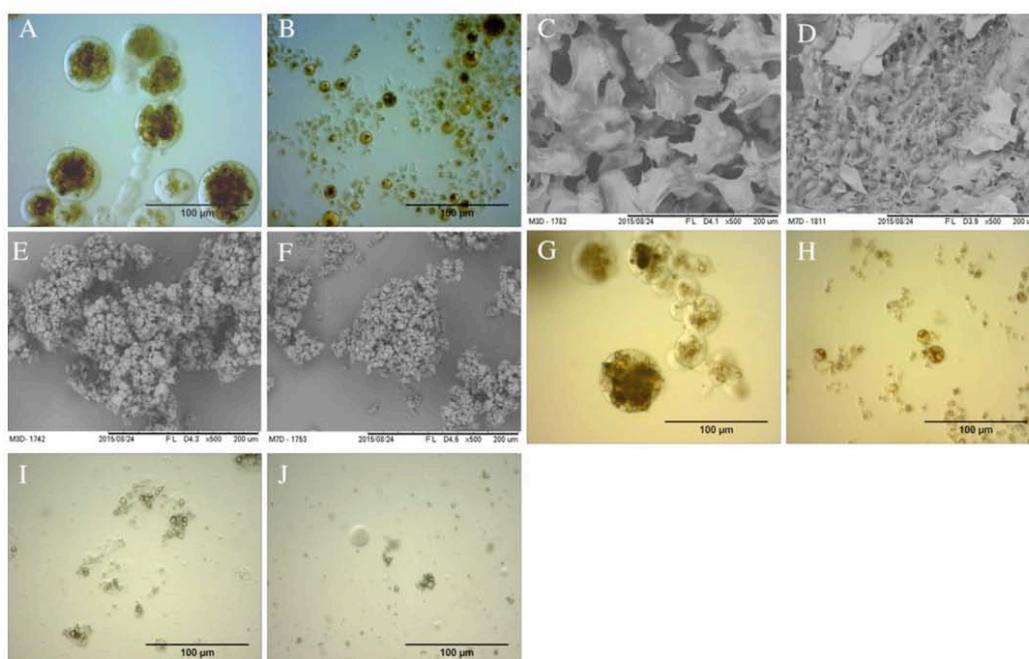


Fig. 2. Optical microscopy of (A) treatment GASb (gelatin-arabic gum as wall materials and sinapic acid added before arabic gum), (B) treatment GCSa (gelatin-cashew gum as wall materials and sinapic acid added after complex coacervation); scanning electron microscopy of (C) treatment GASa (gelatin-arabic gum as wall materials and sinapic acid added after complex coacervation) freeze-dried, (D) treatment GCSa (gelatin-cashew gum as wall materials and sinapic acid added after complex coacervation) freeze-dried, (E) treatment GASa atomized, (F) treatment GCSa atomized; optical microscopy of (G) treatment GASa freeze-dried and rehydrated and (H) treatment GCSa freeze-dried and rehydrated; (I) treatment GASa spray-dried and rehydrated and (J) treatment GCSa spray-dried and rehydrated.

encapsulation procedure, such as concentration of the crosslinker, temperature, time of reaction and spray dryer conditions.

Thus, according to the results presented, lyophilized microcapsules were chosen for the continuation of this research.

3.3. Average particle size and circularity

The particle sizes ranged from 20.53 to 48.72 μm , showing significant differences between treatments (Table 1). The use of cashew gum as a wall material, crosslinked with sinapic acid or transglutaminase, gave an average particle size that was twice as small as that obtained for the treatments with only arabic gum. This smaller particle size was considered a positive result, as it will have less of an effect on food texture. The treatment CG showed an undefined complex, which was impossible to measure. The treatments GCSb, GCSa and GCT also showed an undefined format, but they were possible to measure using the arithmetic average of three different diameters. The application of different compounds as crosslinkers and the different methods of sinapic acid incorporation did not influence the mean size. No research using cashew gum as wall materials for complex coacervation technique was found in the literature. However, this carbohydrate has been used as wall material for ionic gelation technique, obtaining particles size in the range from 600 to 800 μm (Das, Dutta, Nayak, & Nanda, 2014), approximately 20 times larger than the size obtained for the echium oil capsules.

The circularity values are used to represent the roundness of the microcapsule: the closer to 1, the more rounded the capsule. Rounder capsules are better for application in food due to their easy flow. Treatments with arabic gum showed capsules of a rounded

shape, within the range of 0.88–0.92. Treatments with cashew gum formed a complex with an undefined shape, which made measurement of the circularity impossible. Moreover, the different applications of sinapic acid did not significantly influence the circularity. Comunian et al. (2016) studied the protection of Echium oil by microencapsulation with phenolic compounds using complex coacervation technique and gelatin and gum Arabic as wall materials. These authors also obtained circularity in the range of 0.86–0.92, similar to obtained in this work.

3.4. Accelerated oxidation by Rancimat

The values for the oxidative stability index (OSI), measured in hours, are presented in Table 1, and the oxidation behaviors of encapsulated and free oils are presented in Fig. 3. The control treatments GAO and GCO presented values of 9.39 ± 0.06 and 8.73 ± 0.04 h, respectively, showing that different wall materials did not have a significant influence on the OSI. The treatments GASb and GAsa showed values of 20.99 ± 2.8 h and 26.50 ± 1.60 h, respectively. In other words, the application of sinapic acid increased the oxidative stability of encapsulated oil. The treatment in which sinapic acid was incorporated for 1 min and before the arabic gum (GASb) had double the stability, whereas the treatment in which the phenolic compound was incorporated for 12 h (GAsa) increased stability by almost three fold.

Treatments GCSb and GCSa presented OSI values of 19.88 ± 2.99 h and 20.15 ± 3.50 h, respectively. Therefore, the addition of this antioxidant doubled the oxidative stability of the encapsulated oil compared to treatment with only the wall material; moreover, the different incorporations of sinapic acid in

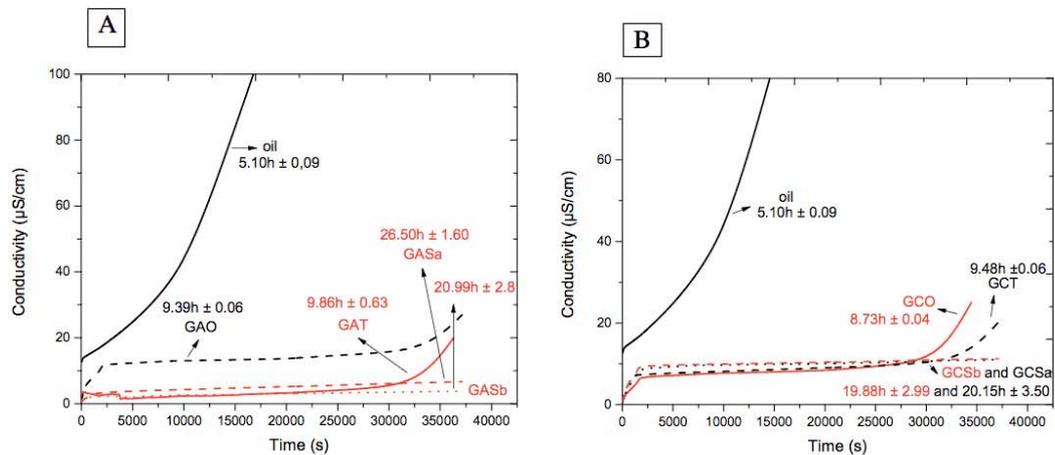


Fig. 3. Accelerated oxidation behavior of microcapsules by Rancimat: (A) treatments with gelatin-arabic gum as wall material; (B) treatments with gelatin-cashew gum as wall material.

the microcapsules with gelatin-cashew gum did not influence the OSI of the oil, as seen in Table 1.

When the transglutaminase enzyme was used as the crosslinker, values of 9.86 ± 0.63 h and 9.48 ± 0.06 h were obtained with GAT and GCT treatments, respectively. As noted, treatment with transglutaminase and control treatments (encapsulated oil with only wall material – GAO and GCO) showed the same OSI; in other words, the addition of transglutaminase as the crosslinker was not more effective than the encapsulation.

Wang, Adhikari, and Barrow (2014) studied the optimization of microencapsulation of tuna oil in gelatin-sodium hexametaphosphate using complex coacervation and observed that the accelerated stability analyzed by Rancimat showed that encapsulated oil stability was more than double that of non-encapsulated oil. In a recent study, Mohammadi, Jafari, Esfanjani, and Akhavan (2016) evaluated the application of nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil using whey protein concentrate and obtained the maximum value of 12 h for Rancimat induction period for the samples, while the material un-encapsulated oxidized in 8 h. Comparing to the results obtained for echium microcapsules, The OSI for the encapsulated echium oil in the treatment GASa was more than five times that of pure echium oil. In other words, the encapsulation method used for echium oil with sinapic acid adequately protected the oil.

3.5. Effect of stress conditions on microcapsules stability

It is known that microcapsules obtained by complex coacervation can be destroyed under certain conditions, which compromises their application in food products. For this reason, the stability of microcapsules produced was studied under certain stress conditions found in food formulations or during food processing, such as different temperatures, pH values, and salt and sucrose concentrations. The rehydrated microcapsules were maintained at different pH values (from 2 to 8), different temperatures (from 2 to 80°C), different concentrations of salt solutions (from 1 to 5% – w/w) and different concentrations of sucrose solutions (from 1 to 20% – w/w) in order to compare the stability of gelatin-arabic gum and gelatin-cashew gum combinations, in addition to the crosslinking agents and different ways of sinapic acid incorporation.

3.5.1. Effect of pH on microcapsules stability

As already mentioned, the complex coacervation process involves interactions that depend on specific pH values. These forces of attraction (called ionic bonds, or electrostatic bonds) are very weak, and they can be changed or eliminated by changes in pH.

Fig. 4 demonstrates that microcapsule resistance was strongly influenced by pH. A low pH value (2.0) was enough to break the microcapsules, except with GASa, GASb and GAT treatments, showing that the wall formed with the gelatin-cashew gum combination was weaker than that of the gelatin-arabic gum combination. Moreover, treatments with a crosslinking agent and the gelatin-arabic gum combination were more resistant than the GCO and GAO treatments (composed of the wall material and oil), proving that sinapic acid has similar crosslinking action to transglutaminase. The same happened at a high pH (8.0); therefore, sinapic acid and transglutaminase mainly acted on extreme pH values. The other pH values (from 3 to 7) did not cause breakage of the microcapsules, while extreme pH values made the capsule wall more fragile, although it remained intact. At pH 4.0, the value used to promote the complex coacervation, the microcapsules were more isolated for treatment GCSb and more agglomerated for treatment GCO. The difference can be explained by the use of sinapic acid as a crosslinker.

The comparison of the crosslinking action of sinapic acid and transglutaminase shows that the two compounds behaved similarly regarding the stability of the material at different pH values. This showed that transglutaminase can be substituted for sinapic acid. Moreover, different incorporations of sinapic acid did not influence the final result.

There are currently no published studies regarding the influence of pH on stability of coacervate microcapsules. However, particles using biopolymers as wall materials have been evaluated by Chung and McClements (2015) who studied the microstructure and physical properties of biopolymer hydrogel particles through modulation of electrostatic interactions. These authors studied the use of sodium caseinate and pectin in the fabrication of particles and compared the pH values within the 3–8 range. They observed that the system was stable at pH values ranging from 3 to 5, while only a few hydrogel particles were visible from pH 6–8. Thus, it is possible to infer that microcapsules produced with gelatin-arabic gum and gelatin-cashew gum crosslinked with sinapic acid are resistant

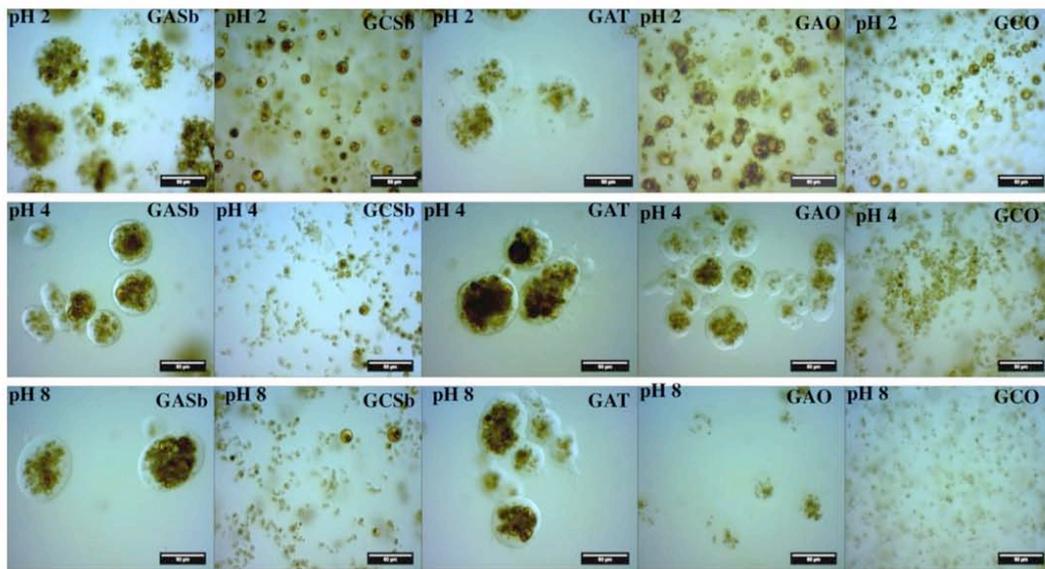


Fig. 4. Optical microscopy of the treatments maintained in solution of pH 2, 4 and 8.

over a wider pH range than the hydrogel particles produced with pectin and sodium caseinate.

3.5.2. Effect of temperature on microcapsules stability

Heating causes agitation among the molecules, so it can destabilize the complex protein-polysaccharide. For this reason, the treatments were subjected to different temperatures (2, 10, 20, 30, 40, 50, 60, 70 and 80 °C).

The walls of the GCO and GAO treatments were destroyed at a temperature of 40 °C, whereas microcapsules containing sinapic acid and transglutaminase remained intact at higher temperatures (up to 80 °C), demonstrating again the crosslinking action of sinapic acid.

The comparison of different wall material combinations showed that the microcapsules prepared with gelatin-cashew gum presented, as already noted, an undefined format at low temperatures. However, temperatures of 40 °C led to the formation of rounded shape microparticles. Fig. 5 shows that the wall materials were not surrounding the core as in the treatments with gelatin-arabic gum, but were instead dispersed in the echium oil. This structure was stable up to 80 °C, at which point the release of oil droplets was observed (Fig. 5). It is important to mention that this rounded structure is more feasible for application in food due to better flow, as already mentioned.

There was no difference between the sinapic acid and transglutaminase treatments, as both kept the capsules intact throughout the temperature range examined. Therefore, this type of microcapsule can be used in products that undergo heat treatment. Moreover, different methods of sinapic acid incorporation did not influence the final result; in other words, it may be incorporated as the crosslinker without affecting the microcapsule structure.

3.5.3. Effect of ionic strength on microcapsules stability

It is known that salt concentration is strongly linked to ionic strength, making it a decisive factor in the stability of the microcapsule. Thus, as seen in Fig. 6, three different concentrations of

salt solutions (NaCl) were tested (1, 3 and 5%) (w/w) and then the resistance of the microcapsules was observed.

All treatments prepared with gelatin-arabic gum were resistant at all NaCl concentrations evaluated. Thus, it can be inferred that different salt concentrations do not affect the strength of microcapsules with gelatin-arabic gum as wall materials. This allows for the application of this microcapsule in various food products, despite the fact that 3 and 5% (w/w) salt solutions are high salt concentrations that are hardly found in foods.

However, free oil droplets were observed in treatments with gelatin-cashew gum in the 3 and 5% (w/w) salt solutions. Furthermore, the crosslinking action did not make the particle more resistant in this case.

