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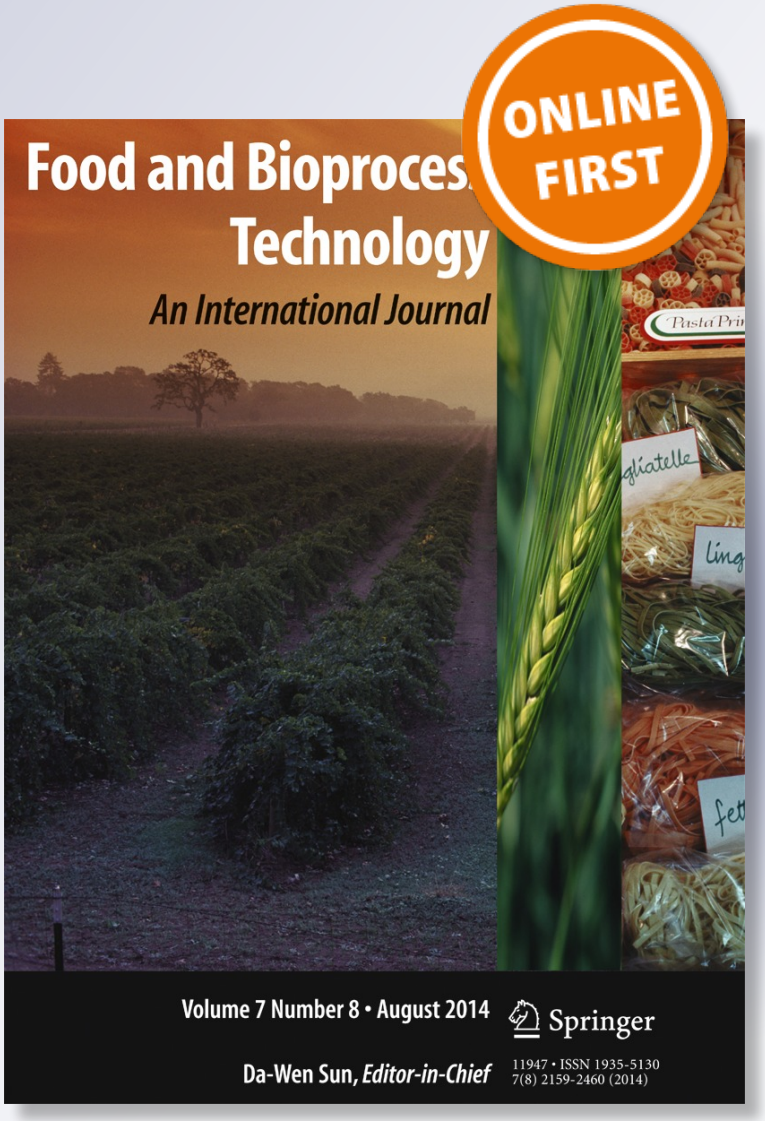
Food and Bioprocess Technology
An International Journal

ISSN 1935-5130

Food Bioprocess Technol
DOI 10.1007/s11947-014-1367-x



Springer

Food Bioprocess Technol
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ORIGINAL PAPER

Physical Properties and Oxidative Status of Concentrated-from-Fish Oils Microencapsulated in Trehalose/Sodium Caseinate Matrix

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Received: 19 April 2014 / Accepted: 30 June 2014

Abstract Foods supplemented with omega-3 fatty acids have attracted much attention in the past decade. However, it is difficult to protect polyunsaturated fatty acids from oxidation. Microencapsulation is a technological process used with the aim to protect oils against oxidation or chemical deterioration, to mask unpleasant flavors or retain aromas, and/or to powder polyunsaturated fatty acids for food fortification purposes. The objective of this study was to analyze physical properties and oxidation status of microencapsulated concentrated-from-fish oils. Powders were prepared from emulsions formulated with 10 wt.% of concentrated-from-fish oils as fat phase and 20 or 30 wt.% trehalose solution that also contained 0.5, 2.0, or 5.0 wt.% sodium caseinate as aqueous phase. Encapsulation efficiency was higher for powders coming from 20 wt.% trehalose emulsions, and the percentage of retention of core material increased with increasing sodium caseinate concentration. The powder prepared from 20 wt.% trehalose and 5 wt.% sodium caseinate showed the highest retention of core material. This powder had lower water content and an amorphous matrix. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry method, used for this new application, allowed proving that trehalose/sodium caseinate matrix was efficient for microencapsulation of polyunsaturated oils and that concentrated-from-fish oils was protected from oxidation in powder form. Spectra were very similar to the original oil without any treatments. Most likely, the oxidation products found when core material was extracted were formed during extraction steps.

Keywords Microencapsulation · Freeze drying · Retention · Particle size distribution · Concentrated-from-fish oils · MALDI-TOF MS method

Introduction

Foods supplemented with omega-3 fatty acids, and especially with long-chain polyunsaturated fatty acids such as docosahexanoic acid (DHA) and eicosapentanoic acid (EPA) from marine sources, have attracted much attention in the past decade. Nutritionists consider the fortification of foods highly desirable and safe, and epidemiological studies suggest that a diet high in DHA and EPA may exert a variety of beneficial health effects such as prevention from atherosclerosis and protection against arrhythmias (Anderson and Ma 2009). However, due to the high degree of unsaturation, it is difficult to protect DHA and EPA from oxidation. Microencapsulation is a technique whereby liquid droplets or solid particles (called core materials) are packed into continuous individual shells (called matrix). This is a technological process used with the aim to protect oils against oxidation or chemical deterioration, to mask unpleasant flavors or retain aromas, and/or to powder polyunsaturated fatty acids for food fortification purposes (Velasco et al. 2003). There are several methods for encapsulating sensitive materials. Spray

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Published online: 10 July 2014

drying is the most common method as the cost of drying is less than freeze drying. Spray drying provides a very large surface area, which enhances oxidation if the wall material is not thick or dense enough to provide a good oxygen barrier. Freeze drying occurs at a low temperature from the frozen state avoiding any water phase reactions and most oxidation because of the vacuum. Thus, it is the preferred method for the flavor industry and is a good choice for oils rich in DHA and EPA. Products obtained in this way are usually stored under a protective atmosphere like nitrogen and typical food applications are low-moisture foods like instant beverages, infant formula, or bread mix.

Although there are a high number of analytical methods available to evaluate lipid oxidation, selection of suitable methods for microencapsulated systems is a difficult task since many factors must be considered. A number of studies on oxidation of fish oil microencapsulated in different matrices and with different methods have been performed (Anwar and Kunz 2011; Drusch et al. 2006; Polavarapu et al. 2011; Tamjidi et al. 2012; Drusch et al. 2012). In those studies, lipid oxidation was monitored through the analysis of hydroperoxide content or/and volatile secondary lipid oxidation products concentration. Some of the reported results are difficult to explain, and others showed a limited description of oxidation products, i.e., oxidation was followed evaluating only one product.

Several authors studying encapsulation of unsaturated lipids have reported that encapsulated oil remained unoxidized while extractable oil underwent rapid oxidation (Shimada et al. 1991; Grattard et al. 2002; Liao et al. 2012; Ng et al. 2013). The methodology used in those studies followed the oxidation of the oil within the matrix without extracting the core material or releasing lipids without using steps that involved heat or agitation for a long time. Although these methods avoided lipid oxidation during sample preparation, some of them are only applicable to specific systems. Picariello et al. (2009) developed a powerful matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) method to analyze lipid mixtures. They simulated deep-frying conditions stressing sunflower and olive oils using heat and described oxidation products formed in those edible oils during heating. Although powerful, this method has not been used yet for encapsulated products. The aim of the present work was to study physical properties and oxidation status of a concentrated-from-fish oils microencapsulated in a trehalose/sodium caseinate (NaCas) matrix, kept at low relative humidity. Oxidation was studied by a MALDI-TOF MS method performed in situ (without extracting the core material). Powders obtained from emulsions with different compositions were analyzed for water content, encapsulation efficiency, matrix crystallinity, and oxidation products. This is a new application of the MALDI-TOF MS method, which was developed for bulk fat.

