



DEVELOPMENT OF AN INFANT FORMULA HIGH IN ALPHA-LACTALBUMIN WITH ONLY A₂ BETA-CASEIN BY SPRAY DRYING, DESIGNED TO RESEMBLE THE PROTEIN COMPOSITION OF HUMAN MILK

DESARROLLO DE UNA FÓRMULA INFANTIL ALTA EN ALFA-LACTALBUMINA CON SOLAMENTE BETA-CASEINA A₂ UTILIZANDO SECADO POR ASPERSIÓN, DISEÑADA PARA ASEMEJAR LA COMPOSICIÓN DE LA PROTEÍNA DE LA LECHE HUMANA

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Abstract

In this work, an infant formula enriched in α -lactalbumin and whose β -casein fraction contains only A₂ β -casein was developed by spray drying. Experimental infant formula (EF) was designed to closely resemble the protein composition of human milk. Response surface method (RSM) with two factors: total solids and inlet air temperature was used for the optimization of the spray drying process. Response variables were solubility, moisture content, luminance and peroxide value. To define optimal drying conditions, a stability test was performed for two treatments (120 °C, 40 wt. % and 170 °C, 30 wt. %), which were obtained from the RSM. The treatment with 170 °C and 30 wt. % concentration of total solids was selected as the best alternative for the spray drying process. The protein composition in the EF was 37.1% casein, 23.1% α -lactalbumin and 21.3% β -lactoglobulin. A decrease of 6% in β -lactoglobulin increased the relative proportion of α -lactalbumin, which favoured the similarity of the EF to breast milk. The homology of the β -caseins with the A₂ β -casein isoform was 99.82%.

Keywords: infant formula, breast milk, spray drying, optimal drying conditions, A₂ β -casein.

Resumen

En este trabajo se desarrolló mediante secado por aspersión, una fórmula para lactantes enriquecida en α -lactalbúmina y cuya fracción de β -caseína contiene únicamente β -caseína A₂. La fórmula se diseñó para parecerse a la composición de proteínas de la leche humana. Para la optimización del proceso de secado por aspersión, se utilizó el método de superficie de respuesta (MSR) con dos factores: sólidos totales y temperatura del aire de entrada. Las variables de respuesta fueron solubilidad, humedad, luminancia y el valor de peróxido. Para definir las condiciones de secado óptimas, se realizó una prueba de estabilidad para dos tratamientos (120 °C, 40% de concentración de sólidos y 170 °C, 30% de concentración de sólidos), que se obtuvieron del MSR. El tratamiento con 170 °C y 30% de concentración de sólidos se seleccionó como la mejor alternativa para el proceso de secado por aspersión. La composición proteica en la fórmula infantil experimental (EF) fue de 37.1% de caseína, 23.1% de α -lactalbúmina y 21.3% de β -lactoglobulina, una disminución del 6% en β -lactoglobulina aumentó la proporción relativa de α -lactalbúmina, lo que favoreció la similitud de la EF con la leche materna. La homología de las β -caseínas con la isoforma β -caseína A₂ fue del 99.82%.

Palabras clave: fórmula infantil, leche materna, secado por aspersión, condiciones óptimas de secado, β -caseína A₂.

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1 Introduction

Breast milk (BM) covers the nutritional needs of term infants during the first six months of life; it is a dynamic fluid that adapts to the needs of the baby and contains multiple bioactive compounds (Andreas *et al.*, 2015). When lactation is not possible, newborns are fed with infant formulas (IFs). Currently, IFs are made from cow's milk to which other compounds are added in order to reach the desired composition (Chávez-Servín *et al.*, 2015).

IFs differ from human milk in quantity and quality of protein. In cow's milk protein, the ratio of whey to casein protein is 20:80. 50% of whey protein is composed of β -lactoglobulin which is absent in breast milk and is responsible for allergic responses commonly observed in infants fed with IF (Murphy *et al.*, 2015). β -casein (β -CSN) accounts for 38-42% of casein in bovine milk (Paterson *et al.*, 1995). There are 13 variants of β -CSN and the most common forms in the dairy breeds are A₁ and A₂. A₁ β -CSN differs from A₂ β -CSN in an amino acid at position 67, where histidine is present in the former, and proline in the latter. This change has important repercussions since during the digestion of A₁ β -CSN a bioactive peptide called β -casomorphin 7 (BCM-7) is released. Different studies *in vitro* demonstrate that this peptide induces expression and secretion of gastrointestinal mucins and regulatory peptides in both human and animal intestinal cells (Zoghbi *et al.*, 2006), as well as affects human lymphocyte proliferation (Raies *et al.*, 2014). BCM-7 has also been reported to slow down gastrointestinal motility similar to the effect of morphine (Shah, 2000) and to exert antihypertensive and cardiotropic effects (Saito, 2008) in rodents. Moreover, BCM-7 has been found to decrease pain sensitivity (Dubynin *et al.*, 2008) and to induce apnea and irregular breathing in adult rats and newborn rabbits (Hedner and Hedner, 1987). Furthermore, consumption of A₁ β -CSN and the consequent release of BCM-7 have been suggested as risk factors for gastrointestinal discomfort and constipation (Brooke-Taylor *et al.*, 2017) in adults, as well as for apnea expressed as apparent life-threatening events (Wasilewska *et al.*, 2011) and for delay in psychomotor development (Kost *et al.*, 2009) in infants.