In addition to pH and temperature influence, there are no studies in the literature regarding the influence of ionic strength on the stability of coacervate microcapsules. Chung and McClements (2015) studied the microstructure and physical properties of biopolymer hydrogel particles through modulation of electrostatic interactions, evaluated the use of sodium caseinate and pectin in the fabrication of the particles and compared treatments with different sodium chloride concentrations within the range of 0–400 mM. They observed that the sodium caseinate-pectin system containing no salt produced small particles, whereas the size of the particles increased with an increase in salt content. The opposite happened to the microcapsules with the gelatin-cashew gum combinations; in other words, the capsules ruptured with an increase in salt concentration, whereas the microcapsules with gelatin-arabic gum remained their original size and format.

3.5.4. Effect of sucrose on microcapsules stability

Different sucrose concentrations were also analyzed (1, 10 and 20%) (w/w), since small carbohydrate molecules are capable of changing the hydration level of proteins and polysaccharides. Thus, sucrose could possibly influence the structure, and therefore the stability, of the microcapsules produced with this kind of material. However, there were no changes in the structures produced with

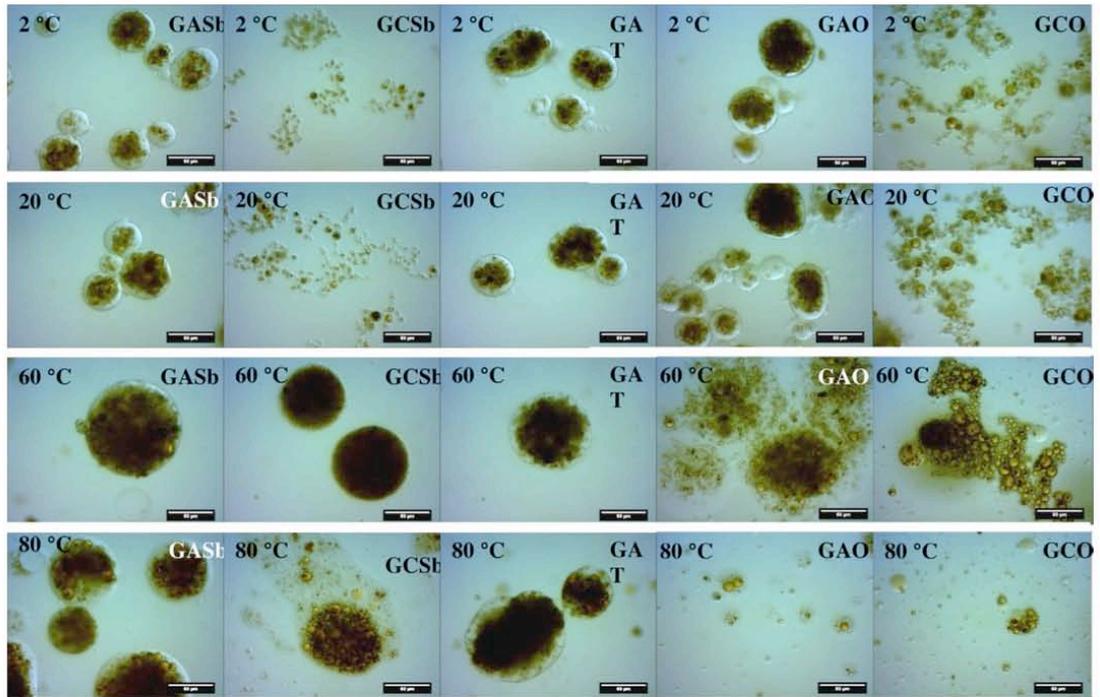


Fig. 5. Optical microscopy of the treatments submitted to temperatures of 2, 20, 60 and 80 °C.

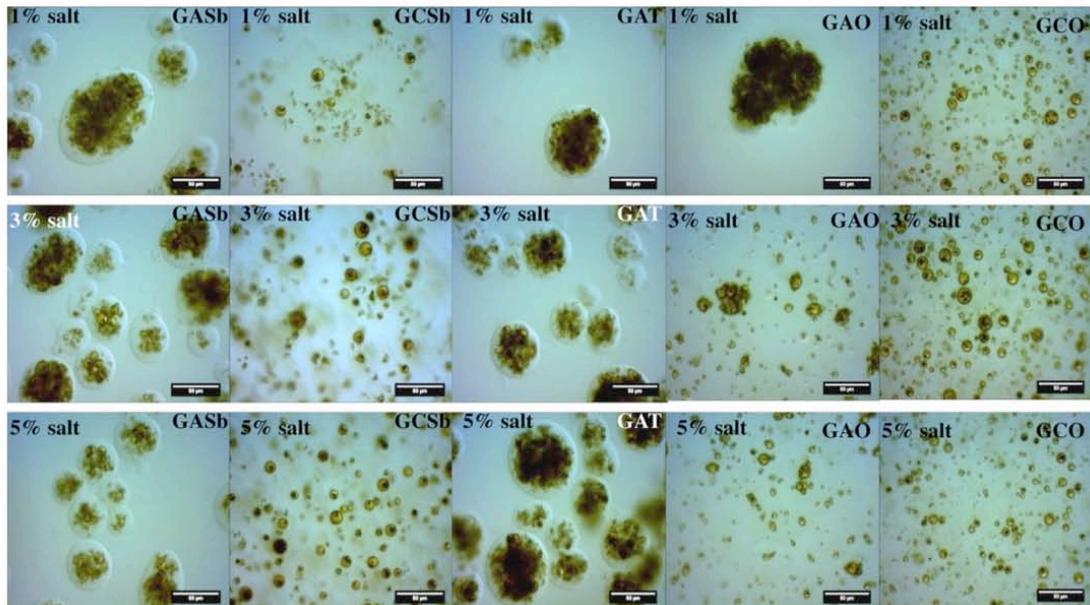


Fig. 6. Optical microscopy of the treatments in solutions of 1, 3 and 5% (w/w) of salt.

any of the sucrose concentrations used, showing that sucrose did not influence microcapsule resistance or stability. According to this result, the microcapsules can be applied to products with different sucrose contents while ensuring their integrity.

The results obtained regarding microcapsule stability under different stress conditions supports the hypothesis that gelatin-arabic gum microcapsules crosslinked with sinapic acid can be applied in a huge range of foods while maintaining their stability.

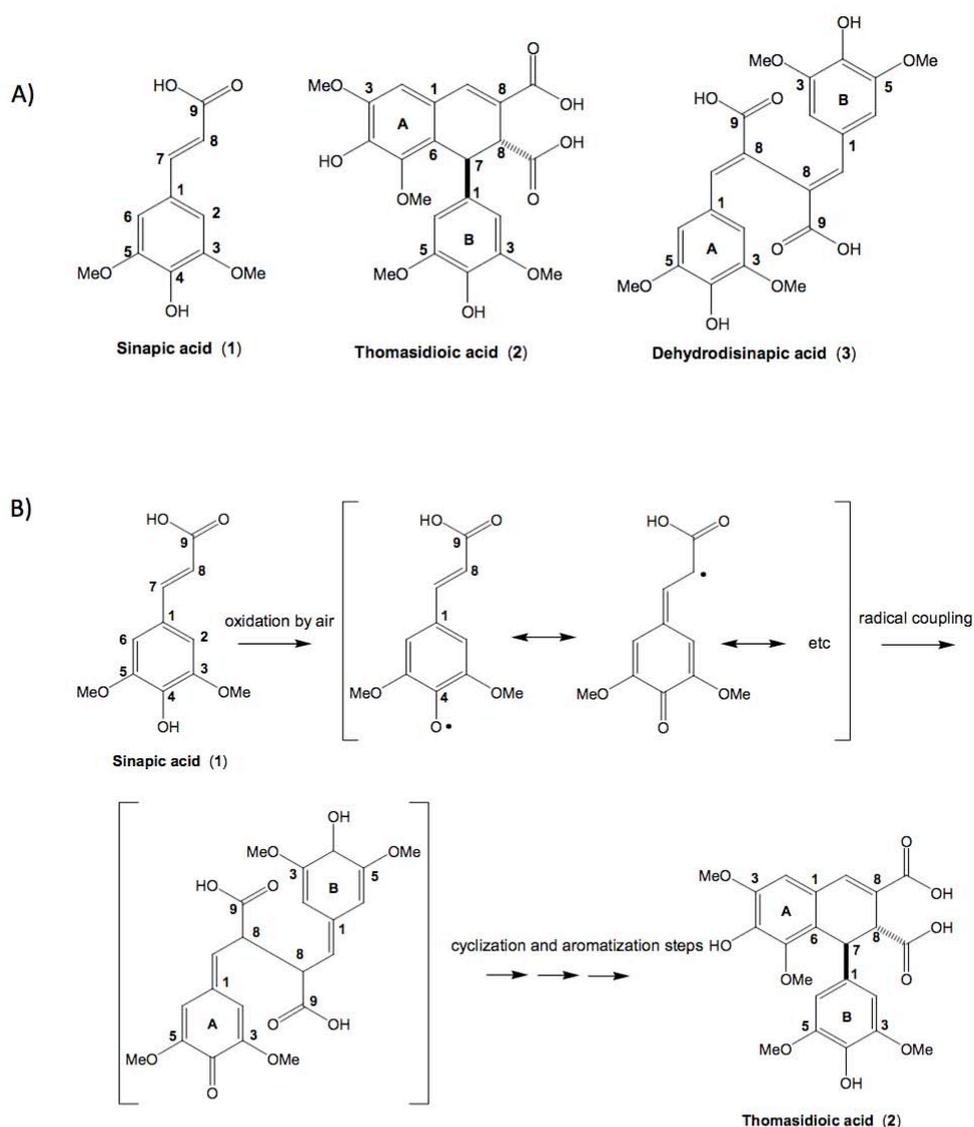


Fig. 7. (A) Sinapic acid (1) and dehydrosinapic acid derivatives thomasidioic acid (2) and compound 3; (B) Oxidative radical coupling dehydrodimerization of sinapic acid to form thomasidioic acid. Adapted from [Bunzel et al. \(2003\)](#).

3.6. Crosslinking process of sinapic acid

Sinapic acid was used as a crosslinker in this work for different reasons. First of all, [Bunzel et al. \(2003\)](#) reported that two sinapic acid compounds, (1) 8-8-coupled dehydromers, compound 3 (the noncyclic isomer) and (2) lignan thomasidioic acid (the cyclic isomer), were recovered from basic hydrolysis of both soluble and insoluble dietary fibers of wild rice ([Fig. 7A](#)). Chemically, oxidative radical coupling dehydrodimerization of sinapic acid can afford two primary products, 8-O-4- and 8-8-dehydromers, only. However the former was not detected at any significant level in cereal grain dietary fibers evaluated by [Bunzel et al. \(2003\)](#). On the other hand, air oxidation of sinapic acid yields thomasidioic acid as the sole

product 100% of the time at pH 8.5 and 30% of the time at neutral pH. Air oxidation of sinapic acid to thomasidioic acid was also described by [Charlton and Lee \(1997\)](#) and [Bunzel et al. \(2003\)](#).

Since sinapic acid 8-8-coupled dehydromers are a naturally occurring pathway for crosslinking polysaccharide chains and that sinapic acid itself is promptly oxidized by air to afford medium to high yields of its dehydromer thomasidioic acid through an oxidative radical coupling reaction ([Fig. 7B](#)), it can be hypothesized that a similar event happens to some extent when cashew gum and/or arabic gum is exposed to sinapic acid or its dehydromer thomasidioic acid.

Therefore, regarding the encapsulation process, it seems likely that the resulting polysaccharide-polysaccharide crosslinkages

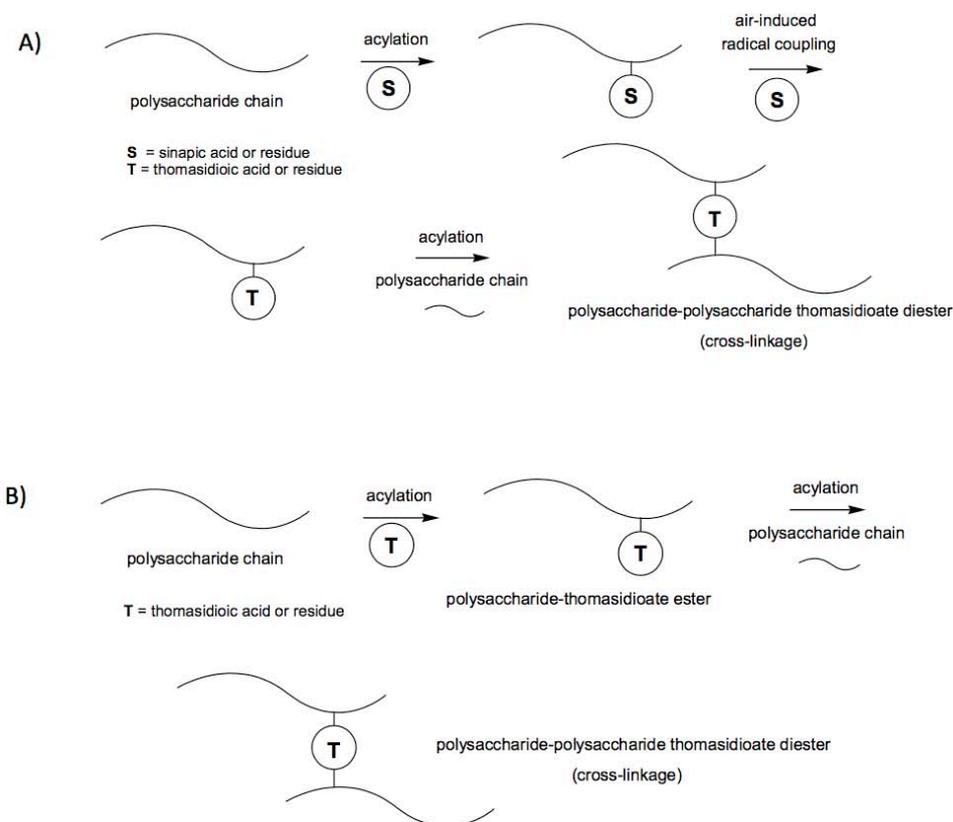


Fig. 8. (A) Proposed polysaccharide-polysaccharide crosslinkage through a polysaccharide-sinapate ester air-induced oxidative radical coupling dehydrodimerization with a free sinapic acid; (B) Proposed polysaccharide-polysaccharide crosslinkage through a polysaccharide-thomasidioate ester.

through a thomasidioic acid moiety would be the major influence for the enhanced mechanical properties of microcapsules formed through the complex coacervation technique with sinapic acid as a crosslinker. The main constraint of this approach is the previously required polysaccharide acylation by free sinapic acid and/or thomasidioic acid without prior acyl group activation by added reagents or catalysts. Even so, given that the whole complex coacervation process was performed under slightly acidic conditions, two possible pathways for crosslinking the cashew gum and/or arabic gum polysaccharides mediated by sinapic and/or thomasidioic acid were envisaged.

The first pathway is based on prior acylation of a polysaccharide chain by free sinapic acid followed by further radical coupling dehydrodimerization and ultimately acylation of another polysaccharide chain (Fig. 8A) as follows:

- Attachment of sinapic acid to a polysaccharide chain through a Fischer esterification-type reaction of the acid's acyl group and an OH group from a suitable sugar residue, under acidic conditions.
- Air-induced oxidative radical coupling dehydrodimerization with free sinapic acid.
- Attachment of the polysaccharide-bound sinapic acid dehydrodimer to another polysaccharide chain through a Fischer esterification-type reaction of the dehydrodimer's acyl group

and an OH group from a suitable sugar residue, under acidic conditions.

The second proposed pathway for polysaccharide-polysaccharide crosslinkage requires previous formation of thomasidioic acid from air-induced oxidative radical coupling dehydrodimerization of free sinapic acid prior to the later polysaccharide chain acylation (Fig. 8B).