Materials and Methods

Starting Materials

α-D-Glucopyranosyl-(1-1)-α-D-glucopyranoside (α,αtrehalose dihydrate) from Hayashibara Co., Ltd., (Hayashibara, New York, USA) was used without any further purification. Deionized water (18 mΩ; Milli-Q Water System, Millipore Corporation, Billerica, MA, USA) was used for all experimental work. NaCas was obtained from ICN Biomedical, Inc. (Aurora, Ohio, USA). Fat phase was a commercial concentrated from blends of fish oils supplied by SPES company (Santiago, Chile), whose main fatty acids were identified as C14:0 (11.81 %), C15:0 (2.64 %), C16:1 (2.08 %), C17:0 branched (2.16 %), C17:0 linear (2.39 %), C18:0 (6.94 %), C20:5 (10.90 %), C20:4 (4.58 %), C20:2 (3.66), C20:1 (7.65 %), C22:6 (7.96 %), C22:5 (5.21 %), C22:1 (5.66 %), and C24:1 (2.06 %).

Emulsion Preparation

Aqueous phase was a 20 or 30 wt.% solution of trehalose, a known cryoprotectant. Emulsions were formulated with 10 wt.% concentrated from blends of fish oils. NaCas was used as emulsifier at 0.5, 2, and 5 wt.%, giving oil-to-protein ratios of 20:2. Fat and aqueous phases were kept at 60 °C during pre-homogenization. They were mixed using an Ultra-Turrax T18 high-speed blender (S 18N-5G dispersing tool, IKA Labortechnik, Janke & Kunkel, GmbH & Co., Staufen, Germany), operated at 20,000 rpm for 1 min. The resultant pre-emulsions were further homogenized for 20 min using an ultrasonic liquid-processing VIBRA CELL, VC750 (power 750 W; 20 kHz frequency) model (Sonics & Materials, Inc., Newtown, CT, USA) with a 13-mm-diameter, 136-mm-length tip using an amplitude of 30 %. The temperature of sample cell was controlled by means of a water bath set at 15 °C. By doing this, sample temperature was around 40±1 °C during ultrasound treatment. Then, emulsions were cooled quiescently to ambient temperature (22.5 °C). Subsequently, initial emulsions were analyzed for particle size distribution. The pH values of the emulsions were 6.63±0.05. No buffer was added to the six analyzed emulsions. Experiments were done in duplicate, and results were averaged.

Powder Production

Emulsions were frozen with liquid nitrogen (−190 °C) and stored overnight at −80 °C before freeze drying to allow the highest amount of freezable water to crystallize. Freeze-thawed emulsions were analyzed for particle size distribution. After freezing, emulsions were dried.

An Alpha 1-2 LD Plus (Christ, Osterode, Germany) freeze dryer was operated at −110 °C and at a chamber pressure of 4.10^{-4} mbar for 48 h. The dried emulsions were placed in a dried box under nitrogen atmosphere. Then, they were broken into powder using a mortar and pestle. Powders were analyzed for water content, thermal behavior, and crystallinity by X-ray diffraction. Emulsions were reconstituted from the powders and were analyzed for droplet size distribution.

Encapsulation Efficiency

To determine encapsulation efficiency (retention), powders were washed with hexane (HPLC grade) to remove nonencapsulated fat. The washing process was repeated twice. Powders were separated from hexane by filtering. Hexane was then evaporated and the fat phase was weighed to determine the initial efficiency of encapsulation (Shimada et al. 1991). In detail, extractable and encapsulated fat was determined as follows: extractable fat was determined by dispersing 2 g powder in 15 mL hexane and shaking for 15 min. The soluble fraction was filtered and the solvent was evaporated, leaving the fat. The weight of the dried material representing the extractable fat was calculated as a percentage of the total fat in the dry powder. Then, the powder, free of extractable fat, was mixed with 15 mL water and 15 mL ethanol. The resulting solution was extracted with 40 mL sulfuric ether. The clear organic phase was collected, and this extract containing the encapsulated fat was dried and weighed. Encapsulation percentage was then calculated.

Analysis Performed on Nonencapsulated and Encapsulated Fat

The extractable fat was also analyzed for hydroperoxide content and oxidation products were determined by MALDI-TOF MS. The washed powder was further dehydrated under vacuum over $Mg(ClO_4)_2$ for 24 h. Then, encapsulated fat was analyzed for hydroperoxide content and oxidation products by MALDI-TOF MS in two ways: extracting the fat from the matrix and "in situ" without extracting the core material. Methods are described below.

Determination of Hydroperoxide Content

Peroxide value was determined by measurement of ion oxidation using the ferrous oxidation/xylenol orange method (Pegg 2001). It is based on the ability of lipid peroxides to oxidize ferrous ions at low pH. The resulting oxidation is quantified by using a dye that complexes with the generated ferric ions to produce a color that can be measured spectrophotometrically. To evaluate absorbance, 0.1000 to 0.1500 g of concentrated-from-fish oils was accurately weighted in 10, 25, or 50 mL volumetric flask. Volume was completed with 7:3 chloroform/methanol solution; 1 mL of this solution, 25 μL of 10 mM of xylenol orange solution, and 150 μL of iron(II) solution (prepared with 0.2 g de $BaCl_2{\cdot}2H_2O$, 0.7 g $FeSO_4{\cdot}7H_2O$, and 1 mL of HCl 10 M) were mixed in a 10-mL volumetric flask. Volume was also completed with chloroform/methanol 7:3 solution. Samples were shaken and absorbance was measured 5 min after addition of iron(II) in a Ocean Optics DT-Mini spectrophotometer (Ocean Optics Inc, USA) at a λ of 560 nm. All measurements were done by triplicate and results were expressed as average values and standard deviation. Peroxide values were expressed as milliequivalents of active oxygen per kilogram of sample.

Oxidation by MALDI-TOF MS

Experiments were carried out on an OMNI FLEX MALDI-TOF MS (Bruker Daltonics Inc., Billerica, USA) in the *m*/*z* (mass/charge) range of 500–2,500. MALDI-TOF MS method was the one developed by Picariello et al. (2009). In brief, a N_2 laser of λ=337 nm was used. Typically, 250 pulses of 3 ns working in the linear mode were acquired in a mass spectrum. The instrument was operated with an accelerating voltage of 19 kV and was calibrated with two standards triphenylene hexa methoxy (HMT; mass=408.16) and hexakis octyloxy triphenylene (HOT; mass=996.81). The matrix solution was prepared by dissolving 0.05 g of DHB (2,5-dihidroxybenzoic acid) in 5 mL of methanol containing 0.1 % TFA (trifluor acetic acid). Fifty microliters of 10 μL/mL solution of standards in $CHCl_3$ were mixed with 50 μL of matrix solution, and 1 μL of the resultant solution was deposited in the sample plate and air dried. Standards were analyzed in triplicate. Spectra were plot with Flex Analysis 2.2 software. In MALDI-TOF MS triacylglicerols (TAG) are detected as alkaline sodium ion adducts in the positive ion mode. Commercial concentrated-from-fish oil, extractable fat (none encapsulated material), and both, extracted core material from powders and washed powders as they were (without fat phase extraction) were analyzed for oxidation products. Commercial concentrated-from-fish oil, extractable fat, and extracted core material were analyzed as follows: they were dissolved in $CHCl_3$ at a concentration of 20 μL/mL. Then, 1 mL of NaCl 1 M was added to the chloroform solution and was shaken. Fifty microliters of organic phase was mixed with 50 μL of the matrix solution, and 1 μL was deposited onto the sample plate for analyses. Washed powders were analyzed as follows: 0.05 g of dried powders free of none encapsulated oil (washed in hexane) were dissolved in 5 mL of methanol (solution A). A 2.925 g NaCl were dissolved in 50 mL of methanol/water 2:1 (solution B). One milliliter of solution A was mixed with 1 mL of solution B (solution C). Then, 50 μL of solution C was mixed with 50 μL of matrix

solution (solution D). One microliter of solution D was deposited onto the sample plate and air dried to perform MALDI-TOF MS analysis. Mass signals were assigned following Picariello et al.'s (2009) work.

