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Research Article

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Muhammad Kaleem[1,2,3], Yves Farizon[1,2,3], Francis Enjalbert[1,2,3] and Annabelle Troegeler-Meynadier[1,2,3]

[1] INRA, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, Castanet-Tolosan, France

[2] Université de Toulouse, INPT ENVT, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, Toulouse, France

[3] Université de Toulouse, INPT ENSAT, UMR1289 Tissus Animaux Nutrition Digestion Ecosystème et Métabolisme, Castanet-Tolosan, France

Heating oilseeds has been shown to improve the milk fatty acid profile when given to dairy cows, compared to raw oilseeds. However, results from published studies are conflicting. The conditions of heating and storage of the oilseeds could be responsible for these differences, probably partly through their effects on lipid oxidation, the products of which could act on ruminal biohydrogenation (BH). Thus, 15 different treatments were applied to ground soybeans: three levels of heating (no heating, 30 min at 110 or 150°C) × 5 ambient storage durations (0, 1, 2, 4, or 6 months). Soybeans were incubated in vitro with ruminal fluid for 6 h. Triacylglycerol (TAG) polymers, hydroperoxides and hydroxyacids (HOA), aldehydes, and fatty acids were assayed in soybeans and ruminal culture. No TAG polymer was detected in any treatment. Soybeans stored for a long time had a high content of HOA, whereas those heated at 150°C, whatever the storage duration, had high aldehyde contents. The percentage disappearance of *cis*-9,*cis*-12 18:2 and *cis*-9,*cis*-12,*cis*-15 18:3 in incubates decreased significantly in cultures with heated soybeans, especially at 150°C, suggesting that this partial protection of polyunsaturated fatty acids (PUFA) from BH was at least in part linked to the aldehyde content of the heated soybeans.

Practical applications: Oilseeds given to ruminants are often heated, and heat treatment is known to generate oxidation products. Knowing what oxidation products influence ruminal biohydrogenation of unsaturated fatty acids could result in technological processes allowing a better transfer of unsaturated fatty acids from oilseeds to ruminant products.

Keywords: Heating temperature / Peroxidation / PUFA / Ruminal biohydrogenation / Storage duration

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1 Introduction

In ruminant production, addition of unsaturated fat to the diet, commonly by addition of oilseeds, is often used to increase polyunsaturated fatty acids (PUFA) concentration of milk fat and so enhance the dietetic quality of milk for human consumers. However, oilseeds contain some anti-nutritional and/or toxic factors, which can be inactivated by heating. Heating is also often used to decrease the ruminal degradability of proteins. On the other hand, heating of fat, especially unsaturated fat, promotes oxidation, leading to the formation of oxidation products like oxypolymers of Triacylglycerol (TAG), hydroperoxides, and terminal products like aldehydes, ketones, and hydroxyacids (HOA). The oxidation of fat during heating is affected by the temperature and duration of heating [1] but also by the percentage of moisture [2]. Thereafter, conditions of storage can also enhance the oxidative status of fat [3].

The effects of heated oilseeds on the milk fatty acid (FA) profile differs from that of raw oilseeds, and depends on the

nature and conditions of heating [4]. Compared to raw oilseeds, PUFA of heated oilseeds are partly protected from ruminal biohydrogenation (BH), and result in a greater ruminal production of BH intermediates in vivo [5], in situ [6], and in vitro [7]. Among these intermediates, conjugated linoleic acid (CLA) [8] and *trans* C18:1 [9] can affect human health but more importantly the production of *trans*-10,*cis*-12 CLA should be avoided because this isomer results in milk fat reduction in dairy cows [10]. However, this partial protection against BH is not always observed [11, 12]. Moreover, some differences could be observed according to the heating process: extruded oilseeds resulted in an increase of BH intermediates, and roasted oilseeds resulted rather in protection against BH in the two studies comparing these heating processes [4, 6]. Nevertheless, most published studies provide little information about heating conditions, which could have directly affected the generation of lipid oxidation products.