4. Conclusions

In this work, microcapsules of echium oil were successfully prepared by a complex coacervation technique using combinations of gelatin-arabic gum and gelatin-cashew gum as wall materials and with the crosslinkers sinapic acid and transglutaminase. Cashew gum can be used as wall material since it protected the oil (treatments provided oxidative stability four times greater than pure oil). Moreover, sinapic acid, besides being a powerful antioxidant, can also be used as a crosslinker instead of toxic and expensive ones. Gelatin-arabic gum microcapsules crosslinked with sinapic acid for 12 h showed better oil oxidation stability (almost three times higher than the non-crosslinked gelatin-arabic gum microcapsules), and all microcapsules exhibited optimal morphologic properties and appropriate average particle size. The crosslinked treatments showed greater resistance to different stress conditions than the treatments with just wall material and oil, thereby con-

firming that these new vehicles of bioactive compounds can be used in food products.

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ATTACHMENT D – PAPER PUBLISHED IN FOOD CHEMISTRY

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Improving oxidative stability of echium oil emulsions fabricated by Microfluidics: Effect of ionic gelation and phenolic compounds



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ABSTRACT

Echium oil is rich in omega-3 fatty acids, which are important because of their benefits to human health; it is, however, unstable. The objective of this work was the coencapsulation of echium oil and quercetin or sinapic acid by microfluidic and ionic gelation techniques. The treatments were analyzed utilizing optical and scanning electron microscopy, encapsulation yield, particle size, thermogravimetry, Fourier transform infrared spectroscopy, stability under stress conditions, and oil oxidative/phenolic compound stability for 30 days at 40 °C. High encapsulation yield values were obtained (91–97% and 77–90% for the phenolic compounds and oil) and the encapsulated oil was almost seven times more stable than the non-encapsulated oil (0.34 vs 2.42 mg MDA/kg oil for encapsulated and non-encapsulated oil, respectively). Encapsulation was shown to promote oxidative stability, allowing new vehicles for the application of these compounds in food without the use of solvents and high temperature.

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1. Introduction

Omega-3 fatty acids (ω -3) are widely used in food and pharmaceutical products due to their benefits to human health (Ghorbanzade, Jafari, Akhavan, & Hadavi, 2017). A source of ω -3 is the echium seed oil (*Echium plantagineum* L.), which contains from 9 to 16% stearidonic fatty acid, an intermediate in the biosynthesis of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), also important for human nutrition (Berti et al., 2007; Clough, 1993; Payne, Lad, Foster, Kholá, & Gray, 2014). Echium oil also has a ratio of omega-3 to omega-6 ideal for health, which is not found in any another type of oil (Berti et al., 2007). The use of this oil in the food industry is hampered due to the instability of its unsaturated fatty acids when they come into contact with light, oxygen and heat. Two strategies that could minimize these limitations, singly or together, are: (1) adding compounds with antioxidant activity—such as phenolic compounds—or (2) microencapsulation.

Phenolic compounds have been of great interest in the food industry due to their activity as antioxidant agents (Abdallah, Salama, Abd-Elrahman, & El-Maraghy, 2011; Lee et al., 2013), in

addition to their other health benefits, such as the prevention of cancer, inflammation and neurodegenerative diseases (Esfanjani & Jafari, 2016). Sinapic acid is the major phenolic acid of canola (Rawel & Rohn, 2010) and has been studied for its neuroprotective effects against Alzheimer's disease (Lee et al., 2012), cardiac hypertrophy and dyslipidemia (Pari & Jalaludeen, 2011; Roy & Prince, 2013). Quercetin, another phenolic compound, is one of the most common flavonoids present in nature and has exhibited anti-stress, anti-inflammatory and anti-cancer properties (Waterhouse, Wang, & Sun-Waterhouse, 2014).

Microencapsulation consists of a broad class of techniques in which one or more bioactive materials are contained or immobilized by one or more polymers or lipids, protecting the encapsulated material against environmental conditions to facilitate handling, application and storage of these materials (Comunian & Favaro-Trindade, 2016). Ionic gelation is one such technique, often used to form microparticles whose wall material is composed of a gel-forming polymer, not requiring the use of solvents or high temperatures. Ionic gelation, however, does not afford good control of particle size (which can subsequently range from μ m to mm), which can negatively affect resultant food texture. By coupling ionic gelation with microencapsulation using microfluidics, this limitation could be overcome.

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Microencapsulation by microfluidics is a promising method for the production of monodisperse droplets—not to mention capsules with multiple compartments—which allows greater control and optimization of the encapsulation efficiency and release of active agents (Zhao et al., 2011; Comunian, Abbaspourrad, Favaro-Trindade, & Weitz, 2014). A microfluidic device consists of coaxial assemblies of a series of rigid glass capillaries resistant to chemicals; their three-dimensional geometry allows for the controlled production of multiple emulsions. This technique has been little explored for the encapsulation of food ingredients.

The objective of this work was the encapsulation of echium oil by using a combination of ionic gelation and microfluidic techniques. In addition, a phenolic compound (quercetin or sinapic acid) was also tested at various concentrations, offering benefits to the consumer's health, protection to the encapsulated compounds and the possibility of controlled release. No research related to the encapsulation of these compounds by the combination of these technologies was found in the literature, which suggests the innovative nature of this study.

2. Material and methods

2.1. Materials

Echium oil (NEWmega™ Echium Oil, Ref.15200, De Wit Specialty Oils, De Waal, Tescel, The Netherlands) was used as core. Sinapic acid and quercetin (Sigma Chemical Co., St. Louis, MO, USA), sodium alginate (Manugel GHB, FMC/Philadelphia, Pennsylvania), corn oil (Mazola, ACH Food Companies), soy lecithin Ultralect P (ADM/Decatur, IL, USA) and Tween 80 (Pepsico/Delaware, USA) were used as antioxidants, wall material, continuous phase, and hydrophobic and hydrophilic surfactant, respectively. Calcium chloride hexahydrate (Fisher Scientific, Waltham, MA, USA) and EDTA disodium salt (Fisher Scientific/Waltham, MA USA) were used for the formation of a complex that would promote the gelation of the polymer.

2.2. Methods

2.2.1. Microencapsulation process

The encapsulation process was performed using a glass microfluidic device (Fig. 1a and b) as described by Comunian et al. (2014). The cylindrical capillaries used (World Precision Instruments, Inc., Sarasota, Florida, United States) had a diameter and length of 1 mm and 6 in, respectively. These capillaries were inserted into a square capillary (Harvard Borosilicate Square Tubing), with outer and inner diameters of 1.5 and 1.05 mm, respectively. An oil-in-water-in-oil (O/W/O) double emulsion was produced using this device, composed of echium oil in the internal oil phase, sodium alginate aqueous solution in the middle phase, and corn oil and soy lecithin in the continuous phase.

Five encapsulation treatments were performed, which either had quercetin added to the inner phase or sinapic acid added to the middle phase (at two different concentrations each), or no phenolic compound added at all (see Table 1). The flow rates used were 1300, 870, and 5500 $\mu\text{L}/\text{h}$ for the inner, middle and continuous phases for both the control treatment and the S050-Alg treatment; 1300, 870, and 5700 $\mu\text{L}/\text{h}$ for the Q500-Alg treatment; 1300, 970, and 5300 $\mu\text{L}/\text{h}$ for the S025-Alg treatment; and 1500, 870, and 5000 $\mu\text{L}/\text{h}$ for the Q800-Alg treatment. The flow rates were determined in preliminary testing, pumped into the microfluidic device using a syringe pump (New Era Pump Systems, Inc/Farmingdale, New York, USA).

In combination with the microfluidic technique, the internal ionic gelation technique was used according to Utech et al.

(2015). In order to prevent the coalescence of the microcapsules and to obtain a faster gelation process for sodium alginate, a calcium-EDTA complex was prepared by mixing a solution of calcium chloride and a solution of EDTA disodium salt, both at 0.1 M. This complex was added to a 2% (w/w) solution of sodium alginate, in equal ratios, resulting in a final alginate concentration of 1% (w/w). The pH of the solution was adjusted to 10. The double emulsion droplets were collected in a plastic beaker containing corn oil, 2% (w/w) soy lecithin and 5% (v/v) glacial acetic acid, with constant stirring, which promoted the gelation of sodium alginate and the formation of microcapsules.

During production of the O/W/O double emulsion (Fig. 1a and b), after the sodium alginate gelation, the microcapsules were transferred from an oil medium to a water medium to facilitate handling and control of the material. After sedimentation of the capsules, they were transferred with a pipette into an aqueous solution with 1% (w/w) Tween 80 and centrifuged at 4000g for 10 min to separate the oil and aqueous phases. Due to the difference in density, the microcapsules remained in the aqueous phase. The aqueous solution was washed several times to remove Tween 80 from the media. For specific characterization analysis, the aqueous phase with the microcapsules was frozen in an ultra-freezer ($-80\text{ }^{\circ}\text{C}$) and lyophilized (LabConco freeze-dryer, LabConco Corporation/Kansas city, MO) for 24 h to produce a powder.

2.2.2. Encapsulation yield of sinapic acid, quercetin and oil

The yield was determined as a function of the mass difference between the total amount used in the encapsulation process and the amount of compound actually retained in the microcapsules after the process. The capsules were ruptured by adding a 0.1 M EDTA disodium salt solution and then it was mixed using a vortex mixer for 1 min. For the encapsulation yield of the phenolic compounds, methanol was added to the mixture in order to extract them. The samples were stirred with vortex again for 1 min and centrifuged (Heraeus Multifuge X1R Centrifuge, Thermo Scientific/Waltham, Massachusetts – USA) at 5000g for 3 min at room temperature. The concentrations of sinapic acid and quercetin were determined in the supernatant using UV-Vis spectrophotometry (UV-Vis Spectrophotometer UV-2600, Shimadzu Scientific Instruments/Marlborough, Massachusetts – USA) at wavelengths of 321 and 371 nm for sinapic acid and quercetin, respectively.

For the encapsulation yield of echium oil, after the capsule rupture, hexane was added to the solution in order to extract the oil, then the solution was mixed with a vortex for 1 min. The sample was centrifuged (Heraeus Multifuge X1R Centrifuge, Thermo Scientific/Waltham, Massachusetts – USA) at 8000g for 3 min at room temperature, and the supernatant containing hexane and oil was transferred to an eppendorf and maintained at $60\text{ }^{\circ}\text{C}$ until complete evaporation of the solvent. The same extraction process was performed twice to ensure that all the oil was quantified.

2.2.3. Morphology of the microcapsules by optical and scanning electron microscopy

The optical images were obtained using an inverted optical microscope (DMIL LED, Leica) connected to a fast camera (MicroLab 3a10, Vision Research). The scanning electron microscopy images of the microcapsules were obtained using a scanning electron microscope (LEO 1550 FESEM (Keck SEM) (Carl Zeiss, New York/USA)). Before taking the SEM images, the wet emulsions were placed on the SEM stub and allowed to dry for 24 h at room temperature.

2.2.4. Particle size and size distribution

The particle size and particle size distribution analyses were performed using ImageJ (NIH, Bethesda, MD), where 100 microcapsules for each treatment were measured individually.

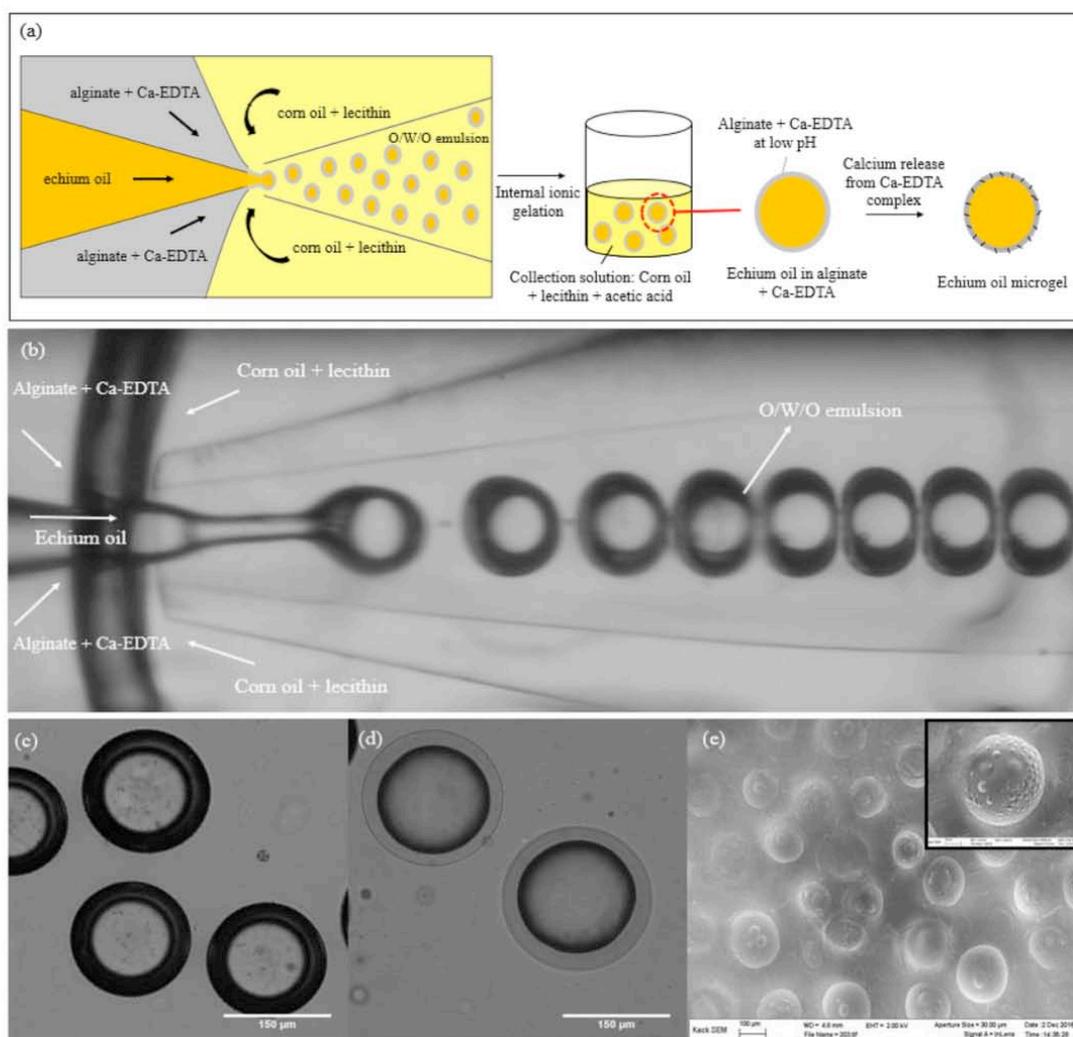


Fig. 1. (a) Graphical representation of the encapsulation process: combination of microfluidic device and ionic gelation; optical microscope image of (b) a glass microfluidic device during the production of double emulsions; (c) Q800-Alg capsules before gelation, (d) Q800-Alg capsules after gelation and (e) scanning electron microscope image of S050-Alg capsules.

The images were obtained by inverted optical microscopy (DMIL LED, Leica).

2.2.5. Thermogravimetric analysis

TGA curves were obtained using a Q500 Thermogravimetric Analyzer (TA Instruments/New Castle, DE). The wet samples were conditioned on a platinum support and nitrogen was used in the atmosphere of the furnace, at a flow rate of 60 mL/min, with a heating rate of 10 °C/min, starting from 25 to 600 °C.

2.2.6. Oxidative stability by FTIR

The spectra of echium seed oil and the microcapsules were obtained in the 4000 to 650 cm^{-1} region, using 16 scans at a resolution of 4 cm^{-1} , using the IRAffinity-1S Fourier Transform Infrared Spectrophotometer (Shimadzu Scientific Instruments/Marlborough, Massachusetts – USA). The freeze-dried samples were stored for 0, 7, 15, 21 and 30 days at 40 °C prior to reading. The formation

of lipid hydroperoxides, the primary oxidation products, was monitored according to Haider, Majeed, Williams, Safdar, and Zhong (2017).

2.2.7. Microcapsule stability under different stress conditions

The wet microcapsules were submitted to various stress conditions, including temperature (10, 20, 30, 40, 50, 60 and 70 °C), pH (2, 4, 6 and 8) and NaCl concentration (1, 3 and 5% w/w) following from work by Comunian, Boillon et al. (2016). In addition, the samples were also freeze-dried and resuspended in water. The structures of the microcapsules were evaluated using an inverted optical microscope (DMIL LED, Leica).