Particle Size Analysis

The particle size distribution of emulsions was determined immediately after emulsion preparation, after freeze-thaw treatment, and in emulsions reconstituted from powders reproducing the original formulation, by light scattering using a Mastersizer 2000 with a Hydro 2000MU as dispersion unit (Malvern Instruments Ltd., UK). The pump speed was settled at 1,800 rpm. Refraction index for the oil phase was 1.4694. Distributions were expressed in differential volume. The particle size data were reported as the volume-weighted mean diameter ($D_{4.3}$), the volume percentage of particles exceeding 1 μm in diameter ($\%V_{d>1}$), and the distribution width (W) expressed as $W=d_{90}-d_{10}$, where d_{10} and d_{90} are the 10 and 90 % volume percentiles, respectively. Determinations were conducted in duplicate, and values were reported as mean and standard deviations.

Determination of Water Content

The water content of the samples kept in the dried box under nitrogen atmosphere for 2 h was determined by difference in weight before and after drying in vacuum oven at 98 °C for 48 h. These conditions had proved themselves to be adequate for assessing constant weight after drying.

Thermal Transitions

Differential scanning calorimetry (DSC) was used to study thermal behavior of powders. Glass transition temperatures (T_g), melting point of trehalose matrix (T_m), and heat of fusion (ΔH_m) were determined for the dried systems. A DDSC Mettler Toledo model 822e (Mettler Toledo, Schwerzenbach, Switzerland) was used with a thermal analysis software Mettler Stare. Calibration was carried out at a heating rate of 10 °C/min using indium proanalysis (p.a.) as standard. Ten to fifteen milligrams of each sample in hermetically sealed aluminum pans were placed in the DSC cell and held at −30 °C for 5 min prior to melting at a heating rate of 10 °C/min from −30 to 120 °C. The concentrated-from-fish oil is a liquid above 0 °C and therefore melting peaks did not overlap trehalose melting peak. A single empty pan was employed as a reference. Two replicates were performed for each sample, and means and standard deviation of peak temperatures and melting enthalpies are reported. The melting endothermic peak that appeared at a temperature almost identical to that corresponding to crystalline trehalose dihydrate (Shimada et al. 1991) was used to indicate the amount of crystalline dihydrate present. The degree of crystallization (DC) was calculated as:

$$\mathrm{DC} = \frac{\Delta H_m}{\Delta H_T}$$

where ΔH_m is the melting endotherm area for a given sample and ΔH_T is the melting endotherm area corresponding to pure trehalose (149 J/g).

Crystallinity by X-ray Diffraction

Samples were analyzed for their crystallinity by X-ray diffraction (XRD). A Philips 1730 X-ray spectrometer fitted with a system for temperature control (Philips Argentina, S.A., Buenos Aires, Argentina). Kα1α2 radiation from copper was used at 40 kV, 20 mA, and a scanning velocity of 1°/min from 5° to 50°. Experiments were performed at ambient temperature (22.5 °C).

Statistical Analysis

Significant differences between means were determined by the Student's t test. An α level of 0.05 was used for significance.

Results and Discussion

Physical Properties of Powders

Particle Size Distribution

Figure 1 shows the particle size distribution for the six emulsions immediately after preparation, the emulsions after freeze-thaw treatment, and the emulsions reconstituted to the original formulation from freeze-dried powders. $D_{4.3}$, $\%V_{d>1}$, and W parameters for emulsions in Fig. 1 are reported in Table 1. Initial emulsions had bimodal distributions when they were stabilized with 0.5 and 2.0 wt.% NaCas and also contained 20 wt.% trehalose in the aqueous phase and when the emulsion was formulated with 0.5 wt.% NaCas and 30 wt.% trehalose. The emulsion formulated with 5.0 wt.% NaCas and 20 wt.% trehalose had a distribution almost monomodal with a small shoulder on the right side indicative of a second minority population of droplets. The other two emulsions (2.0 or 5.0 wt.% NaCas and 30 wt.% trehalose) had monomodal distributions (Fig. 1). $D_{4.3}$ parameter diminished with increase concentration of NaCas and for the same protein concentration with increase sugar content. $\%V_{d>1}$ and W parameters were smaller for emulsions stabilized by 5.0 wt.% NaCas for both sugar contents and for the emulsion with 2.0 wt.% NaCas and 30 wt.% trehalose. These three

