Food Research International 54 (2013) 432–438

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

Evaluation of the fatty matter contained in microcapsules obtained by double emulsification and subsequent enzymatic gelation method

Leilane Costa de Conto [a,*], Gabriel Deschamps Fernandes [a], Carlos Raimundo Ferreira Grosso [a], Marcos N. Eberlin [b], Lireny Aparecida Guaraldo Gonçalves [a]

[a] Faculty of Food Engineering, University of Campinas (UNICAMP), 13083-862 Campinas, SP, Brazil
[b] Thomson Mass Spectrometry Laboratory, Institute of Chemistry, University of Campinas, 13083-970 Campinas, SP, Brazil

ARTICLE INFO

Article history:
Received 3 April 2013
Accepted 3 July 2013

Keywords:
Omega-3 polyunsaturated fatty acids content
Microcapsules
Soy protein isolate
Transglutaminase
Extraction methods

ABSTRACT

The objective of this study was to evaluate encapsulated fatty matter using double emulsification and subsequent enzymatic gelation method using soy protein isolate cross-linked by transglutaminase. For this purpose, six extractions methods (acids, alkaline and enzymatic methods) were used. The extraction methods showed differences, total or partial extraction being observed when using the acid method, alkaline enzymatic method and the acid method with direct determination of the fatty acid composition. The presence of triglycerides and ethyl ester was investigated in the microcapsules by easy ambient sonic-spray ionization mass spectrometry (EASI-MS), high performance size exclusion chromatography (HPSEC) and the fatty acid composition as determined by gas chromatography (GC). The microcapsules were shown to contain 1.07 g omega-3 fatty acids / 100 g microcapsules.

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1. Introduction

Epidemiological and nutritional studies suggest that the consumption of omega-3 polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has several benefits for human health, such as anti-carcinogenic activity, anti-inflammatory effects, reduction in the risk of heart disease, and the prevention of osteoporosis and neurological disorders (Alzheimer's disease, Crohn's disease, etc.), also aiding in the reduction of depression (Conto, Grosso, & Gonçalves, 2013; Riediger, Othman, Suh, & Moghadasian, 2009).

According to Rubio-Rodríguez et al. (2010) and Cohen, Norman, and Heimer (1995), fish and microalgae are the traditional sources of EPA and DHA-rich oils, and the production of concentrated methyl or ethyl ester derivatives from fish oil stands out, since these are more stable to oxidation. However, the application of fish oil and its derivatives in food systems is limited by their oxidative instability during storage, presenting off-flavors (Cho, Shim, & Park, 2003). Therefore techniques have been developed aimed at protecting these compounds against oxidation, such as microencapsulation, which simultaneously facilitates incorporation of these ingredients into food formulations (Ackman, 2006).

Various wall materials and encapsulation methods have been tested for the microencapsulation of fish oil. Protein films are generally excellent oxygen and aroma barriers and are used to produce microcapsules using coacervation techniques (Conto et al., 2013). Others techniques that consist of double emulsions and subsequent reticulation with glutaraldehyde or heat gelation have also been investigated (Lee & Rosenberg, 2000). Although proteins from plant sources display emulsifying and/or film-forming properties, there are few proteins which have been studied as carrier or wall materials in the microencapsulation applications (Nesterenko, Alric, Silverstre, & Durrieu, 2013). This is because their uses for sensitive ingredients are limited by their heat instability and organic solvents sensibility. However, the use of reticulating agents such as enzyme transglutaminase (TGase) to convert the proteins into stabler forms could lead to an increment in their industrial applications (Babiker, 2000).

Many studies have been investigating the microcapsules structural characteristics, process conditions, applications and release methods (Lamprecht, Schafer, & Lehr, 2001; Polovarapu, Oliver, Ajlouni, & Augustin, 2011; Velasco, Dobarganes, & Márquez-Ruiz, 2000). This release can occur by different methods, such as fracture, diffusion, dissolution or degradation. Fractures or breaks can be achieved by pressure, shear and sonication. Diffusion occurs by concentration gradient or attractive forces between chains. Dissolution occurs by the action of heat or solvents. Finally, biodegradation occurs due to susceptibility of the wall material to certain compounds, such as enzymes (Mascarenhas, 2010). However, in this context, few works have been devoted to define the integrity of the core material after its release, which can be accompanied by specific tests, such as gas chromatography — GC and mass spectrometry — MS (Yeo, Bellas, Firestona, Languer, & Kohane, 2005). Another possible analysis is the content of lipid compounds using high performance liquid chromatography size exclusion — HPSEC (Velasco

* Corresponding author. Tel.: +55 019 32891186; fax: +55 019 32893617.
E-mail address: leilanedeconto@gmail.com (L. Costa de Conto).

