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Acceptance Letter

Dear Morteza Kordi,

It is pleased to inform you that your papers entitled "**Effects of extrusion process on physicochemical properties, *in situ* rumen degradability, oxidative stability and α-Linolenic acid retention in Flaxseed during long term storage,** submitted to the journal is acceptable for publication in Research Opinions in Animal & Veterinary Sciences (ROAVS) in its current form.

Thank you for your valued contribution. Please always mention your paper number printed on the right hand side of the letter in your future correspondence. On behalf of the editorial board of Research Opinions in Animal & Veterinary Sciences (ROAVS), we look forward to your continued contributions to the Journal.

Sincerely,

Dr. Rifat Ullah Khan

Managing Editor

Effects of extrusion process on physicochemical properties, *in situ* rumen degradability, oxidative stability and α-linolenic acid retention in flaxseed during long term storage

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Abstract

The current study was conducted to evaluation of the effects of extrusion process on chemical composition, quality parameters, *in situ* rumen degradability, oxidative stability, and fatty acid profile changes of flaxseed during long term storage. In a completely randomized design, flaxseed ground (GF) or mixed with alfalfa hay and pistachio by-products with 80:10:10 (by mass) proportions and extruded (EF). There was no difference between ground and extruded flaxseed for dry matter content (P>0.05). But, the content of crude protein (CP) and ether extract (EE) reduced and the ash content significantly increased after extrusion process (P<0.05). EF treatment significantly had higher oil lost (OL) and bulk density (BD) than GF (P<0.05) and the angle of repose (AR) in GF treatment was slightly higher than EF. Extrusion significantly reduced proportion of unsaturated fatty acids and increased peroxide value (PV) in the flaxseed (P<0.05). Also, PV linearly increased during storage period (P<0.05) and this increase was significantly higher in EF treatment than GF (P<0.05). In addition, slowly degradable fraction (*b*), potential DM degradability (*a*+*b*) and effective DM degradability of flaxseed reduced, but soluble DM fraction (*a*) and the rate of degradation of the slowly degradable DM (*c*) increased by extrusion (P<0.05).

Key words: extrusion, flaxseed, linseed, pistachio by-products.

Introduction

Among oilseeds, flaxseed (*Linum usitatissimum*) has the highest proportion of linolenic acid, averaging 18% of total seed weight and constituting 53% of total fatty acid (Gonthiere et al., 2004). Feeding whole, rolled, or extruded flaxseed to dairy cows increases concentration of milk unsaturated fatty acid and decreases the concentration of saturated fatty acid, particularly C16:0 (Glasser et al, 2008). However, feeding flaxseed produced relatively small changes in the concentration of C18:2 and C18:3 in milk due to the extensive biohydrogenation of these fatty acids in the rumen (Ferlay et al., 2011). Altering the physical structure of oilseeds through heat treatment may help to protect the dietary fatty acid of oilseeds from ruminal biohydrogenation (Mustafa et al., 2002). The application of heat treatment to oilseeds can protect dietary polyunsaturated fatty acid from ruminal biohydrogenation by denaturing the protein matrix surrounding the fat droplets (Kennelly, 1996), and altering the site of nutrient digestion in the gastrointestinal tract of ruminants (Mustafa et al., 2002). The seeds of flaxseed are quite small and the mucilage present in the outer coating lends the seed a very slippery quality; the combination of which makes it difficult to process (Eggie, 2010).