In breast milk, the whey to casein ratio is 60:40 and β -CSN is the major component of the casein fraction. Breast milk is 'A₂-like' since it has proline at position

67 (Lönnerdal *et al.*, 1990). α -lactalbumin (ALA) is one of the main components of whey protein in human milk and it has a high biological value (Murphy *et al.*, 2015). The total amount of protein ingested with IF is also different compared to human breast milk; IF has a protein concentration of 1.4-1.7 g/100 mL (Camilia *et al.*, 2016) and breast milk contains 0.8-1.1 g/100 mL (Lönnerdal *et al.*, 2017). According to Codex Alimentarius, 2007, the concentration of protein recommended for infants in formulas are 1.2-2 g/100mL (Garlick, 2006). Among the consequences of these high protein concentrations is rapid weight gain in infants which may increase the risk of obesity in later life (Koletzko *et al.*, 2009). Some authors have proposed a decrease in the amount of protein in infant formulas (Lönnerdal, 2014; Weber *et al.*, 2014).

In the pharmaceutical, agrochemical and food industries, spray drying is the most widely-used method to transform fluid materials into a solid for the purpose of facilitating storage and prolonging shelf life. The final product has a low water activity due to evaporation during drying. This process enables the manufacture of end products with high physicochemical quality and stability during storage (Murugesan and Orsat, 2012). The physicochemical properties of the final product mainly depend on inlet temperature and total solids concentration (Birchal *et al.*, 2005).

Response surface methodology (RSM) is a tool commonly used in the analysis of experimental data for the optimization of manufacturing processes (Madamba, 1997). The aim of this work was development an infant formula designed to closely resemble the protein composition of human milk by spray drying. The spray drying process was optimized using response surface methodology (RSM). This formula was made with cow's milk that contained 100% A₂ β -CSN, with protein concentration (1.21 g/100mL), it was high in α -lactalbumin and a relatively low β -lactoglobulin protein concentration, with respect to infant formulas which are currently marketed.

2 Materials and methods

2.1 Chemicals

Methanol, chloroform, hexane, iron, ammonium thiocyanate, barium chloride, ferrous sulfate, sulfuric acid, sodium hydroxide, boric acid, potassium acetate,

potassium carbonate, sodium nitrate, potassium chloride, acetonitrile, water and formic acid were purchased from J. T. Baker (Center Valley, PA, USA).

Acrylamide, N,N'-methylene-bis-acrylamide, ammonium persulfate, tris base, tetramethylethylenediamine, glycine, glycerol, 2-mercaptoethanol, sodium dodecyl sulfate, urea, pepsin, trypsin, chymotrypsin, carboxypeptidase, thermolysin enzymes along with α -lactalbumin, β -lactoglobulin and β -casein standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Coomassie blue G-250 was purchased from Serva (Heidelberg, Germany).

2.2 Design of the EF

The EF was designed using skimmed milk contains only A₂ β -casein and was obtained from Jersey cows previously genotyped according to Duarte-Vázquez *et al.*, (2017), for detection of SNP rs43703011[C] (GeneBank) within CSN2 gene that causes the change from proline 67 in A₂ β -CSN to histidine in the same position of A₁ β -CSN, produced by Ecológico Tierra Viva® (Guanajuato, México). The composition of skimmed milk is: 4.77 g protein/100 g dry solids, 0.157 g fat/100 g dry solids, 6.57 g lactose/100 g dry solids; EF formulation also included 5.69 g whey protein concentrate (WPC)/100 g. WPC was enriched with α -lactalbumin and is specially designed for use in infant formulas (Hilmar Ingredients). The composition of WPC is: 78 g protein/100 g dry solids, 12 g fat/100 g dry solids, 3.3 g ash/100 g dry solids, 1.5 g lactose/100 g dry solids. The following ingredients were also added to EF: fat, carbohydrates (lactose, maltodextrin), vitamins mixture (vitamin A, vitamin D3, vitamin E, vitamin K, vitamin B6, folic acid, pantothenic acid, biotin, niacin, riboflavin, thiamine, vitamin B12, vitamin C), minerals mixture (calcium, phosphorus, potassium, zinc, iron, selenium, sodium, chlorine, copper, iodine, magnesium, manganese) and prebiotics (fructooligosaccharides), obtained from standards ingredients commonly used for infant formula preparation.

The fat mixture was made from soybean oil, coconut oil, sunflower oil, Betapol, and ARA and DHA oils. Betapol and coconut oil were melted at 30 °C before blending with soybean, sunflower, ARA and DHA oils. The final fat mixture was heated to 60 ± 5 °C and added to the mixture of all the other ingredients.

Water-soluble materials including maltodextrin, lactose, minerals, serum proteins and fructooligosaccharides, were reconstituted in 114 mL skimmed

milk with A₂ β -CSN at 50 °C and then stirred until completely mixed. The previously-prepared fat mixture was dispersed into this mixture using an Ultra-Turrax homogenizer (T25 digital Ultra-Turrax, IKA, Wilmington, NC, USA) at 17500 rpm for 3 min, after that purified water was added to adjust total solid concentration. Finally, the emulsion was heated to 50 °C and then spray-dried at different temperatures according to the experimental design, using a mini Spray Dryer B-290 (Büchi, Flawil, Switzerland). A mixture of vitamins was added after the spray drying process.

2.3 Process development

EF was dried using a laboratory-scale spray dryer. Inlet air temperature varied from 110 °C to 180 °C, outlet air temperature varied from 60 °C to 80 °C and total solids concentration varied from 28 to 42%. The powder was then vacuum-packed in polyethylene bags and stored at -8 °C until further analysis.