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http://dx.doi.org/10.1016/j.foodres.2013.07.013

et al., 2000). EASI-MS has been extensively used for the analysis of oils and biofuels (Riccio et al., 2010), which makes it a new alternative for determining the integrity of the encapsulated material.

The objective of the present research was to study six extraction methods (acids, alkaline and enzymatic methods) and the composition of the lipid matter extracted of the encapsulated core material by an enzymatic gelation process, using soy protein isolate as the wall material, the enzyme transglutaminase as cross-linker agent and fish oil ethyl ester (EE) as the core material.

2. Materials and methods

2.1. Materials

Fish oil ethyl ester (EE) (62% EPA + DHA, Vital Atman, Uchoa, SP), corn oil (Qualitá, Bunge Alimentos S.A., Gaspar, SC, Brazil), Span 80 (Surfactant sorbitan mono-oleate, Croda do Brasil Ltda, São Paulo, SP, Brazil), soy protein isolate (The Solae Company, Porto Alegre, RS, Brazil, 88% protein w.b.), and active transglutaminase TG-S® microbial (TGase) (Ajinomoto, Limeira, SP, Brazil).

2.2. Methods

2.2.1. Microcapsule production

Microcapsules were produced using the enzyme transglutaminase (TGase) as the gelling agent, using the methodology adapted to Cho et al. (2003). The microcapsules were prepared using a double-emulsification process followed by enzymatic gelation like described below:

(a) A 10% solution of the protein (SPI) was first prepared in 100 mL deionized water, and 0.025% (2.7 UA/g of protein) of the enzyme TGase added at room temperature and pH 6.0 by adding 0.5 mol/L and 0.1 mol/L HCl.
(b) The first emulsion was formed by mixing the EE with the initial protein solution for 10 min in a basic T18 Ultraturrax (Ika-Werk, Brasília, DF) at 11000 rpm. The ratio of wall material to core material was 2:1 (*w*/*w*).
(c) 400 mL of corn oil, pre-heated to 50 °C was used to form the second emulsion, adding the surfactant (1%) Span 80.
(d) Immediately after forming the first emulsion, this was slowly added to the second emulsion with mild magnetic stirring. After mixing, the double-emulsion was maintained at 37 °C for 17 h in a chamber (BOD — Biochemical Oxygen Demand incubator), this time was necessary to form capsules (pretest not showed).

The microcapsules were filtered and washed with ethanol three times and then once with petroleum ether, air-dried, frozen for 12 h and then freeze-dried in a Pirani 50 freeze-dryer (Edwards, USA) for 24 h.

2.2.2. Free lipid content

The free lipid content was extracted using the methodology described by Velasco et al. (2000), with some adaptations of the scale.

To determine the free lipid content, 20 mL of petroleum ether were added to 0.8 g microcapsules and stirred for 15 min at 25 °C. The microcapsules were then filtered through anhydrous Na_2SO_4, the solvent evaporated and the samples dried with nitrogen.

The microcapsules recovered after this determination, were used to determine the extraction methods of core material, and its constitution.

2.2.3. Encapsulation yield and efficiency

The encapsulation yield was determined from the mass of freeze-dried microcapsules (d.b.) obtained divided by the total initial mass used (EE + SPI, d.b.).

The encapsulation efficiency was obtained after the acid extraction of the core, where 0.2 g of sample was added to 4.5 mL of boiling deionized water and 5.5 mL 8 M hydrochloric acid, and maintained in a boiling water bath for 30 min (until complete degradation of the wall material). The mixture was subsequently filtered and washed with 10 mL boiling deionized water. The filter papers with the hydrolyzed samples were dried in an oven and then extracted according to the methodology for the determination of the oil content (AOCS, 2009) of compounds with high protein content. The encapsulation efficiency was determined using Eq. (1) as described by Davidov-Pardo, Roccia, Salgado, León, and Pedroza-Islas (2008).

$$\%\,\text{Encapsulation efficiency} = \frac{(\text{Total lipid content} - \text{free lipid content}) \times 100}{\text{Total lipid content}} \quad (1)$$

2.2.4. Microcapsule morphology

The morphology of the microcapsules was determined using a model TM 3000 high vacuum scanning electronic microscope (SEM) (Hitachi, Tokyo, Japan). Three samples were arranged on aluminum stubs containing a double-faced copper tape, using carbon glue to secure the material. The best fields were selected, where the microcapsules were isolated.

