Effective stabilization of CLA by microencapsulation in pea protein

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A R T I C L E   I N F O

Article history:
Received 5 February 2014
Received in revised form 28 May 2014
Accepted 5 July 2014
Available online 11 July 2014

Keywords:
Encapsulation
Spray-drying
Pea protein concentrate
Maltodextrin
Carboxymethylcellulose
Lipid oxidation

A B S T R A C T

CLA was microencapsulated by spray drying in ten varied wall systems (WS) consisting of pea protein isolate or pea protein concentrate (PPC) alone at varied core:WS ratios (1:2; 1:3 and 1:4), or blended with maltodextrin (M) and carboxymethylcellulose at a pea protein:carbohydrate ratio of 3:1. The physical–chemical properties of the CLA microparticles were characterised by core retention, microencapsulation efficiency (ME), particle size and moisture. CLA:M:PPC (1:1:3) showed the most promising results, thus we evaluated the effect of M addition in the WS on other physical–chemical characteristics and oxidative stability (CLA isomer profile, quantification of CLA and volatile compounds by SPME coupled with CG-MS) during two months of storage at room temperature, CLA:PPC (1:4) was selected for comparisons. CLA:M:PPC (1:1:3) microparticles demonstrated better morphology, solubility, dispersibility and higher glass-transition temperature values. M addition did not influence the oxidative stability of CLA, however its presence improved physical–chemical characteristics necessary for food applications.

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1. Introduction

Conjugated linoleic acid (CLA) is formed by rumen bacterial fermentation combined with mammalian Δ9-desaturation, and therefore is found naturally in dairy products and beef. Much interest has been drawn to CLA due to its anti-atherogenic, anti-carcinogenic, anti-diabetic, immunomodulatory and anti-obesity effects (Whigham, Watras, & Schoeller, 2007). CLA consumption vary greatly according to populations habitual diet (15–1000 mg/day), however, on average, it is lower than typical doses presenting bioactivity (Nunes & Torres, 2010; Whigham et al., 2007). Direct addition of free CLA aiming to supplement food products is limited by CLA hydrophobic nature and oxidative instability. CLA is very polar due to the presence of two unsaturated double bonds and therefore is found naturally in dairy products and beef. Much interest has been drawn to CLA due to its anti-atherogenic, anti-carcinogenic, anti-diabetic, immunomodulatory and anti-obesity effects (Whigham, Watras, & Schoeller, 2007). CLA consumption vary greatly according to populations habitual diet (15–1000 mg/day), however, on average, it is lower than typical doses presenting bioactivity (Nunes & Torres, 2010; Whigham et al., 2007). Direct addition of free CLA aiming to supplement food products is limited by CLA hydrophobic nature and oxidative instability. CLA is very unstable in aqueous media leading to oxidative rancidity and nutritional loss, decreasing products quality and shelf-life (García-Martínez, Márquez-Ruiz, Fontecha, & Gordon, 2009). Therefore, effective food supplementation with CLA should guarantee protection of these bioactive fatty acids and preserve foods physical and chemical stability.

Microencapsulation is used to protect unstable molecules from interaction with food components and the adjacent environment during storage and food processing. Among microencapsulation methods, spray drying is the most commonly used in the food industry, because of its flexibility and low cost (Gharsallaoui, Roudaut, Chambin, Voilley, & Saurel, 2007). Several wall systems (WS) composed of proteins, gums and modified starches protected microencapsulated edible oils. Moreover, the combination of additional highly-soluble material can improve the functionalities of the particles, such as encapsulation efficiency and water solubility (Choi, Ryu, Kwak, & Ko, 2010; Jimenez, Garcia, & Beristain, 2006; Kagami et al., 2003). Nevertheless, the search for new wall materials that are biocompatible and sustainable is a promising area (Gharsallaoui et al., 2007).

Legume seeds are attractive for use in food products, because they are less allergenic than animal proteins and present interesting functional properties, such as emulsification, solubility and film formation (Rangel, Domont, Pedrosa, & Ferreira, 2003). Besides, the use of vegetable proteins as wall materials have been preferred than animal derived proteins as a reflection of the current “green” trend in the food industry. Wall materials derived from plants are emerging due to their more sustainable production and the recent increased consumer concerns over their diets, safety of animal-derived products and the animals’ breeding and slaughter conditions (Nesterenko, Alric, Silvestre, & Durrieu, 2013). In the case of pea proteins, the presence of high quality proteins also adds nutritional value to the food additive (Tome, 2012). Previous works

http://dx.doi.org/10.1016/j.foodchem.2014.07.016
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from our group showed that pea protein was an effective wall material for microencapsulation of ascorbic acid and α-tocopherol (Pereira et al., 2009; Pierucci, Andrade, Farina, Pedrosa, & Rocha-Leão, 2007). For the best of our knowledge, CLA microencapsulation by spray-drying using varied WS consisting of pea protein concentrate (PPC) or pea protein isolate (PPI) as single constituents in different core:WS ratios, or blended with maltodextrin (M) or carboxymethylcellullose (CMC), and to investigate the effects of the WS tested on the physical–chemical properties of the powders and on the oxidative stability of the encapsulated CLA stored at room temperature for 2 months.

2. Materials and methods

2.1. Materials

Microparticles were prepared with CLA (c9,t11/t10,c12, 1:1, w/w; Cognis, Brazil) as core and as wall materials: pea protein concentrate (PPC; Labonatus, Brazil), pea protein isolate (PPI), produced according to Rangel et al. (2003), maltodextrin (M; MOR REX 1910; Corn Products, Brazil), and sodium-carboxymethylcellulose (CMC) (Latinoquímica, Argentina). A commercial mixture of fatty acid methyl esters (37-component FAME mix; Supelco, PA, US) and a commercial standard (glyceryl triheptadecanoate; Sigma–Aldrich, US) were used for identification of volatiles. All reagents used were of chromatographic grade (Tedia, São Paulo, Brazil; Merck, US).