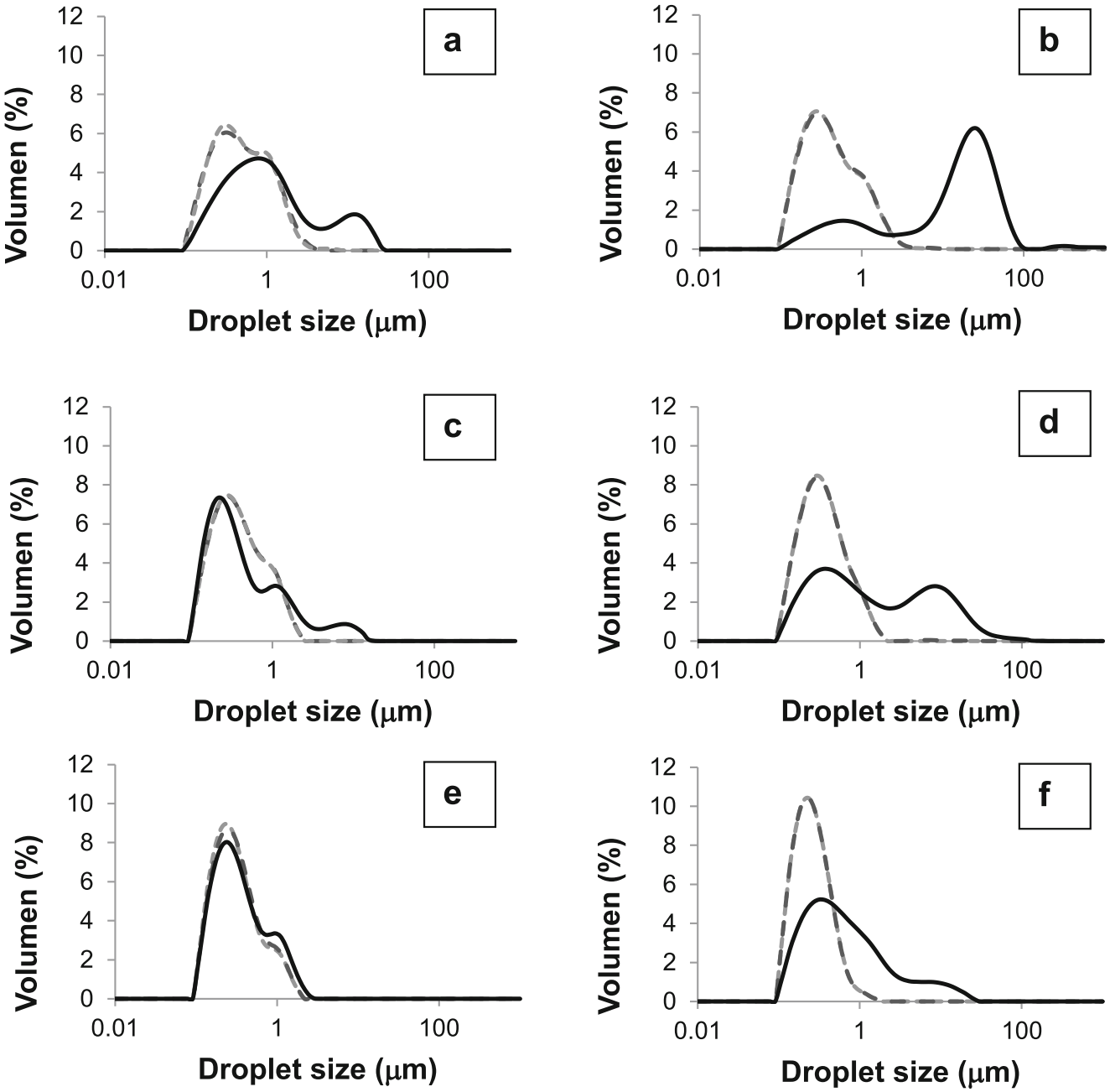

Fig. 1 Particle size distribution of initial (*dotted line*), freeze-thawed (*plotted line*), and reconstituted (solid line) emulsions. Concentrated-from-fish oils as fat phase: **a**, **c**, **e** 20 and **b**, **d**, **f** 30 wt.% trehalose; **a**, **b** 0.5, **c**, **d** 2.0, and **e**, **f** 5.0 wt.% sodium caseinate

emulsions showed narrow distributions of small particles (Table 1).

No significant differences between initial emulsions and freeze-thaw emulsions were found in all cases showing that the six systems remained unchanged after freeze-thaw treatment (Fig. 1; Table 1). It was reported that even relatively small amounts of sucrose (>5 wt.%) added to the continuous phase of oil-in-water emulsions greatly improved their freeze-thaw stability (Thanasukarn et al. 2004). According to Thanasukarn et al. (2004), sucrose increased the fraction of unfrozen water present in the emulsions preventing droplets from being together. In addition, authors proposed that sucrose might protect proteins from dehydration, reduce the tendency of associating with each other, and increase the conformational stability. In agreement with their results, in the six emulsions prepared in this study, trehalose prevented oil particle growth during freeze thaw.

When emulsions were reconstituted to the original formula from the 20 wt.% trehalose dehydrated powders, minor changes were found in droplet size distributions, especially for the 5 wt.% powder. This powder proved to be stable to freeze drying. Emulsions reconstituted from freeze-died powders

Table 1 Volume-weighted mean diameter ($D_{4,3}$, μm), width of the distribution (W), and volume percentage of particles exceeding 1 μm in diameter ($\%V_{d>1}$) of emulsions immediately after preparation, emulsions after freeze-thaw treatment, and emulsions reconstituted to original formulation from dried powders

Samples	Emulsions			After freeze thaw			From dried powders		
	$D_{4,3}$ (μm)	W (μm)	$\%V_{d>1}$	$D_{4,3}$ (μm)	W (μm)	$\%V_{d>1}$	$D_{4,3}$ (μm)	W (μm)	$\%V_{d>1}$
20 T									
0.5 C	0.78±0.12a	0.98	11.15	0.72±0.14a	1.30	19.90	3.03±0.15b	10.13	45.77
2.0 C	0.54±0.05a	0.98	11.37	0.53±0.11a	0.98	11.15	1.15±0.12b	2.23	22.67
5.0 C	0.42±0.09a	0.74	6.37	0.44±0.09a	0.76	9.31	0.51±0.09a	1.00	11.27
30 T									
0.5 C	0.62±0.11a	1.18	15.17	0.64±0.13a	1.20	15.76	26.09±0.21b	48.30	82.82
2.0 C	0.46±0.13a	0.74	5.92	0.51±0.10a	0.76	6.47	5.68±0.11b	15.08	52.48
5.0 C	0.32±0.04a	0.39	1.09	0.32±0.06a	0.39	1.20	1.86±0.10b	4.59	31.45

Significant differences between means were determined by the Student's t test. An α level of 0.05 was used for significance. Data in the same row with the same lowercase letters are not significantly different. All emulsions have 10 % concentrated-from-fish oils

T trehalose *C* sodium caseinate

with 30 wt.% trehalose had droplet size distributions significantly different from initial emulsions (Fig. 1). $D_{4,3}$, $\%V_{d>1}$, and W parameters significantly increased as a result of freeze-drying treatment (Table 1). Most likely, 30 wt.% amount of solids is too high for an efficient retention using this method.

Water Content and Encapsulation Efficiency

Table 2 shows the water content for the six powders immediately after preparation. Samples were freeze dried and then kept in a dried box under nitrogen atmosphere. In those conditions, water contents were expected to be low. Table 2 shows low values for all powders. As these results are intimately related to the crystallinity of the systems, powders were also analyzed for thermal behavior and crystallinity. Results are shown later on.

Table 2 also reports encapsulation efficiency (retention) for the six dried powders formulated in this study. Powders obtained from emulsions containing 30 wt.% trehalose had very low encapsulation efficiency. Most likely, for the formulations described in this study, an amount of 30 wt.% of solids is too high to obtain powders with high retention of core material. The powders obtained from the 20 wt.% trehalose emulsions, however, had greater efficiency of encapsulation. Retention values were higher with increasing protein content. The best matrix was the one formulated with 5 wt.% NaCas and 20 wt.% trehalose.

Table 2 Water content and encapsulation efficiency (retention) of the six dried powders formulated with 10 wt.% of concentrated-from-fish oils and different concentrations of trehalose (T) and sodium caseinate (C)

Samples	Water content (%dry basis)	Retention of core material (%)
20 T and 0.5 C	7.13±0.71a	46.8±2.0a
20 T and 2.0 C	4.93±1.32b	61.0±1.0b
20 T and 5.0 C	5.73±0.67a	79.4±0.9c
30 T and 0.5 C	2.99±1.90b	1.0±0.1d
30 T and 2.0 C	3.80±1.40b	11.5±0.6e
30 T and 5.0 C	3.16±1.47b	18.8±0.3f

Significant differences between means were determined by the Student's t test. An α level of 0.05 was used for significance. Data in the same column with the same superscript are not significantly different

Thermal Behavior and Crystallinity of Matrix

Figure 2 shows as an example DSC thermograms of powders obtained from emulsions containing 20 wt.% of trehalose. Powders from emulsions with 0.5 and 2.0 wt.% NaCas showed no noticeable T_g when analyzed immediately after freeze drying while powder from 5.0 wt.% emulsion showed a T_g at 46.6±1.2 °C. The higher the concentration of NaCas, the lower the DC of powders (Table 3). Powders from emulsions that contained 30 wt.% trehalose had a DC intermediate between powder from the 20 wt.% trehalose to 2.0 wt.% NaCas emulsion and powder from 20 wt.% trehalose to 5.0 wt.% NaCas emulsion (Table 3). As in the case of 0.5 and 2.0 NaCas and 20 wt.% trealose, no T_g was noticeable in those powders.