2.2.5. Size distribution and average size of the microcapsules

The average size and size distribution of the microcapsules freeze-dried were determined using a Mastersizer 2000 (Malvern Instrument LTDA, Worcestershire, UK). Three readings were made giving a total of nine repetitions, with agitation at 3500 rpm and ultrasound to disperse the particles in water.

2.2.6. Extraction of the encapsulated fatty matter

The microcapsules obtained were subjected to six different extraction methods to extract the encapsulated material, so as to determine which method extracted more material with higher quality. The amount of encapsulated fatty matter extracted (weight) was determined using Eq. (2):

$$\%\,\text{Encapsulated Matter} = \frac{g\,\text{fatty material extracted} \times 100}{g\,\text{microcapsules}} \quad (2)$$

In parallel, the physical degradation of the microcapsule wall by each method was determined by optical microscopy (eclipse E800, Nikon, Tokyo, Japan), photographing the images using a digital camera controlled by the image-pro plus 6.0 program, using the 12×, 25× and 40× objectives.

Table 1 shows the methods difference and the code adopted for the samples, the descriptions of methods follow below:

TM1 = alkaline extraction method, using the methodology described by Velasco et al. (2000), where 0.1 g of microcapsules free of surface oil were dispersed in 1 mL deionized water at 65 °C with stirring, and 0.2 mL 25% NH_4OH then added and heated at 65 °C for a further 15 min. The solution was cooled, transferred a beaker by a separating funnel been washed with 10 mL ethanol. The encapsulated material was extracted three times in the following sequence: a) 50 mL of a 1:1 (*v*/*v*) solution of ethyl ether:petroleum ether; b) 5 mL of ethanol, 30 mL of a 1:1 (*v*/*v*) solution of ethyl ether:petroleum ether; c) 30 mL of a 1:1 (*v*/*v*) solution of ethyl ether: petroleum ether. After filtration through anhydrous Na_2SO_4, the solvent was evaporated off with nitrogen to constant weight.

TM2 = enzymatic extraction method using the methodology described by Mascarenhas (2010) with some adaptations, where 0.1 g of microcapsules free of surface oil were dispersed in 4 mL of enzyme solution containing 0.5% Protex 6 L liquid bacterial protease (Genencor, Danisco, Cotia, SP, Brazil) and 0.5% liquid bacterial

Table 1
Description of methods utilized to extract lipid content of the microcapsules obtained by enzymatic gelation.

	TM1	TM2	TM3	TM4	TM5	TM6
Extraction method	Alkaline	Enzymatic	Acid	Alkaline enzymatic	Mechanical/ microwaves	Acid with direct fatty acid determination
Authors	Velasco et al. (2000)	Mascarenhas (2010)	AOCS (2009a,b)	Lamprecht et al. (2001)	Polovarapu et al. (2011)	Ruben and Barclay (1999)
Microcapsule (g)	0.1	0.1	0.2	0.1	0.1	0.3
Temperature (°C)	65	50	boil	40	50	110
Time (min)	15	60	30	overnight	10	60
Breaker capsule agent	NH4OH	Protex 6 L liquid bacterial protease/bacterial α-amylase	hydrochloric acid	NH_4OH/Savinase®	–	methanol/sulfuric acid
Extraction solvents	ethyl ether; petroleum ether; ethanol	petroleum ether	petroleum ether; hexane	hexane	hexane	hexane

α-amylase (Thermamyl 2X, Novozymes, Araucária, PR, Brazil) at pH 8, obtained by adding 0.1 mol/L NaOH. The mixtures were placed in a water bath at 50 °C for 1 h, mixing the contents of the tubes each 10 min for 20 s. The contents were then transferred to a separating funnel and 20 mL of a 1:1 (*v*/*v*) solution of petroleum ether: deionized water added, thus starting the phase separation process. The funnels were left to rest overnight, after which it was possible to separate the aqueous phase from the residue of wall material. The organic solvent phase was run into a weighed flask and the solvent evaporated off in nitrogen to constant weight.

TM3 = acid extraction method as described in item 2.2.2 up to the filtering step, where the samples were filtered without the aid of boiling water and simply transferred and dried in nitrogen for cold extraction. Cold extraction was carried out by adding 20 mL of a 1:1 (*v*/*v*) solution of petroleum ether:hexane, the solvents and fatty material being retained in weighed test tubes, evaporating the solvents off with nitrogen to constant weight.