2.2. Production of CLA microparticles

Ten formulations of feed emulsions were prepared with different core:WS ratios and with varied WS components (Table 1). Feed emulsions were prepared with 8.6% total solids content and 150 mL final volume, except for the formulations containing CMC, which had 3.7% total solids in 350 mL, because of high viscosity. Emulsions were prepared by dissolving the wall materials (PPI or PPC) in distilled water and homogenising with a magnetic stirrer for 30 min. In parallel, M and CMC were dissolved in distilled water and homogenising with a magnetic stirrer for 30 min. CLA was emulsified by slowly dropping into the PPI/PPC emulsion.

The carbohydrate solution was mixed with the emulsion in two steps: (1) agitation (13,500 rpm/2 min) with Ultra-Turrax (T25- IKA®; Labotechnik, USA) and; (2) Ultrasonic (DRH-UP 100H; Hiel scher Ultrasonics, Germany) agitation (90% amplitude/1 cycle/ 2 min). The emulsions were fed immediately into a Mini Spray Dryer Büchi 290 (Büchi Laboratoriums Technik Flawil, Switzerland), at an inlet and outlet air temperature of 184 ± 3.0 °C and 88 ± 1.5 °C, respectively, through a 0.3 mm nozzle, 6 mL min⁻¹ feed rate, 6,900 mL min⁻¹ and 0.3 bar air flow, and 32.5 m³ h⁻¹ aspiration rate. Microparticles’ formulations were selected according to basic microencapsulation performance, assessed through the following factors: moisture, CLA microencapsulation efficiency, CLA retention, and particle size. Further investigations of microparticles were conducted exclusively on selected formulations.

2.3. Characterisation of CLA microparticles

2.3.1. CLA retention

CLA methyl esters were produced by direct transesterification of microparticles containing 7 mg of lipid as described by Lepage and Roy (1986). The methylated CLA were quantified by gas chromatography coupled with flame ionisation detector (GC-FID) in a GC-2010 chromatograph (Shimadzu, Japan) according to Nunes and Torres (2010), with modifications. Briefly, the split ratio used was 1:20 and the oven heating program was modified to optimise separations. Column oven temperature was held at 200 °C for 10 min, temperature programmed at 1.5 °C/min to 210 °C and then held for 25 min. A solution of glyceryl triheptadecanoate in hexane (0.2 µg/µL) was used as internal standard for CLA quantification. Before microparticles analyses, the non encapsulated CLA (raw CLA) was analysed to confirm label information, and the composition of the raw material was 66.0% total CLA (31.6% c9,t11; 31.9% t12,c10; 1% c,c; and 1.5% t,c) and 34% of other fatty acids: 16:0, 1.1%; 18:0, 4.9%; 18:1, 27.6%; 18:2n-6, 0.4% (Supplementary material). As control, we analysed all the wall systems’ components (PPI, PPC, CMC and M) and for calculation of CLA retention these data was considered quantitatively. CLA retention was calculated by the following equation and assuming that 66% of the core was composed of CLA:

$$\text{CLA retention (％) = } \frac{\text{CLA microparticles( per 100 g solids)}}{\text{CLA emulsion( per 100 g solids)}} \times 100$$

2.3.2. Microencapsulation efficiency (ME)

Total CLA content was determined by GC-FID as described in the Section 2.3.1. Extractable CLA was determined according to

### Table 1

<table>
<thead>
<tr>
<th>Formulation (w/w)</th>
<th>Solids (%)</th>
<th>Emulsion volume (mL)</th>
<th>Components quantity (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CLA</td>
</tr>
<tr>
<td>CLA:PPI (1:2)</td>
<td>8.6</td>
<td>150</td>
<td>4.4</td>
</tr>
<tr>
<td>CLA:PPC (1:2)</td>
<td>8.6</td>
<td>150</td>
<td>4.4</td>
</tr>
<tr>
<td>CLA:PPI (1:3)</td>
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<td>150</td>
<td>3.25</td>
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<tr>
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<td>150</td>
<td>3.25</td>
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<tr>
<td>CLA:PPI (1:4)</td>
<td>8.6</td>
<td>150</td>
<td>2.6</td>
</tr>
<tr>
<td>CLA:PPC (1:4)</td>
<td>8.6</td>
<td>150</td>
<td>2.6</td>
</tr>
</tbody>
</table>
| CLA:M:PPI (1:1:3)| 8.6        | 150                  | 2.6 | 7.8 | –   | –   | 2.6
| CLA:M:PPC (1:1:3)| 8.6        | 150                  | 2.6 | –   | 7.8 | –   | 2.6
| CLA:CPC:PPI (1:1:3)| 3.7      | 350                  | 2.6 | 7.8 | –   | –   | 2.6
| CLA:CPC:PP (1:1:3)| 3.7      | 350                  | 2.6 | –   | 7.8 | –   | 2.6


* Total solids were 13 g in all formulations.
(Sankarikutty, Sreekumar, Narayanan, and Mathew, 1988), with modifications (Kouassi et al., 2012). ME was calculated as follows:

\[
\text{ME} \, (\%) = \frac{\text{Total CLA} - \text{Extractable CLA}}{\text{Total CLA}} \times 100
\]

2.3.3. Particle size

Microparticles were dispersed in ultra-pure water (Milli-Q; Millipore, US) and analysed in a particle size analyser (Mastersizer 2000-Malvern Instruments, England) by the scattering pattern of a transverse laser light. Results were reported as D(0.5) and scattering index (span), which are defined as maximum size (μm) of 50% analysed particles and the particles size range, respectively.