A previous in vitro study in our laboratory showed that increasing the duration and temperature of heating of sunflower oil decreased the ruminal production of *c*9,*t*11 CLA and *trans*-11 18:1, while the production of *trans*-10,*cis*-12 CLA and *trans*-10 18:1 was increased, which at least in part was linked to lipid peroxidation [1]. Moreover, in this study, the BH extent of linoleic acid (*cis*-9,*cis*-12 18:2) was also shown to be dependent on temperature and heating duration. The duration and temperature of heating, and the duration and conditions of storage of oilseeds could also affect the production of oxidation products, and could be responsible for the discrepancies between studies regarding BH of oilseed PUFA. Many oxidation products could be responsible for these effects, and a hypothesis could be proposed to explain their action. Oxipolymers of TAG could protect PUFA from BH. Hydroperoxides and HOA of PUFA could interfere with BH reactions because of their structural similarity with PUFA and hydroperoxides because of their toxicity to bacteria, since they have been shown to inhibit the activity of anaerobic microorganisms [13] present in the ruminal ecosystem. Furthermore, aldehydes could have an effect on BH [14], probably because of antimicrobial properties [15].

The aim of the present experiment was to study the effects of heating temperature and storage duration of ground soybeans on ruminal BH of *cis*-9,*cis*-12 18:2, and to investigate the relationship between observed modifications of BH and oxidation compounds of the treated soybeans.

2 Materials and methods

2.1 Preparation of soybean seeds

Soybean seeds of commercial origin were ground using an ultracentrifugal mill (ZM 200, Restch, Düsseldorf, Germany) with a ring sieve of 1 mm at 10,000 rpm, and were assigned to three treatments: no heating, heating at 110 or 150°C for 30 min. For heating, ground oilseeds were spread 1 cm thick in a crystallizing dish (30 cm diameter). After 30 min heating, seeds were cooled at ambient temperature for 30 min. Heated or unheated seeds were then transferred into closed flasks and kept for 0, 1, 2, 4, or 6 months under ambient conditions in order to obtain different levels of lipid oxidation. At the end of this time, the flasks were hermetically sealed and kept at −80°C until use. As the purpose of this study was not to explore the effects of heating temperature or storage duration on soybean oxidation compounds, the three treatments were not replicated. Indeed, these processes were only used as a way to obtain different contents of oxidation compounds in soybeans, to explore the effects of these compounds on BH. Hence there were 15 different seed treatments (i.e., 3 heating conditions × 5 ambient storage durations); six series of in vitro incubations were performed to obtain six replicates per treatment.

2.2 In vitro cultures

Rumen content was collected from two cows fed a corn silage-based diet (20 kg of corn silage, 3 kg of hay, 1.6 kg of soybean meal, and 0.3 kg of a mineral + vitamin supplement as fed) in two equal meals. The cows were adapted to this diet for 16 days before the beginning of the experiment. One liter of ruminal fluid was taken from each cow with a vacuum pump 30 min before morning meal and strained through a metal sieve (1.6 mm mesh). Rumen fluids from the two cows were mixed and quickly transferred to the laboratory in anaerobic conditions at 39°C.

In an Erlenmeyer flask, 0.5 g of one of the 15 treated soybeans was added together with 2 g of hay ground at 2 mm, and 0.5 g of corn ground at 1 mm; for each series of incubations, a control flask with 2 g hay and 0.5 g corn was prepared. In each flask, 80 mL of rumen fluid and 80 mL of bicarbonate buffer (pH 7.0; 19.5 g/L of $Na_2HPO_4 \cdot 12H_2O$, 9.24 g/L of $NaHCO_3$, 0.705 g/L of NaCl, 0.675 g/L of KCl, 0.108 g/L of $CaCl_2 \cdot 2H_2O$, and 0.180 g/L of $MgSO_4 \cdot 7H_2O$) saturated with CO_2 and prewarmed at 39°C were added. The flasks were filled with CO_2 to eliminate oxygen and placed in a water-bath rotary shaker (Aquatron, Infors AG, Bottmingen, Germany). They were closed with a rubber cap through which a plastic tube led into the water to vent fermentation gas without allowing the ingress of oxygen. Flasks were stirred at 130 rpm and kept away from the light during the 6-h incubation. Control flasks were filled with the same quantities of rumen fluid and buffer, the pH was measured and they were immediately frozen to serve for determination of the initial status of cultures.