TM4 = alkaline enzymatic extraction method following the methodology described by Lamprecht et al. (2001), where 0.1 g of microcapsules free of surface oil, were dispersed in 5 mL of a 2 M solution of NH_4OH plus 0.5 mL of the protease Savinase® (Novozymes, Araucária, PR, Brazil) and incubated overnight at 40 °C without shaking. Ten milliliters of hexane were then added and the solvents and fatty material retained in 50 mL weighed test tubes, evaporating off the solvents with nitrogen to constant weight.

TM5 = mechanical extraction method assisted by microwaves following the extraction methodology described by Polovarapu et al. (2011), where 0.1 g of microcapsules free of surface oil were heated in a microwave oven (commercially available 352 W) at maximum power for 10 min, and then macerated in a pestle and mortar. The mixture was subsequently transferred to a 50 mL test tube, washed and dispensed in 2 mL deionized water at 50 °C, mixed using a vortex mixer, 4 mL of hexane added and manually shaken, and finally centrifuged at 1000*g* for 10 min. The hexane layer was separated into a 70 mL tube and a further 4 mL of hexane was added, it was manually shaken and centrifuged again at 1000*g* for 10 min. The hexane layer was removed back to the initial test tube and the solvent evaporated off with nitrogen to constant weight.

TM6 = acid extraction method with the direct determination of the fatty acid composition using the methodology described by Ruben and Barclay (1999). Three microcapsule samples (300 mg) were weighed into 50 mL test tubes and 4 mL of a solution of methanol containing 4% sulfuric acid added and mixed in a vortex for 15 s. The mixtures were deaerated using nitrogen gas and mixed again. The samples were then left on a digestion block for 1 h at 110 °C, shaking every 20 min. The samples were then cooled to room temperature and 1 mL of deionized water plus 1 mL hexane added, shaken for 20 seconds and centrifuged at 1000*g* for 3 min. The supernatant was used to determine the fatty acid composition. This method was only evaluated with respect to extraction method of the core by physical degradation of the microcapsule wall and preservation of the fatty acids, considering that the final product was obtained directly in the form of the fatty acid methyl esters.

2.2.7. Constitution of the free and encapsulated material

2.2.7.1. Easy ambient sonic-spray ionization mass spectrometry (EASI-MS). The encapsulated material was extracted according to Lamprecht et al. (2001) — TM4 for analysis by easy ambient sonic-spray ionization mass spectrometry (EASI-MS) and mass spectra obtained in the positive mode using a mono-quadrupole mass spectrometer (LC/MS 2010, Shimadzu), equipped with an EASI source. This technique is an ambient desorption/ionization method (Alberici et al., 2010) that has been extensively used for the analysis of oils and biofuels (Riccio et al., 2010).

Typical EASI-MS conditions were: N_2 gas nebulization at a rate of 3 L min^{-1}, surface angle ~ 30º, methanol at a rate of 20 μL min^{-1}, and 2 μL of sample placed directly on the Kraft paper surface. Mass spectra were accumulated for 30 s in the *m*/*z* range from 50 to 1000 as described by Simas et al. (2010).

2.2.7.2. Determination of the fatty acid constituents by gas chromatography (GC). The encapsulated material was extracted as described by Ruben and Barclay (1999) — TM6 in order to analyze the fatty acid composition.

The fatty acid composition was determined according to the AOCS (2009) by capillary gas chromatography (GC) using the Agilent 6850 Series GC system, as follows: Agilent DB-23 column (50% cyanopropyl) — methylpolysiloxane, length 60 m, internal diameter 0.25 mm, film 0.25 mm. The chromatographic operational conditions were: column flow = 1.0 mL/min; linear velocity 24 cm/s; detector temperature of 280 °C; injector temperature of 250 °C; oven temperature of 110 °C-5 min/110–215 °C–5 °C/min/215 °C = 34 min; carrier gas: helium; volume injected 1.0 μL; split 1:50.

The fatty acid compositions of one sample of EE and one sample of the corn oil used in the second emulsion were also analyzed to determine which fatty acids might be found inside of the microcapsules. The methyl esters of the fatty acids in these samples were then prepared using the methodology described by Hartman and Lago (1973), adapted for use on a micro-scale, using the equipment conditions previously described.

2.2.7.3. High performance size-exclusion chromatography to determine the lipid group constituents (HPSEC). The extraction of the encapsulated

material for the analysis of the lipid group composition was carried out according to TM4 — Lamprecht et al. (2001).

The presence of EE in the encapsulated and free materials was evaluated according to Moura, Gonçalves, Grimaldi, Soares, and Ribeiro (2006), using HPSEC in a Perkin Elmer 250 liquid chromatograph equipped with a Sicon Analytic refractive index detector and 300 × 7.8 mm JORDI GEL DVB columns with 500 Å and 100 Å. The mobile phase was tetrahydrofuran (THF) with a flow rate of 1 mL/min, and the sample was dissolved in 1% THF, injecting 20.0 μL.