2.3.4. Proximate composition of microparticles

Moisture was determined in all samples with a moisture balance (MA35-Sartorius; Germany), with 500 mg samples in a standardised protocol according to manufacturer’s instructions (105 °C for 90 min) right after production. Protein, carbohydrate and lipid contents were determined on selected CLA microparticles. Protein and carbohydrate were assessed according to Lima, Lima, Tavares, Costa, and Pierucci (2014). Lipid and fatty acid content were determined by gas chromatography, as described in Section 2.3.1.

2.3.5. Infrared absorption spectrometry (FTIR)

The selected CLA microparticles were submitted to infrared absorption spectrophotometry in a Varian Excalibur 3100 FT-IR spectrometer in transmittance mode with 20 scans and resolution of 2 cm⁻¹. Each sample was macerated and homogenised with KBr to form pellets used to obtain the spectra. As control, each wall material and the WS dried without CLA were analysed.

2.3.6. Thermogravimetric analysis (TGA)

A thermogravimetric analyser, from Perkin-Elmer Pyris 1, was used to analyse the thermal stability of the selected CLA microparticles and their components. Samples (5.0 mg) were heated from 25 °C to 800 °C (10 °C/min) under nitrogen gas flow (30 mL/min). As control, each component of the microparticles and the WS dried without CLA were analysed.

2.3.7. Differential Scanning Calorimetry (DSC)

A Differential Scanning Calorimeter, from Perkin-Elmer, model Diamond, was used to examine the glass-transition temperature (Tg) of the selected CLA microparticles. Under a nitrogen gas flow of 20 mL/min, samples (5.0 mg) were scanned at heating and cooling steps. Two heating steps were used from 0 °C to 180 °C at a heating rate of 20 °C/min. One cooling step was used from 180 °C to 0 °C at 100 °C/min. Before and after each run the samples’ pans were weighed and Tg was determined as the middle point of the first jump in the DSC thermogram baseline. As control, each component of the microparticles and the wall system dried without CLA were analysed.

2.3.8. Solubility

The solubility in water of the selected CLA microparticles was determined as described by Lai and Cheng (2004) with modifications. Briefly, 1.25 g of CLA microparticles (dry sample weight), was reconstituted in 15 mL of distilled water by stirring with a magnetic bar at 25 °C for 30 min. The emulsion was centrifuged at 7577.7×g for 30 min; subsequently, a supernatant aliquot (10 mL) was placed in a Petri dish and its weigh was measured after drying at 105 °C for 5 h in an oven (dry supernatant). Solubility in water was calculated by the following equations:

\[
\text{Solubility (\%)} = \frac{\text{dry supernatant in 15 mL (g)}}{\text{dry sample weight (g)}} \times 100
\]

2.3.9. Dispersibility

CLA microparticles samples (50 mg) were mixed with 25 mL of PBS at pH 7.4 during 10 min at 500 rpm. Then 500 μL of the mixture was diluted in 500 μL of PBS solution, stirred at 2200 rpm for 3 min and placed in a cuvette to measure the absorbance at 440 nm wavelength using a spectrophotometer (UV-1800, Shimadzu, Japan) for 20 min (Choi et al., 2010). The results were expressed as the difference between initial absorbance and final absorbance (ΔAbs).

2.3.10. Morphology

Surface morphology of selected CLA microparticles was carried out by scanning electron microscopy (SEM). Samples of microparticles were directly deposited on carbon conductive tape on aluminum SEM stubs, and coated with a thin gold layer, using a gold-sputtering (Blazers Union, FL-9496). The samples were analysed using a JEOL SEM 5310, operated at 20 kV.

2.4. Microparticles stability at room temperature

Right after production, the selected CLA microparticles formulations were placed in plastic bags, sealed, covered with aluminum foil for light protection and stored in desiccators at room temperature (25 °C and below 20% relative humidity). Microparticles were periodically analysed for up 2 months (0, 15, 30, 60 days). The microparticles’ core stability was assessed by: (1) CLA isomer profile, base-catalysed transesterification followed by GC-FID; (2) CLA quantification, acid-catalysed transesterification followed by GC-FID (Section 2.3.1); and (3) Volatile degradation products of CLA, by SPME-GC-FID. In addition, water activity (Aw) of the microparticles was assessed at day 0 and after 15 and 60 days.

2.4.1. CLA isomers profile and CLA quantification

For determination of CLA isomers profile, it is necessary to avoid isomerization during analysis, and therefore CLA methyl esters were produced by base-catalysed transesterification as described by Kramer et al. (1997). The CLA from the microparticles was extracted (Hara & Radin, 1978) and methylated, with modifications proposed by Nunes and Torres (2010). After derivatization, CLA methyl esters were analysed by GC-FID as described in Section 2.3.1. Contents of CLA isomers were determined by peak areas internal normalisation of the following groups of isomers: cis,trans; trans,cis; cis,cis and trans,trans, and the results were presented as g/100 g total CLA.

In contrast, for determination of CLA concentration in microparticles (g CLA/100 g of microparticles), direct acid-based methylation was used as described in Section 2.3.1. Direct derivatization of samples was more appropriate for quantitative analysis, when total CLA concentrations were to be determined.

2.4.2. Volatile degradation products of CLA by SPME-GC-FID

2.4.2.1. Quantification of volatile compounds. Volatile compounds from CLA microparticles were extracted by solid phase microextraction (SPME) using a divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fiber (Supelco, PA, USA) (Larick & Parker, 2001). Extraction fiber was conditioned for 60 min in the GC injection port at 260 °C. An aliquot (1 g) of CLA microparticles was weighed in a headspace vial and dissolved in 3.25 mL 3 M KCl with internal standard (4.4 μg/mL of isopropanol in KCl solution). Vials were capped with a PTFE-lined septum and placed in a glycerol bath (40 °C) under magnetic agitation
A mixture was run under the same conditions during 10 and 3 min, respectively.