To define the optimal conditions for the development of EF and for the maximisation of stability during storage, inlet air temperature and total solids concentration were measured as two independent variables in a factorial design, with four factors and five levels, totalling 13 experiments (RCCD, rotational central composite design) (Table 1). The tests were performed randomly, and data was analysed using Design-Expert software v.6.010 (STAT-EASE, 2003). Experimental error was obtained from the mean and standard deviation of the central points. The following response variables were used to characterise EF: moisture content (%), solubility (%), luminance (%) and peroxide value (meq O₂/kg⁻¹ fat).

2.4 Analysis of EF

2.4.1 Bulk density and tapped density

For determination of bulk density, powdered EF was transferred to a 10 mL graduated cylinder. After the content was weighed, mass was divided by occupied volume. Tapped density was calculated by placing 1 g of powder sample in a 10 mL measuring cylinder. The cylinder was tapped vigorously by hand until no further change in volume occurred.

Table 1. Coded and original values of the independent variables.

Treatment no.	Inlet air temperature		Solids concentration	
	Real value (°C)	Coded value (X2)	Real value (%)	Coded value (X1)
1	145	0	28	-1.414
2	145	0	35	0
3	145	0	35	0
4	120	-1	40	1
5	170	1	30	-1
6	145	0	35	0
7	145	0	35	0
8	110	-1.414	35	0
9	145	0	35	0
10	120	-1	30	-1
11	180	1.414	35	0
12	170	1	40	1
13	145	0	42	1.414

2.4.2 Particle morphology

Particle morphology was evaluated by scanning electron microscopy (SEM) and it was analyzed qualitatively. Powders were attached to a double-sided adhesive tape mounted on SEM stubs, coated with 10 nm gold under vacuum and examined with a scanning electron microscope (EVO50 Carl Zeiss). SEM was operated at 5 kV with magnifications of 2500 to 5000 X.

2.4.3 Total fat

Fat analyses were performed using soxhlet extraction mechanism. 2 g of EF was weighed and placed in a Soxtec Avanti 2055 (FOSS Tecator AB, SE). The extraction was performed for 1.25 h using 75 mL of n-hexane.

2.5 Tests for process optimization

2.5.1 Solubility

Five g of EF was reconstituted in 50 mL of water at 24 °C; the solution was shaken and centrifuged at 3800 rpm in conical graduated tubes in order to evaluate the sediment (Schuck *et al.*, 2012).

2.5.2 Moisture content

Moisture content was evaluated using a vacuum oven (AOAC International 2016; method 927.05; 33.5.02).

A sample of 2 g was weighed in an aluminium pan and kept at 102 °C for 24 h. Moisture content was calculated as mean weight loss after drying.

2.5.3 Luminance

Representative samples of each treatment were placed in a circular container (80 mm diameter and 20 mm depth), and then covered with clear glass (2 mm thickness). Using a tristimulus colorimeter (HunterLab Miniscan XE, Mod 45/0-L, Hunter Associates Laboratory, Inc. Reston, VA, USA) 5 surface points were obtained, and the L value means were measured.

2.5.4 Peroxide value

Lipid oxidation was evaluated by determination of peroxide value according to International Standard ISO-IDF methodology (ISO, 2006). A sample of 0.14 g of powder was weighed into a test tube and suspended in 9.8 mL of a chloroform/methanol (7:3 v/v) mixture. For colour formation, 50 μ L of 3.94 M ammonium thiocyanate and 50 μ L of Fe^{2+} (obtained from a mixture of 0.132 M BaCl_2 and 0.144 M FeSO_4) were added. The tube was centrifuged at 3000 rpm for 3 min and kept in the dark for 6 min. Absorbance was determined at 500 nm using a Genesys 2-V visible spectrophotometer (Spectronic, Rochester, NY). Peroxide value concentration was calculated with a Fe^{3+} calibration curve (Shanta and Decker, 1994).

2.6 Stability during storage

2.6.1 Peroxide value (PV) and hydroxymethylfurfural (HMF)

PV as a predictor or indicator of lipid oxidation and HMF as a component of the Maillard reaction were evaluated in freshly-prepared samples of EF and during storage after 2, 4, 6, 8 and 10 weeks at different percentages of relative humidity (RH): 0, 40, 60 and 80%. PV was determined following the methodology described in section 2.5.4. For determination of HMF, 0.7 g of EF was weighed in a 25 mL centrifuge tube and 15 mL of deionised water was added. The centrifuge tube was shaken vigorously for 1 min and centrifuged for 10 min at 5000 rpm. The supernatant was clarified with 1 mL of Carrez I (potassium ferrocyanide 15% w/v) and Carrez II (zinc acetate 30% w/v) solutions and the resulting mixture was centrifuged. HMF was determined by UPLC and UV detection (276 nm), using the method described by Guerra-Hernández *et al.* (1992), with slight modifications. Briefly samples were centrifuged for 15 min at 7000 rpm and filtered through a 0.22 μ m disc filter. All analyses were performed in duplicate.

2.7 Protein identification in EF

2.7.1 Extraction of total casein

Total casein fractionation was performed using the isoelectric precipitation method described by Hollar *et al.*, (1991), with minor modifications. Briefly, 42 g of EF was rehydrated with 30 mL of purified water. The solution was centrifuged at 4500 rpm for 20 min at 25 °C, and this procedure was repeated three times in order to skim the solution. Then, the skim solution was heated at 30 °C and was adjusted to pH 5.0 with 1M acetic acid. This solution was immediately centrifuged at 4500 rpm for 20 min at 25 °C in order to precipitate the total caseins. Subsequently, the precipitate was washed with water until a pH of 4.5 was achieved. Finally, the caseins were centrifuged again at 4500 rpm for 20 min at 25 °C, 3 times, and the pellet of total caseins was suspended in HPLC-grade water at pH 9.