At the end of the incubations, the flasks were placed into crushed ice. The pH was measured. To determine NH_3 and volatile fatty acids (VFA), 2 × 8 mL were taken from each flask into two vials containing 0.8 mL of 2% mercuric chloride, as a stabilizer. The rest of the flask contents was frozen at −20°C, freeze-dried (Virtis Freezemobile 25, Virtis, Gardiner, USA), weighed, ground and homogenized

in a ball mill (Dangouman, Prolabo, Nogent-sur-Marne, France), and kept at −20°C until analysis.

2.3 Analysis of fermentation parameters

The concentrations of VFA were determined using the GC method of Playne [16], modified as follows: the ruminal samples were first centrifuged at 2880 × *g* for 20 min to separate the liquid phase. For protein removal, 1 mL of supernatant was mixed with 200 μL of metaphosphoric acid solution (25% w/v) and further centrifuged at 20,000 × *g* for 15 min. Then, 100 μL of supernatant was added to 75 μL of 4-methylvaleric acid as an internal standard (0.2% v/v) and 900 μL of water. Analysis was performed by injecting 1 μL of the mixture into a gas chromatograph (Model 5890 Series II, Hewlett Packard, Palo Alto, CA, USA) equipped with a flame ionization detector.

The determination of NH_3 content was based on the modified Berthelot reaction with the Skalar Method, as reported by Julien et al. [17].

2.4 Fatty acid analysis

The FA of feed ingredients, including the 15 kinds of treated soybeans, control flasks and incubated media were extracted and methylated in situ using the procedure of Park and Goins [18], except that the solution of 14% of boron trifluoride in methanol was replaced by a solution of methanol-acetylchloride (10:1). Nonadecanoic acid (19:0) was used as the internal standard at a dose of 0.8 mg. The FA methyl esters were then quantified by GC (Agilent 6890N, Network GC System, equipped with a model 7683 auto injector; Agilent Technologies, Palo Alto, CA, USA) using a fused silica capillary column (100 m × 0.25 mm i.d., 0.20 μm film thickness, CPSil88,Varian, Middelburg, the Netherlands), as described in Zened et al. [19]. Peaks were identified and quantified by comparison with commercial standards (Sigma Co., St Louis, MO, USA), except 18:1 other than *trans*-9 18:1, *trans*-11 18:1 and *cis*-9 18:1, which were identified by order of elution. The integration of chromatograms was done using the software Peak Simple (Peak Simple Data System, version 3.29, SRI, Torrance, CA, USA).

2.5 Lipid-oxidation compound analysis

For each combination of heating treatment × storage duration, one sample of soybeans was analyzed in triplicate for three kinds of putative lipid oxidation products generated during heating: TAG polymers, hydroperoxides and HOAs, ketones, and aldehydes. Prior to these analyses, fat from 15 treated oilseeds was extracted: 6 g of oilseeds were stirred for 1 h with 50 mL of hexane and then strained on filter paper (No. 1, Whatman Inc., Buckinghamshire, UK). An evaporation of hexane under nitrogen at 35–40°C was done for hydroperoxides/HOA and ketones/aldehydes analyses in order to obtain only extracted oils.

For TAG polymer assay, the NF T60-268 official method [20] was used, except for the detector. The analysis was performed by HPLC with an P680 HPLC pump (Dionex, Voisins le Bretonneux, France) coupled with an evaporative light scattering detector (PL-ELS 2100, Polymer Laboratories, Stretton, UK) equipped with a size exclusion PLgel column (particle size 5 μm, pore size 300 Å, 300 × 7.5 mm i.d., Polymer Laboratories, Church Stretton, UK). The mobile phase was tetrahydrofuran. The injected volume was 20 μL. The column oven temperature was set at 30°C. The mobile phase flow rate was 1 mL/min. The conditions for evaporative light scattering detection were 1 mL/min for nitrogen flow rate, 50°C for nebulization temperature and 75°C for evaporation temperature. As there is no standard available for TAG polymer, peaks were identified by order of elution, as indicated by the AFNOR method. Because no TAG polymers were found in heated seeds, pure trilinolein (99%, Sigma Co.) heated at 150°C during 6 h was used to validate our assay.