Qualitative analysis was performed by GC–MS (Shimadzu, Japan). Volatile compounds were desorbed from the SPME fiber in the injection port for 3 min, in splitless mode, and after 3 min sampling time, split purge was open at 3.0 mL/min. The injection and detector temperatures were 260 °C and 280 °C, respectively. Helium was used as the carrier gas and column pressure was set to attain a carrier speed of 25.0 cm/s. Column oven temperature was held at 30 °C for 10 min, temperature programmed at 3 °C/min to 200 °C and held for 25 min. A C18-C30 mixture was run under the same conditions to obtain linear retention index (LRI) values for the volatile compounds (Viegas & Bassoli, 2007). Results were expressed in μg volatile compound/g of microparticles and for comparisons of production of volatile compounds during the stability test, the area under the curve (volatile content (μg/g) vs. time (d)) was calculated.

### 2.4.2.2. Identification of volatile compounds

Qualitative analysis was performed by GC–MS on a GC-17A gas chromatograph coupled to a QP5050A mass spectrometer (Shimadzu, Japan). Chromatographic conditions used were similar to described in the Section 2.4.2.1. The mass spectrometer was operated in electron impact mode at 70 eV. The interface and ion source temperatures were 260 °C. Analyses were performed in full scan acquisition mode, in the mass range 29–500 m/z at 0.5 scan/s. Data were collected by Lab Solutions GC–MS (Shimadzu, Japan). Compounds were identified first by comparison of mass spectra with the National Institute of Standards library and calculation of similarity index by the instruments software (Lab Solutions GC–MS; Shimadzu, Japan), and also by comparing LRI values with published data of fatty acids’ volatile oxidation products.

### 2.4.3. Microparticles’ water activity (A_w)

A water activity analyser (LabMaster-aw; Novasina, Switzerland) was used to assess the microparticles A_w. Briefly, approximately 1 g of sample was used and measurements were performed at 25 °C. Results were recorded after samples did not present temperature and A_w variations during 10 and 1 min, respectively.

### 2.5. Statistical analysis

All statistical comparisons were based on triplicate results, and all data is presented as mean and standard deviation. Comparisons between means were made by t-test or one-way ANOVA with Tukey’s post-test, whenever appropriate. Repeated measures two-way ANOVA with Bonferroni’s post-test was used to investigate time- and formulation-dependent differences in water activity. CLA isomer profile, CLA quantification and volatile products during the stability test. Data were analysed with GraphPad Prism v.5.0 (GraphPad software, Inc, US). Significance was established at P < 0.05.

### 3. Results

#### 3.1. Encapsulation performance of the wall materials

##### 3.1.1. CLA retention

In our laboratory, the use of the pea protein (isolated or concentrated) as wall material in the microencapsulation of ascorbic acid and α-tocopherol has been evaluated (Pereira et al., 2009; Pierucci et al., 2007). The higher core retention presented in these works demonstrated the effectiveness of this material to protect substances during droplet formation and drying and when CLA was used as core, the retention values were similar. All the formulations showed CLA retention near 90% or higher (mean CLA retention: 105 ± 12.3%) and were satisfactory compared to other works that microencapsulated CLA by spray-drying, or by inclusion complex with amylase and different types of cyclodextrins (Jimenez et al., 2006; Yang, Gu, & Zhang, 2009). Tan, Chan, and Heng (2005) and Rosenberg, Kopelman, and Talmon (1990) showed that high core:wall system ratios influence negatively the core retention. Moreover, other researchers have demonstrated the effect of the addition of polysaccharide in the WS on the core retention (Pierucci, Andrade, Baptista, Volpato, & Rocha-Leão, 2006; Pierucci et al., 2007; Rosenberg & Sheu, 1996). In this work the varied WS and core:WS ratios did not influence the CLA retention.

#### 3.1.2. Microencapsulation efficiency (ME)

The PPC microparticles showed higher ME values than PPI, when these materials were used as single wall component. The core:WS ratios influenced the ME of the PPC microparticles and the carbohydrates addition improved this parameter in the two types of microparticles. Choi et al. (2010) and Jimenez et al. (2006) microencapsulated CLA with WS composed by whey proteins and carbohydrates, they showed values of ME in the range of 57–99%. In this study the higher values of ME were demonstrated in the microparticles with CMC in their WS, 41.6 and 40.6% for CLA:CMC:PPI (1:1:3) and CLA:M:PPC (1:1:3), respectively (Table 2).

It is noteworthy that this analysis can also induce an extraction of encapsulated oil to some extent, depending on the porosity of the matrix (Rosenberg & Sheu, 1996), thus resulting in a lower ME than the actual. ME is commonly determined by extracting unencapsulated oil (i.e. free oil) present on the surface of microparticles through washing powders with an organic solvent. The presence of free oil influences adversely the physical properties of spray dried powders, such as flowability, dispersibility, solubility, as well as the stability toward oxidation (Bae & Lee, 2008; Drusch, Serfert, Scampicchio, Schmidt-Hansberg, & Schwarz, 2007).