Extrusion is a heat treatment used extensively in the production of animal feed, and can be useful in protecting oilseeds from ruminal degradability (Sterk et al., 2010). Indeed, during extrusion several operations take place, such as grinding, hydration, mixing, shearing, thermal treatment, gelatinization, protein denaturation, destruction of microorganisms and some toxic compounds, shaping, expanding, and partial dehydration (Riaz et al., 2005). However, there are some inherent problems in the extrusion process of an oilseed-based feed ingredient. In fact, due to the extrusion process of flaxseed, the rapid release of intracellular oil may lead to considerable oil loss. Excess oil not retained by the feed ingredient may be lost during transport and storage, which can be considered an economic loss, as the desired and healthful beneficial ingredient of flaxseed is its oil. In addition, potential production of oxidized compounds and reduced shelf life due to high ALA content of flaxseed is also of concern (Eggie, 2010). Therefore, using an appropriate binder (absorbent) material for production of extruded linseed can be a good resolution to reduce oil losses (Eggie, 2010) and oil oxidation during extrusion. Alfalfa hay and pistachio by-products are materials with high fat absorption capacity for prevention from oil lost from oilseed after extrusion process (Eggie, 2010, Kordi et al, 2015).

Therefore, the objective of this study was investigation the effect of extrusion process on chemical composition, quality parameters, *in situ* rumen degradability, oxidative stability, and fatty acid profile changes of flaxseed during long term storage.

Materials and methods

Processing

Flaxseed was purchased from a local market (Mashhad, Iran). For control treatment, samples were dried in an air oven at 40 ºC for 48 h, then ground to pass through a 3-mm screen and stored for later analysis. For the extrusion trial, a mixture of flaxseed and absorbents (alfalfa hay, pistachio by-products) with a ratio of 80:10:10 (by mass) was prepared. The extrusion was performed in a double screw extruder (DS56-III, Jinan Saixin Machinery Co., Shandong, China), consisting of three independent zones of controlled temperature in the barrel. The temperature profiles in the first and second zones were kept constant at 70 and 80 ºC, respectively, and the die head temperature was about 110 ºC. The extruded material was cut with a die face cutter as it left the extrusion die. After stable conditions were established, about 700 g of extruded product was collected and dried in air oven at 40 ºC for 24 h. Extruded material was stored at 4 ºC in plastic bags for various analyses as describe below.

Analysis for physicochemical properties

Chemical composition of different treatments was analyzed according to AOAC (2005). For quality parameters, Oil lost was measured using the method of Eggie (2010). Bulk density was determined using a modified version of ASAE Standard S269.4 DEC 01 as modified by Eggie (2010). Angle of repose was measured using the established method for the Carr Angle of Repose Carr (1965) described by ASTM D6393-99 (ASTM, 2006). According to Carr (1965), low angles of 30° to 40° indicate a material with relatively easy flowing characteristics, while high angles of 50° to 60° represent difficult flow conditions (Ganesan et al., 2005).

Lipid peroxidation and fatty acid profile

Processed materials were stored in plastic bags and kept in ambient temperature for 90 days. The stored samples were analyzed for peroxide value (PV) and fatty acid profile on days 0, 10, 20, 30, 60, and 90 after processing. Each time, oil was extracted from samples. For oil extraction, dried samples were ground to powder in a grinder. The powders were extracted with n-hexane (1:4 wt/vol) by agitation in a dark place at ambient temperature for 48 h. The solvent was evaporated *in vacuo* at 40 °C to dryness. Peroxide value was assessed by colorimetric determination of iron-thiocyanate according to Shantha and Decker (1994).

Fatty acid profile was determined by gas chromatography. Fatty acid methyl esters (FAME) were prepared according to Wijngaarden (1967). A fused silica capillary column (WCOT Fused Silica Capillary, DANI, Model 1000, Rome, Italy) 120 m in length, 0.32 mm internal diameter, and 0.2 μM film thickness on an HP 6890 GC equipped with flame ionization detector was used to quantify FAMEs. The initial column temperature was set at 180 °C for 20 min, which was increased to 225 °C by increments of 5 °C/min, then to 250 °C by 10 °C/min and held for 12 min. Hydrogen was used as carrier gas with a flow of 1.7 ml/min for the first 10 min. Then, the flow was decreased to 1.3 ml/min, and maintained until the end of the analysis. The detector temperature was set at 300 °C. Identification of FA was performed by comparison with the retention times of FAMEs standards (Sigma-Aldrich, Catalog #18919). Separations of all FAME were obtained with a single chromatographic run (Ferlay et al, 2013).

