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Novel fiber-rich lentil flours as snack-type functional foods: Extrusion cooking effect on bioactive compounds

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ABSTRACT

Novel snack-type functional foods based on extruded lentil flours could convey the related health benefit of their bioactive compounds, provide gluten-free alternative to consumers, and potentially increase the consumption of pulses. Extrusion treatment promoted an increase in galactopinitol, ciceritol, raffinose, stachyose and total α–galactosides content, in most lentil flours. As α–galactosides may act as prebiotics, they could convey beneficial effect to human and monogastric animals. Conversely, extrusion significantly ($p < 0.05$) reduced the inositol hexaphosphate content to less phosphorylated phytates (inositol pentaphosphate and inositol tetraphosphate), which provide health effects. The gluten-free formulation (Control formulation #3) presented the highest significant ($p < 0.05$) drop in the inositol hexaphosphate of 14.7 fold decrease, but had a large increase in inositol pentaphosphate, due to extrusion processing. These two results are desirable in the finished product. Extrusion also caused a significant ($p < 0.05$) reduction in trypsin content and completed inactivated lectin, in all processed samples.

KEYWORDS

Gluten-free formulations, extrusion, lentils, nutritional active factors, oligosaccharides, inositol phosphates.

INTRODUCTION

Pulses, such as lentils (*Lens culinaris*), are currently considered gluten-free functional foods. Since pulses are a rich source of nutritional and healthy compounds such as fiber, proteins, resistant starch, as well as phytochemicals with health-promoting activity. In adequate proportions, these health promoting compounds support important biological functions like the stabilization of glycemic and cholesterol indexes, promotion of intestinal transit, and may also act in the prevention of some cancers and chronic diseases, heart disease, and diabetes[1]. The remarkable nutritional profile present in pulses and their proven impact on human and animals wellbeing make pulses fine functional food ingredients with justified nutritional and health promoting importance.

Additionally, pulses also contain bioactive compounds, such as oligosaccharides, inositol phosphates, protease inhibitors and lectins. Some of these compounds have been associated to undesirable nutritional and physiological factors that have limited the widespread use of pulses as a primary food, mainly in developed countries[2]. Phytic acid and trypsin inhibitors have been indicated to hinder the digestion and absorption of some important nutrients as proteins and some vitamins, while oligosaccharides cause undesirable physiological side effects as flatulence. However, the effect of these compounds, bioactive or anti-nutrient, mostly depends on their concentration in the food products, time of exposure, and their interaction with other dietary components[3]. On the other hand, several number of research has shown evidence of the beneficial effects of many compounds, including bioactive compounds such as trypsin inhibitors, phytic acid, saponins, and pectins which in small quantities, play important role in the prevention of diseases. Particularly, α-galactosides have been identified as prebiotic agents[4], they are fermented by colonic flora to produce a mixture of short-chain fatty acids (SCFA). Their stimulation of bifidobacteria may have several beneficial

implications for health: Potential protective effects against colorectal cancer and infectious bowel diseases through inhibition of some putrefactive and pathogenic bacteria[5]. Moreover, the SCFA could also be responsible for lowering cholesterol and glycemia symptoms, due to their intervention in lypogenesis or gluconeogenesis after being widely (90-95%) absorbed in the colon[6]. In the other hand, inositol phosphates play critical roles in diseases such as cancer, diabetes type 2, Lowe syndrome, myotubular myopathy, and Chaicot-Marie-Tooth disease[7]. While, trypsin inhibitors were effective preventing or suppressing carcinogen-induced effects[8].

Wheat bran is a gluten containing ingredient that is extensively used by the food industry as a source of fiber in a large variety of food products, including bakery products, cookies, and snack foods, among others. Fortification of pulses flours with gluten-free fiber sources such as corn, apple fiber, or similar ingredients, would add nutritional value to pulses products. Moreover, the development of healthy, crunchy extruded snack-type foods from lentil-based formulations fortified with gluten-free fibers would be a good alternative to increase pulse consumption, especially for those individuals suffering of the celiac disease.

Nowadays, the consumption of pulses in European and American population has considerable decreased over the years due to changes in their food habits, especially in children and young adults. Various consumer studies, focused on the acceptance of extruded food products, have indicated that the attractive appearance and texture of snack products are becoming more and more valued and demanded by consumers[2].

Extrusion cooking technology is a high-temperature, short-time, versatile, and modern food operation that converts agricultural commodities, usually in a granular or powdered form, into fully cooked, shelf-stable food products with enhanced textural attributes and flavor[9]. Through extrusion processing food materials are plasticized and

cooked, resulting in molecular transformations and chemical reactions that provide the retention of nutritional compounds[10]. Extrusion processing has also been reported to be a very effective method to improve protein digestibility in pulses[11] and reduce or eliminate the negative effects of some antinutrients[12]. Extruded foods are commercially made from cereal-based formulations. However, pulses could be included as vegetable protein sources in extrusion formulations for making nutritionally enhanced, gluten-free, convenient products, to include in the daily diet as functional foods, as a good alternative to cereal-based gluten-containing products.

Based on the literature reviewed, there is limited information on the effect of extrusion processing on some phytochemicals and main sugars in pulses' extrudates. Moreover, to the authors knowledge there are no information available about extrusion effect on pulse-based formulations fortified with different types of fiber sources as functional foods. Therefore, the aim of this study was to evaluate the effect of extrusion cooking on the bioactive compounds content of different lentil-based, expanded snack-type products made from functional formulations fortified with fiber-rich, gluten-free or gluten-containing ingredients.