2.2.8. The encapsulated fatty matter and EE contents

Encapsulated material was extracted from the microcapsules using the method described by Lamprecht et al. (2001), with an adaptation to the solvent washing step, where the material was transferred to separating funnels with 10 mL of ethanol. The core material was extracted three times using the following sequence: a) 50 mL of a 1:1 (*v*/*v*) solution of ethyl ether:petroleum ether; b) 5 mL of ethanol, 30 mL of a 1:1 (*v*/*v*) solution of ethyl ether:petroleum ether; c) 30 mL of a 1:1 (*v*/*v*) solution of ethyl ether:petroleum ether for better extraction of the material, and determined using Eq. (2) as cited in Section 2.2.6.

The encapsulated EE content was determined by multiplying the value obtained for the per cent EPA + DHA in item 2.2.7.2 by the value for the encapsulated fatty material.

2.2.9. Statistical analysis

The results obtained were submitted to a statistical analysis using the analysis of variance (ANOVA), analyzing the difference between the means using Tukey's means comparison test with the software Statistica 7.0 (StatSoft, Inc., Tulsa, OK, USA) and a 95% level of significance ($p \leq 0.05$).

3. Results and discussion

3.1. Encapsulation yield and efficiency

The results obtained for encapsulation yield and efficiency were 72.26% (± 2.65) and 56.41% (± 3.42), respectively. Davidov-Pardo et al. (2008) obtained 65% encapsulation efficiency for capsules produced by a similar process to that used in the present study. The lower values obtained in the present study could be explained by losses during the formation of the double emulsion or losses during washing of the microcapsules (Cho et al., 2003). Other option is the losses during the incubation time, that was longer than similar works that used 4 h, thus keeping the samples more time in contact with a lipophilic emulsion (17 h).

3.2. Free lipid content

Davidov-Pardo et al. (2008) found values for free fish oil of above 5% (exact value not specified) when working with soy protein isolate in an enzymatic gelation process by a similar conditions, just changing the incubation time (4 h). Velasco et al. (2000), working with microcapsules obtained in a freeze-drying process using mono-hydrated D-lactose and sodium caseinate, obtained results of 9.3 and 8.7 g/100 g, respectively, for microcapsules containing fish oil and sunflower oil. The average result observed in this study to free lipid content was 17.71% (± 0.33). This higher value could be justified by the size of the droplet of microcapsules and the longer time of microcapsules contact with a lipophilic emulsion.

3.3. Microcapsule morphology

Fig. 1 shows the micrograph of the microcapsule produced by enzymatic gelation. Note that the surface of the microcapsules was circular, rough and with smaller particles adhered to the surface. Tang and Li (2012) also observed the presence of roughness on the surface of the microcapsules produced with soy protein isolated by spray drying, justifying this fact by unequal shrinkage of the capsule during the drying process and / or the high protein content of its wall.

3.4. Size distribution and mean size of the microcapsules

The particle size distribution followed a unimodal distribution, with a tendency to normality, as shown in Fig. 1. The value of mean size particle obtained was 76.06 μm (± 6.46). Cho et al. (2003) obtained microcapsules with a narrow particle-size range (30 to 60 μm) with a relatively uniform distribution in the similar process conditions.

3.5. Extraction of the encapsulated fatty matter

According to Kus (2009), food matrices typically undergo treatment for the extraction and fatty acid analysis; these treatments can use alkali and acid to solubilize the proteins or the use of specific enzymes to hydrolyze proteins and polysaccharides.

Fig. 2A shows the effect of each extraction method tested, as shown by optical microscopy. It can be seen that only TM3 showed complete degradation of the wall material, and TM4 and TM6 showed partial degradation, the latter with the visible presence of free oil (indicated by arrows). The methods TM2 showed intact microcapsules. TM1 showed the largest value for the extraction of fatty matter, indicating the presence of ruptured capsules and free oil (figure not presented). TM5 presented aggregation of the microcapsules, possibly due to the action of the microwaves and maceration applied in this method.