α-Linolenic acid retention

The retention of α-linolenic acid in flaxseed products was calculated according to expression given below:

α-linolenic acid retention, % = (the content of ALA after extrusion ÷ the content of ALA before extrusion) × 100

***In situ* rumen dry matter degradability**

A measurement of *in situ* DM degradability of treated samples was performed in 4 rumen-fistulated dairy cows using the nylon bag technique (Ørskov and McDonald, 1979). The nylon bags (9 × 18 cm^2, pore size 50 µm) were filled with 5 g of samples and put into the rumen. Table 5 shows the ingredient composition of the total mixed ration (TMR) offered to the dairy cows in two equal feedings at 08:00 and 16:00 hours.

The bags were removed at 2, 4, 8, 12, 24 and 48 (h) after the start of incubation, and each bag was washed immediately with cold tap water until color disappeared.

For disappearance at t_0 time, the unincubated bags were simply washed in water. All washed bags were dried in a forced-air oven at 65 °C for 48 hours. Disappearance of DM at each incubation time was estimated from the proportion remaining after incubation in the rumen.

Calculations and Statistical analysis

The rate and extent of DM degradation were estimated according to the equation: $p = a + b (1 - e^{-ct})$ where *P* is the disappearance rate at time *t*, *a* is rapidly degradable DM fraction, *b* is slowly degradable DM fraction in the rumen, *c* is the rate constant of degradation of *b*, and *t* represents the time of incubation.

Effective degradability of DM (EDDM) was calculated as $EDDM = a + (b \times c)/(c + Kp)$, where *k* is the fractional outflow rate (*k* =0.05) from the rumen (per hour), with *a*, *b*, and *c* as described above (Ørskov and McDonald, 1979).

All data were analyzed in a completely randomized design using the general linear model procedure of SAS 9.2 (2003).

The statistical model used, was as following:

$$Y_{ij} = \mu + T_i + e_{ij}$$

where Y_{ijk} is the observation; μ is the overall mean; T_i is the effect of treatments and e_{ij} is the random error. Significant differences between individual means were identified using Duncan's multiple range test at a 0.05 probability level.

Results and discussions

Physicochemical properties

Data on physicochemical properties of processed flaxseed are presented in Table 1. These data showed that there were no differences between treatments for DM content (P>0.05), but the content of CP and EE in GF treatment was significantly higher than in EF (P<0.05). Also, the ash percentage in EF was significantly higher than in GF treatment (P<0.05). These decreases in CP and EE content or increase in ash content after extrusion process on flaxseed can be related to added absorbents (10% alfalfa hay and 10% pistachio by-product) to flaxseed before processing, because CP and EE content of flaxseed is more than absorbents and the ash content of flaxseed was less than them. Also, data indicated that OL in EF treatment was significantly higher than in GF (P<0.05). In fact, OL is the ability of the extruded products to retain oil that it is related to the type of absorbent and the ratio of oilseed: absorbent in the extrusion process (Eggie, 2010). In our study, extrusion increased the OL from flaxseed as a result of pressure and heat treatments that exist in the extrusion process.

Extrusion process significantly increased BD of EF treatment (P<0.05), that it can be resulted from more BD of alfalfa hay and pistachio by-product compared to flaxseed that used as absorbents during extrusion. Eggie (2010) reported that there was significant difference between extruded flaxseed products produced with alfalfa, soy hulls, or corn gluten. Extruded material with corn gluten had the highest BD compared to the other two products.

There were slight differences in AR among GF and EF treatments. This index indicated that EF product sample had relatively easy flow characteristics, while the GF had a little more difficulty with flow than the EF. Eggie (2010) observed no differences among extruded flaxseed products containing 25% alfalfa, soy hulls, or corn gluten for the AR parameter and all of these products had acceptable flow characteristics.