Hydroperoxide and HOA analysis were done according to the HPLC method of Browne et al. [21] after saponification of lipids: 100 mg of extracted oil plus 2 mL of potassium hydroxide 1 M (prepared in 95% ethanol) were kept for 16 h with continuous stirring at 20°C. Then, 5 mL of ultra-pure water and 10 mL of hexane were added, and homogeneization was obtained by a vigorous manual stirring. The upper organic phase was eliminated as it contained the non-saponified molecules, and the alcoholic phase was acidified using 5 mL of 6 N hydrochloric acid. Afterwards, this phase was washed three times with 10 mL of hexane and filtered with a phase separator silicone treated filter paper (1PS, Whatman Inc., Buckinghamshire, UK). Hexane was evaporated under vacuum at 35–40°C. The residue was weighed and diluted with ethanol in order to obtain 10 mg of residue per mL of ethanol. The analysis was done using an automated HPLC–UV system with a P680 HPLC pump and diode array detection at 320 nm (Agilent technologies). The column used for separation was an Uptisphere C18 ODB (particle size 3 μm, 15 × 0.46 cm i.d., Interchim, Montluçon, France). The mobile phase was a mixture of 1 g/L acetic acid, acetonitrile and tetrahydrofuran (52:30:18, v/v/v). The injected volume was 20 μL of the ethanolic solution. The column oven temperature was set at 40°C. The mobile phase flow rate was 1 mL/min. Peaks were identified and quantified by comparison with commercial standards (Interchim, Montluçon, France), focusing on products from *cis*-9,*cis*-12 18:2 oxidation, 13(OOH) *cis*-9,*trans*-11 18:2 and 9(OOH) *trans*-10,*cis*-12 18:2, and their respective hydroxy-acids (13HOA and 9HOA).

Aldehyde analysis was done according to the method of Li et al. [22]. Extracted oil (75 mg) was mixed with 10 mL of hexane, and then 1 μL was injected into a gas chromatograph (Agilent 7890A, GC System, equipped with a model 7683 auto injector, Agilent Technologies) using a capillary column (30 m × 0.53 mm i.d., 0.5 μm film thickness, DB-5, Agilent

Technologies). For analysis, the flame ionization detector temperature was maintained at 280°C and the injector at 280°C; the split ratio was 1:5. Nitrogen was used as the carrier gas with a constant flow of 4 mL/min. The initial oven temperature was 60°C, which was increased by 2°C/min to 70°C, then increased by 10°C/min to 250°C and held for 1 min. Peaks were identified and quantified by comparison with commercial standards (Sigma Co.).

Chromatograms for TAG polymers, hydroperoxides/HOA, and aldehydes were integrated using the software Chromeleon chromatography data system (version 6.60, Dionex, Voisins le Bretonneux, France).

2.6 Calculations and statistical analysis

The percentage of PUFA disappearance was calculated as the quantity of unsaturated FA disappeared (initial − final quantity)/initial quantity of this PUFA × 100. The initial quantity of FA was calculated by adding the quantity of FA from the rumen fluid (quantified from control flask) to that from the soybeans. The percentages of PUFA disappearance, FA percentages, NH_3 and VFA concentrations of incubates were analyzed using the General Linear Model of SYSTAT (Version 9, SPSS Inc., 1998, Chicago, USA) and were reported as mean values with their standard errors. The effects of the different oilseed treatments and their interaction were analyzed using the following model:

$$\begin{aligned}\text{Variable} = {} & \text{mean} + \text{Day of incubation effect} \\ & + \text{Heating temperature effect} \\ & + \text{Storage duration effect} \\ & + \text{interaction Heating temperature} \\ & \times \text{Storage duration} + \varepsilon\end{aligned}$$

with six levels for ''day of incubation'', three levels for ''Heating temperature'' (no heating, 110 and 150°C) and five levels for ''Storage duration'' (0, 1, 2, 4, or 6 months). To compare the three levels of heating, Tukey's test was used.