MATERIAL AND METHODS

Lentil flour and formulated flours

Decorticated red chief lentils (*Lens culinaris* Medik) were purchased from a local wholesale distributor in California (USA). Upon arrival, the lentils were blended using a 400 kg per batch capacity paddle-type mixer (Marion Rapid Machinery Co., Marion, IA, USA) operated at 325 rpm for 10 min, to a uniform lot. The lentils were first ground to coarse flour through a 5 mm screen using a Gruendler Model WBB-4 hammer-mill (Pulvco Corp., Tulsa, OK, USA) equipped with a Toshiba Transistor AC Inverter

Model VF Pack-P1 (Toshiba Corporation, Tokyo, Japan) operated at 25 Hz, which controlled the feed rate of the seeds to the mill. The coarse flour was subsequently ground into a fine flour (< 1 mm) using an Alpine Model 160Z pin-mill (Hosokawa Alpine AG, Augsburg, Germany) operated at 3200 rpm. The pin-milled lentil flour was stored in airtight 55 gal steel drums double-lined with plastic bags until use. Wheat bran was provided by ConAgra Company (Oakland, CA, USA), apple pomace was provided by TreeTop Company (Selah, WA, USA), Nutriose® and Hi-Maize Whole Grain Corn Flour were obtained from National Starch (Bridgewater, NJ, USA). Formulations containing at least 68% of lentil flour were blended with the selected gluten-free and gluten-containing fiber sources, indicated above, and with starch and flavouring agents (salt and sugar) on a Hobart Model V-1401 mixer (The Hobart Mfg. Co., Troy, OH, USA) for 10 min, with speed setting 1, to a uniform batch. The prepared formulations (patent pending)[13] shown in Table 1, were stored in airtight 4 gal HDPE buckets until extrusion.

Extrusion process

A Clextral EVOL HT32-H twin-screw extruder (Clextral, Inc., Tampa, FL, USA) with co-rotating and closely intermeshing screws was used. The extruder was equipped with six barrel sections, each 128 mm in length. The screw diameter (D) was 32 mm and the total configured screw length (L) was 768 mm, which gave an overall L/D ratio of 24. Screws were driven by a 74.8 kW variable speed drive, Model ACS600 (ABB Automation, Inc., New Berlin, WI, USA). The screw speed was maintained constant at 500 rpm. A combination of feeding, transporting, compression and kneading elements was used to provide a moderate-shear screw configuration (patent pending)[13]. The temperature of the last barrel section and the die was maintained at 160 ± 1 °C.

The formulated pulse-based fiber-rich mixture was metered into the feed port by a twin-screw, loss-in-weight gravimetric feeder, Model LWFD5-20 (K-Tron Corp., Pitman, NJ, USA) at a rate of 20 kg/h (wwb). Water was supplied to the extruder by a triplex variable stroke piston pump with 12 mm plungers, Type VE-P33 (Bran and Luebbe, Wheeling, IL, USA) to provide final moisture content of 17%. The mixture was extruded through two circular dies each with a 3.5 mm diameter opening. Pressure at the die was monitored using a pressure transducer, Type PT412-5M (Dynisco Instruments, Sharon, MA, USA). A PLC+ Industrial computer (Allen-Bradley, Milwaukee, WI, USA) using Intouch software (FITSYS PLUS ver. 1.23) was used to collect extruder parameter data at 1 s intervals. Data were collected approximately 10 min after the operation conditions of torque and pressure were at a steady state.

Chemical characterization

To obtain representative samples, the lentil flours and formulated lentil-based fiber-rich flours, before and after extrusion cooking, were reduced to uniform powders using a Cyclone mill (Udy Corp., Fort Collins, CO, USA) fitted with a 0.5 mm screen. Then, the flours were stored in air-tight glass jars at room temperature until analyzed.

Bioactive compounds analysis

Soluble sugars

The concentration of soluble sugars in the unprocessed and extruded flours was determined by HPLC, using a modification of the method previously reported by Muzquiz et al.[14]. Each sample (0.1g) was homogenized in aqueous ethanol (50% v/v, 5 mL) for 1 min using an Ultra-Turrax homogenizer (IKA® Works, Inc. Wilmington, NC, USA). The mixture was centrifuged for 5 min at 12,100 *g*. The supernatant was

177 decanted and the procedure repeated twice. The combined supernatants were passed
178 through Sep-Pak C18 cartridges (500 mg, Waters, Milford, MA, USA) and the column
179 was washed with 3 mL of aqueous ethanol (50% v/v). The combined extracts and
180 washings were collected and evaporated to dryness. The residue was redissolved in 1
181 mL of double deionized water, and centrifuged for 8 min at 12,100 *g*. Before injection,
182 samples were filtered through a 0.45 μm Millipore membrane. Aliquots of 20 μL were
183 injected into an HPLC system (Beckman System Gold Instrument, Los Angeles, CA,
184 USA) equipped with a refractive index detector. A Spherisorb-5-NH_2 column (250 x 4.6
185 mm i.d., Waters, Milford, MA, USA) equilibrated with acetonitrile/water 60:40 (v/v)
186 was used, with a flow rate of 1 mL/min. Samples were analyzed in duplicate.
187 Calibration curves were constructed for all standard sugar solutions. Individual sugars
188 were quantified by comparison with external standards of pure sucrose, maltose,
189 raffinose and stachyose (Sigma, St. Louis, MO, USA); Ciceritol, galactinol and
190 verbascose were purified and kindly supplied by Dr. A. I. Piotrowicz-Cieslak (Olsztyn-
191 Kortowo, Poland). A linear response was evident in the range (0-5 mg/mL), with a
192 correlation coefficient of 0.99.