2.7.2 Composition of the β -CSN fraction of the EF by urea-PAGE

PAGE of casein was performed according to Duarte-Vazquez *et al.*, 2018. Briefly, casein sample were mixed 6:1 (v/v) with a 0.12 M Tris base, 0.0025 M

EDTA, 8.2 M urea, 0.2 M 2-mercaptoethanol and 0.01% bromophenol blue 6x sample loading buffer. Casein working solutions were heat denatured at 95 °C for 5 min, then 7.9 μ g of casein protein, were loaded into PAGE wells. The stacking gel solution was prepared to 4% acrylamide, whereas the resolving gel contained 15% acrylamide, 9 M urea at pH 8.9. A Mini PROTEAN cell (Bio-Rad) filled with 0.02 M Tris, 0.19 M Glycine, pH 8.3 running buffer was used. Separation was performed at 4 °C, 0.01 A for 15 min, followed by 0.03 A for 2 h. The gel was stained with Coomassie G-250 and documented on a GelDoc EZ system (Bio-Rad), using the ImageLab 5.2.1 software (Bio-Rad). Standards of A₁/A₁ β -CSN and A₂/A₂ β -CSN were used as a reference to compare the relative mobility with the β -CSN of the EF.

2.7.3 Amino acid sequencing of the β -CSN in EF

Protein identity of the band corresponding to the A₂/A₂ β -CSN was determined by HPLC-MS/MS (Duarte-Vazquez *et al.*, 2018). A₂/A₂ β -CSN protein band from EF was excised from the gel and subjected to enzymatic digestion with thermolysin. The digestion products of β -CSN were desalted with Zip Tip C18 (Merck Millipore) and final peptides were injected into an LC-MS system using an EASY-nLC II nanoflow pump (Thermo-Fisher) coupled to an LTQ Orbitrap-Velos mass spectrometer (Thermo-Fisher) system with nano-electrospray ionisation (ESI).

A capillary column (0.75 μ m ID and 10 cm L RP-C18) was used with mobile phase gradient 10-80% (water/acetonitrile with 0.1% formic acid) for 120 min in the HPLC with a flow rate of 300 nL/min⁻¹. Peptide fragmentation was carried out by both CID (Collision-Induced Dissociation) and HCD (High-Energy Collision Dissociation), where only 1⁺, 2⁺, 3⁺ and 4⁺ ions were selected for fragmentation events. All spectra were acquired in positive detection mode. The capture of the fragmentation data was performed according to the predetermined scanning of ions, with an isolation width of 3.0 (m/z), 35 arbitrary units of normalised collision energy, activation (Q) of 0.250, activation time of 10 ms and maximum injection time of 10 ms by micro-scanning. During automatic data capture, dynamic ion exclusion was used: (i) exclusion list of 400 ions, (ii) pre-exclusion time of 30 s and (iii) exclusion time of 300 s. The spectrometric data were compared to A₁ and A₂ casein isoforms using the sequence search in the Proteome Discoverer 1.4 program.

3 Results and discussion

3.1 Characterisation of EF

3.1.1 Bulk density and tapped density

Table 2 shows that bulk density and tapped density were mainly affected by drying temperature, since it affects the degree of inflation and roughness and also influences the particles' porosity (Nijdam and Langrish, 2005). Higher temperatures induced increases in drying rate, giving rise to smaller and spherical particles (Nikolova *et al.*, 2015). When the water evaporation rate is accelerated, an air vacuole inside the particle is formed, and the pressure exerted by internal vapor promotes sphericity in particles (Nijdam and Langrish, 2006). This situation facilitates the compressibility of the powder and consequently a higher tapped density is observed (Nikolova *et al.*, 2015). This phenomenon was corroborated in this study, as the highest densities were observed at higher experimental temperatures (170 and 180 °C). It was also observed that the lowest density was obtained at the lowest temperature (110 °C). The lower the bulk density, the more occluded air is present within the powder, which increases the possibility for oxidative degradation and reduced storage stability.

3.1.2 Particle morphology

Treatments with lower temperatures during drying produced particles with surface roughness (Fig. 1a); this phenomenon was notable because at low temperatures the evaporation of the water contained in the droplet is slow, which allows contraction of particles, generating rough surfaces (Kim *et al.*, 2009; Borges *et al.*, 2017; Fuentes-Ortega *et al.*, 2017). When temperature was higher during drying (170 and 180 °C), it accelerates the hardening of the outer layer of the droplet, producing a bubble of inner vapor "vacuole" and in this way deflation is avoided, which produces more sphericity in its morphology (Fig. 1b) (Nikolova *et al.*, 2014). Kim *et al.*, (2009), evaluated the effect of dryer temperature in the particle of milk powder by scanning electron microscopy, and found that higher temperature the drying accelerated the droplet drying rate, promoting rapid formation of a crust on powder surface, which caused the milk particle present a spherical and smooth appearance. However, when the drying temperature was lower, the particle remained moist and flexible for a longer time, emptying and wrinkling as it cooled, with wilted appearance. With respect to the size of most particles, was less than 10 μm , because the total solids content was low (Fig. 1b).

Table 2. Bulk and tapped density of the EF.