Fatty acid composition

Fatty acid composition of flaxseed influenced by extrusion process (Table 2) and data showed that the concentration of saturated fatty acids C16:0 and C18:0 significantly increased after extrusion process ($P<0.05$). But, unsaturated fatty acids concentration (C16:1, C18:1n9, C18:2n6, and C18:3n3) significantly decreased by extrusion ($P<0.05$) that it can be resulted from heat treatment during extrusion process.

Eggie (2010) reported that, the proportions of fatty acid of extruded flaxseed with different absorbents (alfalfa, soy hulls, and corn gluten) were affected by storage period and it is logical that the concentration of fatty acid be affected. Indeed, Eggie (2010) indicated that the concentration of unsaturated fatty acids in extruded flaxseed during storage period significantly reduced.

Peroxide value and α-linolenic acid retention

Results of PV and ALA retention evaluation (Table 3) confirmed the data of fatty acid composition. In fact, EF treatment had higher PV and less ALA retention than GF ($P<0.05$). It means that a part of unsaturated fatty acids, especially ALA, oxidized during extrusion process. Also, during storage period, by increasing time of storage PV of treatments linearly increased too ($P<0.05$), and this increase was significantly higher in EF treatment ($P<0.05$).

A PV of less than 5 cannot be rancid for cattle and probably handled fat should not exceed a PV of 10 (Zinn, 1995). Although, the PV of EF treatment is significantly higher than GF, but it can be appropriate for using in dairy cows diet. However, during storage period, PV of EF treatment increased more than 10, and maybe it decrease the palatability of this ingredient in dairy cows diet.

During the oilseed extrusion process, the rapid release of intracellular oil may lead to considerable oil losses (Akraim et al., 2007). Furthermore, when this oil is extracted, it is exposed to high temperatures and air, which may probably increase the rate of oxidation. In fact, potential production of oxidized compounds and reduced shelf life due to high ALA content of flaxseed is a problem with extrusion process of this oilseed (Eggie, 2010).

In situ **rumen dry matter degradability**

The data of DM degradation parameters for treatments are given in Table 6. Dry matter degradability parameters were different among treatments (P<0.05); soluble DM fraction (*a*) and rate of degradation of the slowly degradable DM (*c*) were significantly higher, but slowly degradable DM fraction (*b*) was lower in GF treatment than EF treatment (P<0.05). In addition, potential DM degradability was significantly higher in GF treatment compared to EF treatment (P<0.05). Effective DM degradability significantly affected by extrusion process and it was lower in EF treatment than GF (P<0.05).

According to our results, Mustafa et al (2003) reported that extrusion increased *in situ* soluble DM fraction (*a*) and decreased the slowly degradable DM fraction (*b*) of flaxseed. Also, they indicated that rate of degradation of the slowly degradable DM (*c*) was higher for unheated than extruded flaxseed. But, disagreement with our study, Mustafa et al (2003) demonstrated that extrusion increased effective ruminal degradability of DM. Maybe the reason of this different is that they did not use any absorbent for mixing with flaxseed before extrusion process. Eggie (2010) indicated that the disappearance of DM in the rumen significantly increased by extrusion. In agreement with our results, several studies have reported a reduction in ruminal nutrient degradability and effective degradability of flaxseed (Mughetti et al, 2007); soybean (Orias et al, 2001) and lupin seed (Aufrere et al, 2001) as a result of extrusion. In addition, Mughetti et al (2007) reported that both *b* and *a*+ *b* fractions were decreased by extrusion. This different effect seems dependent on the fat content and the industrial processing system (Pena et al., 1986).

Conclusion

In conclusion, the oil lost and bulk density of flaxseed increased by extrusion process. In addition, extrusion reduced the content of unsaturated fatty acids and ALA retention, and increased peroxide value in the flaxseed. Also, peroxide value of flaxseed linearly increased during storage period. Furthermore, slowly degradable fraction, potential DM degradability and effective DM degradability of flaxseed reduced and soluble DM fraction increased by extrusion.

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Table 1. Physicochemical properties of flaxseed processed with different methods.