Figure 3 reports X-ray patterns of powders in Fig. 2. The sharp peak that appeared at 2θ value of 23.8° corresponded to trehalose crystallization. For powders from emulsions with 0.5 and 2.0 wt.% NaCas, DC were 20.3±1.3 and 14.9±1.1, respectively (Table 3). In agreement with a low value of DC (2.4±0.9), powder from emulsion with 5 wt.% trehalose showed a pattern with no sharp signals indicative of an amorphous system. For the powders obtained from the 30 wt.% trehalose emulsions, patterns were very similar to the 2.0 wt.%

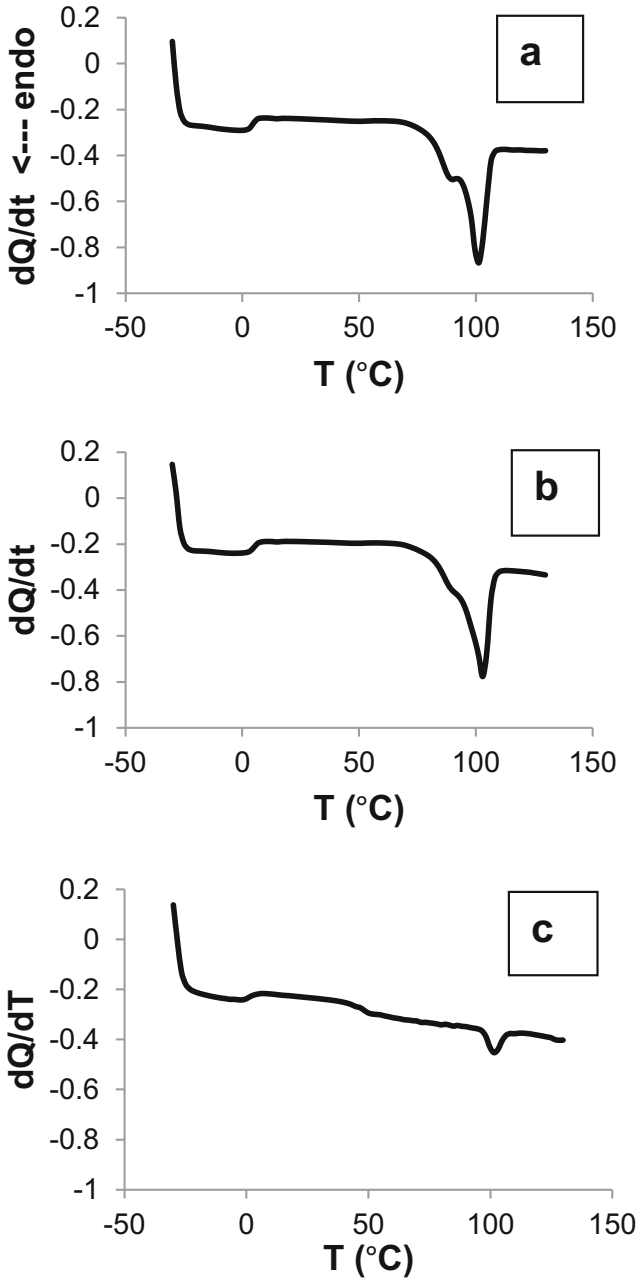

Fig. 2 DSC melting thermograms of dried powders formulated with 10 wt.% concentrated-from-fish oils, 20 wt.% trehalose, and different concentrations of sodium caseinate: **a** 0.5, **b** 2.0 and **c** 5.0 wt.%

NaCas powder (data not shown). In those powders, DCs calculated from DSC diagrams were close to the 2.0 wt.% NaCas powder. Summarizing, there was only one amorphous powder and the others had although low, some degree of crystallinity. It was reported that both, caking or collapse and crystallization may lead to a release of the encapsulated substance from the matrix. Several authors claimed that the protective action of the solid matrix is lost when crystallization occurs (Buera et al. 2005). Other authors observed a compromise between structural collapse (favorable for retention of encapsulated compounds) and matrix crystallinity (promoting the release of encapsulated compounds) (Buera et al. 2005). In the systems selected in this study, the highest retention value corresponded to the amorphous system. However, retention values did not exactly correlate to DC of samples; 30 wt.% trehalose powders had lower DC than the 20 wt.% trehalose/2.0 wt.% NaCas powder but retention was lower for the formers than for the later. These results showed the importance of complex interactions that took place among matrix, stabilizer and lipid phase and the relevance of solid content of systems regarding drying process.

Table 3 Peak temperatures (T_p), melting enthalpies (ΔH), and degree of crystallization (DC) of the six dried powders formulated with 10 wt.% of concentrated-from-fish oils and different concentrations of trehalose (T) and sodium caseinate (C)

Samples	T_p (°C)	ΔH (J/g)	DC (%)
20 T and 0.5 C	99.5±2.0a	30.2±1.7a	20.3±1.3a
20 T and 2.0 C	99.3±1.7a	22.2±1.1b	14.9±1.1b
20 T and 5.0 C	99.0±1.8a	3.5±0.1c	2.4±0.9c
30 T and 0.5 C	95.9±2.2a	14.9±0.2d	10.0±1.5d
30 T and 2.0 C	98.8±0.7a	14.7±0.6d	9.9±1.0d
30 T and 5.0 C	99.0±0.8a	12.6±0.2e	8.4±0.7d

Significant differences between means were determined by the Student's *t* test. An α level of 0.05 was used for significance. Data in the same column with the same superscript are not significantly different

Oxidation Studies

Bulk Oil

Figure 4 shows a MALDI-TOF MS of concentrated fish oil without any treatment (a) and after heating it for 6 h at 90 °C (b). The main signals assignments are reported in Table 4. Signals corresponding to diacylglycerols (DAG) had *m/z* values from 576 to 745 while signals coming from triacylglycerols (TAG) values from 763 to 1,075 (Table 4). Spectrum of original concentrated-from-fish oils showed low fragmentation of TAG (a). Only two fragments were formed by β-scission mechanism: one with a *m/z* value of 853 and the other with a value of 947. Dimers (*m/z*=2,000) were not formed. The signal at 901 had the greatest intensity and corresponded to the TAG composed by miristic, gadoleic, and eicosapentaenoic acids (MGaEPA; Table 4). In agreement with MALDI-TOF MS, peroxide value of original concentrated-from-fish oils was 3.9±0.9 mEq/kg, indicating a low degree of oxidation. When the oil was heated (b), fragmentation occurred and the main *m/z* signal appeared at 699. Figure 5 shows reactions that occurred including intermediate steps. As may be noticed peroxides were the first oxidation products formed and as they are unstable easily decomposed to more stable secondary oxidation

