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Optimization of Vegetable Almond Beverage Mixture Enriched with Omega-3 Fatty Acids by Adding Brown Flaxseeds (*Linum usitatissimum L.*) Using D-Optimal Mixing Diagram Method

Running title: Almond Beverage with Flaxseeds

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SUMMARY

Research background. The almond beverage with flaxseed is a significant source of alpha-linolenic acid, an omega-3 fatty acid that our bodies cannot produce. As such, its consumption is essential for vegans and vegetarians to maintain their health and help reduce the risk of nutritional deficiencies. While fortified products are available for this demographic, this drink offers a natural way to provide omega-3. Its versatility allows it to be easily incorporated into daily diets and combined with various recipes. Therefore, this study aimed to optimize a pattern formula for a plant-based almond drink with flaxseed, making it rich in omega-3 and entirely vegan.

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Experimental approach. The initial formulation of the drink comprised 75 % raw almonds, 25 % flaxseed, a 1:6 ratio of water, and 4 % sweetener. Following preliminary tests, the beverage was optimized using the triangle mixing methodology to determine the ideal proportions of the ingredients. This process resulted in 16 samples, each with varying minimum and maximum levels of each ingredient, which were replicated and analyzed. One of the formulations achieved an optimal value of 4.27 mg/g of omega-3 and 6.03 mPa·s of viscosity. The optimized beverage was assessed for its physicochemical characteristics, bioactive compounds, fatty acid composition, and lipid profile.

Results and conclusions. The addition of flaxseed significantly impacted the lipid profile, increasing the alpha-linolenic acid content in the beverage by 1960 times, ultimately reaching 3.92 %. This optimization enriched the beverage with omega-3 by current legislation and enhanced the concentration of antioxidants and carotenoids. Importantly, these improvements did not significantly affect the color and viscosity of the final product, resulting in an affordable option that can benefit the vegan and vegetarian community.

Novelty and scientific contribution. This study shows that the response surface model effectively identified the ideal composition for the beverage, leading to an optimized formulation of a plant-based drink. This composition may have promising applications in the food industry.

Keywords: response surface methodology; optimization; almond vegetable drink; linseed; omega-3

INTRODUCTION

Plant-based diets (PBDS) have become a trend in recent years due to their environmental and health impacts (*1*). PBDS encompass several dietary patterns that are based on either the complete exclusion of animal products consumption (vegan), the exclusion of meat and fish with the inclusion of dairy and eggs (egg-dairy-vegetarian), or the exclusion of all types of meat or eggs while maintaining dairy consumption (dairy-vegetarian), or the reverse of one (egg-vegetarian), which includes eggs but not dairy (*2*).

As veganism spreads, there is a reduction in speciesism, that is, discrimination against animals based on the species to which they belong. In Brazil, 14 % of the population declares themselves vegetarian, according to a survey by IBOPE conducted in April 2018. In the metropolitan regions of São Paulo, Curitiba, Recife, and Rio de Janeiro, this percentage rises to 16 %. The statistic represented a 75 % increase in 2012 when the same survey indicated that the % of the Brazilian population in the metropolitan regions declared themselves vegetarian was 8 %. In 2018, almost 30 million Brazilians declared themselves vegetarian – a number greater than the populations of all of Australia and New Zealand combined (*3*).

Although vegetarian diets are associated with several beneficial health benefits, there are significant concerns, especially when the diet is poorly planned (*4*). These concerns may arise from inadequate varieties and quantities of foods consumed, which can predispose individuals to nutritional deficiencies, such as vitamin B12, minerals like calcium, vitamin D, iodine, iron, zinc, selenium, as well as vitamin A, protein, and omega-3 fatty acids (*5*).

Adopting a vegetarian diet (VD) causes a deficiency of omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in cold-water fish, due to their absence in the diet (*6*), α-linolenic acid (ALA) is the leading omega-3 fatty acid in VD, being a forerunner of EPA and DHA, its consumption is necessary because it is an essential fatty acid, not synthesized by the human body, and an indispensable component for a healthy and balanced diet (*7*).

Walnut, flaxseed, chia seed, canola, hemp, echium, and perilla seeds are plant-based sources of ALA, which have been associated with many health benefits, including anti-inflammatory, antibacterial, antidiabetic, antioxidant, antihypertensive, and neuroprotective activity. When included in a balanced diet, ALA may also have a beneficial effect on body mass reduction (*8*).

Flaxseed, known as linseed (*Linum usitatissimum L.* of the Linaceae family) or linseed oil, is the best source of omega-3 in the form of ALA (*9*), the 18:3n-3 in flaxseed corresponds to 55 %, being more than half of its overall fat content, obtaining a higher percentage when compared to other vegetable sources of ALA (*10*). ALA can be converted to long-chain PUFAs (EPA and DHA) in the presence of desaturase enzymes by gradual desaturation and chain elongation, its conversion efficiency is minimal (<8 % of ALA to EPA and <4 % of ALA to DHA) because it competes with linoleic acid for the same conversion pathway, even so, its role remains significant for long-term dietary intake (*11*).

Foods and beverages fortified with plant-based omega-3 are gaining attention from the industry as they cater to consumers who follow a plant-based diet and seek delicious drinks with natural, healthy, and nutritious ingredients (*12*). The increase in this audience contributes to new challenges for the food industry and new market possibilities. The global dairy alternatives sector was valued at US$ 27 billion in 2023 and is likely to grow at a CAGR of 10.1 %, reaching US$ 43.6 billion by 2028 (*13*).

Almond vegetable drink is a product intended for consumption by individuals who follow a plant-based diet. It is a colloidal dispersion obtained after processing water with almonds and filtering and homogenization of the resulting milky white liquid (*14*).

Lipids (1.99 g), proteins (0.47 g), carbohydrates (6.02 g), and minerals such as calcium, potassium, magnesium, phosphorus make up 100 g of almond vegetable drink (*15*), with a lower number of calories ranging from 45 to 56 kcal. The drink is deficient in omega-3, because almonds contain an insignificant amount of α-linolenic acid (C18:3n3) (*16*), but at the same time are rich in oleic fatty acid (C18:1n−9; 63 %) and linoleic fatty acid (C18:2n-6; 24 %) (*17*).

In recent years, flaxseed has become increasingly popular as a key ingredient in functional food products such as breads, dairy products, beverages, and ready-to-eat meals. That is due to its high nutritional value and numerous health benefits (*18*). Several studies have addressed the fortification of products with flaxseed-derived omega-3 fatty acids, such as dairy milk fortified with flaxseed oil (*19*), yogurt incorporated with flaxseed mucilage and oil-free or encapsulated flaxseed (*20*). Flaxseed oil single encapsulated and co-encapsulated in CA-CMC hydrogel spheres by the extrusion-drip technique was used for the production of functional orange juice (*21*). These studies concluded that flaxseed successfully serve as a potential issue system for omega-3 fatty acids in the form of ALA.