Vicilin is the predominant protein and component of the two microparticles in this study (Pereira et al., 2009) and proteins are porous wall materials (Drusch et al., 2007; Rosenberg & Sheu, 1996). According to Rosenberg and Sheu (1996), the additions of lactose in a proteinaceous wall system diminish the diffusion of the solvent through the matrix. This phenomenon could be explained by the formation of a dry crust and a continuous glass phase in which the proteins are dispersed. The dextrose equivalent (DE) and the molecular weight of the carbohydrate influenced greatly the porosity of the matrix (Hogan, O’Riordan, & O’Sullivan, 2003; Sheu & Rosenberg, 1998). Kagami et al. (2003) microencapsulated fish oil in WS composed by a mixture of sodium caseinate (NaCas) and maltodextrin with different DE (4, 11 and 18), and found that maltodextrins with higher DE increased the oxidative stability and decreased the unencapsulated oil of the microparticles. In addition the presence of carbohydrates in the WS could also increase the hydrophilic nature of the WS, and therefore diminished the interaction with the extraction solvent (Rosenberg & Sheu, 1996). PPC is the concentrated form of vicilin, thus this material also has lipids and carbohydrates in its composition. We suggested that the presence of this minor components and their interaction with CLA, could explain the higher ME values of the PPC microparticles.

The effect of varied core:WS ratios on ME has already been investigated by Rosenberg et al. (1990) and Tan et al. (2005), they showed that high core:WS ratios decreased ME. According to these authors, the amount of WS is insufficient to encapsulate the core. However Hogan, O’Riordan, and O’Sullivan (2003) did not find
any relation with core:WS ratio and ME, when they microcapsulated menhaden oil with a WS composed by proteins and carbohydrates. In this study, the different core:WS ratios only influenced the PPC formulations and the microparticle with the lower core:WS value, CLA:PPI (1:4) (Table 2), showed the least ME value. Therefore, the determination of an optimal ratio of core:WS is necessary for high ME values.

3.1.3. Particle size (PS)

The PS analysis showed that the microparticles are in the micro-scale, with median particles sizes between 5 and 11 μm for CLA:CMC:PPI (1:1:3) and CLA:PPI (1:4), respectively (Table 2). In the PPI microparticles, CLA:PPI (1:2) showed the biggest particle size, this result is in accordance with Tan et al. (2005), they have demonstrated that the oil load influenced the PS. However the PPC microparticles did not follow this trend. Pierucci et al. (2007) showed that the addition of carbohydrate in the WS increased particle size. However, in this study we observed the opposite effect, in the microparticles with the same core:WS ratio (1:4) (Table 2), carbohydrates diminished the median size particles in the size range of microparticles. The conflicting results could be associated with physical–chemical characterises of the encapsulated molecule.

Although the addition of CMC decreased the mean particle size, the microparticles with this carbohydrate showed a bigger variation in particles sizes, indicated by the highest span 18.2 and 11.9 ± 0.5%, and the two types of microparticles demonstrated a 1:1 mass ratio. The conflicting results could be associated with the sustained released of the core and stability (Walton & Mumford, 1999).

3.1.4. Moisture

Microparticles showed expected values of moisture according with Reineccius (2004), ranging from 2.6% for CLA:PPI (1:3) to 5.2% for CLA:CMC:PPI (1:1:3) and can thus be considered to possess long-term stability against microbiological spoilage (Drusch et al., 2007) (Table 2). Pierucci et al. (2006) showed a relation between the amount of solids in the emulsion and the moisture of the microparticles. Here all the feed emulsions had the same solids content, and we just found a statistical difference when CLA:M:PPI (1:1:3) and CLA:M:PPC (1:4) were compared.

We selected the PPC microparticles for further investigations, because they were more homogeneous regarding to their size and showed higher ME values (Table 2). CLA:PPI (1:4) and CLA:M:PPC (1:1:3) were the formulations chosen, as we aimed at to evaluate the addition of carbohydrate in the WS on other physical–chemical parameters and the oxidative stability of the microparticles. M was the preferred carbohydrate, as the microparticles containing CMC showed great variation in their particle size and the drying process was longer, compromising future applications. CLA:PPI (1:4) was elected because it had equivalent core:WS ratio, moreover we also wanted to determine the effectiveness of PPC, when used isolated in the WS and the influence of a smaller ME in the oxidative stability of the microparticles. From this point, all the results are related to the microparticles selected (CLA:PPI, 1:4; and CLA:M:PPC, 1:1:3), and the composition of formulations will be suppressed for clarity.

3.2. Characterisation of the selected microparticles

3.2.1. Proximate composition

The microparticles presented protein content of 58.2 ± 0.1% and 42.5 ± 0.1% for CLA:PPI and CLA:M:PPC, respectively. As expected, CLA:M:PPC showed higher carbohydrate content (30.4 ± 1.0% vs. 11.9 ± 0.5%), and the two types of microparticles demonstrated similar lipids contents (CLA:PPI, 26.3 ± 0.6%; CLA:M:PPC, 24.2 ± 1.0%). According to the fatty acid composition estimation, cis,trans CLA (≈33%) and trans,cis CLA (≈34%) were the major fatty acids in the particles, and presented a 1:1 mass ratio.

3.2.2. FTIR spectrometry

FTIR analysis (Fig. 1A) was used to evaluate if the drying process could promote interactions between microparticles compounds. When the FTIR spectra of M:PPC (1:3) dried by spray-drying was compared with the compounds’ physical mixture, any absorption peak appeared or disappeared. This same trend was observed when the FTIR spectra of the selected CLA:M:PPC (1:1:3) and CLA:PPI (1:4) were compared with the isolated wall materials (M and PPC).