2.2.8. Stability of quercetin and sinapic acid

The levels of sinapic acid and quercetin were monitored by UV-Vis spectrophotometry as mentioned in Section 2.2.2. Analyses were performed in triplicate, at 0, 7, 15, 21 and 30 days after

Table 1
Composition of each treatment, encapsulation yield for the phenolic compounds and for the oil and average particle size before and after gelation of sodium alginate.

Treatments	Inner phase	Middle phase	Continuous phase	Encapsulation yield for the phenolic compounds (%)	Encapsulation yield for the oil (%)	Average particle size before gelation (μm)	Average particle size after gelation (μm)
Control	Echium oil	Sodium alginate	Corn oil + 2% (w/w) soy lecithin	–	85.39 \pm 10.74 ^a	117.22 \pm 12.32 ^c	115.56 \pm 19.27 ^d
S025-Alg	Echium oil	Sodium alginate + 0.025 g sinapic acid/g alginate	Corn oil + 2% (w/w) soy lecithin	91.33 \pm 18.74 ^a	83.29 \pm 10.56 ^a	213.86 \pm 25.84 ^a	227.55 \pm 21.40 ^a
S050-Alg	Echium oil	Sodium alginate + 0.05 g sinapic acid/g alginate	Corn oil + 2% (w/w) soy lecithin	94.04 \pm 23.19 ^a	77.81 \pm 1.80 ^a	148.03 \pm 24.21 ^c	155.56 \pm 26.71 ^c
Q500-Alg	Echium oil + 500 ppm [*] of quercetin	Sodium alginate	Corn oil + 2% (w/w) soy lecithin	97.90 \pm 4.60 ^a	90.61 \pm 3.99 ^a	135.97 \pm 15.33 ^d	155.71 \pm 17.89 ^c
Q800-Alg	Echium oil + 800 ppm [*] of quercetin	Sodium alginate	Corn oil + 2% (w/w) soy lecithin	93.19 \pm 13.98 ^a	82.47 \pm 1.41 ^a	185.34 \pm 15.98 ^b	196.77 \pm 22.91 ^b

* 500 and 800 ppm in relation to the oil weight. Equal letters in the same column do not differ statistically at a 5% level by the Tukey test. control: echium oil encapsulated without phenolic compounds; S025-Alg: echium oil encapsulated with 0.025 g of sinapic acid/g of sodium alginate; S050-Alg: echium oil encapsulated with 0.055 g of sinapic acid/g of sodium alginate; Q500-Alg: echium oil encapsulated with 500 ppm of quercetin in relation to the mass of oil; Q800-Alg: echium oil encapsulated with 800 ppm of quercetin in relation to the mass of oil.

encapsulation, with the wet material stored in glass vials, protected from light, in the presence of O₂ at 40 °C. In addition to the levels of phenolic compounds inside the microcapsules, the free phenolic compounds were also analyzed in solution, submitted to the same storage conditions.

2.2.9. Oxidative stability by thiobarbituric acid reactive substance (TBARS)

The formation of malonaldehyde, the second oxidation product, was monitored by TBARS analysis, according to Gray, Payne, McClements, Decker, and Lad (2010) and Gomez-Estaca, Comunian, Montero, Ferro-Furtado, and Favaro-Trindade (2016) with the wet samples stored at 0, 7, 15, 21 and 30 days at 40 °C. First, the microcapsules were ruptured as mentioned in Section 2.2.2. The samples were then added to an aqueous solution containing 15% (w/w) trichloroacetic acid, 0.375% (w/w) thiobarbituric acid and 2% (w/w) HCl, and kept in a water bath at 90 °C for 15 min. An ice bath was used in order to stop the reaction. Butanol was added to the solution, stirred with vortex for 1 min and then centrifuged at 5000g for 10 min at room temperature. The supernatant was analyzed by UV–Vis spectrophotometry (UV–Vis Spectrophotometer UV-2600, Shimadzu Scientific Instruments/Marlborough, Massachusetts – USA) at 532 nm. The same was also done for pure echium oil. The standard curve was made using 1,1,3,3-tetraethoxypropane and the solution used as a blank was submitted to the entire process without the addition of the microcapsules.

2.2.10. Statistical analysis

Data were analyzed using SAS statistical software (Statistical Analysis System, SAS Institute, Cary, NC), version 9.0, using ANOVA and post-hoc Tukey's tests, with statistical significance assumed at $p < 0.05$.

3. Results and discussion

Encapsulation by ionic gelation technique occurs because alginate crosslinks with divalent cations. This process can occur in two ways: internal or external gelation. In the case of internal gelation, the method used in this work, a dispersion of insoluble calcium particles is added into the alginate solution and the release

of these ions is induced by the reduction of pH via the addition of acid, resulting in the gelation of alginate (Leong et al., 2016).

For the production of echium oil microcapsules, a Ca-EDTA complex was added into the sodium alginate solution. The gelation of alginate occurred when the droplets were collected in corn oil (the same material used as continuous phase for the O/W/O emulsion) containing acetic acid. According to Utech et al. (2015), the acid diffuses into the droplets, causing the pH reduction and consequently the dissociation of calcium ions from the Ca-EDTA complex. The released calcium ions react with the alginate chains, promoting the gelation of the polymer and then the formation of the microcapsules containing echium oil as core and sodium alginate as the wall material (Fig. 1a).

3.1. Morphology of microcapsules by optical and scanning electron microscopy

Optical microscope images for the Q800-Alg treatment (addition of 800 ppm quercetin) before and after gelation, and a SEM image for the S050-Alg treatment (0.05 g of sinapic acid/g alginate) are shown in Fig. 1c, d and e, respectively. The treatments did not show any morphological differences, that is, the addition of the phenolic compounds in the inner oil phase or in the middle aqueous phase did not influence the formation of the spherical and mononucleate microcapsule. The SEM image shows the presence of particles adhering to the surface of the microcapsule. These particles can be explained by the presence of soy lecithin, which was used as an emulsifier in the continuous oil phase during the formation of the O/W/O emulsion. The emulsifiers have a hydrophilic and a hydrophobic part, which enables them to act at the water-oil interface in order to stabilize the emulsion. Thus, soy lecithin, used in the continuous oil phase, acts at the alginate-corn oil interface and—after the gelation of the polymer and the transfer of the microcapsules from an oil medium to a water medium—the lecithin remains in the wall material. The lecithin did not affect the functionality of the microcapsule, as demonstrated in later results.

Ren, Ju, Xie, and Chu (2010) produced similar microcapsules when they studied the formation of monodisperse alginate microcapsules with an oil core generated with a microfluidic device; however, because they used a combination of three devices, our control over, and stability of emulsions would be more precise.

3.2. Encapsulation yield of sinapic acid, quercetin and oil

The values of encapsulation yield for the phenolic compounds and oil are presented in Table 1. Values from 91 to 97% were obtained for sinapic acid and quercetin and from 77 to 90% for oil. For the two phenolic compounds, we hypothesized that the yield for sinapic acid would be lower than that for quercetin because sinapic acid was added to the aqueous phase—middle wall—and thus its release would be more likely, however this was not the case. There were no significant differences among treatments, showing that addition of a phenolic compound to either the oil phase or the aqueous phase does not influence their encapsulation yield. Considering the oil encapsulation yield, no significant differences among the treatments were observed. The presence of phenolic compounds did not interfere with the amount of encapsulated oil, which shows that the entire encapsulation process, used in combination with the addition of phenolic compounds, was efficient and suitable for the task.

So far, no research has reported the encapsulation of sinapic acid and quercetin using a combination of the microfluidic and ionic gelation techniques. A study conducted by Comunian, Boillon et al. (2016) has reported the encapsulation of echium oil and sinapic acid or rutin by complex coacervation using gelatin and arabic gum as wall materials and obtained an encapsulation yield for sinapic acid of 39 to 50%, values almost half of those obtained using the microfluidic and ionic gelation combination employed in this study. In the case of quercetin, Vidal et al. (2016) studied the synthesis and characterization of nanoparticles for the encapsulation of quercetin using polyhydroxybutyrate-co-hydroxyvalerate as wall material by the double emulsion technique and obtained an encapsulation yield of 51% for quercetin, again around half of that obtained in this work.

Several authors have studied the encapsulation of oil; however, only two papers report the encapsulation of oil by microfluidic devices using alginate as the wall material; moreover, these studies did not examine encapsulation yield or stability of the capsules (Liu et al., 2013; Ren et al., 2010). Comunian, Boillon et al. (2016) obtained an encapsulation yield for echium oil of 73 to 99% when it was encapsulated by the complex coacervation technique using gelatin and arabic gum as wall materials, values similar to those obtained in this work. Moreover, Ghorbanzade et al. (2017) were able to encapsulate fish oil in nano-liposomes, obtaining a yield of 92.22%, which corroborates that obtained in this study for the Q500-Alg treatment.

3.3. Average particle size and particle size distribution

Values for average particle size were in the range of 117 to 213 μm before alginate gelation and 115–227 μm after gelation (Table 1). There were significant differences among treatments, suggesting the amount of added phenolic compounds had an influence on the average particle size. The control treatment produced a smaller average particle size compared to the other treatments, which was expected, as the presence of phenolic compounds in the inner or middle phases causes an increase in solution viscosity, which may influence particle size. For the sinapic acid treatment, a higher concentration was expected to lead to a larger average particle size, because it would trigger the destabilization of the structure (Section 3.5), however this did not occur. On the other hand, an increase in quercetin concentration did lead to a parallel increase in the particle size. It is important to note that even with a significant difference among the treatments, the particle size distribution for all treatments was unimodal, which demonstrates the homogeneity of the microcapsules produced.

The addition of sinapic acid, a phenolic compound, to the microcapsule wall (S025-Alg treatment) resulted in a bigger particle size,

as compared to the control and the treatments with quercetin. This can be explained by the influence of surface charges. Alginate has a negative charge, and both sinapic acid and calcium ions have a positive charge. For this reason, sinapic acid and the calcium ions compete for alginate, thus affecting the gelation of this polysaccharide, making the microcapsules less stable and, consequently, bigger.

Particle size values within the range of 256–337 μm were obtained by Ren et al. (2010) when they studied the formation of monodisperse alginate microcapsules with an oil core using a microfluidic device with a combination of three capillaries, obtaining particle sizes bigger than those for echium oil.

3.4. Thermogravimetry analysis (TGA)

The thermogravimetric behavior of the treatments and the non-encapsulated echium oil is shown in Fig. 2a. All treatments displayed mass loss in three steps. The first step occurred in the 25–120 $^{\circ}\text{C}$ range, which corresponds to the loss of water. This step was not shown in Fig. 2a since the wet microcapsules were analyzed. Thus, for the purpose of comparison, the mass after this first step was considered 100%. The other two steps were observed from 200 to 350 $^{\circ}\text{C}$ and from 350 to 450 $^{\circ}\text{C}$, with variation for some treatments. They can be attributed to the decomposition of the alginate and oil, respectively. This result is in accordance with Yang, Gao, Hu, Li, and Sun (2015), who reported the encapsulation of poppy-seed oil by complex coacervation using gelatin and arabic gum as wall materials.

Comparing the control (echium oil encapsulated without phenolic compound) with the S025-Alg and S050-Alg treatments (oil encapsulated with 0.025 and 0.05 g of sinapic acid/g of alginate), it appears that the treatment with a higher sinapic acid concentration was less thermally stable than both the control and the treatment with lower sinapic acid concentration. Sinapic acid, a proton donor, competes with calcium, a cation, in its interaction with alginate, a negatively charged carbohydrate. Thus, a higher concentration of sinapic acid in the capsule wall would lead to the capsule's destabilization. This higher concentration will then provide less oil protection, as will be discussed further in Sections 3.7 and 3.8. When sinapic acid was added at a lower concentration, however, it did not influence the microcapsule structure.

As for the Q500-Alg and Q800-Alg treatments (oil encapsulated with 500 and 800 ppm of quercetin in relation to the total mass of oil), it was shown that the treatment with a higher concentration of quercetin was more thermally stable than both the control and the treatment with a lower concentration.

Between the sinapic acid and quercetin treatments, it appears that the Q800-Alg treatment was the most thermally stable, followed by the S025-Alg treatment. This behavior can be related to the concentration of quercetin and its function as an antioxidant in comparison to sinapic acid. The Q500-Alg and S050-Alg treatments showed similar behavior. Thus, how much and in which phase a phenolic compound is added are important factors to consider when designing a thermally stable microcapsule.

3.5. Stability of quercetin and sinapic acid

Retention of the encapsulated phenolic compounds was 25–30% after a 30-day storage, while for the non-encapsulated compounds, the retention values were 16 and 24% for sinapic acid and quercetin, respectively (Fig. 2b and Supplementary Table 1).

There were significant differences in retention among all the treatments; it was not possible, however, to obtain a relationship between a phenolic compound's concentration and its retention. However, non-encapsulated sinapic acid and quercetin were less stable than their encapsulated counterparts, that is, the encapsulation of these compounds protected them against degradation.

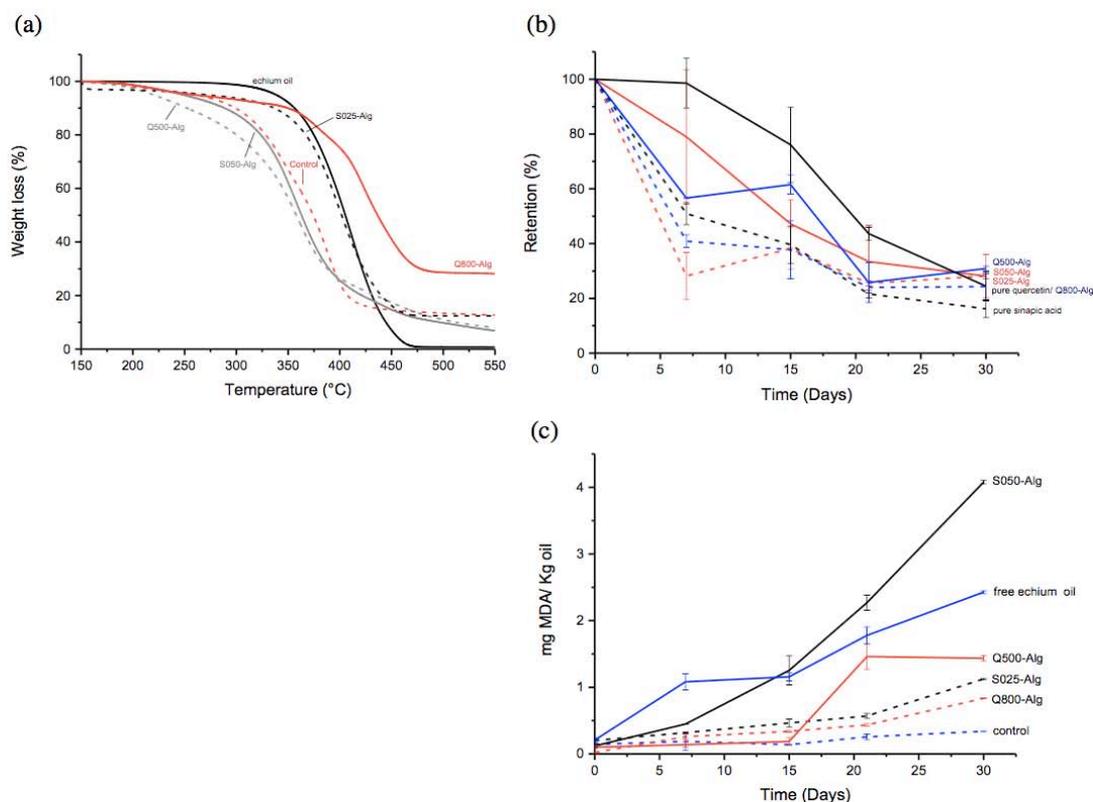


Fig. 2. (a) TG curves for each treatment and pure echium oil; (b) Retention (%) of encapsulated and non-encapsulated quercetin and sinapic acid for the samples stored for 30 days at 40 °C and (c) oxidative stability of the encapsulated and non-encapsulated echium oil by thiobarbituric acid reactive substance (TBARS).