Treatment no.	Inlet temperature (°C)	Solids concentration (wt.%)	Total fat (%)	Bulk density (g/mL)	Tapped density (g/mL)
1	145	28	14.8 ± 0.2 ^e	364.0 ± 0.4 ^{cdef}	520.1 ± 10.0 ^{bcd}
2	145	35	11.5 ± 0.1 ^{bc}	339.7 ± 2.0 ^{abc}	499.7 ± 13.4 ^{abcd}
3	145	35	13.1 ± 0.0 ^d	351.0 ± 5.3 ^{abcde}	512.4 ± 2.4 ^{bcd}
4	120	40	10.9 ± 0.0 ^b	351.5 ± 4.5 ^{abcde}	540.8 ± 4.9 ^d
5	170	30	12.2 ± 0.2 ^{cd}	370.6 ± 14.3 ^{ef}	501.6 ± 38.5 ^{abcd}
6	145	35	11.2 ± 0.1 ^{bc}	357.2 ± 2.6 ^{bcdef}	463.9 ± 5.2 ^{ab}
7	145	35	15.0 ± 0.3 ^e	343.3 ± 12.9 ^{abc}	480.3 ± 22.7 ^{abc}
8	110	35	14.5 ± 0.4 ^e	328.8 ± 6.3 ^a	444.4 ± 8.5 ^a
9	145	35	14.8 ± 0.2 ^e	378.5 ± 3.8 ^f	498.0 ± 5.0 ^{abcd}
10	120	30	15.4 ± 0.1 ^e	365.4 ± 2.8 ^{def}	514.8 ± 14.2 ^{bcd}
11	180	35	12.8 ± 0.7 ^d	382.2 ± 1.0 ^f	530.8 ± 1.4 ^{cd}
12	170	40	6.4 ± 0.0 ^a	448.8 ± 5.0 ^g	680.0 ± 7.5 ^e
13	145	42	11.0 ± 0.3 ^b	333.0 ± 2.3 ^{ab}	520.3 ± 3.6 ^{bcd}

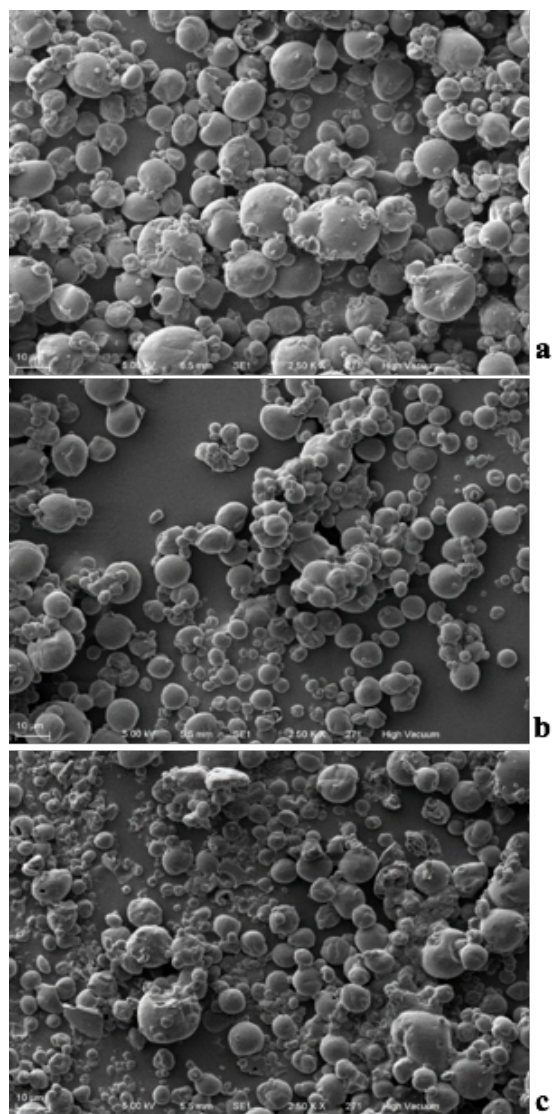


Fig. 1. Microstructure of spray-dried EF. (a) Treatment 4: 120 °C and 40 wt.%. (b) Treatment 5: 170 °C and 30 wt.%. (c) Treatment 12: 170 °C and 40 wt.%.

In conditions of higher solids concentration and higher temperatures, the hardening of the outer layer of the particle was so rapid that some particles were fragmented and others developed hardness in the outer layer, so total fat extraction was lower (Fig. 1c). According with Park *et al.*, (2013), an increased feed solids concentration during drying result in an increase in the average particle size and a reduction in the surface free fat, because the droplets generated contained a higher concentration of solids and thus the powder particles are larger upon drying. The increase in average particle size because of feed

solids concentrations is consistent with previously published literature with whole milk powders (Nijdam and Langrish, 2006).

3.2 Regression coefficients and statistical analysis

Four response surface models were obtained, considering four response variables. The experimental results were statistically analyzed in order to obtain the regression models. ANOVA was used in order to evaluate the influence of each variable on the optimization model. The response variables solubility and moisture content, were analyzed with a quadratic model and the response variables luminance and peroxide value were analyzed with an interaction model with two factors. The Table 3 showed the values of R^2 were between 0.36-0.77. The statistical analysis (Table 3) indicated that the A was the factor that showed the more highly significant effect ($p < 0.05$) in the linear and quadratic terms on solubility. The factor B exhibited a significant effect ($p < 0.05$) in its quadratic terms on moisture content. Also, the interaction AB had significant effect on the variables of response moisture content, and luminance.

3.2.1 Solubility and moisture content

An important characteristic in infant formula is the need to dissolve instantly when incorporated into water. Solubility is an important parameter that influences of this process. If a powder has a solubility index below 95% it is considered insoluble (Schuck *et al.*, 2012). Effect of the process variables on solubility is related to their effect on residual moisture content of powder samples. The lower the moisture content of the powder sample, the more soluble it is (Goula and Adamopoulos, 2005). The percentage of solubility found in all treatments was above 99%, which is consistent with data reported by Schuck *et al.*, (2012), in powders obtained from skimmed and semi-skimmed milk. The data found in this study showed that solubility increased when solids concentration and temperature increased (Fig. 2a), due to their negative effect time on moisture content of the powder sample. This result agrees with the data reported by Bansal *et al.*, (2014), who suggested that the solubility index rises when there is an increase in the air inlet temperature during the drying process. Increase in water solubility with increase in blower speed is also related to its negative effect on powder moisture content (Muzaffar *et al.*, 2016).