Table 2

<table>
<thead>
<tr>
<th>Formulation (w/w)</th>
<th>ME (%)</th>
<th>PS</th>
<th>Moisture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D(0.5) (μm)</td>
<td>Span</td>
<td></td>
</tr>
<tr>
<td>PPI</td>
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<tr>
<td>CLA:PPI (1:2)</td>
<td>N.D.</td>
<td>8.96 ± 0.18</td>
<td>1.98 ± 0.04</td>
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<td>6.66 ± 0.10</td>
<td>2.31 ± 0.03</td>
</tr>
<tr>
<td>CLA:CMC:PPI (1:1:3)</td>
<td>41.6 ± 1.4</td>
<td>5.08 ± 0.06</td>
<td>18.22 ± 3.18</td>
</tr>
<tr>
<td>PPC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLA:PPC (1:2)</td>
<td>32.0 ± 1.2</td>
<td>9.22 ± 0.25</td>
<td>2.13 ± 0.02</td>
</tr>
<tr>
<td>CLA:PPC (1:3)</td>
<td>35.5 ± 0.6</td>
<td>7.16 ± 0.47</td>
<td>2.41 ± 0.05</td>
</tr>
<tr>
<td>CLA:PPC (1:4)</td>
<td>14.7 ± 1.3</td>
<td>11.07 ± 0.59</td>
<td>2.06 ± 0.04</td>
</tr>
<tr>
<td>CLA:M:PPC (1:1:3)</td>
<td>36.4 ± 0.6</td>
<td>9.71 ± 0.80</td>
<td>2.08 ± 0.02</td>
</tr>
<tr>
<td>CLA:CMC:PPC (1:1:3)</td>
<td>40.6 ± 2.0</td>
<td>8.12 ± 0.30</td>
<td>2.96 ± 0.06</td>
</tr>
</tbody>
</table>

CLA: conjugated linoleic acid; PPI: pea protein isolate; PPC: pea protein concentrate; M: maltodextrin; CMC: carboxymethylcellulose; ME: microencapsulation efficiency; PS: particle size; D(0.5): particle median size; span: particle size scattering index. Different capital letters within the same column indicate significant differences (P < 0.05, T-test).
The presence of peaks corresponding to the hydroxyl (≈3200 cm\(^{-1}\)), carbonyl (≈1640 cm\(^{-1}\)) and conjugated double bonds (≈1530 cm\(^{-1}\)) indicate the presence of CLA in the microparticles. Yang et al. (2009) produced self-assembled CLA complex with amylose and β-cyclodextrin, and found similar FTIR spectra. We also found 2 peaks (≈2920 cm\(^{-1}\) and ≈2850 cm\(^{-1}\)) corresponding to C–H simple bonds that vibrate by stretch, that could be related to the CLA in the microparticles (Fig. 1A).

Kosaraju, Dath, and Lawrence (2006) observed interactions between olive leaf polyphenolic compounds with a chitosan matrix microencapsulated by spray-drying. In contrast, our results support that the microencapsulation by spray-drying is a physical process, which did not change the chemical structure of the core substance.

### 3.2.3. Thermal stability

Differences in thermal stability among the microparticles and their components were examined by thermogravimetric analysis (Fig. 1B). All the substances, except CLA, showed an initial weight loss at temperatures under 100 °C, which is caused by sample dehydration. The initial weight loss of CLA:M:PPC and CLA:PPC microparticles were very similar to the moisture content assessed by the moisture balance. In addition, the absence of this initial weight loss in the CLA sample confirmed that this material was anhydrous (Table 2). Wall materials had similar degradation rates and degradation temperatures (M: 186.3 ± 1.8 °C and PPC: 158.5 ± 0.2 °C), thus the mixture of M with PPC did not increase the thermal stability of the WS. CLA:M:PPC and CLA:PPC microparticles showed lower degradation temperatures (P < 0.05), 131.0 ± 0.6 °C and 133.9 ± 0.4 °C, respectively, than CLA (145.5 ± 0.5 °C), suggesting that the microencapsulation process did not increase CLA thermal stability. The microparticles showed two stages of weight loss; the first stage corresponding to CLA and their components (mean ± SD).

### 3.2.4. Differential Scanning Calorimetry (DSC)

The addition of M in the WS increased the Tg value of the CLA microparticles (CLA:M:PPC vs. CLA:PPC), however there were no differences when the wall materials were compared (PPC vs. M:PPC), suggesting that the differences observed could be related to the presence of CLA (Table 3). In the present work, we could not determine the CLA Tg. The addition of high molecular weight carbohydrates tended to increase Tg values of spray-dried fruit juices (Yousefi, Emam-Djomeh, & Mousavi, 2011).

Spray-drying products should be kept under their Tg to maintain quality during storage. At temperatures above Tg, an amorphous solid exists in a “rubbery” state in which the molecular mobility of the wall system is higher and the reactions are accelerated, resulting in physical–chemical changes and nutritional losses (Bhandari & Howes, 1999; Reineneccius, 2004).

The microparticles produced showed satisfactory values of Tg compared to previous works (Kouassi et al., 2012; Yousefi et al., 2011). From the economical point of view, microparticles with high Tg are more appealing, because they are stable at ambient temperature, not requiring refrigeration during storage. However these results must be interpreted carefully, because other factors during storage, such as relative humidity, tend to influence core stability. In addition, even at temperatures below the Tg some reactions might occur at low rates, such as non-enzymatic browning, and some small molecules such as oxygen and water might diffuse through the glassy matrix (Andersen, Risbo, Andersen, & Skibsted, 2000; Bell & Hageman, 1994).

### 3.2.5. Solubility

Rangel et al. (2003) have demonstrated the high solubility of vicilin, the major component of PPC, in a wide range of pH values. However the solubilities of PPC and the microparticles (CLA:PPC, 1:4; and CLA:M:PPC, 1:1:3) (Table 3) were lower compared to previous data (>75%) of microencapsulated CLA in whey proteins (isolate or concentrate), M and Maillard reaction products (Choi et al., 2003).