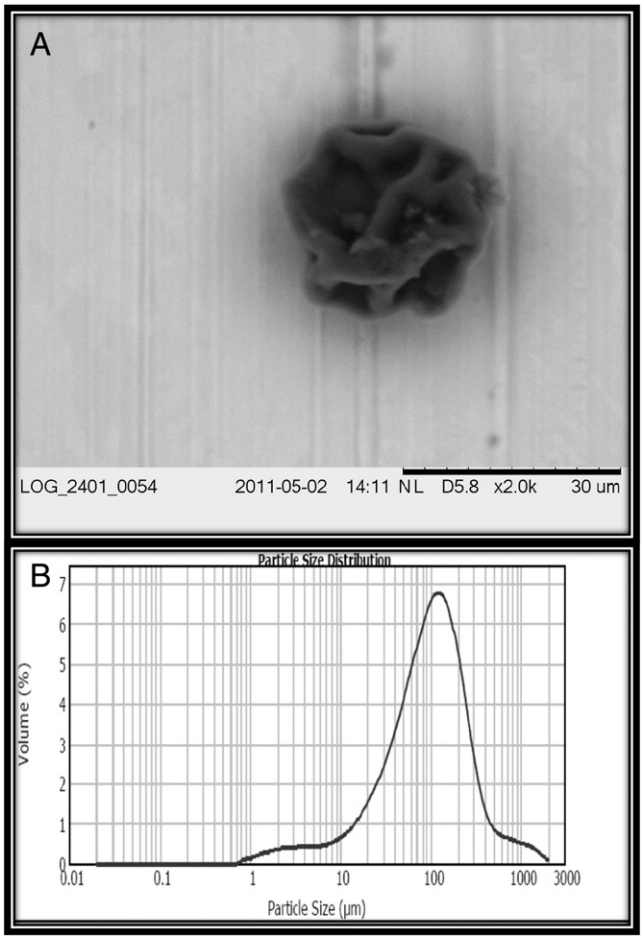

Fig. 1. Scanning electronic microscopy (A) and size distribution (B) of the particles of the EE microcapsules produced by enzymatic gelation.

Mascarenhas (2010) found results up to 76% of encapsulated material after full visualization of wall degradation by the methodology described as TM2, when used gelatin and gum Arabic as wall material to encapsulate linseed oil by complex coacervation. Lamprecht et al. (2001), obtained 15.7% to 55.2 % encapsulation rates EPA ethyl ester capsules produced by complex coacervation of gelatin and gum Arabic under different process conditions, utilizing the TM4. The results obtained in this study for this methodologies (3.37 and 3.77 to TM2 and TM4, respectively) were much lower, and could be explained by different proteins utilized, been gelatin compound constituted by linear proteins and soy proteins by a mixture of globular proteins, less accessible to enzymes.

Velasco et al. (2000), used a method similar to TM1, obtained results around 70% microencapsulation oil, working with fish oil encapsulated into milk proteins by freeze-drying. The values obtained for the encapsulated fatty matter extracted by TM1 (alkaline degradation) and TM3 (acid degradation) did not differ statistically ($p > 0.05$), and the values obtained by the other methods varied from these two ($p \leq 0.05$) but not from each other (Fig. 2B). Although acid degradation (TM3) presented elevated values for the extraction of the encapsulated material and total degradation of the microcapsule walls, it cannot be used for the steps of evaluating the encapsulated material, since this method degrades the fatty matter. However, this method is more adequate for analyses such as to measure the encapsulation efficiency.

The alkaline enzymatic degradation method (TM4) showed lower recovery of the encapsulated fatty matter, which may be due to lower temperature utilized in this method. The acid degradation method with direct determination of the fatty acids maintained the fatty acids present unaltered, and is therefore recommended for the analysis described in Section 2.2.7.3.

Kus (2009) testing acids and alkaline methods to extract polyunsaturated oil of food matrixes concluded that alkaline method was more reliable than the acid method, because they use milder reagents, not compromising the lipid material.

3.6. Constitution of the encapsulated and free fatty matter

3.6.1. Easy ambient sonic-spray ionization mass spectrometry

Fig. 3 shows the mass spectra obtained in the analysis of the EE components (A) and fatty materials extracted (B). It can be seen that the compounds found are due to a mixture of lipid compounds, a fact indicating the presence of corn oil from the second emulsion on the inside of the microcapsules, as shown by the detection of DHA (m/z 379) and OOLn (C54:5, m/z 903).

Ions of the ethyl esters $[EE + Na]^+$ of EPA (m/z 353) and of DHA (m/z 379) were identified in Fig. 3A. According to Simas et al. (2010), the main triacylglycerol ions identified in B were PLL (C52:4, m/z 877), PLO (C52:3, m/z 879), POO (C52:2, m/z 881), LLL or OLLn (C54:6, m/z 901), OLL or OOLn (C54:5, m/z 903), OOL (C54:4, m/z 905) and the presence of oxidation products $[TAG + OOH + Na]^+$ m/z 935 (OLL), where: O = oleic acid, L = linoleic acid, Ln = linolenic acid and P = palmitic acid. These interpretations can be confirmed together with the results obtained for the fatty acid composition (see below).