Table 3. Analysis of regression coefficients in the optimization process.

	Solubility	Moisture content	Luminance	Peroxide value
Intercept	99.9505	13.4168	93.3258	2.72687
Linear				
A	0.0202535 (0.047)	-2.2256 (0.064)	-	-
B	-	-	-	-
Quadratic				
A ²	-0.0216563 (0.047)	-	-	-
B ²	-	-2.72771 (0.04)	-	-
Interactions				
AB	-	-4.73279 (0.013)	-0.94625 (0.032)	0.445484 (0.092)
R ²	0.68	0.77	0.50	0.36
<i>poF</i> (model)	0.0854	0.0308	0.08	0.27

$$Solubility : 99.950 + 0.0202535A - 0.0216563A^2 \quad (1)$$

where: A: Total solids

B: Inlet drying temperature

Goula and Andamopoulos, (2008), related solubility with powder moisture, arguing that the former increases when humidity decreases; this tendency was observed during this study, since solubility was maximised at the lowest humidity conditions (Fig. 2a).

With regard to moisture content, the coefficients from Eq. (2) show that humidity decreased as the temperature and the total solids concentration increased. It was found that 99% of the moisture percentage depended on the interaction between the total solids concentration and the air inlet temperature. It was observed that the lowest moisture percentages

were obtained in conditions of higher temperature and total solids concentrations (Fig. 2b). A similar behaviour was reported by Nikolova et al, (2014). The decrease in moisture content at higher inlet air temperatures is due to the greater temperature gradient between the atomized feed and the drying air, resulting in a greater driving force for water evaporation and thus produces powders with lower moisture content. Muzaffar and Kumar (2015), also observed similar results during spray drying of watermelon juice. Increase in feed flow rate offers shorter contact time between the feed and the drying air, making the heat transfer less efficient and resulting in lower water evaporation.

$$Moisture : 13.4168 - 2.2256A - 4.73279AB - 2.72771B^2 \quad (2)$$

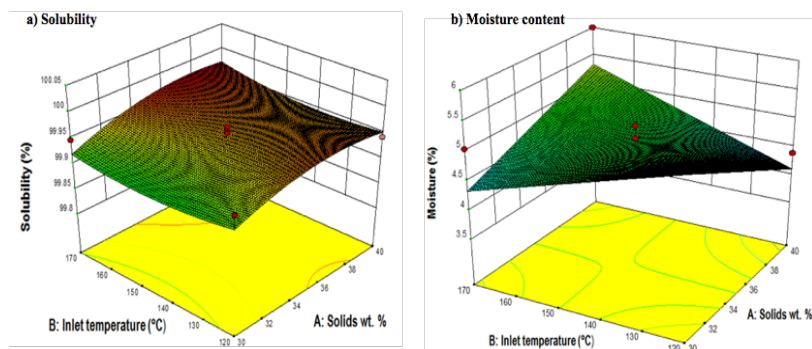


Fig. 2. Effect of inlet air temperature and total solids on a) solubility and b) moisture content.

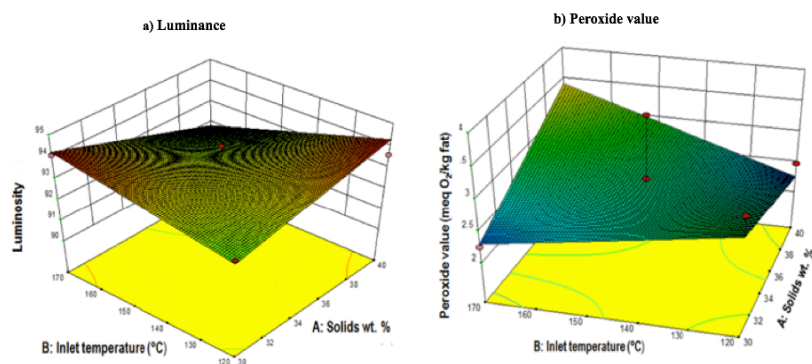


Fig. 3. Effect of inlet air temperature and total solids on a) luminance (L) and b) peroxide value.

3.2.2 Luminance (L) and peroxide value (PV)

The decrease in luminance represents an increase of the Maillard reaction (MR). This reaction depends mainly on the infant formula's heat treatment conditions during drying, milk composition and storage conditions (Van Boekel, 1998). Eq. (3) shows that the luminance decreased when the concentration of solids and the inlet air temperature increased.

$$\text{Luminance} : 93.3258 - 0.94625AB \quad (3)$$

Two areas were found in which luminance was maximised: the first when there was a higher temperature and a lower total solids concentration, and the second when there was a lower temperature and a higher total solids concentration.