193 **Inositol phosphates**

194 Individual inositol phosphates (IP4-IP6) were extracted following Burbano et al.
195 method[15] (described below). A 0.5 g of sample was extracted with 5 mL of 0.5 M HCl
196 for 1 min, using an Ultra-Turrax homogenizer. The extract (2.5 mL) was diluted with 25
197 mL of deionized water and placed onto a SAX column (Varian, Lake Forest, California,
198 USA). The column was washed with 2 mL of deionized water, and the inositol
199 phosphates eluted with 2 mL of 2 M HCl. The eluate was evaporated to dryness and the
200 residue dissolved in a buffer solution. The solution were centrifuged at 12,100 *g* for 6
201 min, to remove any suspended material, prior to injection into the HPLC (Beckman

System Gold Instrument, Los Angeles, CA, USA). The column consisted of a macroporous polymer PRP-1 (150 x 4.1 mm i. d., 5 μm, Hamilton, Reno, Nevada, USA), maintained at a temperature of 45ºC. The mobile phase was a mixture of methanol/H_2O (51.4/48.5; v/v) with 0.8% of tetrabutylammoniun Hydroxide (Fluka, 40% in water), 0.1 % of 5M H_2SO_4, 0.05% of 91% formic acid (Fluka), and 100 μL of a phytic acid hydrolysate (6mg/mL) and the pH was adjusted to 4.3. The mobile phase was run at a flow rate of 1 mL/min. Samples were analyzed in duplicate.

Trypsin inhibitors activity

For sample preparation, 0.25 g of unprocessed and extruded flours was extracted in 10 mL of 50 mM HCl for 1 h under continuous stirring, followed by centrifugation at 12,100 *g* for 10 min. The supernatants were frozen until used. Trypsin inhibitor activity measurements were made on the sample extracts using a small-scale quantitative assay described by Domoney and Welham[14], where one unit (TIU) is defined as that which would give a reduction in A_{410} nm of 0.01, relative to trypsin control reactions, using a 10 mL assay volume. All assays were performed at 4 ºC, in triplicate.

Lectins

Unprocessed and extruded flours were extracted according to the procedure of Cuadrado et al.,[17]. Samples were extracted with 0.1 M Phosphate Buffered Saline (PBS) at pH 7.4, containing 0.1 mM D-glucose at a concentration of 200 mg/mL, using an Ultra-Turrax homogenizer (2 min). The homogenized sample was centrifuged at 4300 *g* for 20 min at 4 ºC, the supernatants were diluted 4 or 100 times, and used for the haemagglutination test and ELISA assays.

Haemagglutinating activity was estimated in the PBS extracts by a serial dilution procedure, using trypsin treated rat blood cells[18]. The amount of material causing 50% agglutination of erythrocytes was defined as that, which contained one

haemagglutinating unit (HU). For comparison, values were expressed as HU/Kg flour. The assays were reproducible to ± 1 dilution and the final values were the mean of four separate measurements. *Phaseolus vulgaris* cvs. Processor and Pinto were included in each assay as positive and negative controls, respectively. Pure lentil lectin (LCA), previously obtained[15], diluted in PBS (0.01 M PBS, pH 7.4), was used as standard.

Competitive Indirect ELISA was performed according to Cuadrado et al.,[17] to estimate the lectin content of the samples from a calibration curve. Plates were coated overnight at 4ºC with 0.5 μg/mL of LCA in 0.05 M sodium carbonate-bicarbonate buffer, pH 9.8. Coated plates were washed four times with PBST (0.1 M PBS containing 0.01% v/v Tween 20, pH 7.4). Then, 0.2 mL of PBSG (0.1 M PBS containing 0.5% w/v gelatine, pH 7.4) was added. After incubation for 1 h at 37ºC, the plates were washed for another four times with PBST. After this, 0.05 mL of pure rabbit anti-LCA D-glucose or lentil samples (also diluted in 0.05 mL of PBS containing D-glucose) with unknown content of lentil were added, followed by 0.05 mL of rabbit anti-LCA IgG antibody (diluted 1:100 in PBS). After incubation for 1 h at 37ºC, the plates were washed four times with PBST. Then, 0.1 mL of horseradish peroxidase (HRP)-conjugate goat anti-rabbit IgG (Human, Hungry) diluted with PBS (1:10000, v/v) was added to each well. The plates were incubated for one additional hour at 37ºC and 0.1 mL of substrate solution (0.34 mg/mL *o*-phenylendiamine in 0.05 M phosphate citrate buffer pH 5.0, containing 0.03% v/v hydrogen peroxide) was added to each well. After 5 min, the reaction was stopped by adding 0.05 mL of 3M H_2SO_4 and the optimal density was measured at 492 nm using a DYNATECH plate reader. The lectin content of the samples was estimated from the calibration curve (0.001-1000 μg/mL of pure LCA standard). Determination was performed in triplicate for each data point. Results were expressed in percentage of LCA on a dry matter basis.

252

253 **Statistical analysis**

254 The results were expressed as mean values ± standard deviation. The significant
255 difference of their mean values was assessed with one-way analysis of variance
256 (ANOVA) followed by Duncan`s test (significant level $p<0.05$) using Minitab 16 for
257 windows software (State College, PA, USA). Moreover, Principal Components
258 Analysis (PCA) was performed, using Statgraphics Plus 5.1 software (StatPoint
259 Technologies, Inc., Warrenton, VA, USA) with a multivariable analysis. A PCA is used
260 to analyze multivariate data and to generate new sets of variables, these being linear
261 combinations of the original ones[19]. PCA was applied as pattern recognition
262 unsupervised classification method to identify properties that underline group
263 differences in terms of bioactive components (oligosaccharides, inositol phosphates,
264 trypsin inhibitors and lectins) for each lentil-based extrudate. PCA transforms the
265 original, measured variables into new uncorrelated variables called principal
266 components (PC). The basic purpose of this discriminate analysis is estimating the
267 relationship between a single categorical dependent variable (formulation effect and
268 extrusion) and a set of quantitative independent variables (each bioactive compounds
269 value obtained in all the performed assays). The number of dimensions considered for
270 PCA was chosen in order to allow meaningful interpretations and to ensure their
271 reliability.