To the best of our knowledge, no studies have investigated the enrichment of almond-based vegetable drinks with omega-3 through the addition of flaxseed during processing. Therefore, this study aimed to develop an almond-based vegetable drink incorporating flaxseed, using the mixture triangle chemometric methodology to determine the optimal ingredient levels. Additionally, the study examined the effects of flaxseed addition on the lipid profile, fatty acid composition and physicochemical characteristics of the drink.

MATERIALS AND METHODS

Materials and reagents

Sodium hydroxide, methanol, sulfuric acid, hexane, acetone, isopropyl alcohol, and sodium sulfate were purchased from (Synth Lab, São Paulo, Brazil). The standards methyl tricosanoic acid (23:0), analytical standard mixture of fatty acid methyl esters (FAMEs C4-C24), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from (MilliporeSigma, Saint Louis, USA).

The ingredients required for the drink preparation were obtained from the local mart in Maringá, Paraná, Brazil. These included 1000 g of raw almond seeds (*Prunus dulcis*), 500 g of brown flaxseed (*Linum usitatissimum L.*), 300 g of xylitol, and 300 g of erythritol. All ingredients were obtained from a unique batch from Terra Verde Company in Maringá, Paraná. They were then transported to the Laboratory of Applied Analytics for Lipids, Sterols, and Antioxidants at the State University of Maringá (APLE-A, UEM) for further testing.

Preliminary test

To prepare an almond vegetable drink fortified with omega-3 and determine the standard formulation, an industrialized almond vegetable drink already commercialized and available for consumption was used as a reference. The priority was given to a product with natural ingredients without colorings and preservatives. The almond vegetable drink of Iracema (Dobrada, São Paulo, Brazil) brand, composed of water, almonds, natural flavors, and natural sweeteners erythritol and stevia, was chosen due to its equal composition to the prepared sample.

Initially, a preliminary test was done to determine the needed amount of flaxseed and consequently increase the omega-3 content in the drink without altering its flavor and viscosity. After the test, the following formulation was chosen: 75 % almond seed, 25 % flaxseed, 1/6 water, and 4 % xylitol and erythritol.

Sample preparation was performed according to Manzoor *et al.* (*22*). After that, the omega-3 fatty acid content of the sample was analyzed by gas chromatography. The results showed that adding 25 % flaxseed to the drink increased the omega-3 content of the sample, a value consistent with the data in Regulatory Instruction No. 28, dated July 26, 2018, from the National Health Surveillance Agency (ANVISA) (*23*). This regulation establishes minimum limits for nutrients, bioactive substances, enzymes and probiotics that must be provided in foods according to the daily consumption recommendations and the population group indicated by the manufacturer. According to this regulation, for a food to be considered a source of alpha-linolenic acid, it must contain between 0.24 g and 2.4 g per serving to meet the daily requirements for individuals nine years and older. Additionally, Resolution of the Collegiate Board No. 54, dated November 12, 2012, from ANVISA (*24*), which provides for the Technical Regulation on Supplementary Nutritional Information establishing a minimum limit of 300 mg of alpha-linolenic acid per 100 mL in prepared foods for it to be considerate a source.

Therefore, the vegetable drink made from this percentage of flaxseed can be recognized as fortified, enriched, or a source of omega-3.

Preparation of almond and flaxseed vegetable drink

The almond-flaxseed vegetable drink was prepared following the method described by Manzoor *et al*. (*22*), with some modifications. Initially, (100.000±0.001) g of almond seed was hydrated in distilled water at (4.0±0.0) °C in a ratio of 1:3 (*m*/*V*) for 16–19 h. After immersion, the seeds were drained, washed, and weighed again to determine the amount of the absorbed water. The drinks were prepared from 100 g of almond seed, 25 % or 25 g were replaced by natural brown flax seed, and 75 % (75 g) corresponded to flax seed.

The seeds were mixed with distilled water in a 1:6 (*m*/*V*) ratio and ground in a blender (Philips Walita Serie 5000 RI2240/9-1200*W*, São Paulo, Brazil) for 150 s. After this process, the beverage was filtered through a cloth strainer. A mixture of 4 % (4 g) of xylitol and erythritol was added to the obtained liquid and homogenized in the blender for 2 min.

The beverage was then bottled in glass bottles, previously sterilized in hot water steam at 100 °C for 10 min, followed by pasteurization at 75 °C for 15 s in a water bath, according to the methodology of Nagarajappa *et al.* (*25*). The temperature was monitored during the process with a digital thermometer and then rapidly cooled in an ice bath at 4–5 °C. Thus, the obtained beverage was stored under refrigeration at 4 °C in a previously sterilized glass bottle, and later used for the analysis.

Experimental design using the D-optimal mixture triangle methodology

The mixture triangle methodology and numerical and graphical optimization techniques were applied to optimize the ingredient composition of the almond-based vegetable drink. The goal was to determine the ideal proportion of almonds, flaxseed and sweeteners to produce a beverage enriched with omega-3, while maintaining desirable viscosity, considering the gelling properties of flaxseed upon hydration. The Design-Expert v. 7.0.0 software (D-optimal mixing design) was used (*26*).

From the preliminary test, the amount of flaxseed of 25 % was found ideal for the preparation of the drink with increased omega-3 content. A mixture diagram was applied to optimize its preparation and provide information regarding the influence of each of the independent variables in the preparation of the drink. The extraction conditions according to parameters and levels for D-optimal design are minimum levels of 15, 5 and 2 g and maximum levels of 17, 6 and 5 g of almond, flaxseed, and sweetener, respectively.

Sixteen samples were analyzed using the central composite face design, considering three independent variables and two responses. The experimental design followed the central composite design matrix in standardized order (Table 1), with the conditions presented in Fig. S1.

Physical and chemical characteristics

The viscosity of the almond vegetable drink was measured at 25 °C using a HAAKE MARS controlled tension rheometer (Thermo Fisher Scientific Inc, Waltham, Massachusetts, USA) with a conical geometry-parallel steel plate (35 mm, separated by a fixed distance of 0.052 mm). The samples were carefully placed on the lower plate and allowed to return to equilibrium for at least 1 min before starting the analysis. A gradient range from 0 to 500 was used.

Titratable acidity was determined according to AOAC method 947.05 (*27*). A sample volume of 10 mL was transferred to a 125 mL Erlenmeyer flask, and 10 mL of water and 4 drops of the acid-base indicator 1 % (*m*/*V*) phenolphthalein were added. The sample was titrated using a standardized 0.1 mol/L sodium hydroxide (NaOH) solution and shaken until a permanent pink coloration was observed for 30 s. The results were expressed as a percentage of lactic acid.

To determine the ash content, the AOAC 940.05 method was used (*28*), which consists of incinerating 10 g of the sample in a porcelain crucible in a muffle furnace at a controlled temperature of 600 °C, which induces the evaporation of water, volatile substances and oxidation of organic matter.