### Table 3

<table>
<thead>
<tr>
<th>Samples</th>
<th>Tg (°C) (^{a})</th>
<th>Solubility (%)</th>
<th>Dispersibility (Δ Abs, × 10(^{-3})) (^{a})</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microparticles</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLA:PPC (1:4)</td>
<td>41.4 ± 1.0a</td>
<td>22.4 ± 0.1a</td>
<td>38.8 ± 2.3a</td>
</tr>
<tr>
<td>CLA:M:PPC (1:1:3)</td>
<td>67.8 ± 2.2a</td>
<td>38.5 ± 1.8a</td>
<td>7.1 ± 0.8a</td>
</tr>
<tr>
<td><strong>Materials</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CLA</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>PPC</td>
<td>74.1 ± 2.1b</td>
<td>22.4 ± 0.1a</td>
<td>NA</td>
</tr>
<tr>
<td>M</td>
<td>156.3 ± 1.9c</td>
<td>86.3 ± 0.7c</td>
<td>NA</td>
</tr>
<tr>
<td>M:PPC</td>
<td>76.6 ± 5.4a</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>


\(^{a}\) Different letters mean significant difference in the same column (P < 0.05, ANOVA).

\(^{a}\) Different letters mean significant difference in the same column (P < 0.05, T-test).

Maltodextrin showed the highest solubility and addition of this carbohydrate in the WS improved microparticles solubility (Table 3). This high molecular weight carbohydrate is one of the most used wall materials in spray-drying processes because of its physical properties, such as high solubility and high Tg (Yousefi et al., 2011).

3.2.6. Dispersibility

Dispersibility is primarily influenced by particle size, density and the WS used (Reineccius, 2004). The small values of $\Delta_{\text{abs}}$ observed for the microparticles indicate good dispersibility (Table 3). When the particles settle down rapidly, high values of $\Delta_{\text{abs}}$ are found, and vice versa.

In the present work we only evaluated the influence of the WS, because the two formulations had similar particle size (Table 2). CLA:M:PPC presented higher dispersibility than CLA:PPC, possibly because of addition of a soluble carbohydrate (M). According to Choi et al. (2010) the interactions protein-water and sugar-water are responsible for differences in microparticles’ dispersibility. The results of dispersibility and solubility in the present work are consistent, and taken together suggest that the CLA microparticles produce stable emulsions when dispersed in water.

3.2.7. Morphology

Scanning electron micrograph (SEM) images showed that all microparticles presented spherical geometry but differed in surface topography. CLA:PPC was characterised by intense invaginations and roughness (Fig. 2A and B). In contrast, CLA:M:PPC surface topography was less rough, and did not present agglomeration problems (Fig. 2C and D). These results are in contrast with Pierucci et al. (2007), that showed maltodextrin combined with pea protein as WS did not affect particle surface morphology.

Microstructure of spray-dried microcapsules is affected by wall composition and properties, core-to-wall ratio, drying parameters, and storage conditions (Jimenez et al., 2006; Sheu & Rosenberg, 1998). Invaginations formation has been attributed to effects of drying rate, viscoelastic properties of the wall matrix and uneven shrinkage at early stages of drying (Sheu & Rosenberg, 1998). Additionally, protein-to-sugar ratio in the WS affected morphology of spray-dried CLA microparticles (Choi et al., 2010). Protein content in microparticles was negatively associated with surface invaginations, and therefore formulations with higher protein contents presented less invaginations (Choi et al., 2010). Another factor explored by Choi et al. (2010) was protein extract purity, especially concerning carbohydrates, because lactose seemed to stimulate invaginations’ formation. Accordingly, the protein concentrate used in the present work presented carbohydrates.

Invaginations adversely affect powder floatability, reconstitution properties and core material stability (Rosenberg & Sheu, 1996). Microparticles with rough and/or invaginated surfaces present accelerated release of encapsulated material due to a greater surface area (Walton & Mumford, 1999). In the present work, we did not observe cracks and open pores in CLA:PPC and CLA:M:PPC microparticles, which according to Sheu and Rosenberg (1998) might result in appropriate encapsulating ability of the core material.

3.3. Stability test

3.3.1. CLA isomer profile

CLA biological effects are isomer-specific (Whigham et al., 2007), thus the production of microparticles that could keep initial CLA isomer profile during storage is a relevant issue. Microencapsulated CLA isomer profile remained fairly stable during storage.

![Fig. 2. Structure of the microparticles produced. CLA:PPC, bar: 30 μm and 10 μm for A and B, respectively. CLA:M:PPC, bar: 30 μm and 10 μm for C and D, respectively.](image-url)
at room temperature for 2 months (Fig. 3A and B). Slight changes of the two minor groups of CLA isomers (cis,cis and trans,trans) were observed in the two microparticles. In the CLA:PPC cis,cis isomers decreased from zero to 15 days, and stabilized thereafter. In contrast, trans,trans isomers distribution was stable during the first 30 days, and decreased thereafter. Concerning CLA:M:PPC microparticles, cis,cis isomers decreased from zero to 15 days, stabilized from 15 to 30 days, and increased back to initial values at the end of the study. However, trans,trans isomers remained constant during the stability test.

These results are in accordance with other studies that showed a decrease in the minor CLA isomers during thermal processing and storage (Giua, Blasi, Simonetti, & Cossignani, 2012; Rodríguez-Alcalá & Fontecha, 2007). We also evaluated the influence of the drying process on the CLA profile of the microparticles (data not shown). Although high temperatures (150–220 °C) are used for microparticles production by spray-drying, CLA isomer profile was not influenced. Inside the drying chamber, drying takes place almost instantaneously and an intense evaporation takes place at the surface of each droplet. Consequently to the high evaporation rate, droplet remains at moderate temperatures (50–80 °C) until dry state is reached (Gharsallaoui et al., 2007).