272

273 **RESULTS AND DISCUSSION**

274 **Soluble sugars**

275 Soluble sugar content (mg/g) in the unprocessed and extruded lentil flours and
276 formulated fiber-rich lentil flours are shown in Table 2. The following eight soluble

sugars were identified and quantified in the lentil and formulated flours by HPLC: disaccharides (sucrose and maltose), oligosaccharides (raffinose, stachyose, and verbascose). The disaccharides and trisaccharide-type-sugars-alcohol galactinol and galactopinitol, and ciceritol were also identified and quantified in the flours. The most interesting group of the soluble sugar fractions in pulses, from a physiological point of view, is the oligosaccharides raffinose and stachyose. The effect of these α-galactosides as responsible for flatulence, due to its fermentation by intestinal bacteria, is well known[20,21]. The unprocessed lentil flour (CR) presented values of sucrose (10.22 mg/g), maltose (3.09 mg/g), galactopinitol (1.82 mg/g) and ciceritol (23.05 mg/g) similar to those reported by Sanchez-Mata[22]. As happened in other pulses, stachyose (22.42 mg/g) and verbascose (16.56 mg/g) were the predominant α–galactosides[23], while raffinose was also present but in lower content (2.72 mg/g).

In general, the content of soluble sugars in the unprocessed (CR) and extruded lentil (CE) flours presented the following pattern: ciceritol > stachyose > verbascose > sucrose > maltose > raffinose > galactopinitol > galactonitol. This pattern changed for all formulated samples, due to inclusion of the different food ingredients used in the mixes. A large increase in sucrose was observed in all the formulated flours compared to the lentil flours, due to the addition of sucrose and other sucrose-containing food ingredients into the mixes such as starch and corn, among others, stated under materials and methods (*Lentil flour and formulated flours* section*)*. The sucrose content within the unprocessed formulations varied in the range of 43.82 to 79.36 mg/g and within the extruded formulations varied from 50.06 to 79.48 mg/g. Moreover, the extruded formulated flours presented higher sucrose content than the unprocessed formulated flours, due to hydrolysis of some soluble sugars as effect of extrusion processing.

Extrusion treatment, as occurred with other high-pressure techniques, as instant controlled pressure drop (DIC) treatment[24,25] increased the soluble sugar content in the final product. This effect could be largely attributed to a mechanical-structure modification of the product matrix, including cell wall breakage, increased porosity and specific surface area, improving the diffusion of solvent inside the matrix and subsequently increased the extraction of the soluble sugars. Therefore, comparing control extruded lentil flour (CE) to control raw lentil flour (CR) an increase in sucrose, maltose, galactinol, galactopinitol, ciceritol, raffinose, and stachyose was observed (Table 2). Also, the total α–galactosides content significantly ($p < 0.05$) increased in most the extruded lentil flour, compared to their control counterpart. An exception to this pattern was observed for samples CE and EF4, which presented total α–galactosides content similar to their respective controls. The observed small reduction of alpha-galactoside in the CE and EF4 samples could be attributed to a mechanical-structure modification of these product matrix during extrusion, which reduced the extraction of these sugars. Lajolo et al.[21] have reported that α–galactosides may act as prebiotics, increasing bifidobacteria population in the colon and subsequently conveying beneficial effect to human and monogastric animals by stimulating their immune system, increasing resistance to infection, and reducing constipation and diarrhea. Therefore, the increased in available α–galactosides, generated as a consequence of extrusion processing, may be consider as a added-value ingredient with potential in functional superfood and would be an added-value attribute of the fiber-rich lentil extrudates.

In sample CE, raffinose and stachyose showed an increase of 10 and 55.14%, respectively, while verbascose showed a decrease of 17.53%. Verbascose is a pentaoligosaccharide that under the extrusion conditions of high temperatures and pressure, could be partially hydrolyzed and subsequent increase the content of raffinose

and stachyose, as observed in this study. These results are in agreement with those previously reported by other authors, under similar extrusion conditions applied to different pulses seeds, in which extrusion process caused an increase in maltose, ciceritol and stachyose in lentil flours, but not in peas nor in chickpea flours[7]. This indicated that different pulse flours are differently affected by extrusion processing conditions.

Guillamon et al.,[26] observed an increase of total α-galactosides of 15.18%, mainly in stachyose and verbascose content, in pea flours after extrusion treatment. Whereas, in other pulses such as kidney bean, lupin, chickpea and faba bean, the same authors reported a decrease from 5 to 50% in total α-galactosides content after processing. Frías, et al.,[27] reported only a slight decrease in total α-galactosides content after the extrusion process of pea flour at 142 ºC. Similarly, Berrios et al.,[19] reported that the total oligosaccharide levels in black bean flours, were not significantly affected under extrusion conditions of 160 ºC and 20% feed moisture. Therefore, based on the present and previously indicated studies, the extent of oligosaccharide reduction is dependent on the extrusion conditions employed during processing, the type of grain and/or formulation used in the development of extruded products.

Lentil flours formulated with wheat-bran (EF1 and EF2) presented a significant increase in total α-galactoside content of 31.05 and 22.48%, respectively. The amount of verbascose in these samples did not change significantly, however stachyose and raffinose showed a significant increased. Whereas, lentil flours formulated with corn and apple fiber (EF4), was the only sample that presented no significant decrease in total α-galactosides content after processing, with values around 25 mg/g. These results highlight the importance of selection of food ingredients that may resist the effect of extrusion processing to retain α-galactosides in the finished product.