Comparing the different concentrations of sinapic acid, it can be affirmed that the higher the concentration of this compound, the greater the retention within 30 days of storage. The same was not observed for quercetin: the higher the quercetin concentration, the lower its retention during storage, probably because it acted better as an antioxidant than sinapic acid did.

Wang, Waterhouse, and Sun-Waterhouse (2013) studied the encapsulation of canola oil and the effect of quercetin addition to the oil core by co-extrusion. After 30 days of storage at 20 °C, no quercetin was found in the encapsulated beads with alginate and pectin as wall materials. That is, even with quercetin acting as antioxidant (and thus decreasing its concentration) in the microcapsules in the present study with echium oil, its retention was still greater than in the system used by Wang et al. (2013). As for sinapic acid, no research was found about its stability when encapsulated, so we can state that the encapsulation of echium oil and sinapic acid by using a combination of the microfluidic and ionic gelation techniques provided good protection for this phenolic compound, because the retention for the non-encapsulated sample was lower after the 30-day storage period.

3.6. Oxidative stability by FTIR

Oxidative stability can be analyzed by Fourier transform infrared spectroscopy according to hydroperoxide formation in the ranges 3200–3600 cm^{-1} and 1730–1750 cm^{-1} . According to

Haider et al. (2017), peaks in these wavelength ranges are related to the formation of hydroperoxides (first oxidation product) and the formation of ketones and aldehydes, respectively.

Hydroperoxide formation was analyzed in the encapsulated echium oil of the lyophilized samples stored for 30 days at 40 °C. In Fig. 3a, the control (microcapsule without phenolic compound) showed a significant increase in absorbance for these wavelength ranges (3200–3600 cm^{-1} and 1730–1750 cm^{-1}) at 21 days of storage, which then decreased until the 30th day. It is known that hydroperoxides are the first products of oxidation and are unstable compounds; thus, they decompose rapidly after they are produced, forming aromatic compounds. This suggests that the formation of the second product of oxidation begins after 21 days of storage. This result corroborates those obtained in Section 3.8 (Oxidative stability by thiobarbituric acid reactive substance), which shows that the formation of malonaldehyde (the second product of oxidation) for the control was significant after 21 days.

In contrast, for the non-encapsulated echium oil and all other treatments with added phenolic compounds, no significant increase in absorbance for these wavelength ranges was found, because the oxidation and formation of malonaldehyde occurred more rapidly. In other words, the formation and decomposition of hydroperoxide occurred so rapidly that it could not be detected. The same behavior was observed by Haider et al. (2017) when they studied the encapsulation of krill oil by emulsion and later electrostatic interaction of chitosan with tripolyphosphate.

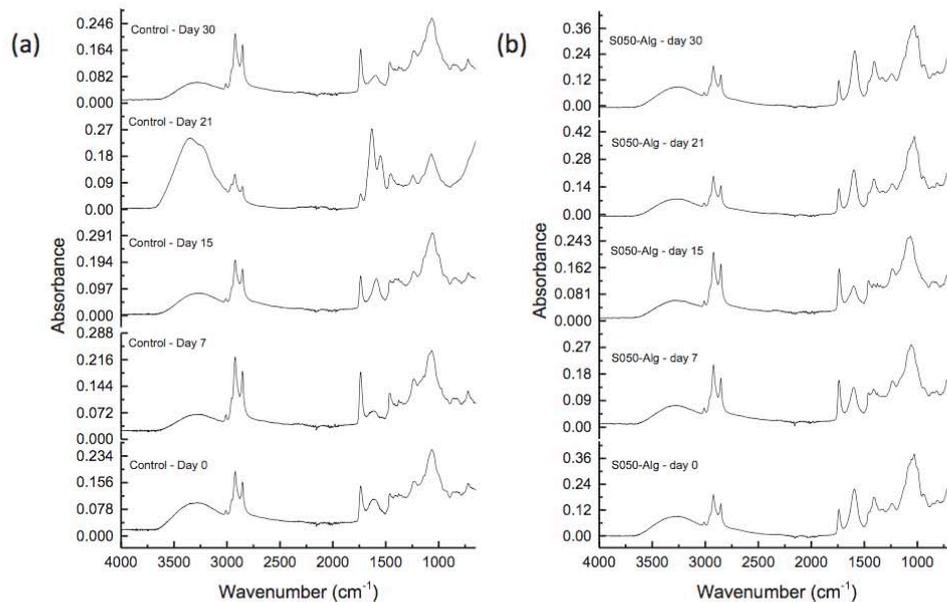


Fig. 3. Fourier Transform Infrared Spectrophotometry spectra for the following treatments: (a) control and (b) S050-Alg stored for 30 days at 40 °C.

3.7. Oxidative stability by thiobarbituric acid reactive substance (TBARS)

Oxidative stability by thiobarbituric acid reactive substance was measured with wet samples stored for 30 days at 40 °C. The formation of malonaldehyde (MDA) (second product of oxidation) provides a more comprehensive idea about the oxidation of the encapsulated oil compared to the non-encapsulated sample (Fig. 2c and Supplementary Table 2). A significant difference in MDA formation was observed between treatments during 30 days of storage, the non-encapsulated echium oil having a higher formation of malonaldehyde (2.4 mg MDA/kg of oil) compared to the other treatments (from 0.3 to 4 mg MDA/kg of oil), with the exception of the S050-Alg treatment (0.05 g sinapic acid/g alginate). This behavior for the S050-Alg treatment was expected since the presence of a higher concentration of sinapic acid in the capsule wall destabilized its structure, affecting the protection of the oil.

The oxidative stability of echium oil has also been studied by Gray et al. (2010) in emulsions using SDS or Tween 20 as emulsifiers. Authors found a higher formation of malonaldehyde for non-encapsulated echium oil (15 mmol/kg of oil) compared to emulsions (from 1 to 3 mmol/kg of oil), with samples stored for 7 days at 40 °C. Comparing the results, echium oil encapsulated by a combination of microfluidic devices and ionic gelation techniques promoted greater oxidative stability since the formation of malonaldehyde for the control (oil encapsulated without the presence of phenolic compounds) started to be significant only at 21 days of storage (0.25 mg MDA/kg of oil), and not at 7 days of storage, as in the results obtained by Gray et al. (2010). Jiménez-Martín, Gharsallaoui, Pérez-Palacios, Carrascal, and Rojas (2015) encapsulated fish oil using monolayered and multilayered emulsions by spray drying with maltodextrin and chitosan as wall materials. They analyzed the oxidative stability by TBARS at three different temperatures, but only for 12 days. Mohammadi, Jafari, Esfhanjani, and Akhavan (2016) studied the application of a

nano-encapsulated olive leaf extract in controlling the oxidative stability of soybean oil, evaluating the TBARS values within a span of 20 days, demonstrating a significant increase in oxidation by the first week of storage. In comparison, the technique used in the present work for the encapsulation of echium oil prevented a significant formation of malonaldehyde in the first 21 days (especially for the control), which argues for its effectiveness. In addition, Pourashouri et al. (2014) also studied the oxidative stability of spray-dried microencapsulated fish oils with different wall materials—storing their samples for 60 days at 20 °C and evaluating TBARS. Comparing their approach to our own, the combination of the microfluidic device and ionic gelation used in our study provided more protection against oxidation, since our samples (stored at 40 °C) showed the same TBARS values as the samples stored at 20 °C from this study.

Comparison of the control with treatments where phenolic compounds were added (Q500-Alg, Q800-Alg and S025-Alg) revealed that the control had lower malonaldehyde formation, which was not expected. This could be explained by the possible action of quercetin as pro-oxidant along with destabilization of the microcapsule structure by the presence of sinapic acid in the microcapsule wall. According to Behling, Sendão, Francescato, Antunes, & Bianchi (2004), flavonoids can act as pro-oxidants, depending on the concentration and total number of hydroxyls. In addition, between quercetin and sinapic acid, it was evident that the treatment with higher quercetin concentration (Q800-Alg) protected the oil better than the treatments with sinapic acid (S025-Alg and S050-Alg).

It follows that the combination of microfluidic devices and ionic gelation techniques was enough to protect the oil, as the control (without quercetin or sinapic acid) showed better oxidative stability. However, even with the addition of these interfering compounds, oil protection was still guaranteed because they underwent a lower rate of malonaldehyde formation compared to non-encapsulated echium oil.

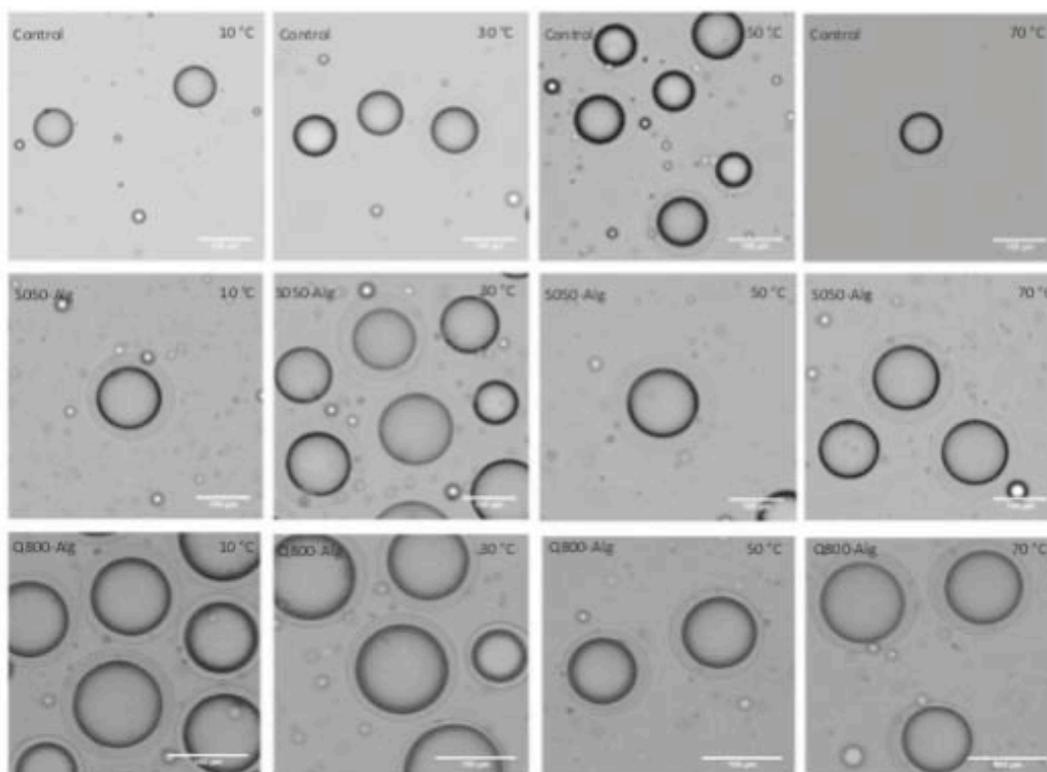


Fig. 4. Optical microscope images of the following treatments: (a) Control, (b) S050-Alg and (c) Q800-Alg submitted to different temperatures.

3.8. Microcapsule stability under different stress conditions

In the temperature range tested, all treatments retained their structure. With increasing temperature however, there was an increase in shell thickness for the treatment involving sinapic acid (Fig. 4). This can be explained by the presence of this compound in the sodium alginate solution. As already mentioned, sinapic acid affects the ionic gelation process and, consequently, the structure of the capsule. Comunian, Gomez-Estaca et al. (2016) studied the effect of sinapic acid as a crosslinking agent on echium oil microcapsules obtained by complex coacervation using the combinations gelatin-arabic gum and gelatin-cashew gum as wall materials, and observed a high potential of this phenolic compound for binding to polysaccharides and proteins. The same was expected to occur with sodium alginate; however, due to the nature of the ionic gelation process, the sinapic acid in this case did not produce a robust effect on the formation of the microcapsule and consequently on the protection of echium oil. Regardless, the control and treatment with quercetin both showed good stability, with intact microcapsules within the temperature range tested, showing that treatments obtained by combining microfluidic devices and ionic gelation techniques can be applied to products over a wide temperature range, ensuring the protection of the encapsulated oil.

In relation to different pH values and salt concentrations, a similar pattern of behavior was observed. In this case, the control and treatment with sinapic acid showed an increase in shell thickness at pH 8 and in all concentrations of salt analyzed (1, 3 and 5% w/w),

whereas the treatments with quercetin retained the same structure. The structure of the microcapsules, however, was not affected. Thus, submitting the treatments to different stress conditions did not affect oil protection.

After freezing, lyophilization and resuspension of the microcapsules in water, it was possible to confirm that the treatments with quercetin were the most stable, since they presented an intact structure even after all these steps (Fig. 5). The S050-Alg treatment (with the higher concentration of sinapic acid) produced an undefined shape after resuspension in water, showing that the presence of sinapic acid, as already mentioned, can destabilize the structure of the microcapsule, causing the loss of its initial form. This result corroborates those obtained for thermogravimetric analysis and oxidative stability by thiobarbituric acid reactive substance in Sections 3.4 and 3.7.

4. Conclusions

Microencapsulation of echium oil by a combination of microfluidic and ionic gelation techniques is feasible, since high values of encapsulation yield and oxidative stability of echium oil during storage at 40 °C were obtained. The control (microcapsules without addition of phenolic compounds) provided high oil oxidative stability, and the control, all treatment with quercetin, and the treatment with a low concentration of sinapic acid were stable under different conditions of stress. Moreover, the Q800-Alg treatment (microcapsule with higher quercetin concentration) showed

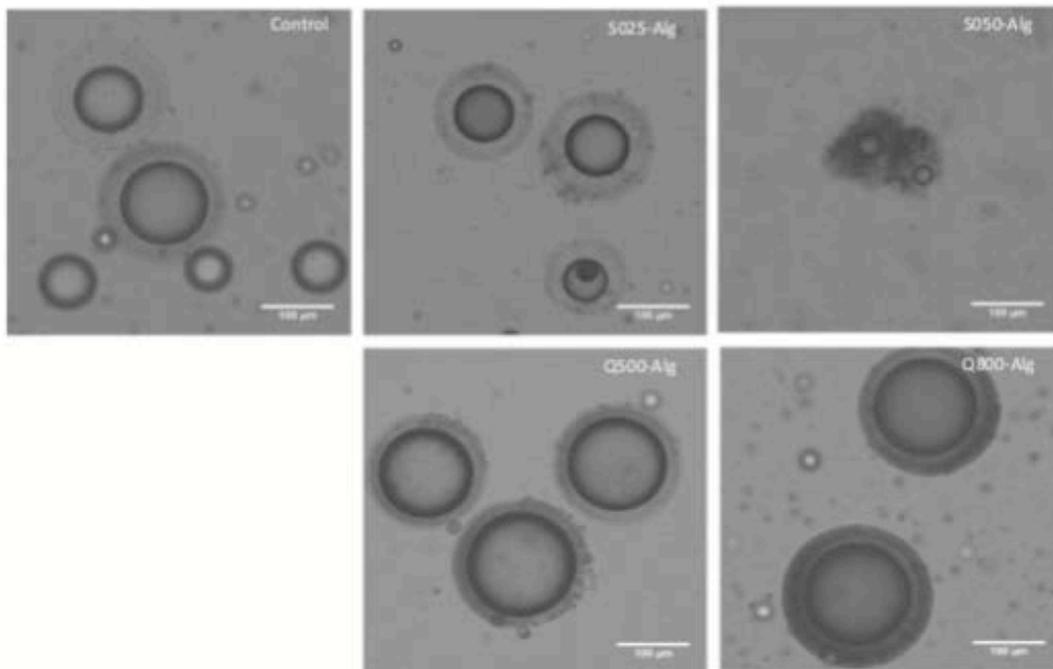


Fig. 5. Optical microscope image of each treatment freeze-dried and resuspended in water.

the best thermal stability, followed by the control, suggesting that this type of microcapsule can be applied to different products. Thus, the combination of the microfluidic and ionic gelation techniques was sufficient to protect the oil. In the future, this combination of techniques can be further explored for the encapsulation of food ingredients, since no solvent or high temperature was used during the process.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.04.085>.