This occurs because when the outer layer is formed, an increase in its internal temperature is observed (Birchal *et al.*, 2006), causing the synthesis of brown pigments in the EF. The time of exposure to high temperature depends on the water content in the drops. The lower the solids concentration in the emulsion, the shorter the exposure time of the particle at higher temperatures, hence L is affected to a lesser extent. On the other hand, when drying temperature was increased, L decreased considerably (Fig. 3a); this effect was also reported by Sulieman *et al.*, (2014). Peroxide values are a good indicator of the quality of lipid compounds in foods because the long-chain polyunsaturated fatty acids (LC-PUFAs) added to infant formulas (IFs) are susceptible to oxidation during manufacturing and storage. Eq. (4) shows that the peroxide value decreased as the inlet air temperature and total solids decreased.

$$\text{Peroxide value} : 2.72687 + 0.445484AB \quad (4)$$

The exposure time of the particle at higher temperatures produced an effect on lipid oxidation. Two areas with lower peroxide values were found: lower temperatures and higher total solids concentration, or higher temperatures and lower total solids concentration (Fig. 3b). In the first condition, we can observe that although the amount of water contained in the droplets was lower, the temperature increase in the particle had a minimal impact on the degree of oxidation of the EF because the drying temperature was lower. In the second condition, the water content to be evaporated was higher, so the time elapsed to evaporate the water content in the droplet was sufficient to avoid the overheating of the particles due to the effect of high temperature, thus avoiding a high degree of lipid oxidation in the EF. Peroxide value increased under conditions of higher temperature and higher total solids concentration during the spray drying process.

Regarding peroxide values, Roumeu-Nadal *et al.*, (2007), found values below 1 meq O₂/kg⁻¹ fat, in freshly prepared IF, although the values found during the optimization in this study were higher (2.40-2.43 meq O₂/kg⁻¹ fat). WHO recommends a limit of 10 meq O₂/kg⁻¹ fat.

3.3 Optimal conditions

To determine the optimal drying conditions, we sought to minimise the peroxide value and the moisture content while maximising luminance and solubility; the response variables solubility and moisture content, were analysed with a quadratic model and the response variables luminance and peroxide values were analysed with an interaction model with two factors.

Table 4. Solutions found.

No.	Total solids (wt.%)	Temperature (°C)	Solubility (%)	Moisture (%)	Luminance (%)	Peroxide value (meq O ₂ /kg)	Desirability
1	40.000	120.000	99.953	4.111	94.294	2.258	0.896
2	39.921	120.000	99.953	4.122	94.293	2.263	0.892
3	40.000	121.917	99.951	4.158	94.290	2.302	0.872
4	30.000	170.000	99.921	4.343	94.262	2.305	0.826

The optimization process was performed using the numerical method with the Design-Expert software v.6.010 (STAT-EASE, 2003).

The desirability for each variable response were chosen as summarized in Table 4. The simultaneous optimization of the responses variables is done based on this parameter. Stability tests were performed on the desired goals that resulted from the optimization process (Treatment 4: 120 °C, 40 wt.% and Treatment 5: 170 °C, 30 wt.%).

3.4 Stability during storage

3.4.1 Peroxide value (PV)

Increases in lipid oxidation were observed when the EF was subjected to different relative humidities (RHs) during storage. The samples with 20% RH showed slight changes during storage; this behaviour showed that with a water activity (a_w) of 0.2, lipid oxidation is very low, as reported by Labuza *et al.*, (1972).

Fig. 4 shows that with a water activity (a_w) of 0.6 to 0.8, the maximum levels of oxidation occurred, which coincides with the findings of Labuza *et al.*, (1972); there may have been polymerisation since the first week, because peroxides are formed continuously and are transformed into byproducts through secondary oxidation (Shahidi and Zhong,

2005). In Treatment 4, polymerisation of peroxides was greater starting at the second week, unlike Treatment 5 in which the same condition was observed after the fourth week, but there was not a treatment effect.

In general, when a_w increases in EF, peroxide value increases. This most likely occurs because the increase in a_w decreases the glass transition temperature in EF (Grattard *et al.*, 2002). Under a_w conditions greater than 0.4, it was observed that the matrix structure showed humidity caking and this facilitated the migration of fat from the interior to the exterior of the particles, producing a greater oxidation of the lipid fraction (Fig. 4).

3.4.2 Hydroxymethylfurfural (HMF)

Higher HMF values were observed under conditions of 0.2 a_w . This does not mean that under this condition HMF formation is greater, so in conditions of higher a_w , increases in polymerisation of HMF and the amount of HMF identified is minor (Fig. 5). The polymerisation of the intermediate products of the Maillard reaction produces brown pigments generated by the synthesised melanoidins (Van Boekel, 1998). When higher water activities were observed, the HMF was polymerised to form melanoidins (Fig. 5).

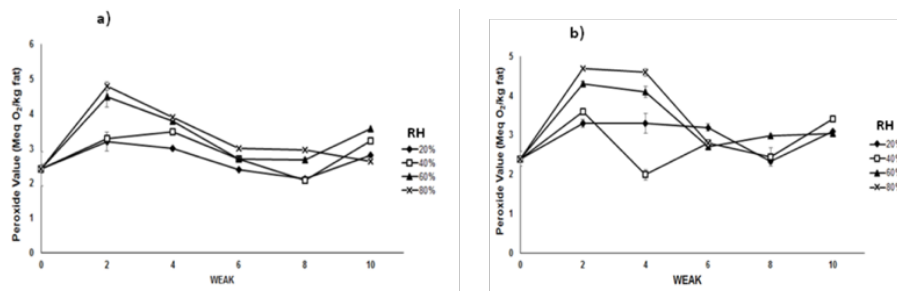


Fig. 4. Peroxide values during storage at 37 °C and different RHs. (a) Treatment 4: Tinlet 120 °C and 40 wt.% (b) Treatment 5: Tinlet 170 °C and 30 wt.%.