Protein content was determined using AOAC 947.05 method (*29*). This method consists of three main steps: (*i*) sample digestion, (*ii*) distillation, and (*iii*) titration. Protein content is determined based on nitrogen content, where nitrogen usually accounts for 16 % of the mass of the protein sample. Carbohydrate content is calculated by subtracting the sum of the percentages of other components such as water, protein, fat, and ash from 100 (*30*).

The total caloric value was determined using the Atwater conversion values of 4.07 kcal per g of proteins, 3.47 kcal per g of carbohydrates, and 8.37 kcal per g of lipids (*31*). The pH was determined using a benchtop pH meter with a combined glass electrode (PHS3BW; Bel Engineering, Milano, Italy). Before sample analysis, the pH meter was calibrated using standard pH=4.0 and 9.0 at 20 °C.

Soluble solids were determined by refractometry, using a portable refractometer (Abbe RTA-100, São Paulo, Brazil) with a °Brix scale of 0–30 (*32*). The humidity was measured on an infrared scale (Bel Engineering Model i-Thermo 163m, Monza, Italy). The analysis was performed in triplicate, and in each run approx. 0.500 g of sample was used.

The color parameters were analyzed using a digital colorimeter Inc, CR-400 (Konica Minolta Sensing Americas, São Paulo, Brazil). Measurements were taken at three points on each side of the plant-based beverages, and the results were expressed according to the CIE system as *L** (luminosity), *a** (variation from red to green), and *b** (variation from yellow to blue) (*33*).

Lipid extraction

Lipids were extracted from the samples according to the methodology developed by Bligh *et al*. (*34*), performed in triplicate. In the first step, approx. 100 g of sample were weighed and 100 mL of chloroform and 200 mL of methanol were added. The mixture was then homogenized for 2 min in a model 653 magnetic stirrer (Fisatom, São Paulo, Brazil). Next, 100 mL of chloroform was added, and the mixture was stirred again for 30 s. Another 100 mL of water was added to the mixture to separate the two phases. The mixture was stirred for another 30 s. The sample was filtered using Whatman filter paper no. 1 (Pró Analysis, Porto Alegre, Brazil) in a Büchner funnel under vacuum, and the filtrate was transferred to a separatory funnel. After separation, the organic (lower) phase was collected in a 250 mL flat-bottomed flask (Duran Glass-Schott, Barcelona, Germany), and the solvent was evaporated in a rotary evaporator model 802 (Fisatom, São Paulo, Brazil). The lipid samples were collected and kept at −18 ºC in a freezer until analysis.

Direct derivatization

Direct derivatization was performed according to the methodology proposed by Sinosaki *et al.* (*35*). To the vegetable beverage sample (100 mg), 2.0 mL of NaOH/MeOH (1.25 mol/L) were added, the mixture was homogenized and placed in an Elmasonic P ultrasonic bath (Elma, São Paulo, Brazil) with a frequency of 37 kHz for 5 min. Subsequently, 2.0 mL of H_2SO_4/MeOH (1.5 mol/L) were added and the mixture was placed in the ultrasonic bath for 5 min. Finally, 1 mL of hexane was added, stirred for 30 h in a vortex tube shaker at 2800 rpm (Kasavi, Adria Laboratory, Londrina, Paraná, Brazil) and centrifuged at 2.000 rpm for 1 min (Kasavi, Adria Laboratory, Londrina, Paraná, Brazil). Finally, 500 µL of internal standard (23:0) were added. Subsequently, the supernatant was collected and injected into the GC-FID.

Fatty acid determination by GC-FID

Fatty acids methyl esters (FAMEs) were separated in the GC-2010 Plus (Shimadzu, São Paulo, Brazil) gas chromatograph equipped with flame ionization detector (FID), split/splitless injector, and CP-7420 fused silica capillary column (Select FAME, 100.0 mm long, 0.25 mm inner diameter and 0.25 µm cyanopropyl film as stationary phase). The gas flows were 1.2 mL/min for carrier gas (H_2), 30.0 mL/min for make-up gas (N_2), and in the FID 35.0 and 350.0 mL/min of gas (H_2) and synthetic air, respectively. The samples were injected in split mode, with a ratio of 1:20, and the injection volume was 1.0 µL. A heating ramp was applied to the column, starting at 65 °C for 4 min, then heated to 185 °C at 16 °C/min and held for 12 min, and finally heated to 235 °C at 20 °C/min and held for 9 min, with a total time of 35 min. FAMEs were identified by comparing the retention times of the samples with the constituents of the analytical standard of FAMEs (C4-C24). Theoretical FID correction values were applied to obtain the fatty acid mass fractions according to Visentainer (*36*), using the following equation:

$$w = \frac{A_\mathrm{X} \cdot m_P \cdot \mathrm{F_{CT}}}{A_\mathrm{P} \cdot m_\mathrm{A} \cdot \mathrm{F_{CEA}}} \qquad /1/$$

where *w* is the mass fraction of the fatty acid (mg/g of sample), A_X is the peak area of the fatty acids, A_p is the peak area of the internal standard (23:0), M_p is the mass of internal standard (23:0) added to the sample (mg), M_A is the mass of the sample (g), $\mathrm{F_{CT}}$ is the theoretical correction factor of the flame ionization detector (FID), and $\mathrm{F_{CEA}}$ is the conversion factor from methyl ester to fatty acid.

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay

The antioxidant activity *i.e.* the DPPH free radical scavenging method was performed according to the methodology described by Rodrigues *et al.* (*37*). To perform the antioxidant assay, the beverage extracts were previously prepared, 2.5 g of samples were weighed, and 4.0 mL of 50 % methanol were added. This solution was then vortexed for 1 min and left to stand for 60 min at room temperature (25 °C). Afterward, it was centrifuged at 3,500 rpm for 25 min. The supernatant was collected and transferred to a 10 mL volumetric flask. A volume of 4 mL of 70 % acetone was added to the residue from the first extraction, vortexed for 1 min, and left to stand for 60 min at room temperature (25 °C). Then, it was centrifuged at 3,500 rpm for 25 min, the supernatant was added to the other supernatant in the 10 mL volumetric flask, and finally, the volume of the flask was measured with distilled water. The beverage extracts (25 µL) were added to 2.0 mL of the methanolic DPPH solution (6 µg/250 mL). Subsequently, the mixture was gently shaken and kept in the absence of light for 30 min at room temperature (25 ºC). The wavelength (λ) of 517 nm was used. Using a UV-VIS spectrophotometer (Genesys 10-S, Madison, USA). To calculate the antioxidant activity standard curve with Trolox (0–0.3 mg/mL) was used, and the result was expressed in µmol/g of the sample.