Inositol phosphates

While it is known that phytic acid or inositol hexaphosphate, has a negative effect on zinc and calcium absorption, other inositol phosphates are considered to have very important role in human health and diseases. Harland & Morris[28] (1995) reported that less phosphorylated phytates forms IP4, IP3, IP2 and IP, promoted intestinal absorption of minerals decreasing ferrous and zinc chelation, and prevented the formation of kidney stones because it reduces the formation of hydroxyapatite crystals. The content of inositol phosphates (IP) in different lentil flours and formulated fiber-rich lentil flours (control and extruded) are shown in Table 3. The unprocessed lentil flour (CR) contained mainly IP6 (3.26 mg/g), followed by IP5 (0.99 mg/g) and IP4, which presented the lowest content (0.28 mg/g). This presented results are in agreement with other authors[29] that reported IP6 and IP5 forms of inositol phosphates accounted for 90% of total phytate content in the raw cereal flours.

Heat treatment reduced the inositol hexaphosphate (IP6) content leading to less phosphorylated forms (IP5 to IP)[30]. A significant reduction ($p< 0.05$) in total inositol phosphates content was observed in the extruded cooked lentil flours EF1, EF2 and EF3, compared to the unprocessed flours. A reduction of total inositol phosphates was also observed in extruded cooked lentil flours CE and EF4, but the reductions were not significant. These overall reduction in total inositol phosphates is a consequence of extrusion cooking are in agreement with those reported by Alonso et al.,[31] in extruded pea flours at 155ºC, in which a significant ($p< 0.05$) reduction of 20.54% was observed. While, smaller decreased in total inositol phosphates have been reported in extruded pulses by various researchers. Butrón [32] reported a decrease of inositol phosphates of 13.7% in Faba bean and 22% in Kidney bean, processed under the same extrusion conditions (160ºC). Guillamon et al.[26] also reported a decrease of inositol phosphates

around 7.74% in Kidney bean flour, 8.46% in chickpea flour, and a much larger decrease of 35.78% in Faba bean flour, after extrusion cooking of the flours at 150ºC.

The effect of extrusion processing was more pronounced in decreasing the content of inositol hexaphosphate (IP6), which had a direct effect on the observed decreased values in total IP (Table 3). The gluten-free formulation CF3 presented the highest significant ($p < 0.05$) drop in IP6 of 14.7 fold decrease, due to extrusion processing. Follow by the gluten-containing formulations CF1 and CF2, which presented 2.42 and 1.65 fold decrease in IP6, respectively. While, the IP6 content in the extruded formulation EF4 was similar to the unprocessed flour (CF4). Frontela et al., [33] reported a higher reduction in IP6 content in wheat flour (76.9%) than in corn flour (30.5%), after roasting treatment at 120 ºC. They concluded that IP6 present in corn flour were more stable to thermal treatment than the ones present in wheat flours. In the other hand, extrusion cooking induced a significant ($p < 0.05$) increase in IP4 in formulations CE and E4 as well as a significant ($p < 0.05$) increase in IP5 in the gluten-free formulations EF3 and EF4.

The present study analyzed formulations containing at least 68% of lentil flour blended with selected gluten-free and gluten-containing fiber sources, namely wheat brand, apple fiber, corn fiber and/or nutriose, before and after extrusion processing. Therefore, the various formulations, due to their different matrix and visco-rheological behavior under extrusion, widely influenced the extrusion effect on the inositol phosphates contents, as supported by research reports of the indicated previous authors.

Extrusion processing promoted a considerable reduction of inositol hexaphosphate (phytic acid). Therefore, extrusion could be a good alternative to reduced phytic acid content and maintain beneficial, less phosphorylated inositol phosphates, in the extrudates.

401 **Trypsin inhibitor activities**

402 Clemente et al.,[6] reported that the natural bioactive substances Bowman-Birk inhibitors
403 (BBI) were effective in preventing or suppressing carcinogen-induced effects, as the
404 digestive enzymes inhibition decreased the availability of nutrients to tumour cells; as
405 well as inhibiting the formation of oxide and peroxide radicals, and stimulation of T
406 lymphocytes production, due to their antioxidant action.

407 Table 4 summarizes the extent of trypsin and lectin inhibition determined in the
408 different lentil-based formulations, before and after extrusion cooking of the samples.
409 The highest trypsin inhibitor activity (TIA) were observed in CR (11.43 TIU/mg)
410 followed by the flours formulated with wheat-bran (CF1 and CF2, 4.97 and 4.86
411 TIU/mg, respectively). These bioactive compounds are found largely in wheat bran[32].
412 After the extrusion treatment, a significant ($p< 0.05$) reduction in trypsin content in all
413 samples analyzed was observed. These results demonstrate the heat-sensitive nature of
414 these compounds. Also, these results confirmed previous results presented by Armour et
415 al.,[34] who indicated that heat treatments have been shown to be very effective in
416 destroying trypsin inhibiting activity. With regard to trypsin reduction, as observed with
417 α-galactosides and inositol phosphates, extrusion processing did not affect all
418 formulations to the same extent as observed for lectin. The greatest losses were
419 observed in the extruded samples formulated with apple fiber and/or corn flour (EF3
420 and EF4). Their content was reduced around 97.59 and 97.61% respectively, compared
421 to the extruded control sample (CE), which presented a 96.85% of TIA reduction.
422 Similar reduction in TIA were reported different authors in Kidney and Faba beans
423 flours[32], and in corn flours enriched with 45% of Kidney bean seeds (Navy and Small
424 red varieties), after extrusion cooking[2].

425 Reduction of TIA in formulations containing wheat bran (EF1 and EF2), were around

93.2-93.5 %, compared to their controls. This reduction was less extensive than those determined in all other samples, under study. Since, none of the studied samples showed a total inactivation of TIA after extrusion treatment, this may indicate that the trypsin inhibitors present in lentil, as well as wheat bran, may be of the Bowman-Birk type. This trypsin type present some thermal inactivation resistance due to their high SH-SH bond configuration, and may also be due to protection of crystallized starches formed during extrusion, as previously indicated by Butrón[32] working with mixtures of faba and kidney beans with corn flours.