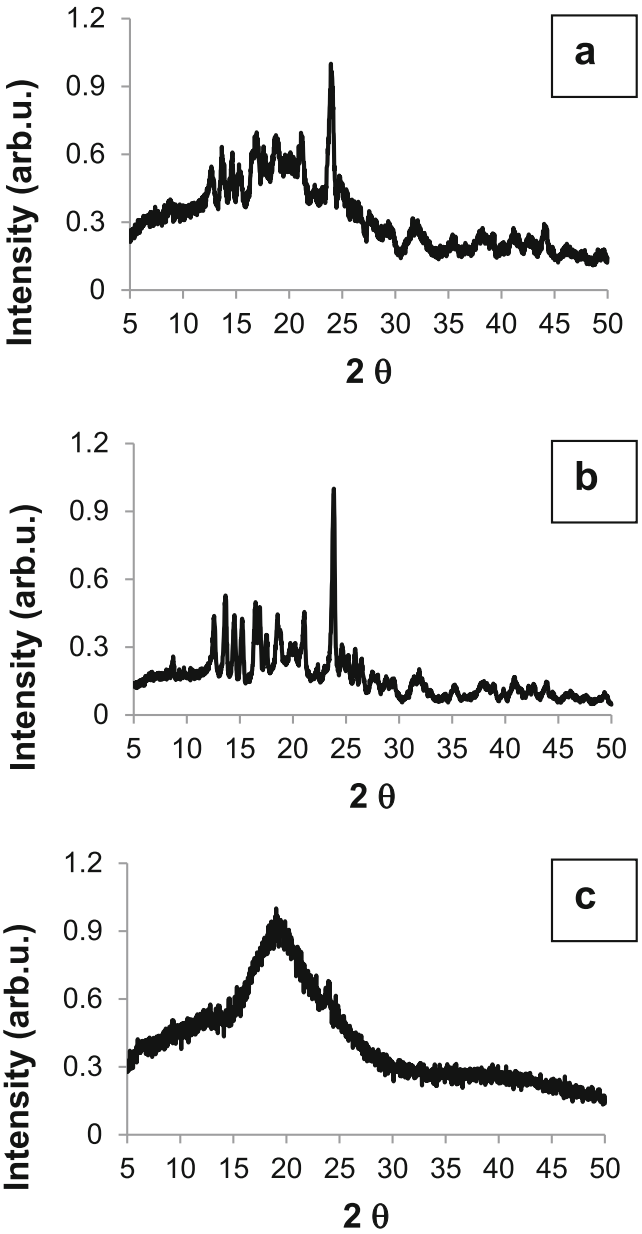

Fig. 3 X-ray diffractograms of dried powders formulated with 10 wt.% concentrated-from-fish oils, 20 wt.% trehalose, and different concentrations of sodium caseinate: **a** 0.5, **b** 2.0, and **c** 5.0 wt.%

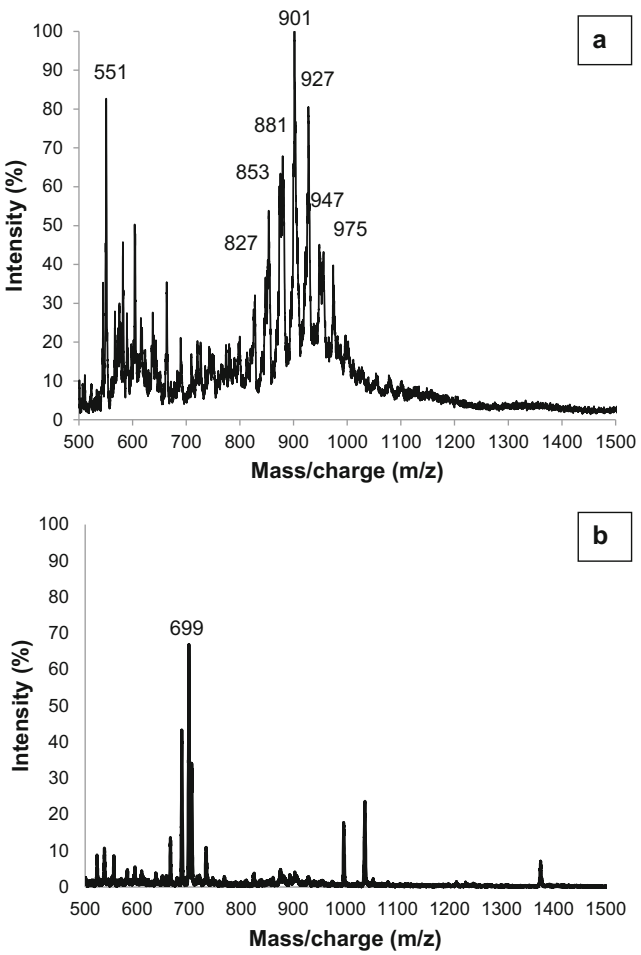

Fig. 4 MALDI-TOF mass spectrum of commercial concentrated-from-fish oils: **a** without any treatment and **b** heat for 6 h at 90 °C

products. TAG with *m*/*z*=901 added oxygen at the double bond closer to the glycerol molecule. Then, a β-scission took place. Picariello et al. (2009) proposed a similar mechanism for oxidation of sunflower oil and olive oil. As was expected, peroxide value for the heated oil was high, 32±2.1 mEq/kg. This value corresponded to oxidized oil.

None Encapsulated Oil

Figure 6 shows, as an example, MALDI-TOF MS of oil extracted by washing powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils, 20 wt.% trehalose and 0.5, 2.0, or 5.0 wt.% NaCas as stabilizer. Slightly significant differences in *m*/*z* signals and intensities were found between oil in Figs. 4a and 6a–c ($p<0.05$). Although these are none encapsulated oils, they were kept 48 h in a dried box at ambient temperature before analysis. In those conditions, oils were not expected to be oxidized in a great degree. Peroxide values for oils in Fig. 6a–c were 16.3±1.9, 10.0±1.7, and 3.5±0.9 mEq/kg, respectively. It was reported that the amount and composition of wall material was of great importance in fish-oil encapsulation (Ramakrishnan et al. 2013; Pourashouri et al. 2014). Despite the analyzed oils were extractable, that is, none encapsulated lipids, matrix exerted some protection against oxidation which increased with protein concentration. Peroxide values indicated that the three oils were still suitable for consumption. Powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils and with 30 wt.% trehalose showed no significant differences with the ones reported in Fig. 6a–c (data not shown). Peroxide values for 0.5, 2.0, and 5.0 wt.% NaCas were 16.0±0.8, 10.5±0.4, and 4.6±1.0 mEq/kg, respectively, indicative of a low oxidation degree.

Table 4 Assignment of the primary mass signals detected in the MALDI-TOF MS spectra of commercial concentrated-from-fish oils without any treatment

Molar mass	Assignment
551	DHB Matrix
576	DAG: MEi$^+$
578	DAG: MGa$^+$
606	DAG: MCe$^+$
615	DAG: MEi
635	DAG: MDHA
665	DAG: EEPA
691	DAG: EPAGa
706	DAG: CeDHA$^+$
745	DAG: CeDHA
763	TAG: MSM
827	TAG: MGaM
853	β-scission of TAG MGaDHA
881	TAG: SMEi
901	TAG: MEPAGa
927	TAG: MGaDHA
947	β-scission of TAG EPAGaDHA
975	TAG: EPAGaEPA
993	TAG: CeGaM
1,001	TAG: EPAGaDHA
1,011	TAG: CeEPAGa o CeDHAS o CeGaDHA
1,021	TAG: CeSCe
1,051	TAG: DPADPADPA
1,075	TAG: CeCeCe

DAG diacylglycerol, *TAG* triacylglycerol, *M* miristic acid (C 14:0), *Ei* eicosadienoic acid (C 20:2), Ga gadoleic acid (C 20:1), *Ce* cetoleic acid (C 22:1), *DHA* docosahexaenoic acid (C 22:6), *S* stearic acid (C 18:0), *EPA* eicosapentaenoic acid (C 20:5), *DPA* docosapentaenoic acid (C 22:5)