Carotenoid content determination

Carotenoid contents were determined by the Higby method (*38*). The carotenoids were extracted by the hexane layer, and isopropyl alcohol was added to obtain a single phase. Initially, 5 g of the sample was weighed in an amber Erlenmeyer flask, and 15 mL of isopropyl alcohol and 5 mL of hexane were added. Then, this solution was stirred for 1 min. After that, the contents were transferred to a 125 mL separatory funnel covered with aluminum foil, and its volume was completed with water and left to rest for 30 min. After this period, the material was washed by opening the funnel tap to remove the aqueous phase, leaving only the yellow phase. After three rests of 30 min, the contents of the funnel were filtered through cotton sprayed with anhydrous sodium sulfate, and the filtrate was collected in a 25 mL amber volumetric flask. Subsequently, 2.5 mL of acetone was added to the flask, and the volume was completed with hexane. The wavelength (λ) of 450 nm was used for reading using a UV-VIS spectrophotometer (Genesys 10-S, Madison, USA). The carotenoid content was expressed in µg/100 g of sample, determined by the following equation:

$$\frac{A_{450\,nm} \cdot V}{125 \cdot m} \div 1000 \qquad /2/$$

where A_{450} is the absorbance value of the beverages, V is the volume of the balloon used (mL), and m is the mass of the sample (g).

Statistical analysis

The experiment followed a completely randomized design, and analyses were performed in triplicate. Data were subjected to Analysis of Variance (ANOVA) and Tukey's mean comparison test ($p<0.05$) using XLSTAT 2018.5 software (Adinsoft, Paris, France) (*30*).

RESULTS AND DISCUSSION

Variability, optimization, and validation by model triangle model of mixtures

The almond-based vegetable drink enriched with plant-derived omega-3 from flaxseed is an ideal option to meet the omega-3 nutritional needs of vegan individuals, integrating it into a healthy and balanced diet. In the present work, through numerical optimization of multiple responses, the optimal formulation in the overall optimization was identified as containing15.426 g of almond seed, 6 g of flaxseed, and 3.571 g of xylitol and erythritol, achieving the highest omega-3 concentration of 4.27 mg/g. In approach, the formulation with 16.568 g of almond seed, 5 g of flaxseed, and 3.432 g of xylitol and erythritol resulted in the lowest omega-3 concentration of 2.06 mg/g (Table 1). The 3D surface plot illustrating the maximum desirability selected under the utter optimal condition showed a peak omega-3 concentration of 4.27 mg/g (Fig. S2).

Table 1

With the modified quadratic model, the experimental and predicted omega-3 concentrations are shown in (Fig. S3). The figure shows the great proximity of the model prediction to the trial dice, signifying the validity of the regression model.

The optimization process was verified using the graphical optimization technique (Fig. S4), where the Triangle Graph for Mixture Optimization was generated to identify the optimal regions of the omega-3-rich formulation. According to the graph, all three variables significantly affected the omega-3 concentration. The omega-3 level was at its minimum at the medium concentration of flaxseed and increased as the flaxseed concentration increased. The green region in the triangle graph represents an optimal combination of the beverage with the highest concentration of omega-3 within a percentage of 100 %, and the blue represents the lowest composition.

Numerical optimization was conducted to determine the optimal ingredients for the production of the almond vegetable drink fortified with omega-3 through the addition of flaxseed after the analysis of variance (ANOVA) test. Three replicates of experiments were performed to ensure the accuracy of the model (Table 2).

Table 2

As shown in Table 2, the values predicted by the model and those obtained experimentally are within the model's coefficient of variation, demonstrating that it accurately predicts the results obtained (Fig. S5).

Therefore, sample 6, shown in Table 1, was the best sample for preparing the almond and flaxseed vegetable drink. It had the highest omega-3 (4.27 mg/g) and an acceptable viscosity value (6.03 mPa·s). We performed the analyses to characterize the drink from this sample.

Fatty acid composition

A total of 17 fatty acids were identified in both beverages, with a total sum of fatty acids of 90.24 and 78.42 for the almond and almond plus flaxseed beverages, respectively (Table 3). The main difference between the two was observed in fatty acid 18:3n-3, where the addition of flaxseed led to a 1960-fold increase in concentration.

Table 3

The significant increase is because flaxseed is a well-known source of unsaturated fatty acids, which account for more than 30 % of its total composition. Linolenic acid (18:3n-3) is the most abundant making up nearly half of the total fatty acids, followed by oleic acid (C18:1-n6) (24.33 %) and linoleic acid (C18:2-n9) (15 %) (*39*).In contrast, studies have shown that lipids constitute more than 50 % of the total net mass of almonds, with a higher proportion of oleic (79.9 %) and linoleic (6.7 %) fatty acids, along with low levels of saturated fatty acids (*40*). By adding flaxseed to the almond vegetable drink, it is possible to increase the content of alpha-linolenic acid (18:3n-3) in the drink (Table 3), whereas, in the almond drink, the content of alpha-linolenic acid (ALA) was 0.20 %. Therefore, by replacing only 25 % of almonds with flaxseeds in the preparation of almond drinks to obtain a drink that is a source of omega-3 of plant origin, we were able to increase the ALA content to 3.92 mg/g of sample. Thus, this result shows that we were able to enrich the almond vegetable drink by 1960 times with ALA, meeting the legislation on food enrichment for alpha-linolenic acid according to Resolution of the Collegiate Board No. 28, dated July 26, 2018, from ANVISA (*23*), which states that food enriched with ALA must have at least 0.24 g, the recommended amount of this fatty acid for a healthy adult. Similar fortification results have been reported by Correa *et al*. (*41*).

According to the results in Table 3, the percentage of palmitic acid (C16:0) did not show a significant difference when comparing the two drinks on the contrary, the fatty acids (18:1n-9) and (18:2n-6) decreased in the enriched drink where oleic acid reduced by around 10 % and linoleic acid by around 2 %, this is because flaxseed contains a lower percentage of these fatty acids compared to almonds. Consequently, when comparing the saturated, monounsaturated, and polyunsaturated fatty acids between the almond drink and the enriched drink, we can observe in Table 3 a decrease of 2, 10 and 1 % in order. Considering the cardioprotective benefits and additional advantages for mental and physical health in humans, such as a reduced risk of chronic diseases and cognitive decline associated with omega-3 consumption (*42*), we can conclude that fortifying the almond-based vegetable drink with omega-3 from flaxseed will be beneficial for vegetarians and vegans. These individuals require an adequate daily intake of ALA to ensure sufficient levels of EPA and DHA, as their consumption of omega-3-rich animal-based foods is low (*43*). Additionally, this fortification can help reduce the cost of the beverage, as flaxseed is more affordable than almonds.