3.6.2. Determination of the fatty acid constituents by gas chromatography

In the gas chromatographic analysis of the fatty acids, the values for EPA and DHA in the samples after extraction were much lower than expected, since the EE presented a total of 61.56% for EPA + DHA,

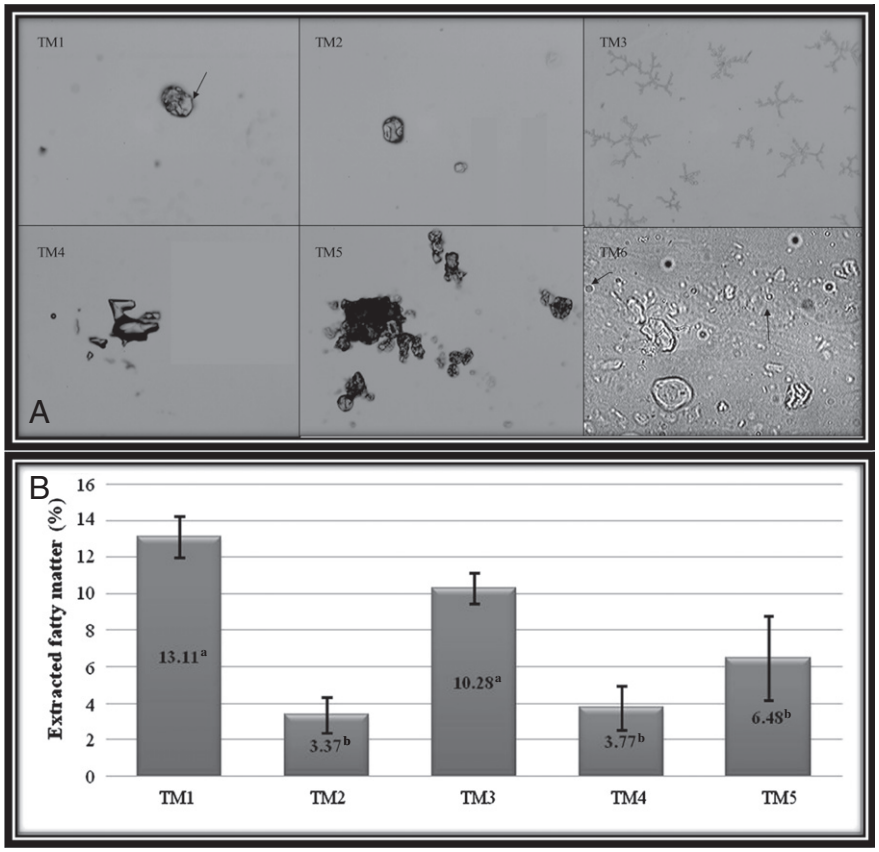

Fig. 2. Micrographs of the fish oil ethyl ester microcapsules after the wall degradation tests and the respective results for the fatty matter extracted. Where: TM1 = alkaline degradation; TM2 = enzymatic degradation; TM3 = acid degradation; TM4 = alkaline enzymatic degradation; TM5 = mechanical degradation; and TM6 = acid degradation with direct determination of the fatty acid composition.

whereas value found after extraction by GC was 7.6% (± 0.5%) for EPA + DHA. It was probably by the incorporation of corn oil into the capsules, which decreased EPA + DHA concentration, as well as a relevant increase in the values for the principal fatty acids found in corn oil, such as oleic acid (C18:1): 31.54%; and linoleic acid (C18:2): 40.97%. These data also confirm the presence of corn oil on the inside of the microcapsules. Moura et al. (2006) found a value of 34.87% for EPA + DPA + DHA in fish oil ethyl esters. The values obtained could be explained by losses during the formation of the double emulsion or losses during washing of the microcapsules (Cho et al., 2003). Also could be explained by losses during the incubation time (17 h).

3.6.3. High performance size-exclusion chromatography of the lipid group constituents

According to Moura et al. (2006), in studies aimed at obtaining fish oil ethyl esters, the HPSEC technique allows the separation the esters from the other compounds present in the reaction medium according to their molar mass, being the order of elution triacylglycerols, diacylglycerols, monoacylglycerols, esters and free fatty acids.

In the present study, triacylglycerols were present in the greatest proportions with values over 60% and 21.34% for ethyl esters, facts which indicates the presence of corn oil from the second emulsion inside the microcapsules.