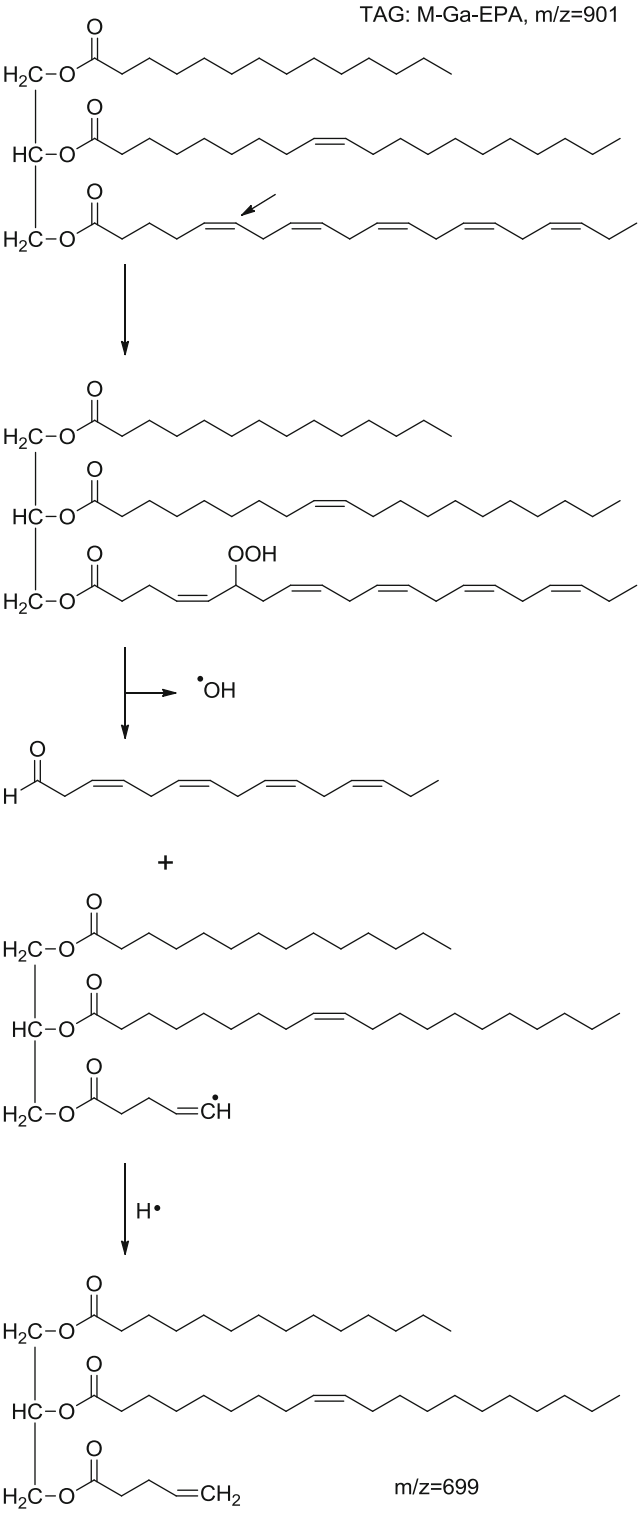

Fig. 5 Reaction mechanism proposed to obtain the *m/z*=699 fragment from the main triacylglycerol signal (*m/z*=901) assigned to the TAG MGaEPA

Encapsulated Oil

Releasing Core Material

Figure 7 reports MALDI-TOF MS of encapsulated oil after being extracted from powders as explained in "Materials and Methods." As an example, powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils and 20 wt.% trehalose were selected. Powders stabilized with 0.5 wt.% (Fig. 7a) and 2.0 wt.% (Fig. 7b) NaCas showed the same *m/z* signals in the 800–1,000 range as the original oil (Fig. 4a) and as the none encapsulated oils (Fig. 6a–c) but with intensities significantly lower ($p<0.05$). An intense signal with *m/z*=699 not present in the original oil appeared in the spectrum. The proposed mechanism of reaction is shown in Fig. 5. Oil release form 5.0 wt.% NaCas stabilized powder did not shown signals in the *m/z* range of 800–1,000 (Fig. 7c). As in the case of 0.5 and 2.0 wt.% powders, a very intense signal at *m/z* 699 was present in the spectrum. The presence of this signal at 699 in the MALDI-TOF MS of the three oils and of signals with *m/z* smaller, in the DAG zone, indicated that oils

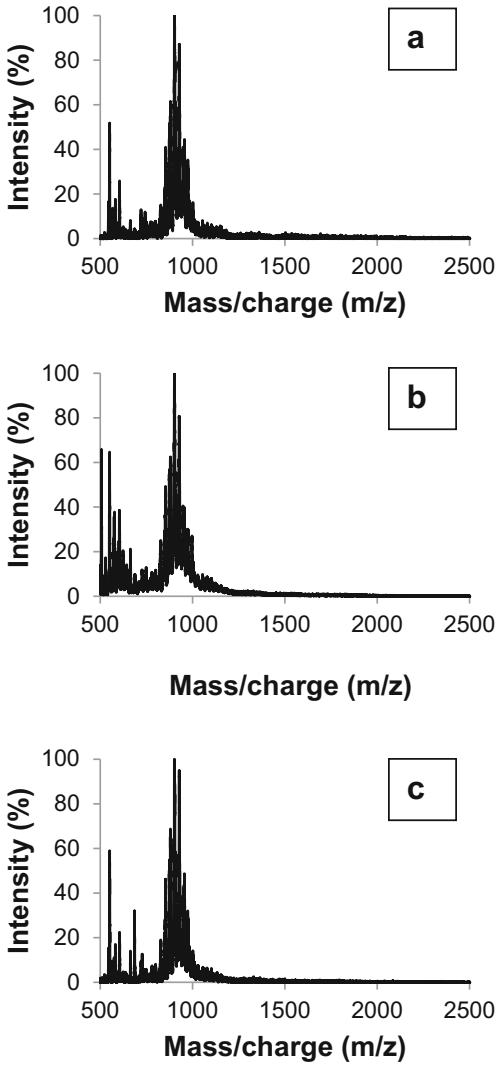

Fig. 6 MALDI-TOF MS spectra of oil extracted (none encapsulated oil) by washing powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils and 20 wt.% trehalose and stabilized with **a** 0.5, **b** 2.0, and **c** 5.0 wt.% sodium caseinate

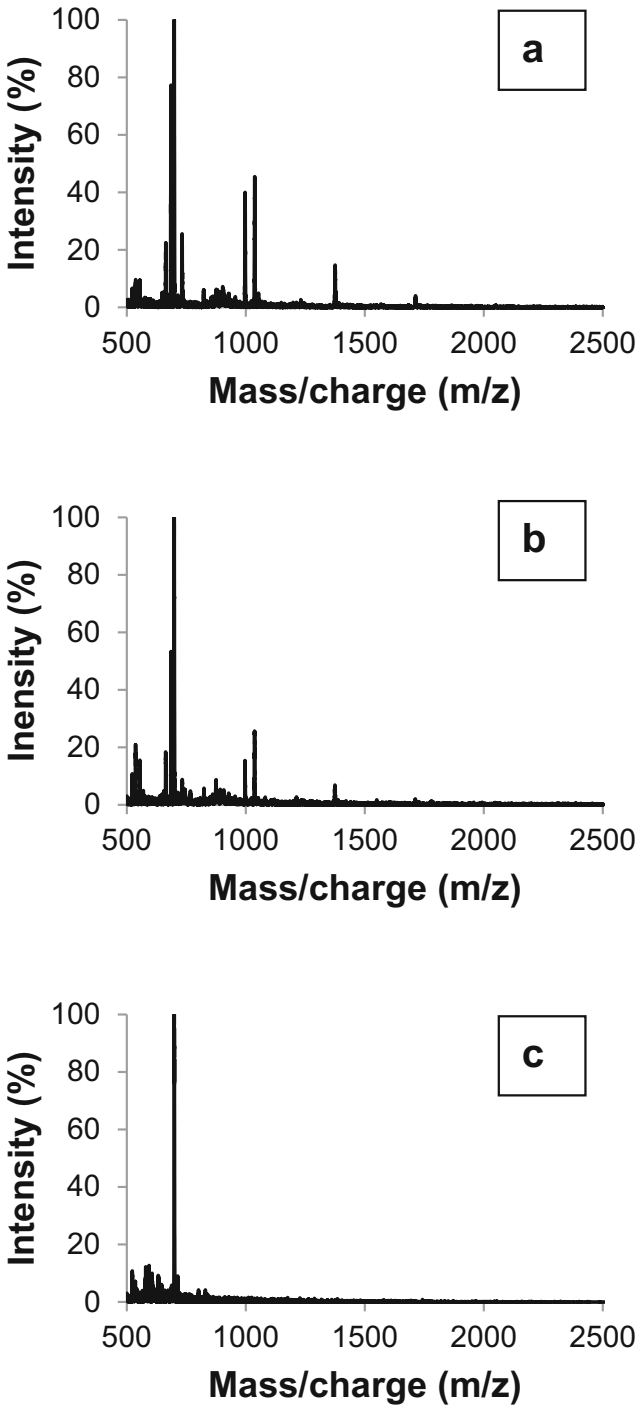

Fig. 7 MALDI-TOF MS spectra of encapsulated oil (releasing core material as explained in "Materials and Methods") for powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils, 20 wt.% trehalose and **a** 0.5, **b** 2.0, and **c** 5.0 wt.% sodium caseinate as emulsifier