Other thermal processing, as high pressure cooking process (DIC), were able to promote up to 96% TIA reduction in lentils[25] while cooking and autoclaving (15 min, 121ºC at 1.4 bars) could promote up to 100 % of TIA inactivation [35].

Lectins

The high resistance to proteolytic *in vivo* degradation of the lectins and their ability to recognize and bind to sugar moieties of intestinal epithelial cells may results in hyperplasia. However, a small amount of lectins may be beneficial in stimulating gut function, limiting tumour growth and ameliorating obesity.[36]

The content of lectins in the different lentil-based formulations (Table 4) were evaluated by ELISA indirect assay. In preliminary results, obtained through hemaglutination assay (data not shown), the control lentil flour (CR) showed the highest amount of lentil lectins (LCA) of 167.67 HU/Kg, while the lowest content was determined in CF4 (2.56 HU/Kg). In all cases, extrusion treatment induced a reduction in LCA content was observed, higher than 91.86% (data not shown). ELISA indirect assay, a more specific and sensitive than hemaglutination assay, was used to report the content of lectins. The result of ELISA revealed that the control lentil flour sample (CR) presented the highest value of LCA of 1.36 %, while lentil flour with apple and corn fiber (CF4) presented the

lowest content of LCA of 0.67%. After extrusion processing, the results of ELISA indirect method showed a 100% reduction on all samples under study. These results demonstrate the heat-sensitive nature of these compounds. Also, these results confirmed previous results presented by Armour et al.[34] who indicated that heat treatments have been shown to be very effective in destroying lectin (haemagglutinating) activity. This result is in agreement with the one reported by Butrón[32] and Alonso et al.[31] who reported total inactivation of lectins in chickpeas, peas, faba and kidney beans extruded flours. Moreover, Leontowicz et al.[37] concluded that extrusion at 150 ºC is adequate to eliminate lectins in peas and faba beans.

Moreover, the developed value-added extrudate could provide a suitable way to increase pulse consumption in the general population, particularly in children and youngsters, suffering of celiac disease or gluten sensitivity related conditions, as well as potentially prevent diabetes type 2, as previously indicated by Shi et al.[10] The gluten-free formulations EF3 and EF4, would be appropriated for these particular populations.

Principal components analysis (PCA)

A multivariate analysis was applied to characterize and classify the different lentil-based formulations according to their bioactive compounds (oligosaccharides, inositol phosphates, trypsin inhibitors and lectins). A principal component analysis (PCA) was performed to reduce the multidimensional structure of the data, providing a three-dimensional map to explain the observed variance. Only the two main dimensions, namely Components 1 and 2, are illustrated in the Biplot showed in Fig. 1, which explain the higher total variance (68%).

The three components of the PCA performed explain 84.22 % of the total variance (42.70 % for the first principal component, 25.30% for the second and 16.14% for the

476 third component). All the lentil-based flours as well as their extrusion treatments were
477 plotted in three separated groups within the defined area of principal component 1 and
478 2, as shown in Fig. 1. The first main group formed by all raw formulated lentil-based
479 flours (CF1, CF2, CF3 and CF4) were positively characterized (1.244; 2.207; 1.244 and
480 1.527) by the first principal component (α-galactosides), and positively characterized
481 (1.368; 2.190; 1.368 and 0.375) by the second component (IP5, IP6, total inositol
482 phosphates, LCA and TIA). The second main group, which corresponded to extrusion
483 treatment effect, were formed by the extruded lentil-based formulations EF2, EF3 and
484 EF4, that showed higher galactosides content, were positively characterized by the first
485 component (0.233; 1.005 and 1.436). Additionally, the extruded lentil-based
486 formulations EF1, EF2, EF3 and EF4, that showed lower content in total inositol
487 phosphates, LCA and TIA, were negatively characterized by the second component (-
488 2.959; -0.756; -3.170 and -0.125), which was positively correlated by IP, LCA and
489 TIA..
490 In this way, the analysis showed that extrusion process effect was well described by
491 PCA, being mainly characterized by the second principal component. Furthermore,
492 these results may also serve as an indicator for processing other pulse-based
493 formulations by extrusion, under similar conditions.
494

495 **CONCLUSIONS**

496 The results of the present study provide relevant information about effect of extrusion
497 processing and oligosaccharides) and reduce or partially inactivate compounds (trypsin
498 inhibitors, lectins, phytic acid) commonly present in pulses. Extruded snack-type foods
499 from lentil-based formulations enriched with fiber sources, would be a good alternative
500 to commercially available gluten-containing and low nutritional value snacks. Since,

501 This novel formulation could be a good and healthy alternative to increase pulse
502 consumption.
503
504 **COMPETING INTERESTS**
505 The authors declare no competing financial interest.
506
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622 **Table 1.** Coded samples of lentil flours and lentil-based formulations containing fiber-
623 rich food ingredients.
624

Sample		Characteristics
CR	Control raw flour	Unprocessed lentil flour
CE	Control extruded flour	Extruded lentil flour (CR)
CF1	Control formulated 1	Unprocessed lentil flour + wheat bran + Apple fiber
EF1	Extruded formulated 1	Extruded CF1
CF2	Control formulated 2	Unprocessed lentil flour + Wheat bran + Nutriose ®
EF2	Extruded Formulated 2	Extruded CF2
CF3	Control Formulated 3	Unprocessed lentil flour + Apple fiber + Nutriose ®
EF3	Extruded formulated 3	Extruded CF3
CF4	Control formulated 4	Unprocessed lentil flour + Apple fiber + Corn fiber
EF4	Extruded formulated 4	Extruded CF4

625

626 Gluten-containing formulations: CF1, EF1, CF2 and EF2; gluten-free formulations:
627 CF3, EF3, CF4 and EF4.
628

Table 2. The effect of extrusion treatment on soluble sugars and α-galactosides (mg/g dwb) of different lentil and lentil-based formulations.