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Development of functional yogurt containing free and encapsulated echium oil, phytosterol and sinapic acid



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ABSTRACT

The consumption of omega-3 fatty acids and phytosterol promotes the reduction of cholesterol and triacylglycerol levels. However, such compounds are susceptible to oxidation, which hampers their application. The objective of this work was to coencapsulate echium oil, phytosterols and sinapic acid (crosslinker/antioxidant), and incorporate the obtained microcapsules into yogurt. The microcapsules were evaluated for particle size, accelerated oxidation by Rancimat, and simulation of gastric/intestinal release. The yogurts were assessed for morphology, pH, titratable acidity, color, rheology and sensory analysis. The microcapsules (13–42 μm) promoted protection against oil oxidation (induction time of 54.96 h). The yogurt containing microcapsules, presented a pH range from 3.89 to 4.17 and titratable acidity range from 0.798 to 0.826%, with good sensorial acceptance. It was possible to apply the microcapsules in yogurt, without compromising the rheological properties and physicochemical stability of the product.

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1. Introduction

Omega-3 fatty acids (ω -3) are compounds widely used in food, pharmaceutical and cosmetic products, due to their beneficial effects on health (Kralovec, Zhang, Zhang, & Barrow, 2012). The oil extracted from the echium seed (*Echium plantagineum* L.) contains 9–16% stearidonic acid (18:4 ω -3), which is a long-chain polyunsaturated ω -3 fatty acid. Stearidonic acid is rare in plants and very important in human nutrition because it is an intermediate in the biosynthesis of eicosapentaenoic acid (EPA, C20:5 ω -3) and docosahexaenoic acid (DHA, C22:6 ω -3), which are also associated with valuable human benefits (Berti, Johnson, Dash, Fischer, & Wilckens, 2007; Zanetti, Monti, & Berti, 2013). Sterols, also known as steroid alcohols, are an important class of organic molecules that occur naturally in fungi (e.g. ergosterol), animals (e.g. cholesterol) and plants (phytosterols), and play essential roles in the physiology of eukaryotic organisms (Fennema, Damodaran, & Parkin, 2010).

The consumption of both, ω -3 fatty acids and phytosterol, is effective in lowering the blood cholesterol and triacylglycerol

levels (Espinosa, Inchingolo, Alencar, Rodriguez-Estrada, & Castro, 2015). However, these compounds are highly susceptible to oxidation, which hampers their use and application. Two strategies used to minimize this problem include the addition of a compound with antioxidant function and microencapsulation.

Microencapsulation by complex coacervation, is caused by electrostatic interactions between two or more oppositely charged colloids (Rocha-Selmi, Bozza, Thomazini, Bolini, & Favaro-Trindade, 2013). Besides the formation of resistant microcapsules by complex coacervation, the use of a crosslinking agent is being explored, in order to stabilize the structure of the complexes (Chen, Li, Ding, & Suo, 2012).

Sinapic acid (SA) is both an antioxidant (Espinosa et al., 2015) and, as demonstrated in a previous work by our research group, displays a crosslinking effect on microcapsules produced by complex coacervation, using gelatin-arabic gum and gelatin-cashew gum combinations, as wall materials (Comunian, Gomez-Estaca, et al., 2016). SA is the main phenolic acid of canola (Rawel & Rohn, 2010) and has been studied in relation to its neuroprotective effects against Alzheimer's disease (Lee et al., 2012), cardiac hypertrophy and dyslipidemia (Roy & Prince, 2013). Its structural formula (Supplementary Fig. 1), possesses only one phenolic group.

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It is known that functional foods are those containing bioactive compounds which help in protecting the body against disease and maintaining health. Microencapsulation is of extreme importance for the development of functional foods because several bioactive compounds are unstable when in contact with light, oxygen and heat (Favaro-Trindade, Comunian, Souza, Santos, & Oliveira, 2016). Coencapsulation of the bioactive compounds echium oil and phytosterols, has not been previously described in the literature and publications regarding the application of this type of microcapsule in food are scarce. Therefore, in this work, echium oil and phytosterols were coencapsulated, using SA, as a crosslinking and antioxidant agent. The obtained microcapsules were evaluated for their gastrointestinal release properties and their application in yogurt, to produce a functional product, by physicochemical, sensorial and rheological analyses. In order to produce a functional product, the proportion of echium oil and/or microcapsules added to yogurt, followed the standards of Brazil's National Health Surveillance Agency (ANVISA) Directors' Collegiate Resolution (RDC) No 54 of 12 November (2012), ANVISA Technical Report No 56 (2014), and the minimum daily recommendation of EPA and DHA (200 mg/day), as well as equivalent ratio of EPA and stearidonic acid, according to Decker, Akoh, and Wilkes (2011).

2. Materials and methods

2.1. Material

Oil extracted from the *E. plantagineum* L. seed, was used as the ω -3 source (NEWmega™ Echium Oil, Ref. 15200, De Wit Speciality Oils, De Waal, Tescel, The Netherlands). Gelatin, arabic gum, cashew gum and SA, were obtained from Gelnex (Santa Catarina, Brazil), Nexira (São Paulo, SP, Brazil), The Brazilian Agricultural Research Corporation (EMBRAPA) Tropical Agribusiness (Fortaleza, Ceará, Brazil) and Sigma Chemical Co. (St. Louis, MO, USA), respectively. The mixture of phytosterols, comprised of β -sitosterol (70–80%), β -sitostanol (0–15%), campesterol (0–15%), stigmasterol (0–2%) and campesterol (0–5%), was obtained from DuPont Danisco (Barueri, SP, Brazil). The pulp, strawberry essence and cochineal carmine dye, used in the production of yogurt, were obtained from Borsato Industrial Ltda (Farroupilha, RS, Brazil), Frutarom do Brasil (São Paulo, SP, Brazil) and Fuchs Gewurze do Brasil Ltda (Itupeva, SP, Brazil), respectively. The yogurt starter culture Granoferm BY220 (20 U), was obtained from Granolab do Brasil SA Tecnologia Para a Indústria Alimentícia (Curitiba, Paraná, Brazil). Whole milk was donated by the prefecture of “Fernando Costa” campus of the University of São Paulo (Pirassununga, SP, Brazil).

2.2. Microencapsulation

The microcapsules were prepared, as described by Comunian, Gomez-Estaca, et al. (2016). The echium oil and phytosterol (0.132 g phytosterols/g oil) mixture was used as the core and added at 50% (w/w) in relation to the total mass of polymers (gelatin-arabic gum and gelatin-cashew gum, respectively), to 5% (w/w) gelatin solution and homogenized (Ultra-Turrax T25, IKA, Germany), at 10,000 rpm for 3 min, obtaining an oil-in-water (O/W) emulsion. Solutions of 5% (w/w) arabic gum or cashew gum were, respectively, added to the O/W emulsion, under magnetic stirring at 40 °C. For complex coacervation, the respective emulsions were adjusted to pH 4.0 and the temperature reduced to 10 °C, in an ice bath.

Ratios of 1:1 gelatin-to-arabic gum and 1:2.5 gelatin-to-cashew gum, were used. The final percentages obtained were 32, 32, 32 and 4% (w/w, dry basis) of gelatin, arabic gum, echium oil and phytosterols, respectively, for the treatments with the combination of

gelatin and arabic gum as wall materials. For the gelatin-cashew gum combination as the wall materials, 18.24, 45.62, 31.93 and 4.21% (w/w, dry basis) of gelatin, cashew gum, echium oil and phytosterols, respectively, were present in the final microcapsules. Treatments were prepared at four concentrations of SA as crosslinker, totaling eight treatments, as shown in Table 1.

Based on Comunian, Gomez-Estaca, et al. (2016), for the crosslinking process, SA (concentrations presented in Table 1) was added to the solution containing the microcapsules (after the complex coacervation process, pH adjustment and temperature reduction) and maintained under low magnetic stirring at 15 °C for 12 h, in a BOD TE-391 incubator (Tecnal, Piracicaba, São Paulo, Brazil).

The coacervate material was stored at 7 °C for 24 h, to promote decantation. Then, the samples were frozen (–18 °C) and lyophilized in a Terroni freeze-dryer (São Carlos, SP, Brazil) for 24 h, at a pressure of 1–0.1 kPa, and initial and final temperature of –20 and 30 °C, respectively.

2.3. Characterization of microcapsules

2.3.1. Average particle size

The mean particle size of 100 microcapsules from each treatment was measured, using ImageJ, and the geometric mean of each treatment was calculated. Images of the microcapsules were obtained, using a light microscope (Bel Photonics BIO3, Italy).

2.3.2. Accelerated oxidation by Rancimat

Accelerated oxidation tests for pure echium oil and the microcapsules, were performed with a Rancimat apparatus (model 873, Metrohm, Switzerland). Samples (4 mL of pure echium oil and/or 1.5 g of microcapsules) were heated under a purified air flow rate of 20 L/h at 90 °C. The sample induction time, measured in hours, was used as the oxidative stability index.

2.3.3. Simulation of the release of SA in simulated gastric and intestinal fluids

The release of the SA present in the microcapsules was monitored for 0, 15, 30, 60, 90, 120 and 180 min in simulated gastric and intestinal fluids, under constant magnetic stirring at 37 °C. The aqueous gastric fluid solution contained 0.9% (w/w) NaCl and 0.3% (w/w) pepsin, at pH 1.8. The aqueous intestinal fluid contained 0.9% (w/w) NaCl, 1% (w/w) pancreatin, 1% (w/w) trypsin, and 0.3% (w/w) bile salts, at pH 7.8 (Cbassi, Vendamme, Ennahar, & Marchioni, 2009). A 0.2-g aliquot of microcapsule was added to 100 mL of each fluid and 1-mL aliquots were removed at each time interval, then centrifuged (Centrifuge 5430R, Hamburg, Germany) at 7500 rpm for 5 min at 4 °C. The supernatant was filtered and analyzed by high-performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan). A C18 column (Amide) was used, with a 45:55 water:methanol (pH 3.5) mobile phase, at a flow rate of 1 mL/min, and detection at 325 nm. The sample injection volume was 10 μ L.

2.3.4. Simulation of the release of the oil and the mixture of phytosterols in the gastric and intestinal systems

The release of the oil and phytosterol present in the microcapsules was monitored for 0, 15, 30, 60, 90, 120 and 180 min, in simulated gastric and intestinal fluids, under constant magnetic stirring, at 37 °C, as described in Section 2.3.3. Nile Red dye (Sigma-Aldrich) (1.5 mg) was added to the oil and phytosterols mixture, in order to stain the material, allowing detection by spectrophotometry at 547 nm. A 1.5-g aliquot of microcapsule was added to 100 mL of each fluid and a 5-mL aliquot was removed at each time interval, followed by addition of 2 mL butanol. The mixture was vortexed for 1 min and centrifuged at 7500 rpm for

Table 1

Composition of treatments, values of average particle diameter and oxidative stability index for each treatment.

Treatments	Wall materials combination	Proportion of polymers	Concentration of phenolic (g/g gelatin)	Average particle size (μm)	Oxidative stability index (hours)
GA00	Gelatina: GA	1:1	0.000	32.61 \pm 14.09 ^c	10.13 \pm 0.26 ^f
GA025	Gelatina:GA	1:1	0.025	42.96 \pm 10.86 ^a	13.27 \pm 0.17 ^{de}
GA050	Gelatina:GA	1:1	0.050	37.16 \pm 10.07 ^b	28.06 \pm 0.35 ^b
GA075	Gelatina: GA	1:1	0.075	40.79 \pm 13.06 ^{ab}	54.96 \pm 1.52 ^a
GC00	Gelatina: GC	1:2.5	0.000	13.38 \pm 2.58 ^c	9.63 \pm 0.17 ^f
GC025	Gelatina: GC	1:2.5	0.025	15.28 \pm 3.06 ^{cd}	14.11 \pm 0.65 ^{cd}
GC050	Gelatina: GC	1:2.5	0.050	14.88 \pm 3.06 ^{cd}	16.57 \pm 0.16 ^c
GC075	Gelatina: GC	1:2.5	0.075	17.88 \pm 4.75 ^d	17.02 \pm 0.25 ^c
Echium oil	–	–	–	–	5.10 \pm 0.09 ^a

Equal letters in the same column do not differ statistically at level of 5% by the Tukey test.

GA00, GA025, GA050 and GA075: treatments with gelatin-arabic gum combination as wall materials added of 0, 0.025, 0.050 and 0.075 g sinapic acid/g gelatin. GC00, GC025, GC050 and GC075: treatments with gelatin-cashew gum combination as wall materials added of 0, 0.025, 0.050 and 0.075 g sinapic acid g gelatin.

3 min (Centrifuge 5430R, Hamburg, Germany). The butanol phase was used for spectrophotometric (Thermo Scientific, Genesys 10S UV-Vis, Shanghai, China) quantification, using butanol as the blank.

2.4. Preparation of yogurt

The microcapsule corresponding to the best treatment (GA075) was chosen for incorporation into yogurt. The yogurt was made in the dairy plant of the Campus "Fernando Costa", at the University of São Paulo (Pirassununga, SP). The following steps were involved in the yogurt manufacture: (1) heat treatment of milk (90 °C for 30 min); (2) addition of sugar; (3) cooling to 45 °C; (4) addition of yogurt starter culture; (5) incubation at 45 °C for 3 h; (6) cooling and maturation for 24 h, and (7) mixing of ingredients (strawberry pulp, strawberry essence and carmine dye) and of microcapsules and/or non-encapsulated echium oil, phytosterols and SA. The yogurts were packed in 200-mL plastic bottles and stored at 4 °C, for 0, 7, 15, 21 and 30 days. The basic yogurt formulation contained 10% (w/w) sugar, 2% (v/v) strawberry pulp, 0.075% (v/v) strawberry essence, and 0.025% (v/v) carmine dye. The three different treatments were differentiated in relation to the presence or absence of microcapsules and free bioactive compounds, as follows: T1: control yogurt, composed of the basic yogurt formulation; T2: yogurt containing the non-encapsulated bioactive compounds: 6% (w/w) echium oil, 0.1% (w/w) phytosterol mixture and 0.05% (w/w) SA; T3: yogurt containing 2% (w/w) microcapsules. The treatments T2 and T3, presented an echium oil concentration equivalent to 15% of the minimum daily intake of EPA (100 g portion).

2.5. Characterization of yogurt

2.5.1. Morphological characterization

The yogurt was morphologically characterized, using an optical microscope (Bel Photonics, Italy) and by scanning electron microscopy (SEM), using a Hitachi SEM TM-3000 Tabletop Microscope (Hitachi High-Technologies Corp., Tokyo, Japan), equipped with the TM-3000 program. For the optical microscopy, an aliquot of yogurt was placed on a glass slide, without any preparation. For the SEM, the yogurt was frozen (–25 °C) and then lyophilized, as described in Section 2.2.

2.5.2. Determination of pH and titratable acidity

The pH of the samples was analyzed using a pH meter (Marte MB-10, São Paulo, Brazil). Titratable acidity was determined by titrating up to pH 8.2, with 0.1 N NaOH and expressed as a percentage of lactic acid (AOAC, 1995, chap. 37; Brazil & Decretos, 1986),

for the yogurt samples stored at 4 °C, for 0, 7, 15, 21 and 30 days, respectively.