Centesimal composition

In the centesimal composition of almond vegetable drinks in Table 4, it can be observed that the obtained moisture values (88.21 to 88.32 %) did not change with the addition of flaxseed, this was expected because the product is liquid, and the moisture content of both seeds is alike. Similar moisture values were reported by Hussein *et al*. (*44*). Protein values (2.37 to 2.29 %) also showed stability by the addition of flaxseed stability in the amount of protein occurs because flaxseed and almond seeds have the same protein content (about 20 %) in their composition according to studies by Kouamé *et al*. (*9*), In contrast, the addition of flaxseed resulted in a decrease in the lipid content (from 6.53 to 4.52 %), this is because flaxseed contains a lower lipid content than almonds, therefore, when we reduced the number of almonds in the preparation and replaced it with flaxseed, the lipid percentage decreased (*45*). Regarding the carbohydrate content, an increase (from 2.64 to 4.68 %) was observed for the almond and flaxseed vegetable drinks, which occurred because flaxseed contains more carbohydrates than almond drinks (*45*,*46)*. No significant differences were observed in the ash content (0.25 to 0.19 %). The composition of carbohydrates, proteins, and lipids that each drink presents influences the caloric value (Table 4), therefore, the almond and flaxseed vegetable drink presented a lower caloric value (68.54 %) when compared to the almond vegetable drink (83.81

%), which is a positive point for the mixed drink because, in addition to being a source of omega-3, it would be an option combined with a balanced diet.

Table 4

pH and soluble solids analysis

The pH and acidity are crucial factors in assessing food quality. The pH values of the almond and almond-flaxseed vegetable drinks are shown in Table 5. It was noted that the inclusion of flaxseed did not affect the pH or acidity of the enriched drink, as the values remained consistent. This finding is consistent with the results of Veena *et al.* (*46*), who reported no changes in pH or acidity when cow's milk was fortified with flaxseed to increase its omega-3 content.

Table 5

Colorimetric analysis

The results of the color parameters are shown in Table 6. A significant difference ($p<0.05$) was observed among the vegetable beverages, with the addition of flaxseed being the main factor responsible for these changes. When evaluating the L^* parameter (luminosity), the samples were closer to 100, indicating a greater tendency towards white. In the study by Zheng *et al.* (*47*), which developed an almond-based milk analog, the L^* value obtained was 71.40±0.14, which is similar to the values found in the present study (75.25±0.16 and 76.29±0.11), demonstrating comparable clarity.

For the parameters a^* and b^*, the values found are positive (+), which shows a tendency towards red and yellow colors, justified by the presence of carotenoids in the raw materials used, with the beverage with added flaxseed having a greater tendency and explained by the analysis of total carotenoids, in which the sample presented a higher concentration than the almond beverage. The increase in the parameter a^* was observed by Lima *et al.* (*48*), who added pre-emulsified flaxseed oil to beef sausages and observed that the higher the concentration of flaxseed oil, the higher the value of a^*.

Table 6

DPPH and carotenoid analysis

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method is the most widely used to evaluate the antioxidant capacity of a product through spectrophotometric measurement of the decrease in DPPH radical absorbance after the start of the reaction (*49*). Although not nutritionally essential, carotenoids serve as precursors of vitamin A and play a role in mitigating oxidative stress and free radical damage (*50*). Flaxseed, in addition to being rich in fatty acids and fiber, contains a high concentration of phenolic acids, including chlorogenic acid, p-hydroxybenzoic acid, ferulic acid, vanillic acid, and coumaric acid, which are among its primary bioactive compounds. In addition, it also has lignans, such as matairesinol, pinoresinol, diphylline, and secoisolariciresinol, which specify a smaller amount. Flaxseed is also an excellent source of vitamins, including tocopherol and tocotrienols, as the most abundant forms of vitamin E, which are responsible for enhancing its antioxidant power (*51*).

Therefore, the inclusion of flaxseed in foods enhances antioxidant activity. In the optimized formula of the almond and flaxseed drink, the DPPH and carotenoid values increased considerably with the addition of flaxseed, going from 6.54 μmol/g and 0.41 μg/100 g in the drink composed only of almonds to 17.84 μmol/g and 1.81 μg/100 g in the mixed version with flaxseed. These results demonstrate that the combined drink has a significantly higher antioxidant capacity than the exclusively almond drink, with a statistically significant difference (p0.05). Thus, the incorporation of flaxseed in the formulation proves to be an effective strategy to enhance the antioxidant benefits of the drink.

Flaxseed has previously been used as a supplement to enhance the nutritional value of food products. Additionally, studies have reported that flaxseed supplementation increases omega-3 fatty acid content, thereby improving the antioxidant potential of foods (*52*). A study involving soy and flaxseed soy and flaxseed supplementation in a South African beverage called mahewu showed that these two ingredients contributed to antioxidant activity and phenolic content (*53*). Another instance of flaxseed fortification was reported by Jiang *et al*. (*54*), in the development of high-yield wheat bread. In this study, flaxseed bagasse was added, which is a byproduct of oil extraction and has high nutritional value. Thus, the bread produced did not lose its quality. The added flaxseed bagasse flour had a double effect on the gluten network of the wheat flour, which improved its yield. It also promoted antioxidant activity, glucose reduction capacity, water mobility, free water distribution of the bread, and slowed down food degradation.

Although the increased antioxidant and carotenoid activity in the formulated beverage may help inhibit lipid oxidation of omega-3 fatty acid, the primary cause of rancidity in oil, this oxidation occurs due to the reaction of oxygen with unsaturated fatty acids, negatively affecting the aroma, flavor, and overall sensory quality of the beverage (*55*).

Future studies will be necessary to assess the impact of factors such as shelf life, storage temperature, heating, pasteurization, type of packaging, the addition of natural or synthetic antioxidants, and oil encapsulation on the oxidative stability of omega-3 fatty acids. Research in this area is essential for enhancing oxidative stability and extending the shelf life of the product.

CONCLUSIONS

This study successfully demonstrated the feasibility of optimizing a plant-based beverage based on almond and flaxseed rich in alpha-linolenic acid. Through the mixing triangle methodology, it was possible to identify the ideal formulation, containing 15.426 g of almonds, 6.000 g of flaxseeds, and 3.571 g of sweeteners (xylitol and erythritol). This combination not only significantly enhanced the omega-3 content but also increased the levels of antioxidants and carotenoids. With a remarkable 1960-fold increase in alpha-linolenic acid, the beverage emerges as a natural and cost-effective alternative. Additionally, the application of the response surface model proved effective in optimizing the desired formula and emphasized the scientific importance of this research, indicating that an optimized formulation can positively influence the food industry by promoting healthier and more sustainable options. Therefore, this drink not only benefits consumer health but also marks a significant advancement in the availability of plant-based products in the market.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Z.E.H. Hussein conducted the bench analyses and was responsible for writing and editing the original manuscript draft. J.M. Silva supervised the entire analysis process. B.H.F. Saqueti directed the color analysis. M.C. Castro performed software and statistical analyses. N.E. Borges focused on data visualization and participated in the review. All authors reviewed and approved the final version of the manuscript before its submission for publication.

SUPPLEMENTARY MATERIALS

All supplementary material is available at www.ftb.com.hr.

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Table 1. Experimental executions from the D-optimal design and the respective responses.