Sample	Sucrose	Maltose	Galactinol	Raffinose	Ciceritol	Stachyose	Galactopinitol	Vebascose	Total α-galactosides
CR	$10.22 \pm 0.54^{a,A}$	$3.09 \pm 0.18^{a,E}$	$0.32 \pm 0.01^{a,A}$	$2.72 \pm 0.20^{a,A}$	$23.05 \pm 0.59^{a,G}$	$22.42 \pm 1.74^{a,C}$	$1.82 \pm 0.05^{a,B}$	$16.54 \pm 0.41^{b,E}$	$41.67 \pm 2.01^{a,F}$
CE	$12.82 \pm 0.63^{b,B}$	$5.51 \pm 0.46^{b,F}$	$1.36 \pm 0.16^{b,B}$	$4.22 \pm 0.26^{b,E}$	$26.65 \pm 0.77^{b,H}$	$24.67 \pm 0.76^{a,D}$	$2.84 \pm 0.18^{b,D}$	$13.64 \pm 0.46^{a,D}$	$42.52 \pm 0.46^{a,F}$
CF1	$49.78 \pm 4.03^{a,DE}$	$1.14 \pm 0.08^{a,B}$	nd	$2.83 \pm 0.10^{a,AB}$	$11.87 \pm 0.65^{a,A}$	$20.44 \pm 1.45^{a,B}$	$0.44 \pm 0.03^{a,A}$	$7.00 \pm 0.19^{a,C}$	$30.27 \pm 1.52^{a,C}$
EF1	$57.86 \pm 0.71^{b,F}$	$2.31 \pm 0.21^{b,D}$	nd	$3.06 \pm 0.15^{a,BC}$	$20.22 \pm 0.20^{b,D}$	$29.69 \pm 1.74^{b,E}$	$0.53 \pm 0.15^{a,A}$	$6.91 \pm 0.50^{a,C}$	$39.67 \pm 2.25^{b,E}$
CF2	$43.82 \pm 0.46^{a,C}$	$0.30 \pm 0.02^{a,A}$	nd	$2.98 \pm 0.25^{a,AB}$	$10.71 \pm 0.71^{a,A}$	$17.60 \pm 0.33^{a,A}$	nd	$6.02 \pm 0.26^{a,B}$	$26.60 \pm 0.73^{a,AB}$
EF2	$51.87 \pm 1.31^{b,E}$	$1.57 \pm 0.14^{b,C}$	nd	$4.18 \pm 0.09^{b,E}$	$15.26 \pm 0.57^{b,C}$	$22.18 \pm 0.66^{b,C}$	$0.41 \pm 0.02^{b,A}$	$6.22 \pm 0.17^{a,B}$	$32.58 \pm 0.85^{b,D}$
CF3	$79.36 \pm 1.90^{a,G}$	nd	nd	$3.51 \pm 0.32^{b,D}$	$10.30 \pm 0.60^{a,A}$	$17.37 \pm 0.09^{a,A}$	$0.50 \pm 0.04^{a,A}$	$5.20 \pm 0.35^{a,A}$	$26.08 \pm 0.67^{a,A}$
EF3	$79.48 \pm 0.64^{a,G}$	$1.19 \pm 0.19^{b,DC}$	nd	$2.99 \pm 0.19^{a,AB}$	$12.92 \pm 0.34^{b,C}$	$19.74 \pm 0.36^{b,B}$	$0.78 \pm 0.03^{b,B}$	$5.72 \pm 0.14^{b,B}$	$28.45 \pm 0.50^{b,BC}$
CF4	$48.42 \pm 1.59^{a,D}$	nd	nd	$2.92 \pm 0.07^{a,AB}$	$16.13 \pm 0.36^{a,E}$	$16.95 \pm 0.31^{a,A}$	$0.51 \pm 0.02^{a,A}$	$6.09 \pm 0.41^{b,B}$	$25.96 \pm 0.77^{a,A}$
EF4	$50.06 \pm 0.28^{a,DE}$	nd	nd	$3.33 \pm 0.24^{b,CD}$	$17.80 \pm 0.36^{b,F}$	$17.70 \pm 0.36^{a,A}$	$0.57 \pm 0.00^{a,a}$	$4.77 \pm 0.01^{a,A}$	$24.60 \pm 2.14^{a,A}$

Values are expressed as mean (standard deviation, n-1). In each column, different letters mean statistically significant differences ($p < 0.05$) compared by Duncan test, small superscript letter means difference due to extrusion treatment for the same formulation, whereas capital superscript letter means differences between all samples analyzed.

nd: Non detected

Table 3. The effect of extrusion treatment on inositol phosphates content (mg/g dwb) of different lentil and lentil-based formulations.