2.5.3. Determination of instrumental color

The color of the stored yogurt samples (4 °C, for 0, 7, 15, 21 and 30 days) was analyzed, using a HunterLab Mini Scan XE Plus colorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA). The CIE L* a* and b* color parameters obtained may vary from white (100) to black (0), from green (–) to red (+), and from blue (–) to yellow (+), respectively. The total color difference (ΔE), at 30 days of storage, was obtained by Eq. (1).

$$\Delta E = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2} \quad (1)$$

2.5.4. Rheological characterization

The viscosity of the formulations was determined using a rotational rheometer AR 2000, TA Instruments (New Castle, Delaware, USA), with parallel plate geometry (60 mm, gap 500 μm) and the software Rheology Advantage Instrument control AR and Rheology Advantage Data Analysis. The rheological measurements were obtained at 6 °C, according to the method of Sah, Vasiljevic, McKechnie, and Donkor (2016). A 3-mL aliquot of yogurt was pre-sheared for 30 s, at a shear rate of 500 s^{-1} , maintained in equilibrium for 300 s, and then analyzed for 4 min. For the frequency sweep analysis, an oscillatory stress of 0.2 Pa was set (this value was in the region of linear viscoelasticity) and a frequency ramp from 0.1 to 10 Hz, was used. The analyzed results of this test, were storage modulus (G'), loss modulus (G''), loss tangent (C''/G') and complex modulus $\{[(G')^2 + (G'')^2]^{1/2}\}$. Also, the flow curve was analyzed at a shear rate from 0.1 to 100 s^{-1} and the results modeled, according to the Herschel-Bulkley equation (Eq. (2)):

$$\sigma = \sigma_0 + k \cdot \dot{\gamma}^n \quad (2)$$

where, σ_0 , k and γ represent the yield stress (Pa), consistency index (Pa s) and shear rate (s^{-1}), respectively. Values of n are necessary to define the type of fluid: $n = 1$: Newtonian fluid; $n < 1$: pseudoplastic fluid and $n > 1$: dilating fluid (Sah et al., 2016).

2.5.5. Consumer acceptance test

Sensory evaluation is a valuable tool for the development and quality evaluation of products with added polyunsaturated oils. The sensory acceptance analysis was performed in individual cabins, according to the method described by Meilgaard, Civille, and Carr (1999). The samples were evaluated using a 9-point hedonic scale (1 – "I highly disagree" and 9 – "I liked it very much"), for the attributes of taste, texture, color, flavor and overall acceptance. A total of 126 untrained tasters, of both genders and of various age, participated in the test. The tasters were selected on the basis of being regular consumers of yogurt and for their willingness to par-

ticipate in the trials. The sample was presented monadically, in coded cups and in a random manner. This study was approved by the Research Ethics Committee of the College of Animal Science and Food Engineering, of the University of São Paulo (number of the Certificate of Presentation for Ethical Assessment (CAAE): 52954916.6.0000.5422).

2.6. Statistical analysis

The data were statistically analyzed using the statistical program SAS (Statistic Analysis System), version 9.0, by analysis of variance (ANOVA) and Tukey's test, at the significance level of 5%.

3. Results and discussion

3.1. Characterization of microcapsules

3.1.1. Average particle size

The average particle sizes obtained for the treatments ranged from 13 to 42 μm (Table 1). There was a significant difference among all the treatments. The treatments with gelatin-arabic gum presented particle size values almost three times higher than the treatments with gelatin-cashew gum. Ifeduba and Akoh (2015) also studied the microencapsulation of stearidonic acid by complex coacervation, using gelatin and arabic gum as wall materials and reported average particle sizes ranging from 286 to 296 μm for microcapsules produced using a low homogenization speed (600 rpm) and 45 to 56 μm for microcapsules produced at high homogenization speed (19,400 rpm). This broad range of average particle sizes obtained, can be attributed to several factors, such as differences in the wall materials, the concentration of polymers and encapsulated material, temperature and homogenization speed of the process. According to Comunian and Favaro-Trindade (2016), the average particle size is a critical factor to be considered when incorporating the microcapsules into a food product, because they may negatively interfere with the texture of the final product. The microcapsules obtained in this work, can be applied in food because they presented average diameters below 100 μm .

3.1.2. Accelerated oxidation by Rancimat

Table 1 presents the induction time (hours), which was used to represent the oxidative stability index of the encapsulated and pure oil. The oxidative stability index ranged from 9.95 to 54.96 h for treatments and 5.10 h for pure echium oil. A comparison of the pure echium oil with the same encapsulated oil, without the crosslinking agent (treatments GA00 and GC00), shows that it was possible to increase the oxidative stability of the oil two-fold. Namely, encapsulation by complex coacervation, using gelatin-arabic gum (GA) and gelatin-cashew gum (GC) as wall materials, respectively, promoted greater oxidative stability to echium oil.

For treatments with addition of various SA concentrations, the oxidative stability index increased with increasing phenolic compound concentration, obtaining up to 10-fold more oxidative stability for the oil when encapsulated with the treatment GA075 and 3.5-fold more in the case of treatments GC050 and GC075. Thus, the encapsulation of echium oil by complex coacervation, using SA as a crosslinking and antioxidant agent, is an effective technique to promote increased stability to the encapsulated material.

Wang, Vongsivut, Adhikari, and Barrow (2015) studied the microencapsulation of tuna oil fortified with multiple lipophilic ingredients, such as vitamins A, D3, E, and K2, as well as curcumin and coenzyme Q10, using gelatin and sodium hexametaphosphate

as wall materials. These authors obtained oxidative stability index values ranging from 5.59 to 6.23 h, which are similar to pure echium oil. In comparison, the technique used in this work, provided considerably greater oxidative stability to the oil. However, a direct comparison with the current study is not practical given that a different oil was studied by Wang et al. (2015).

Previously, Comunian, Boillon, et al. (2016), Comunian, Gomez-Estaca, et al. (2016) studied the coencapsulation of echium oil and phenolic compounds by complex coacervation using gelatin-arabic gum and gelatin-cashew gum combinations as wall materials, respectively, and obtained oxidation index values ranging from 9.97 to 18.77 and 8.73 to 26.50 h, respectively. The difference between these values and that obtained for the microcapsules with echium oil, SA and phytosterols, can be explained by the different concentrations of SA applied, which were 200 ppm SA relative to the mass of oil (Comunian, Boillon, et al., 2016; Comunian, Gomez-Estaca, et al., 2016) and 0.05 g SA/g gelatin for the microcapsules, respectively.

3.1.3. Simulation of the release of SA

The behavior of the release of SA from each treatment, under conditions simulating the gastric and intestinal system, is presented in Fig. 1. It can be stated that the higher the concentration of SA, the greater its release in the system, both for the gelatin-arabic gum and the gelatin-cashew gum combinations as wall materials, ranging from 6 to 20 and 2.5 to 25% for gelatin-arabic gum treatments in the gastric and intestinal system, respectively, and from 7 to 16 and 2.5 to 17%, for treatments with gelatin-cashew gum in the gastric and intestinal system, respectively, for 180 min.

Initially, the release rates were higher in both fluids, due to diffusion through the wall of the rehydrated microcapsule. Over the analyzed time, the rate of release of the phenolic compound decreased, until reaching a plateau. Considering that SA has beneficial health properties, it would be better to have a higher release of SA with echium oil and phytosterols, however, this did not occur. Furthermore, these low release values can be explained by the crosslinking reaction of the SA with the protein and the polysaccharide, according to Comunian, Gomez-Estaca, et al. (2016).

By comparing the same treatments in the different fluids, it can be affirmed that there was greater release in the intestinal system. However, by comparing different combinations of wall materials, with the same SA concentrations and in the same fluid, it can be stated that the microcapsules with the gelatin-arabic gum combination showed a higher phenolic compound release than the treatments with the gelatin-cashew gum combination. Again, this higher release rate for treatments with the gelatin-arabic gum combination can be attributed to the stronger crosslinking bonds between SA and cashew gum. Thus, it could be hypothesized that the composition of the polysaccharides affects the extent of crosslinking by SA. Both arabic gum and cashew gum are polysaccharides composed of D-galactose, L-arabinose, and L-rhamnose, in addition to D-glucuronic acid and 4-O-methyl-D-glucuronic acid (Mothé & Correia, 2002), differing only in the presence of mannose, glucose and xylose, as terminal residues in cashew gum. According to Comunian, Gomez-Estaca, et al. (2016), one of the ways for the crosslinking reaction to occur between SA and a polysaccharide is by the Fischer esterification reaction, with an OH group from a sugar residue, which was present mainly in cashew gum. This suggests that the presence of mannose, glucose and xylose, in the composition of cashew gum, promoted a greater crosslinking reaction with SA.

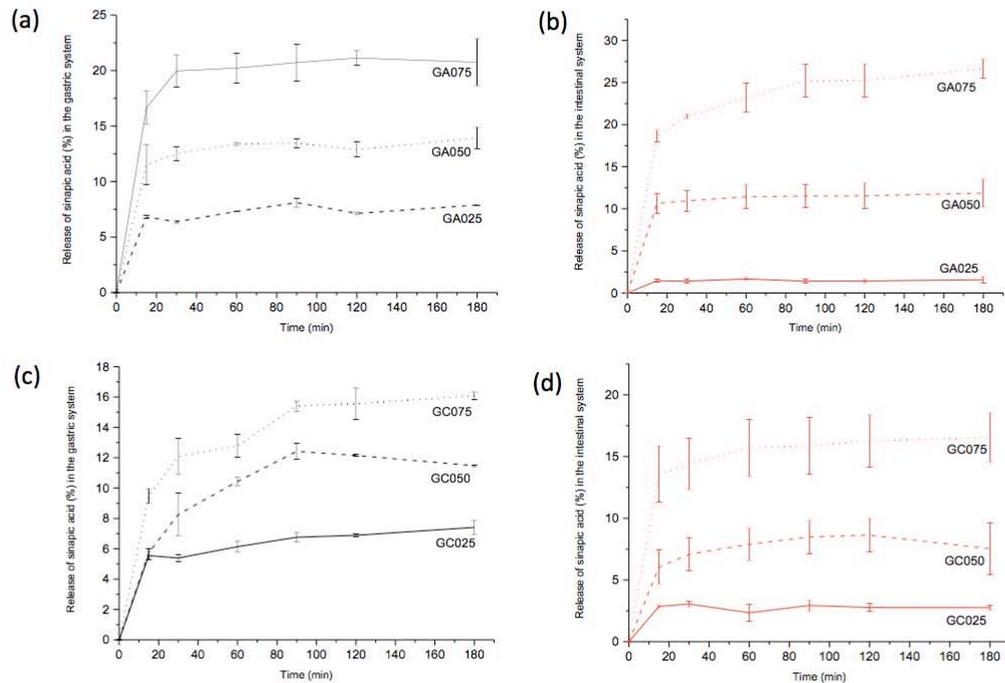


Fig. 1. Simulation of the gastric (a and c) and intestinal (b and d) release of sinapic acid over 180 min, from the treatments prepared with gelatin-arabic gum (a and b) and gelatin-cashew gum (c and d).

3.1.4. Simulation of release of oil and phytosterols mixture

The study of the release behavior of echium oil and the phytosterols mixture, was done with four treatments that were chosen based on the results of accelerated oxidation by Rancimat. Thus, two treatments with the gelatin-arabic gum combination (GA075 and GA00) and two treatments with the gelatin-cashew gum combination (GC050 and GC00) were investigated, to compare the effect of the presence and absence of the crosslinking agent on the release of the core. The treatments GA075 and GC050 were chosen due to the better oxidative stability afforded to echium oil.

The behavior of the release of the oil and phytosterols from these treatments, under conditions simulating the gastric and intestinal system, is shown in Fig. 2. The core release rates ranged from 24 to 45 and 32 to 76% for the gastric and intestinal fluids,

respectively, in 180 min. Treatments with the presence of SA, presented a higher rate of release of the core in the gastric fluid than the treatments without the phenolic compound, which was not expected and a reasonable explanation cannot be provided for this observation. In the case of the intestinal fluid, no clear trend was detected in the association between the presence or absence of the phenolic compound, and the release of the core.

Tello, Prata, Rodrigues, Sartoratto, and Grosso (2016) studied the controlled release of soybean oil containing paprika oleoresin, in microcapsules obtained by complex coacervation, using gelatin and arabic gum as wall materials, and transglutaminase and glutaraldehyde as crosslinking agents. These authors documented oil release values in the gastric system (pH 1.2, at 37 °C for 2 h) that ranged from 17 to 32.3% for microcapsules with transglutaminase

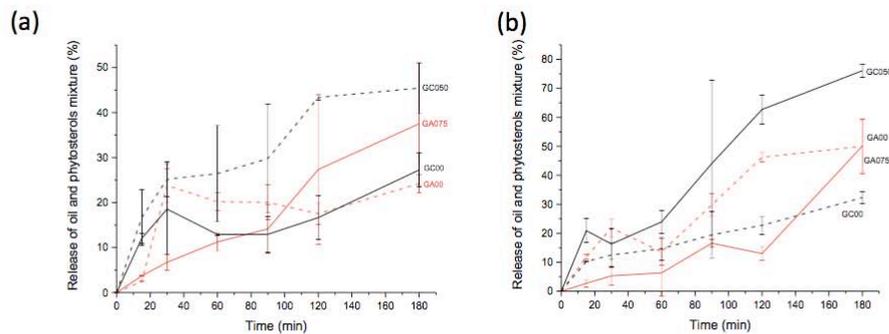


Fig. 2. Simulation of the release of the mixture of oil and phytosterols in (a) gastric fluid and (b) intestinal fluid, during 180 min.

and from 9.1 to 10.3% for microcapsules with glutaraldehyde. These authors obtained lower release rates for paprika oleoresin compared to the values obtained in the release of echium oil and phytosterols, besides a shorter analysis time (2 h). Furthermore, glutaraldehyde is toxic and not allowed for food applications (Comunian & Favaro-Trindade, 2016).

3.2. Yogurt characterization

Based on the results obtained for the different microcapsules, the treatment GA075 was chosen for the application in strawberry flavored yogurt. The yogurt control and yogurt with the added non-encapsulated echium oil, phytosterols and SA, were also produced, totaling three treatments, as mentioned in Section 2.4.

3.2.1. Morphological characterization

Fig. 3 shows the micrographs obtained by optical microscopy and SEM of the three yogurt formulations. It is possible to observe the presence of the droplets of oil and phytosterols, in the treatment with the non-encapsulated bioactive compounds (Fig. 3b). The mixture of phytosterols at room temperature, shows a viscous behavior, however, when at lower temperatures, such as in yogurt (cooling temperature), the phytosterols solidify, which makes the homogenization of the final product difficult. In the case of treatment T3, in which microcapsules were added to the yogurt, it is possible to observe the structure of the microcapsules in the final product (Fig. 3c). It is inferred that the encapsulation of phytosterol helps, even in its application in food, because it allows a homogeneous dispersion of the ingredient. These characteristics were not observed in Fig. 3a, corresponding to the treatment T1 (yogurt control) because it is composed only of the basic formulation (refer to Section 2.4).

SEM was performed to visualize changes in the microstructure of the yogurt gel, due to the variation in the composition of the three treatments. Morphological differences were observed in the yogurt gel structures (Fig. 3d–f), revealing a more open and larger pore sized network was present in treatment T3, corresponding to the yogurt with added microcapsules (Fig. 3f) than the remaining treatments. According to Sah et al. (2016), this can be explained due to the thermodynamic incompatibility between the polysaccharide present in the wall of the microcapsule and the milk proteins. However, this incompatibility did not damage the structure and the physicochemical and sensory characteristics of the final product were retained.

3.2.2. Determination of pH and titratable acidity

The values of pH and titratable acidity, measured as a percentage of lactic acid, for each treatment stored at 4 °C for 30 days, are shown in Table 2. The pH values ranged from 4.14 to 4.05 and from 4.10 to 4.02, for samples stored for 0 and 30 days, respectively. Titratable acidity ranged from 0.789 to 0.826 and 0.782 to 0.802%, for samples stored for 0 and 30 days, respectively.

There was a significant difference ($p < 0.05$) in the values of pH and titratable acidity among treatments stored for the same duration and, also, for the same treatment throughout the 30 days of storage. However, it was not possible to establish an association for pH and titratable acidity as a function of the presence and/or absence of the microcapsules and the non-encapsulated bioactive compounds in the yogurt. These results show that the incorporation of the microcapsules of echium oil, phytosterols and SA affected the pH and the titratable acidity of the yogurt, however, they did not influence the quality of the final product. Thus, pH around 4.0 is ideal for maintaining the structure of the microcapsules intact, preventing the release of the encapsulated materials during the storage of the yogurt.