	Independent variable			Response	
Sample	*m*(almond)/g	*m*(flaxseed)/g	*m*(sweetener)/g	*w*(omega-3 fatty acids)/(mg/g)	*η*/(mPa·s)
1	16.254	6.000	2.746	3.71	6.43
2	15.714	5.000	4.286	3.44	4.78
3	16.568	5.000	3.432	2.06	4.83
4	15.000	5.984	4.016	3.59	6.95
5	15.906	5.600	3.494	2.82	5.97
6	15.426	6.000	3.571	4.27	6.03
7	17.000	5.082	2.918	2.19	4.82
8	17.000	5.562	2.438	3.70	5.59
9	15.154	5.417	4.428	3.02	5.62
10	16.961	6.000	2.039	3.62	5.71
11	15.000	5.001	4.999	2.55	4.49
12	15.000	5.984	4.016	3.33	5.33
13	16.961	6.000	2.039	3.63	5.07
14	15.000	5.001	4.999	3.32	5.35
15	15.906	5.600	3.949	2.30	4.98
16	17.000	5.082	2.918	3.05	4.82

Table 2. Numerical optimization of the results obtained through ANOVA

Sample	Independent variable			Predicted	Answer
	m(almond)/g	*m*(flaxseed)/g	*m*(sweetener)/g	*w*(omega-3 fatty acid)/(mg/g)	*w*(omega-3 fatty acid)/(mg/g)
1	15.744	5.103	4.153	4.03	4.22
2	17.000	5.254	2.746	4.35	4.96
3	16.017	5.197	3.876	3.98	3.52

Table 3. Fatty acid composition (mg/g sample) of almond vegetable drinks

Fatty acid	Almond	Almond and flaxseed
6:0	(0.40±0.01)[a]	(0.08±0.01)[b]
8:0	(0.19±0.02)[a]	(0.04±0.01)[b]
10:0	(1.10±0.03)[a]	(0.08±0.01)[b]
12:0	(0.08±0.00)[a]	(0.10±0.01)[b]
14:0	(0.05±0.00)[a]	(0.09±0.01)[a]
16:0	(5.57±0.08)[a]	(4.66±0.16)[b]
16:1	(0.55±0.01)[a]	(0.56±0.04)[a]
17:0	(0.04±0.00)[a]	(0.03±0.00)[a]
17:1	(0.07±0.00)[a]	(0.04±0.00)[b]
18:0	(1.14±0.01)[a]	(1.05±0.01)[b]
18:1n-9	(57.24±0.78)[a]	(46.79±2.77)[b]
18:2n-6	(18.47±0.24)[a]	(15.90±1.05)[a]
18:3n-3	(0.20±0.02)[a]	(3.92±0.19)[a]
20:1n-9	(0.05±0.00)[a]	(0.02±0.00)[b]
22:0	(0.06±0.01)[a]	(0.07±0.00)[a]
24:0	(5.04±0.05)[a]	(5.04±0.03)[a]
Saturated	(13.66±0.41)[a]	(11.25±0.42)[b]
Monounsaturated	(57.91±3.75)[a]	(47.42±3.38)[b]
Polyunsaturated	(18.66±1.05)[a]	(19.81±1.19)[a]
Total	(90.24±3.14)[a]	(78.42±2.95)[b]

Results are expressed as mean value±standard deviation (S.D.) of triplicate. Values with different letters in superscript in the same row are significantly different (p<0.05) by Tukey's test

Table 4. Centesimal composition of vegetable beverages

Parameter	*w*/%	
	Almond	Almond and flaxseed
Humidity	$(88.21\pm0.84)^a$	$(88.32\pm0.40)^a$
Ash	$(0.25\pm0.01)^a$	$(0.19\pm0.01)^b$
Lipid	$(6.53\pm0.90)^a$	$(4.52\pm0.28)^b$
Protein	$(2.37\pm0.12)^a$	$(2.29\pm0.15)^a$
Carbohydrate	$(2.64\pm0.20)^b$	$(4.68\pm0.81)^a$
E/kcal	$(83.81\pm0.11)^a$	$(68.54\pm1.59)^b$

Results are expressed as mean value±S.D. of triplicate. Values with different letters in the same row are significantly different (p<0.05) by Tukey's test

Table 5. pH and soluble solids analysis of vegetable beverages.

Centesimal	Almond	Almond and flaxseed
pH	$(6.43\pm0.02)^a$	$(6.48\pm0.03)^a$
Soluble solids	$(7.1\pm0.00)^b$	$(8.1\pm0.00)^a$
Titratable acidity	$(0.02\pm0.00)^a$	$(0.02\pm0.00)^a$

Results are expressed as mean value±S.D. of triplicate. Values with different letters in the same row are significantly different (p<0.05) by Tukey's test

Table 6. Color parameters of vegetable beverage formulations

Color parameter	Almond	Almond and flaxseed
*L**	$(75.25\pm0.16)^b$	$(76.29\pm0.11)^a$
*a**	$(3.38\pm0.05)^b$	$(3.95\pm0.05)^a$
*b**	$(5.12\pm0.12)^b$	$(6.87\pm0.03)^a$

Results are expressed as mean value±S.D. Values with different letters in superscript in the same row are significantly different (p<0.05)