Sample	IP4	IP5	IP6	Total IP
CR	$0.28 \pm 0.01^{a,C}$	$0.99 \pm 0.09^{a,CD}$	$3.26 \pm 0.03^{b,E}$	$4.53 \pm 0.09^{a,DE}$
CE	$0.38 \pm 0.02^{b,E}$	$0.91 \pm 0.06^{a,BC}$	$3.06 \pm 0.11^{a,D}$	$4.36 \pm 0.14^{a,EF}$
CF1	$0.28 \pm 0.01^{b,C}$	$1.02 \pm 0.05^{b,DE}$	$4.30 \pm 0.16^{b,G}$	$5.61 \pm 0.18^{b,G}$
EF1	$0.22 \pm 0.01^{a,AB}$	$0.46 \pm 0.01^{a,A}$	$1.78 \pm 0.06^{a,B}$	$2.46 \pm 0.07^{a,B}$
CF2	$0.28 \pm 0.02^{a,C}$	$1.09 \pm 0.07^{a,D}$	$5.02 \pm 0.06^{b,H}$	$6.39 \pm 0.04^{b,H}$
EF2	$0.34 \pm 0.02^{b,D}$	$1.24 \pm 0.09^{a,E}$	$3.04 \pm 0.08^{a,D}$	$4.62 \pm 0.16^{a,F}$
CF3	$0.26 \pm 0.01^{b,BC}$	$0.84 \pm 0.03^{a,B}$	$3.53 \pm 0.06^{b,F}$	$4.63 \pm 0.09^{b,F}$
EF3	$0.21 \pm 0.01^{a,A}$	$0.93 \pm 0.03^{b,BC}$	$0.24 \pm 0.01^{a,A}$	$1.38 \pm 0.05^{a,A}$
CF4	$0.43 \pm 0.04^{a,F}$	$1.12 \pm 0.08^{a,D}$	$2.58 \pm 0.18^{a,C}$	$4.12 \pm 0.25^{a,C}$
EF4	$0.52 \pm 0.04^{b,G}$	$1.29 \pm 0.07^{b,E}$	$2.52 \pm 0.03^{a,C}$	$4.34 \pm 0.05^{a,D}$

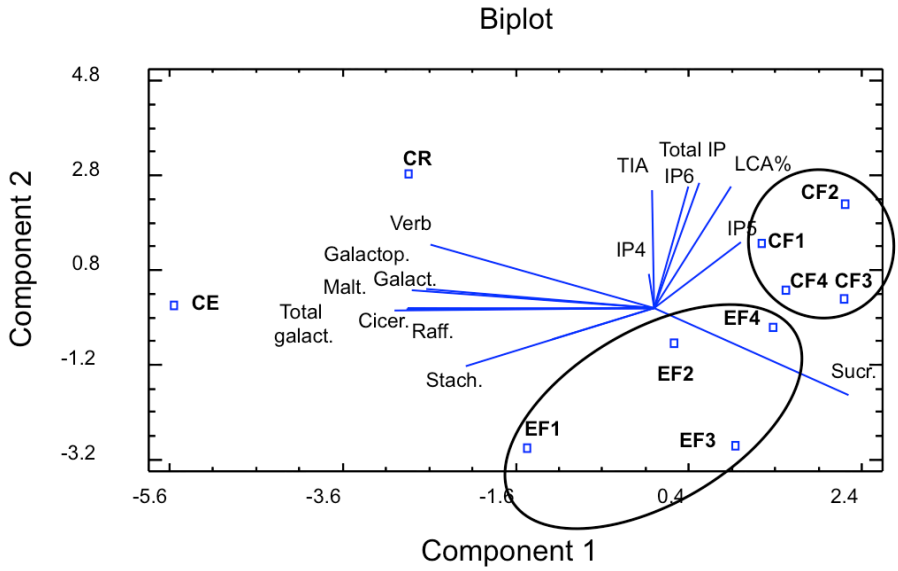
Values are expressed as mean (standard deviation, n-1). In each column, different letters mean statistically significant differences ($p < 0.05$) compared by Duncan test, small superscript letter means difference due to extrusion treatment for the same formulation, whereas capital superscript letter means differences between all samples analyzed.

Table 4. The effect of extrusion treatment on trypsin inhibitiors (TIU/mg dwb) and lectin content (HU (g/kg dwb) for hemaglutination assay and % LCA for ELISA assay) in different lentil and lentil-based formulations.

Sample	Trypsin inhibition	Lectin content
CR	$11.43 \pm 0.52^{b,F}$	$1.36 \pm 0.14^{b,D}$
CE	$0.36 \pm 0.02^{a,B}$	$0.00 \pm 0.00^{a,A}$
CF1	$4.97 \pm 0.17^{b,E}$	$1.09 \pm 0.18^{b,BC}$
EF1	$0.34 \pm 0.02^{a,AB}$	$0.00 \pm 0.00^{a,A}$
CF2	$4.86 \pm 0.22^{b,DE}$	$1.21 \pm 0.10^{b,CD}$
EF2	$0.32 \pm 0.02^{a,AB}$	$0.00 \pm 0.00^{a,A}$
CF3	$4.61 \pm 0.32^{b,D}$	$1.04 \pm 0.01^{b,CD}$
EF3	$0.11 \pm 0.01^{aa,B}$	$0.00 \pm 0.00^{a,A}$
CF4	$3.51 \pm 0.07^{b,C}$	$0.67 \pm 0.13^{b,B}$
EF4	$0.08 \pm 0.00^{a,A}$	$0.00 \pm 0.00^{a,A}$

Values are expressed as mean (standard deviation, n-1). In each column, different letters mean statistically significant differences ($p < 0.05$) compared by Duncan test, small superscript letter means difference due to extrusion treatment for the same formulation, whereas capital superscript letter means differences between all samples analyzed.

Figure 1. Principal component analysis (PCA) projection of two first principal components.



Lentil raw flours (control and formulated): (CR) control raw flour, (CF1) control formulated 1, (CF2) control formulated 2, (CF3) Control formulated 3, (CF4) Control formulated 4. Extruded flours (control and formulated): (CE) control extruded flour, (CE1) extruded formulated 1, (EF2) extruded formulated 2, (EF3) extruded formulated 3, and (EF4) extruded formulated 4. Parameters: Suc. (sucrose); Malt. (maltose); Galact. (galactinol); Raff. (raffinose); Cicer. (ciceritol); Stach. (stachiose), Galactop. (galactopinitol); Verb. (verbascose); Total galact. (total α-galactosides); IP4 (tetra-inositol phosphate); IP5 (penta-inositol phosphate); IP6 (hexa-inositol phosphate); Total IP (Total inositol phosphates); TIA (trypsin inhibitiors activity) and LCA (lectin).