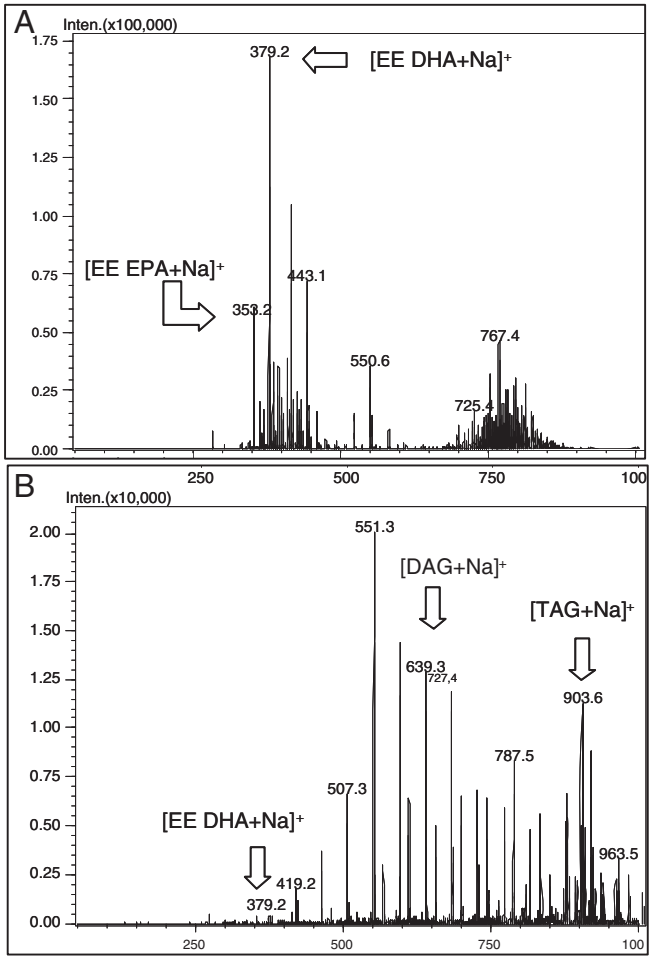

Fig. 3. EASI(+)-MS from the samples of the original raw material (ethyl ester—A) and extracted lipid material (B) of microcapsules produced by enzymatic gelation, showing the Triacylglycerols (TAG), diacylglycerols (DAG), EPA ethyl esters (EE EPA), DHA ethyl esters (EE DHA), and dimmers.

3.7. The encapsulated fatty matter and EE contents

The microcapsules produced with 5% EE at 11,000 rpm with 10% SPI showed the largest amounts of encapsulated EPA + DHA, with values of 7.60 g EPA + DHA/ 100 g lipid matter, indicating that the use of a smaller concentration of surfactant was influenced the quality of the fatty matter encapsulated using the enzymatic gelation process.

Microcapsules produced with 5% EE at 11,000 rpm with 10% SPI, these also showed the highest values for EPA (0.65 g / 100 g microcapsules) and DHA (0.42 g / 100 g microcapsules), giving a total of 1.07 g omega-3 fatty acids / 100 g microcapsules. Therefore, according to the Brazilian National Agency for Sanitary Vigilance (ANVISA, 2012), 3.74 g of the microcapsules would be required per portion or in 100 g or 100 mL of food, in order to warrant the appeal as omega-3 source, since, according to the legislation, a food product must contain at least 0.04 g EPA and/or DHA per portion or per 100 g or 100 mL, to permit this allegation (ANVISA, 2012).

4. Conclusions

The tests for microcapsule wall degradation showed differences between the various methods, the highest values for extracted fatty matter being obtained by alkaline (TM1) and acid (TM3) degradation. Total wall degradation was visible for the acid degradation method (TM3), and partial degradation for alkaline enzymatic degradation (TM5) and acid degradation for the determination of the fatty acid composition (TM6).

The presence of triacylglycerols inside the microcapsules was revealed indicating that corn oil from the second emulsion was encapsulated. For the microcapsules produced with 5% EE at 11,000 rpm with 10% SPI, 3.74 g of microcapsules would be required per portion or in 100 g or 100 mL of food for the product to warrant the appeal of having a functional property, based on the regulation of the National Agency for Sanitary Vigilance (ANVISA, 2012), Brazil.

Acknowledgments

The authors are grateful for the financial support from the Brazilian Governmental Agency, CNPq, in the form of a doctorate scholarship for the first author, FAPESP, and to the following companies, who supplied the raw materials used in this research: Vital Atman, The Solae Company, Croda do Brasil LTDA and Ajinomoto.

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