SUPPLEMENTARY MATERIAL

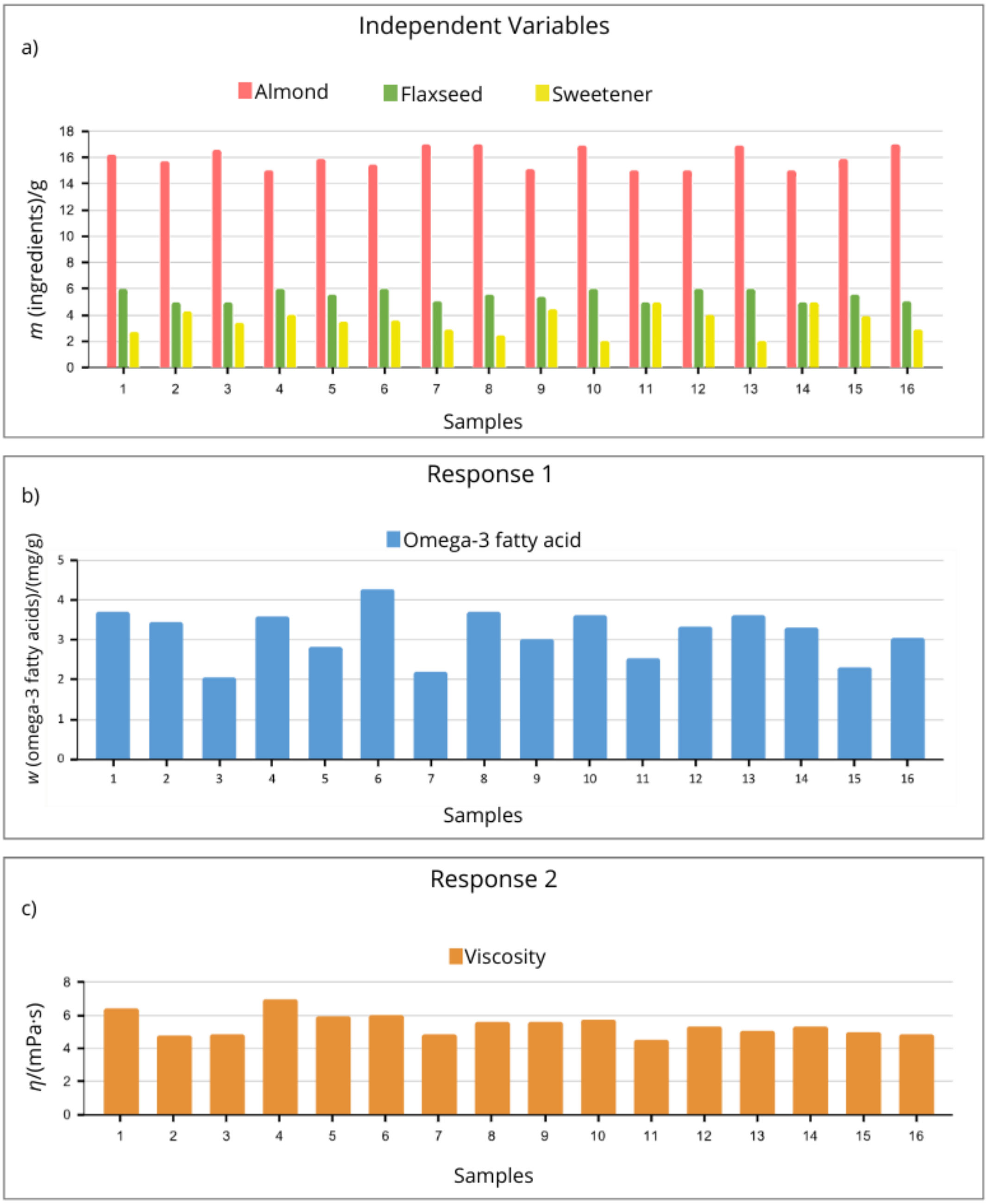

Fig. S1. Experimental executions from the D-optimal design and the respective responses: a) the independent variables of the samples analyzed, b) response 1 (omega-3) and c) response 2 (viscosity)

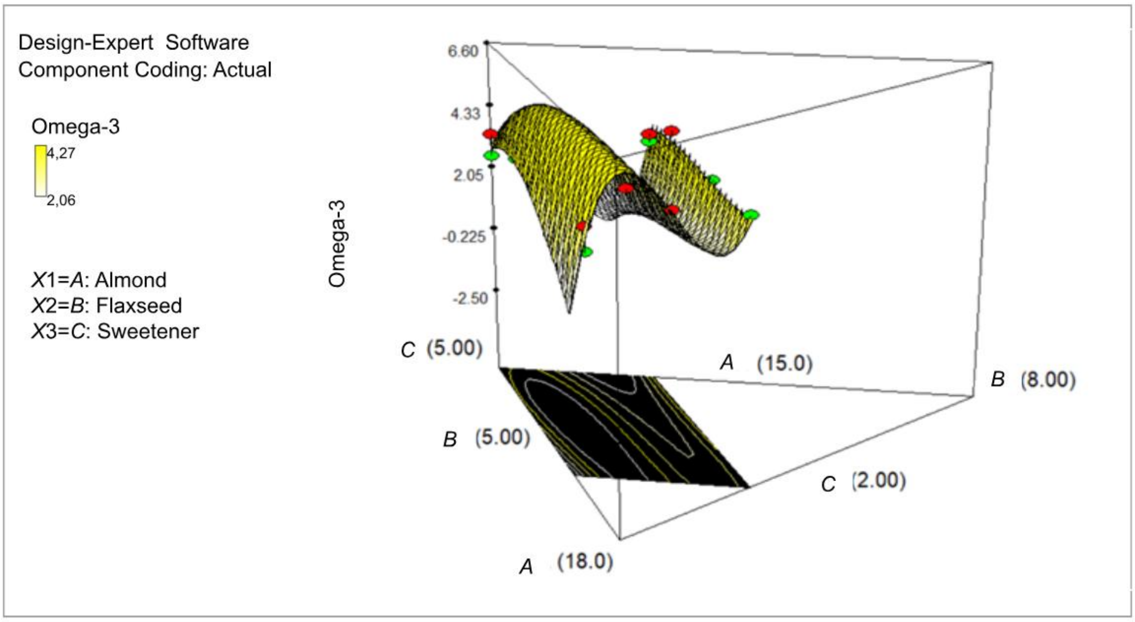

Fig. S2. 3D surface plot showing the desirability function of each combined response variable for mixture optimization. A: almond B: flaxseed C: sweetener