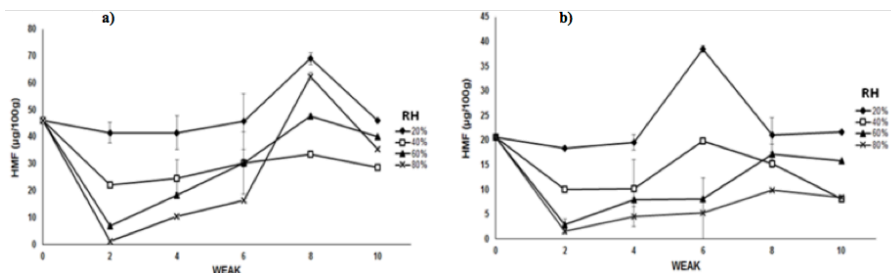


Fig. 5. HMF during storage at 37 °C and different RHs. (a) Treatment 4: Tinlet 120 °C and 40 wt.% (b) Treatment 5: Tinlet 170 °C and 30 wt. %.

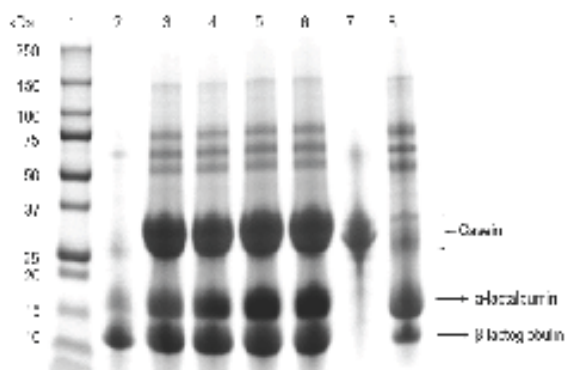


Fig. 6 Protein profiles in the EF obtained by SDS-PAGE. (1) Molecular weight marker; (2) Standard of BLG and ALA; (3,4) Treatment 4; (5,6) Treatment 5; (7) Standard casein; (8) Holstein cow milk whey.

Upon analysis of the 0.2 a_w in both treatments (where there were no brown pigments) it can be observed that the highest HMF value occurred during the eighth week of Treatment 4 (46.8 to $69.0 \mu\text{g}/\text{kg}^{-1}$) and during week six of Treatment 5 (20.7 to $30.5 \mu\text{g}/\text{kg}^{-1}$). However, values returned to baseline due to the polymerisation of the HMF produced. HMF values in Treatment 5 remained below $40 \mu\text{g}/\text{kg}^{-1}$, lower than those produced in Treatment 4 (Fig. 5). As inlet air temperature increases during drying, the rate of water evaporation increases considerably (Fu *et al.*, 2013); for this reason drying was faster in Treatment 5 than in Treatment 4.

a) Experimental infant formula (EF)

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQTEDELQDKIHPFAQTQSLVY**FPFGPI**PNLSL
 PQNIPPLTQT PVVVPFLQP EVMGVSKVKE **AMAPKHKEMP** **FPKY**PVEPFT ESQSLTLTDV
 ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY **QRDMP**IQAF
 LLYQEPVLGP **VRGPFPIIV**

b) Holstein Friesian's milk

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQTEDELQDKIHPFAQTQSLVY**FPFGPI**PNLSL
 PQNIPPLTQT PVVVPFLQP EVMGVSKVKE **AMAPKHKEMP** **FPKY**PVEPFT ESQSLTLTDV
 ENLHLPLPLL QSWMHQPHQP LPPTVMFPPQ SVLSLSQSKV LPVPQKAVPY **QRDMP**IQAF
 LLYQEPVLGP **VRGPFPIIV**

Fig. 7. Protein sequence in β -CSN fraction a) EF and b) Holstein Friesian milk; bold letters represent high certainty.

3.5 Testing for EF protein composition

3.5.1 Identification of EF proteins

To determine the relative abundance of the different proteins in the EF, Bio-Rad Image Lab software was used. The relative abundance was based on the optical density of each band. Quantification of the protein bands showed that the ratio was approximately 40:60 (casein to whey protein). The percentage of casein was 37.1%, α -lactalbumin (ALA) 23.1%, β -lactoglobulin (BLG) 21.3%, 6.5% bovine serum albumin (BSA), 6.0% immunoglobulins and 5.8% lactoferrin. A decrease of 6% in BLG increased the relative proportion of ALA and lactoferrin, which favoured the similarity of the EF to breast milk (Fig. 6).

3.5.2 Sequencing of β -CSN in EF

With regard to amino acid composition, the β -CSN of A₁ and A₂ isoforms have a similar sequence, except for a change of histidine to proline at position 67, for the A₂ isoform. It was found that the β -casein of the EF produced corresponds to the A₂ β -casein isoform with a relative abundance of 92.87%, and it also contained the amino acid proline at position 67, like the proteins in breast milk (Fig. 7). This was expected since the EF was made exclusively with milk from cows typified for the BCA2 gene.

Conclusions

The composition of the infant formula produced in this work was similar to that of breast milk in relation to protein concentration and amino acid composition i.e. casein type. The factors that influenced the characterization of powder infant formulas are drying process temperature and percentage of solids in the emulsion. High inlet temperatures during drying in combined with high total solids concentrations favoured oxidation in the fat fraction. The stability studies suggested, that peroxide value polymerise secondary oxidation compounds at water activities greater than 0.4. The hydroxymethylfurfural polymerised melanoidins under conditions the water activity greater than 0.4, so it is recommended that the formula be stored in humidity not greater than 40%. Finally, according to the results obtained, the recommendations for the spray-drying process in order to obtain an infant formula of higher nutritional quality and greater stability during storage were 170 °C inlet temperature and 30 wt.% total solids concentrations.

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