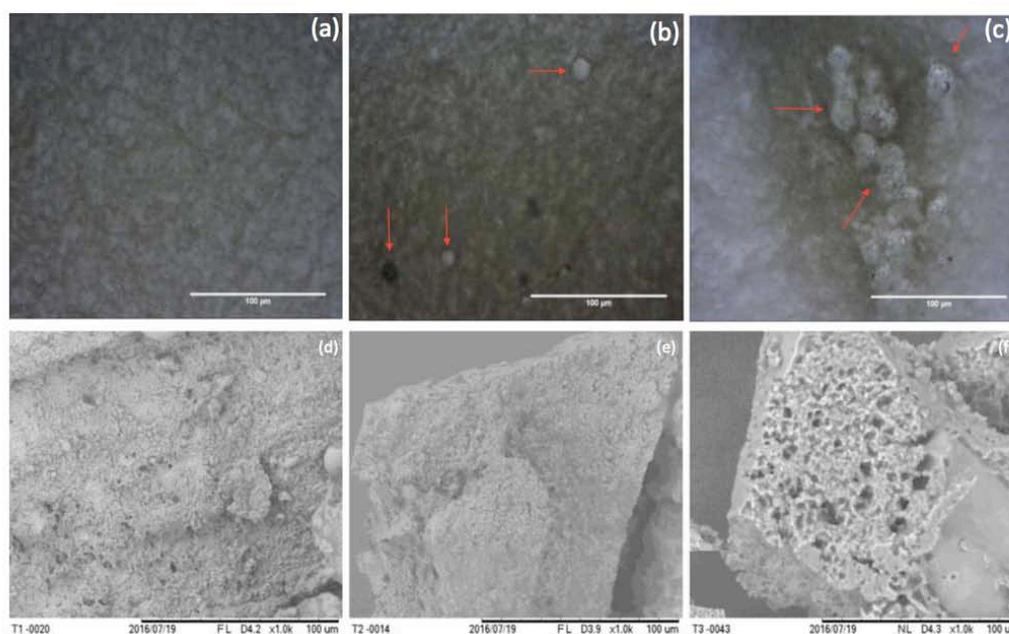


Fig. 3. Optical microscopy of the yogurts (a) T1 (yogurt control - without bioactive compounds added), (b) T2 (yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added), and (c) T3 (yogurt with added microcapsules). Scanning electron microscopy of the yogurts (d) T1 (yogurt control - without bioactive compounds added), (e) T2 (yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added), and (f) T3 (yogurt with added microcapsules).

Table 2
Values of L*, a*, b*, pH and titratable acidity for each treatment in the period of 30 days.

Treatments/ days	0	7	15	21	30
L*					
T1	62.59 ± 0.01 c, A	62.77 ± 0.08 a, A	62.69 ± 0.03 b, A	62.83 ± 0.01 a, A	62.65 ± 0.04 bc, B
T2	62.43 ± 0.03 d, B	62.71 ± 0.03 b, A	62.72 ± 0.02 b, A	62.79 ± 0.01 a, B	62.64 ± 0.02 c, B
T3	62.43 ± 0.10 b, B	62.81 ± 0.23 a, A	62.69 ± 0.04 a, A	62.74 ± 0.02 a, C	62.75 ± 0.04 a, A
a*					
T1	8.99 ± 0.08 c, A	9.18 ± 0.03 ab, A	9.03 ± 0.12 c, A	9.26 ± 0.04 a, A	9.11 ± 0.08 bc, A
T2	8.99 ± 0.04 cd, A	9.14 ± 0.07 ab, A	9.07 ± 0.09 bc, A	9.25 ± 0.04 a, A	8.93 ± 0.10 d, B
T3	8.63 ± 0.14 c, B	9.04 ± 0.08 a, B	8.83 ± 0.09 b, B	8.92 ± 0.07 ab, B	8.94 ± 0.09 ab, B
b*					
T1	-0.89 ± 0.05 c, B	-0.76 ± 0.09 bc, B	-0.79 ± 0.07 bc, B	-0.73 ± 0.06 ab, B	-0.62 ± 0.12 a, A
T2	-0.89 ± 0.08 ab, B	-0.86 ± 0.09 a, B	-1.02 ± 0.08 b, C	-0.93 ± 0.05 ab, C	-0.87 ± 0.09 a, B
T3	-0.62 ± 0.04 b, A	-0.45 ± 0.03 a, A	-0.61 ± 0.07 b, A	-0.53 ± 0.03 ab, A	-0.47 ± 0.08 a, A
pH					
T1	4.14 ± 0.03 a, A	4.13 ± 0.03 a, A	4.16 ± 0.02 a, AB	3.93 ± 0.07 c, A	4.05 ± 0.02 b, A
T2	4.11 ± 0.01 b, B	4.09 ± 0.01 b, B	4.15 ± 0.01 a, B	3.88 ± 0.01 d, A	4.02 ± 0.01 c, B
T3	4.10 ± 0.01 b, B	4.07 ± 0.01 c, B	4.17 ± 0.01 a, A	3.89 ± 0.01 e, A	4.05 ± 0.01 d, A
Titratable acidity (% of lactic acid)					
T1	0.789 ± 0.03 b, A	0.776 ± 0.01 b, B	0.800 ± 0.03 ab, B	0.825 ± 0.01 a, A	0.795 ± 0.01 ab, A
T2	0.799 ± 0.01 bc, A	0.803 ± 0.01 b, A	0.805 ± 0.01 b, B	0.828 ± 0.01 a, A	0.782 ± 0.01 c, A
T3	0.826 ± 0.03 ab, A	0.798 ± 0.01 b, A	0.835 ± 0.00 a, A	0.825 ± 0.01 ab, A	0.802 ± 0.01 b, A

Equal lowercase letters in the same row and capital letters in the same column do not differ statistically at the level of 5% by the Tukey test.

T1: yogurt control (without bioactive compounds addition);

T2: yogurt added of non-encapsulated echium oil, phytosterols and sinapic acid;

T3: yogurt added of microcapsules.

Sah et al. (2016) obtained pH 4.48–4.50 at 1 day of storage and pH 4.23–4.29 at 28 days of storage at 4 °C, for probiotic yogurt enriched with pineapple peel rich in fiber. These values corroborate with those obtained in the current study, for yogurt with the added microcapsules of echium oil, phytosterols and SA.

For yogurt enriched with fish oil microcapsules and stored for 21 days, Tamjidi, Nasirpour, and Shahedi (2011) obtained titratable acidity values ranging from 0.77 to 0.97%. Similar values in the current study were recorded for yogurt with added microcapsules of echium oil, phytosterols and SA but only early on in the storage. According to Tamjidi et al. (2011) and Serra, Trujillo, Guamis, and Ferragut (2009), the decrease in pH and the increase in titratable acidity of the yogurt samples during storage, can be explained by the metabolic activity of the dairy culture used in yogurt production, which implies the fermentation of lactose with the production of lactic acid. These changes in pH and titratable acidity were not observed in yogurts with added microcapsules of echium oil, phytosterols and SA, ensuring that the incorporation of these capsules into yogurt does not interfere with the stability of the product during the evaluated storage duration.

3.2.3. Color

The color parameters L* (brightness), a* (green (-) to red (+)), and b* (blue (-) to yellow (+)), for each treatment stored at 4 °C for 30 days, are presented in Table 2. The total color difference (ΔE) values were obtained, by comparing the values recorded at 0 and 30 days of storage.

The L* values ranged from 62.43 to 62.59 and 62.64– to 62.75, for yogurts at 0 and 30 days of storage, respectively. There was a statistical difference among treatments for the same day and among different days for the same treatment, however, this difference may be considered small. Due to the slight variation in these values, it can be affirmed that all the samples were bright during the analyzed period. However, there was no evident trend of behavior among the samples. Namely, no relation was apparent between the presence and/or absence of microcapsules and non-encapsulated bioactive compounds and brightness values. Sah et al. (2016) obtained L* values ranging from 84.53 to 90.43 and 84.84 to 91.54 at 1 and 28 days storage at 4 °C, for probiotic yogurt

enriched with pineapple peel rich in fiber. The values obtained by these authors were higher than those obtained for the treatments of this work, due to the different compositions, mainly in relation to the presence of fibers.

The a* values ranged from 8.63 to 8.99 and 8.93 to 9.11, for the yogurts stored for 0 and 30 days, respectively. These values originated from the pink/red strawberries used to produce the yogurt. With the small variation presented among the treatments and over the 30 days, it can be reaffirmed that the incorporation of the microcapsules did not interfere with the yogurt coloration compared to the yogurt control.

Likewise, the b* values ranged from -0.89 to -0.62 and from -0.87 to -0.47 for yogurts stored for 0 and 30 days, respectively. There were statistical differences among the treatments for the same time evaluated and also for the same treatment over the 30 days. This difference can be attributed to the compositions of each treatment because the microcapsules are also composed of gelatin and arabic gum as wall materials. However, even though there was a statistical difference in the instrumental color of the final product, this difference did not influence the quality of the yogurt.

Tamjidi et al. (2011) obtained a* values from -2.94 to -1.69 and b* values from 11.12 to 16.87 over the 21-day storage period for yogurt enriched with microcapsules of fish oil. These values are considerably different to those obtained in the current study, for yogurt with the microcapsules of echium oil, phytosterols and SA because the latter yogurt was made with strawberry pulp and carmine dye.

Regarding the total color difference (ΔE), values of 0.32 ± 0.11, 0.26 ± 0.05 and 0.47 ± 0.11 were obtained, for the treatments T1 (yogurt control), T2 (yogurt with non-encapsulated bioactive compounds added) and T3 (yogurt with added microcapsules), respectively. Treatments T1 and T2 were statistically different ($p < 0.5$) to treatment T3. Thus, the addition of microcapsules promoted a greater color change during storage than the yogurt control, probably due to the presence of gelatin and arabic gum as wall materials. However, this change did not interfere with the acceptance of the product, as observed in Section 3.2.5.

3.2.4. Rheological characterization

Rheological characterization analyzes allow studying the deformations and flows of matter, when submitted to a certain stress rate. In the current study, the apparent viscosity of the yogurts decreased with increased rate of deformation during the shear (Fig. 4a and b) and remained stable, without significant difference throughout the storage (Supplementary Table 1). However, there was a significant difference among the treatments from the first day of analysis, with values ranging from 0.526 to 0.689 and 0.539 to 0.663 Pa.s, for the samples stored for 0 and 30 days, respectively, and a deformation rate of 20 s^{-1} . The treatment T3, presented higher values than the treatments T1 and T2, as expected, due to the addition of the microcapsules, which increases the solids content of the final product. This higher viscosity influenced the evaluation of the attribute “texture”, in the sensorial analysis (refer to Section 3.2.5). However, despite this significant difference, the addition of the microcapsules did not influence the quality of the final product.

In the case of the yield stress (Supplementary Table 1), only treatment T1 (yogurt control) showed a significant difference during the 30 days. When comparing treatments on the same day, there was a statistical difference, as expected, considering the varied compositions presented visually different firmness. Also, according to Sah et al. (2016), yield stress is positively correlated to firmness. In the case of consistency index values (Supplementary Table 1), there was a significant difference for treatment T2 over time and among treatments stored for the same duration. It was not possible to establish an association regarding the increase in the consistency index values for treatment T2, however, the difference among the treatments was expected, due to the variation in the compositions and apparent viscosities, as above-mentioned.

The G' values (elastic modulus or storage modulus), which measures the ability of the material to store energy, and G'' (viscous or loss modulus), which measures the ability of the material to dissipate energy, were also obtained, in order to calculate the complex modulus (G^*) for each treatment stored at 4°C for 30 days, as presented in Supplementary Table 1. The G^* values obtained, ranged from 65.20 to 83.45 and 57.99 to 99.18 Pa at 0 and 30 days of storage, respectively. There was no significant difference for treatments T1 (yogurt control) and T3 (yogurt added of microcapsules) during the time analyzed. However, there was a significant difference between treatments on the same day, with higher values for treatment T3. That is, the addition of the microcapsules promoted the formation of a weak gel, as per the other treatments, however, with increased elastic behavior compared to the other yogurt samples.

3.2.5. Consumer acceptance test

The scores obtained for the flavor, color, texture, taste and overall acceptability attributes are presented in Supplementary Table 2. There was a significant difference among the treatments for all attributes, except for color.

For the flavor attribute, the treatment with the added microcapsules (T3) presented similar acceptance to treatment T1 (yogurt control), with average scores of 6.73 and 6.65, respectively, followed by treatment T2 (yogurt with non-encapsulated bioactive compounds added), with a mean score of 6.21. Thus, the incorporation of the encapsulated bioactive compounds into the product improved the flavor in comparison to the addition of their non-encapsulated counterparts because the echium oil may present an unpleasant flavor, after a certain time of exposure to oxygen.

Regarding the color attribute, there was no significant difference in the average scores attributed to the samples. Therefore, the addition of the microcapsules did not influence the visual color of the final product.

In the case of the texture attribute, treatments T1 (yogurt control) and T2 (yogurt with the non-encapsulated bioactive compounds added) presented better acceptance than treatment T3 because the yogurt viscosity increased with the addition of microcapsules, as observed in the rheological characterization (refer to Section 3.2.4). Nevertheless, the yogurt with added microcapsules, obtained a score corresponding to “I liked it lightly”, while the other treatments obtained scores corresponding to “I liked it regularly”, proving that the incorporation of the capsules did not have a negative influence on the acceptance by the tasters.

The texture had a marked influence on the scores obtained for the taste and overall acceptability attributes. In this case, the treatments T1 and T2 showed better acceptance than treatment 3 (yogurt with added microcapsules). However, treatment T3 maintained the score corresponding to “I liked it slightly”, and treatments T2 and T3 received scores corresponding to “I liked it regularly”. Notably, treatment T2 showed droplets of oil and particles of the phytosterol mixture on the surface, promoting the rejection by several tasters.

4. Conclusions

Efficient coencapsulation of echium oil and phytosterols was achieved, using gelatin-arabic gum and gelatin-cashew gum combinations, respectively, as wall materials and the phenolic compound SA, as the crosslinking and antioxidant agent. Specifically, the microcapsules provided oxidative stability to the encapsulated bioactive compounds, and the average particle size and release of the bioactive compounds into the simulated gastric and intestinal

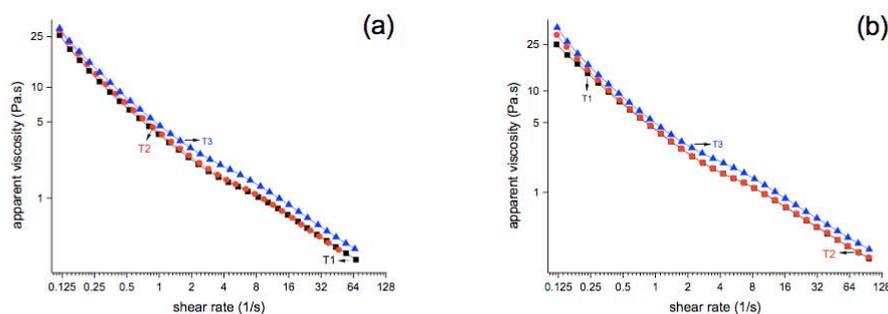


Fig. 4. Effect of storage time on apparent viscosity as a function of deformation rate, for treatments T1 (black curve), T2 (red curve), and T3 (blue curve), at (a) 7 and (b) 30 days of storage. T1 refers to the yogurt control (without bioactive compounds added); T2 refers to yogurt with non-encapsulated echium oil, phytosterols mixture and sinapic acid added, and T3 refers to the yogurt with microcapsules added.

fluids, indicate their suitability for food applications. Additionally, the process of encapsulation by complex coacervation was effective for the application of these bioactive compounds into yogurt, considering that the physicochemical, rheological and sensorial properties of the yogurt with added microcapsules were similar to the yogurt control (without bioactive compounds addition) and superior to the yogurt with the non-encapsulated bioactives added. Furthermore, the improved oxidative stability of the oil, induced by microencapsulation, guarantees the quality of the final product.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.06.071>.

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