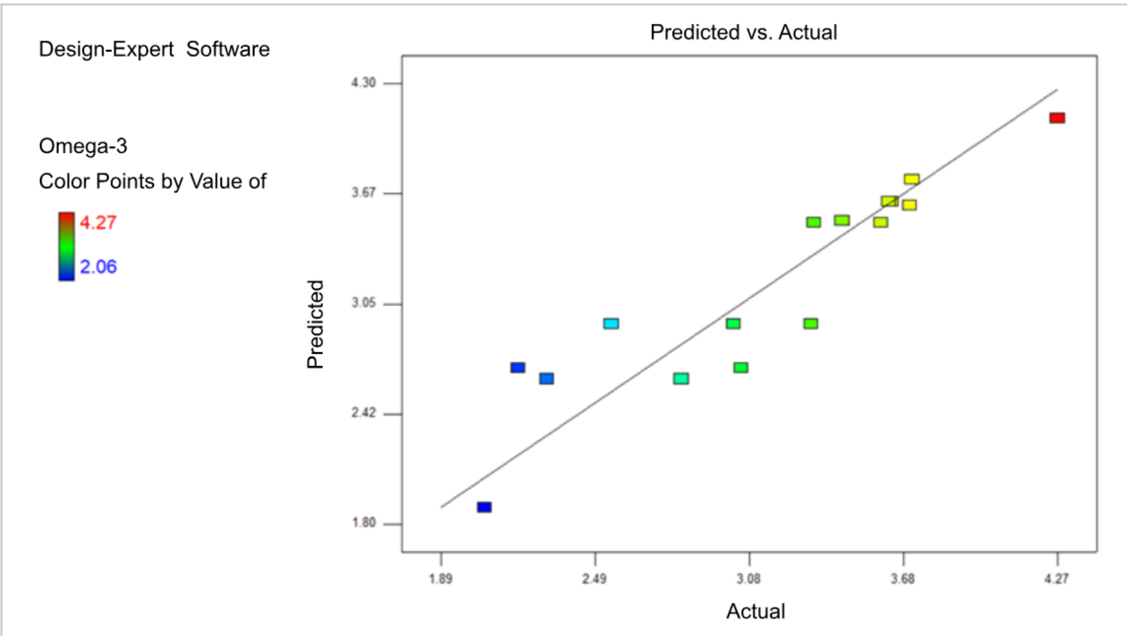

Fig. S3. Graph of predicted versus actual values

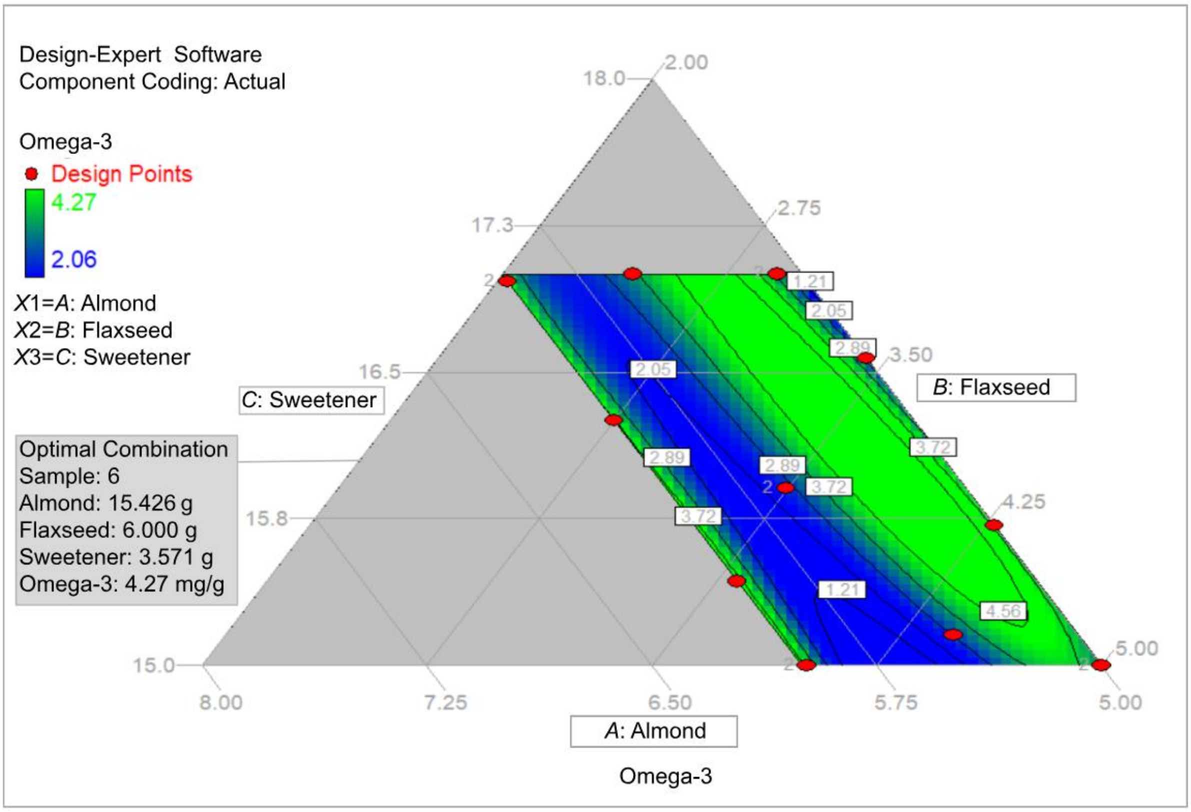

Fig. S4. Triangle graph for mixture optimization

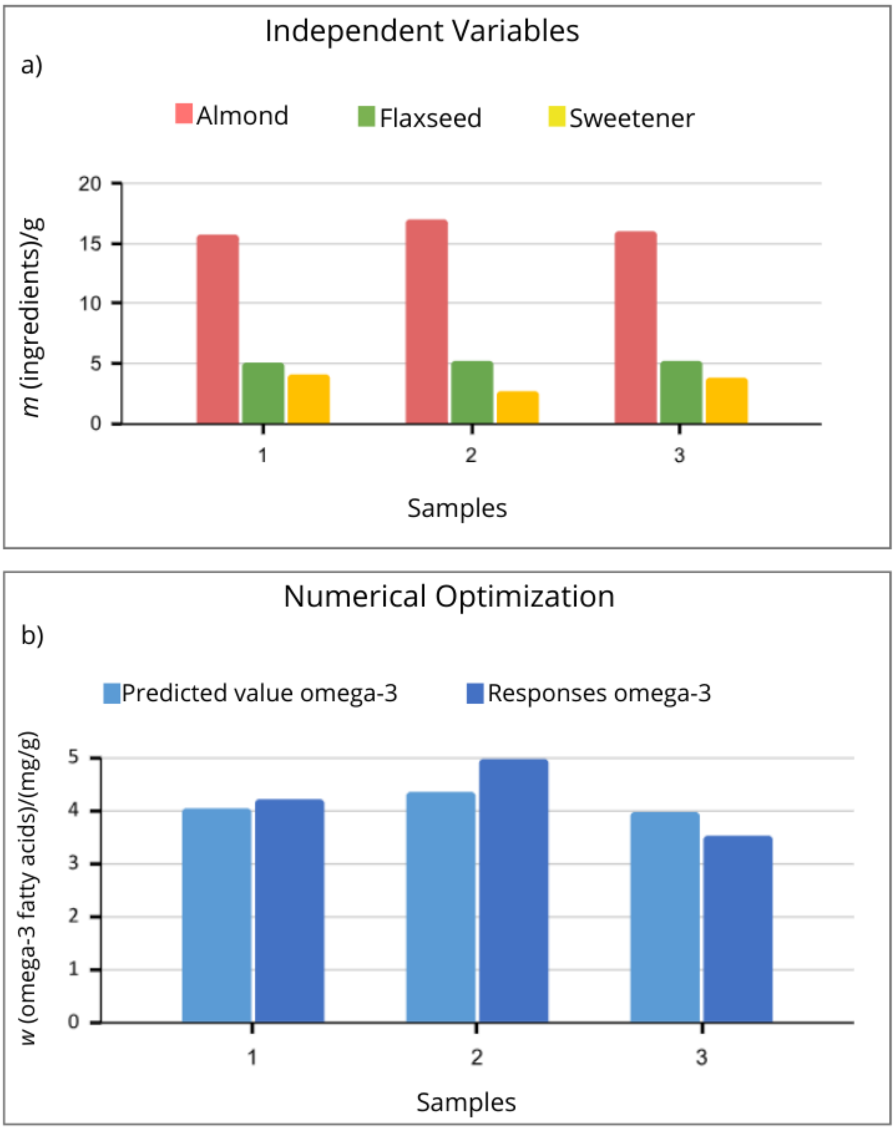

Fig. S5. Numerical optimization of the results obtained through ANOVA: a) the independent variables of the samples analyzed, and b) the numerical optimization of omega-3