3.3.2. CLA quantification

To avoid misleading interpretations that could be caused by CLA isomer profile estimated by internal normalisation, we also evaluated CLA concentration in the microparticles during the stability test (Fig. 3C). CLA contents did not vary between CLA:PPC and CLA:M:PPC microparticles, and were stable in both powders during storage for 60 days (Fig. 3C). Furthermore, water activity...
(A_w, 25 °C) of the microparticles was determined during storage to assess if powders were absorbing or losing water. Water activity of CLA:M:PPC powder was stable between zero and 60 days of storage (0.322 ± 0.007). In contrast, the CLA:PPC microparticles presented a slight increase in A_w (25 °C) during storage for 60 days (day 0: 0.203 ± 0.001 A_w vs. day 60: 0.306 ± 0.003 A_w; Fig. 3E). Although moisture was not determined during storage and sorption isotherms of the microparticles were not determined in the present work, based on samples' A_w variation during storage and on published sorption isotherms of powders with similar composition (Hardy, Scher, & Banon, 2002) it might be inferred that moisture variation was only slight and lead to a weight variation in the range of 2–3%, which is negligible to microcapsules composition during the stability test. Therefore, results of CLA quantification (Fig. 3C) were in accordance with stability of CLA isomer profile (Fig. 3A and B) and were consistent with the efficacy of the two wall systems for CLA protection.

3.3.3. Volatile oxidation products

Although five volatile compounds were identified in the microparticles (hexanal, 2-heptanone, heptanal, 2,2-dimethyl-decane and decane), these were also present in similar amounts in the wall material, with the exception of heptanal. The occurrence of these volatile compounds in the wall material was probably due to the presence of residual pea lipids, which were probably oxidised. Therefore, only heptanal was considered as a marker of CLA oxidation during storage. Heptanal was previously found as oxidation product in CLA-rich oil, and not in control (safflower oil), indicating that it might be a useful specific marker of CLA oxidation progress (García-Martínez et al., 2009). In the present study, heptanal concentrations increased up to 30 days and stabilized thereafter (Fig. 3D). After 60 days of storage, heptanal contents in the two types of microparticles (CLA:M:PPC, 31.7 ± 2.4; CLA:PPC, 35.1 ± 4.5) were smaller than the concentration of this volatile in a CLA-rich oil after ten days at 60 °C demonstrated by García-Martínez et al. (2009). These results are consistent with CLA stability (Fig. 3A–C) and indicate only minor changes in CLA by oxidation.

In order to evaluate the influence of microparticles WS composition in oxidative stability, AUC was calculated and reflect total production of heptanal by CLA oxidation (Fig. 3D). Heptanal increased up to 30 days and stabilized thereafter to the end of the study and this behaviour was similar between microparticles, and also total volatiles produced (CLA:M:PPC, 1412.7 ± 49.7 vs. CLA:PPC, 1442.3 ± 94.5) were not significantly different (P > 0.05) (Fig. 3D). Although we did not find influence of WS on total volatile production, Jimenez et al. (2006) showed that aldehyde accumulation was associated with WS composition of microencapsulated CLA. In this study microparticles solely with whey protein showed the lowest aldehyde accumulation and the best microstructure (spherically-shaped, smooth-surface and free of visible cracks and pores) (Jimenez et al., 2006). However, in other studies with WS comprising only protein(s) presented high oxygen permeability that could be reduced by addition of carbohydrates (Bae & Lee, 2008; Kagami et al., 2003). This may be partly due to the hydrophobic nature of oxygen gas and therefore, the presence of hydrophilic components, such as carbohydrates, could reduce wall matrix permeability to oxygen (Bae & Lee, 2008).

In contrast, oxygen diffusion across the wall greatly depends on dried WS porosity and integrity (Jimenez et al., 2006), and according to the morphological analysis in the present study the two types of microparticles produced had a surface topography devoid of open pores and cracks. CLA:PPC microparticles presented surface topography with more invaginations and roughness than CLA:M:PPC microparticles. According to Walton and Mumford (1999) these characteristics could increase core release, because of larger surface area and consequently higher interaction with medium. However, the two formulations showed effective core protection, despite of surface roughness and variation in solubility, demonstrating that in this case microparticles’ morphology did not influence core stability. Possibly, microparticles’ surface topography in this case is related to the material properties of pea protein preparations, as previously shown (Pierucci et al., 2007).

4. Conclusion

PPC microparticles showed higher microencapsulation efficiency values and more homogeneous particles. In this sense, we selected the CLA:M:PPC (1:1:3) formulation for further characterisations and stability testing. CLA:PPC (1:4) was also used for comparisons. Carbohydrate addition to the WS improved morphological properties, such as surface topography, dispersibility, solubility, and increased microparticles Tg values. Microencapsulation in the two WS was able to keep CLA stable at room temperature for 60 days, as both CLA:PPC and CLA:M:PPC microparticles showed similar and satisfactory results in stability tests. Although carbohydrate addition did not affect core stability in the proportion tested, M improved WS characteristics, such as morphological properties, dispersibility, solubility and Tg, that could favour food applications. This work was the first in which a highly hydrophobic molecule, CLA, was microencapsulated with pea proteins as the WS major component. Besides, the results here presented contribute to confirm the versatility of wall systems composed by more than one material and to increase the application range of pea protein, a natural, low cost, sustainable and hypoallergenic wall material.

Acknowledgements

This work was supported by CNPq, CAPES and FAPERJ (Brazil), A.M.M.C. was a recipient of a CAPES MSc studentship, and B.N.N.L. and J.C.N. were recipients of a CAPES DSc studentship. The authors are grateful to Maír Machado Medeiros and Leonardo Andrade for morphology analyzes assistance.

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.2014.07.016.

References