in Fig. 7a–c were oxidized. Peroxide values for 0.5, 2.0, and 5.0 wt.% NaCas were 27.8±8.1, 49.2±4.7, and 89.1±5.3 mEq/kg, respectively. Oils released from 30 wt.% trehalose powders showed the same behavior as the 20 wt.% powders (data not shown). Peroxide values for 0.5, 2.0, and 5.0 wt.% NaCas powders were 55.0±9.9, 66.0±3.2, and 95.4±7.2 mEq/kg, respectively, indicating a high degree of oxidation.

It is well known that encapsulation process protects oils from oxidation. However, these results seemed to indicate the opposite. Some other reports in literature showed similar results, unexpected results, or results difficult to explain. It was reported that when fish oil was encapsulated by spray-freeze-drying, spray-drying, and freeze-drying methods some oxidation occurred during powder preparation (Karthik and Anandharamakrishnan 2013). According to these authors, air contact or high temperatures during processing led to oxidation of fish oil. In the method selected for extraction of oil in this study, the release of core material from matrix involved heating, shaking, or solvent evaporation. Although it is a usual way to extract encapsulated oils,

oxidation could have taken place in those steps. To check this hypothesis, we developed a method of sample preparation that did not need oil release from matrix and thus did not involve steps where fish oil could be oxidized. Results are shown in the following section.

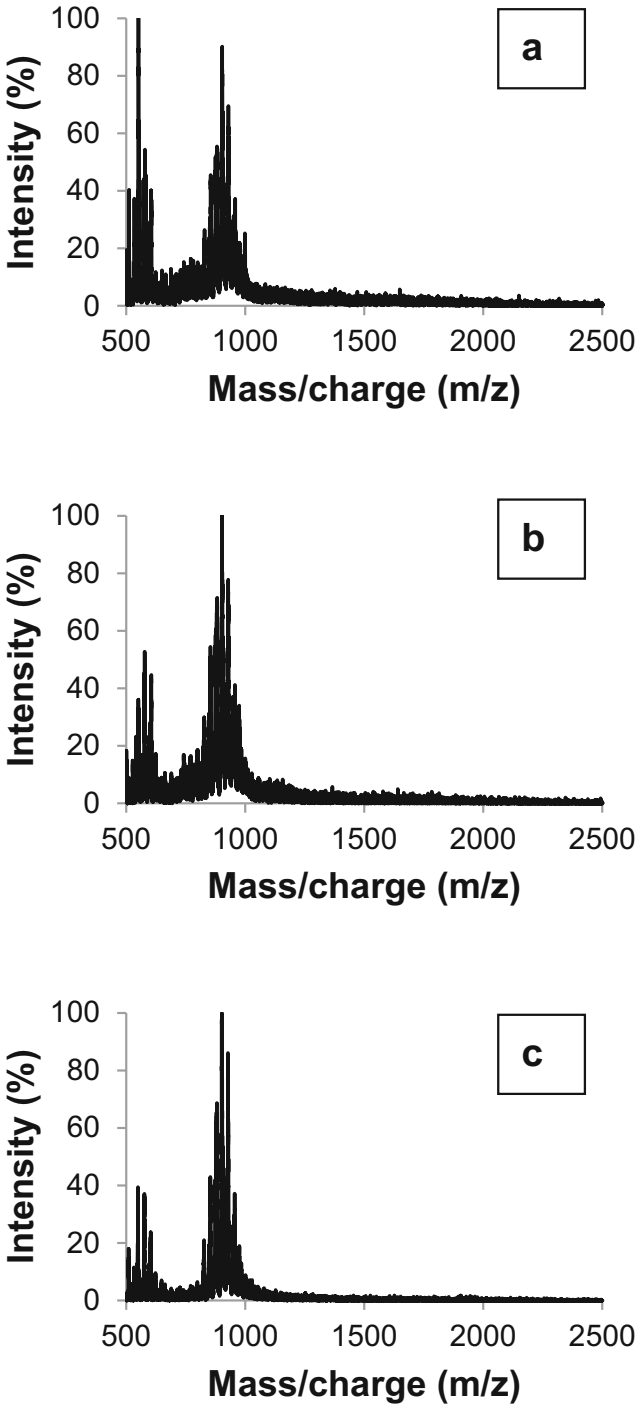

Fig. 8 MALDI-TOF MS spectra of encapsulated oil (without releasing core material) for powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils, 20 wt.% trehalose and **a** 0.5, **b** 2.0, and **c** 5.0 wt.% sodium caseinate as emulsifier

Without Releasing Core Material

MALDI-TOF MS of washed powders obtained from emulsions formulated with 10 wt.% concentrated-from-fish oils, 20 wt.% trehalose, and different concentrations of NaCas are reported in Fig. 8. The direct analysis (without extracting core material) of powders gave spectra that did not differ significantly from the one of the original oil. The *m*/*z* zone in the range 500–2,500 showed the same signals as in Fig. 4a. Trehalose and NaCas signals did not interfere with concentrated-from-fish oils spectra signals since they appeared in a different range. These results proved that encapsulated oil did not suffer much oxidation during liofilization or during 48 h in a dried box and therefore encapsulation by freeze drying is a suitable method to protect polyunsaturated oils from oxidation. Most likely, the oxidation products shown in Fig. 7 were formed during extractions steps where oxygen reacted with double bonds. The 30 wt.% powders gave similar results (data not shown). Spectra were similar to the original oil indicating a low degree of oxidation.

Most of the methods used to evaluate oxidation in edible oils are based primarily on the determination of oxidized compounds such as the peroxide value (PV), thiobarbituric acid (TBA) value, *para*-anisidine value, amount of conjugated dienes, or analysis of volatile oxidation products. These traditional analytical parameters are frequently used as quality indicators of fats and oils. However, some of these compounds are unstable and are only intermediate steps in oxidation process. MALDI-TOF MS method has the advantages that it allows determining the oxidized species actually present in samples and not products selected by the operator to follow oxidation. Therefore, more information for proposing mechanisms of reaction is obtained.

Conclusions

The 20 wt.% trehalose/5 wt.% NaCas matrix was the most efficient formulation to retain concentrated-from-fish oils. In this system, particle size distribution did not significantly change from emulsion to reconstituted powder. This matrix was mostly amorphous with a low degree of crystallinity. However, for the selected formulations, retention values did not exactly correlate to crystallization degree (DC); 30 wt.% trehalose formulations had low DC but poor encapsulation properties. This lack of correlation showed the importance of complex interactions that took place among matrix, stabilizer, and lipid phase, and the relevance of solid content of systems regarding drying process.

MALDI-TOF MS method applied directly to powders allows proving protective effect of encapsulation on lipid oxidation giving results that were in agreement with well-

established methods. In addition, actual products of oxidation could be described for oxidized oils.

Acknowledgments This work was supported by the National Research Council of Argentina (CONICET) through Project PIP 11220110101025, by the National Agency for the Promotion of Science and Technology (ANPCyT) through Project PICT 0060, and by the University of Buenos Aires through Project 20020100100467.

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