	Processed flaxseed*

Items	GF	EF	SEM†
DM (%)	95.82	94.53	0.288
CP (%)	18.71^{a}	17.63^{b}	0.052
EE (%)	41.06^{a}	33.75^{b}	0.815
Ash (%)	2.96^{b}	5.62^{a}	0.217
OL (g)‡	0.038^{b}	0.071^{a}	0.001
BD (gcm^{-3})	0.59^{b}	0.74^{a}	0.019
AR (°)	48.28	43.11	-

*GF, ground flaxseed; EF, extruded flaxseed.

†SEM, standard error of mean.

‡ OL, Oil lost; BD, Bulk density and AR, Angle of repose.

Table 2. Fatty acid composition of ground and extruded flaxseed.

Item	Processed flaxseed*		
FA(g /100g FA)	GF	EF	SEM†
C16:0	5.02^{b}	5.37^{a}	0.021
C16:1	0.16^{a}	0.05^{b}	0.014
C18:0	3.53^{b}	4.29^{a}	0.021
C18:1 cis-9	20.83^{a}	20.22^{b}	0.019
C18:2 cis-9, 12	17.13^{a}	16.53^{b}	0.022
C18:3 cis-9, 12, 15	52.57^{a}	52.16^{b}	0.020

*GF, ground flaxseed; EF, extruded flaxseed.

†SEM, standard error of mean; means whit different superscripts (a, b, c) are significantly different (P<0.05).

Table 3. Peroxide value and α-linolenic acid retention of ground and extruded flaxseed.

	Processed flaxseed*		
Item	GF	EF	SEM†
Peroxide value (meq O_2/kg oil)	3.61^{b}	8.23^{a}	1.408
α-linolenic acid retention (%)	100^{a}	99.22^{b}	0.007

*GF, ground flaxseed; EF, extruded flaxseed.

†SEM, standard error of mean; means whit different superscripts (a, b, c) are significantly different (P<0.05).

Table 4. Peroxide value of ground and extruded flaxseed during storage period.

	Processed flaxseed*												SEM†	P-value		
	GF:storage day						EF:storage day							treat	time	treat×time
Item	0	10	20	30	60	90	0	10	20	30	60	90				
‡Peroxid value	3.61	3.84	4.51	4.58	4.71	5.02	8.23	16.71	19.36	29.68	32.13	41.55	0.625	0.002	<0.0001	<0.0001

*GF, ground flaxseed; EF, extruded flaxseed.

†SEM, standard error of mean.

‡meq O_2/kg oil

Table 5. Ingredient of the total mixed ration for fistulated dairy cow.

Ingredients	% DM
Alfalfa	20
Corn silage	17
Extruded Flaxseed mixture	8
Corn grain	18
Barley grain	10
Soybean meal	10.5
Rapeseed	7
Meat powder	3.5
Sugar beet pulp	4.6
Calcium carbonate	0.5
Vitamin-mineral mix	0.7
Sodium chloride	0.2
Chemical composition, g/kg DM	
CP	17.5
NDF	30
Forage NDF	17.8
ADF	19
Ether extract	4.6
Ca	1.2
P	0.6

Table 6. Degradation parameters of flaxseed process with different methods.

Degradation parameter[‡]	Processed flaxseed[*] GF	Processed flaxseed[*] EF	SEM[†]
a (mg/g)	7.52[b]	8.08[a]	0.032
b (mg/g)	73.91[a]	69.42[b]	0.046
c (h^{-1}%)	0.070[b]	0.078[a]	0.0005
Potential degradability (a+b) (mg/g)	81.43[a]	77.50[b]	0.017
Effective degradability (%)	50.77[a]	50.42[b]	0.029

*GF, ground flaxseed; EF, extruded flaxseed.

†SEM, standard error of mean; means whit different superscripts (a, b and c) are significantly different (P<0.05).

‡*a* is rapidly degradable DM fraction, *b* is slowly degradable DM fraction in the rumen, *c* is the rate constant of degradation of *b*.