Correlation coefficients between oxidation compounds and percentage of disappearance of PUFA were computed. Significance was set at $p \leq 0.05$.

3 Results

The initial quantities of C18 FA in the flasks were nearly the same whatever the soybean storage duration. The cultures with heated soybeans initially had larger amounts of 18:0 and *cis*-9 18:1, and smaller amounts of *cis*-9,*cis*-12 18:2 and *cis*-9,*cis*-12,*cis*-15 18:3 than cultures with unheated soybeans (Table 1). No TAG polymer was detected in soybeans, whatever the heating treatment and the storage duration: Fig. 1 shows the chromatogram obtained with one of the treated soybeans compared to heated pure trilinolein. All other treatments resulted in similar chromatograms.

Table 1. Initial quantities of total and C18 fatty acids, and of lipid oxidation products in in vitro cultures, according to soybean treatment ($n = 5$ storage durations for each heating process; mean ± SD)

	Unheated soybeans	Heated soybeans	
		110°C	150°C
Fatty acids (mg/flask)			
Total fatty acids	202.6 ± 0.1	203.1 ± 0.1	203.1 ± 0.1
18:0	51.2 ± 0.1	52.2 ± 0.1	52.2 ± 0.0
18:1 *cis*-9	23.4 ± 0.4	26.6 ± 0.2	26.8 ± 0.1
18:2 *cis*-9,*cis*-12	59.3 ± 0.2	57.4 ± 0.1	57.2 ± 0.1
18:3 *cis*-9,*cis*-12,*cis*-15	15.1 ± 0.3	12.2 ± 0.2	12.3 ± 0.1
Lipid oxidation products (μg/flask)			
Total HOA	77.6 ± 32.4	103.8 ± 51.7	78.8 ± 33.8
Total aldehydes	4.5 ± 0.4	5.0 ± 0.2	5.9 ± 0.7

Total HOA, sum of all hydroxyacids assayed; Total aldehydes, sum of all aldehydes assayed.

The analysis of hydroperoxides only revealed HOAs (example in Fig. 2): 13HOA (84% of total HOA, on average) content was always greater than that of 9HOA (16% of total HOA, on average). The concentration of total HOA in soybeans apparently increased with storage duration, from around 0.48 μg/g of fat for no storage to an average of 1.37 μg/g of fat after six months storage. Besides, this, increase seemed to be more pronounced for 110°C heating compared to other treatments, and after six months of storage, it was 1.24 μg/g on average for non-heated and soybeans heated at 150°C compared to 1.64 μg/g for soybeans heated at 110°C (Fig. 3).

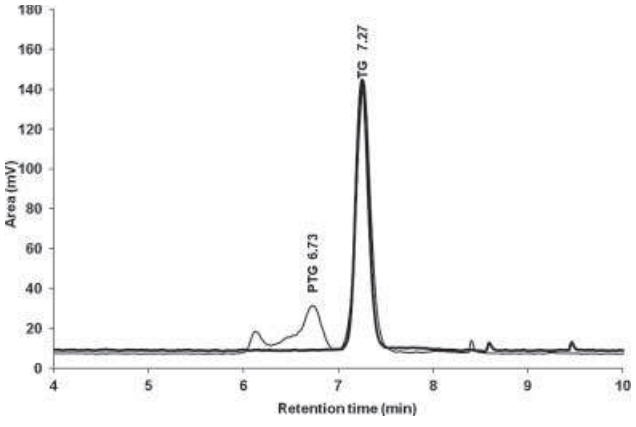

Figure 1. Chromatogram of polymers of TAG (PTG) obtained from soybeans heated at 150°C for 30 min and stored under ambient conditions for 1 month (thick line) compared to pure trilinolein heated at 150°C for 6 h (thin line).

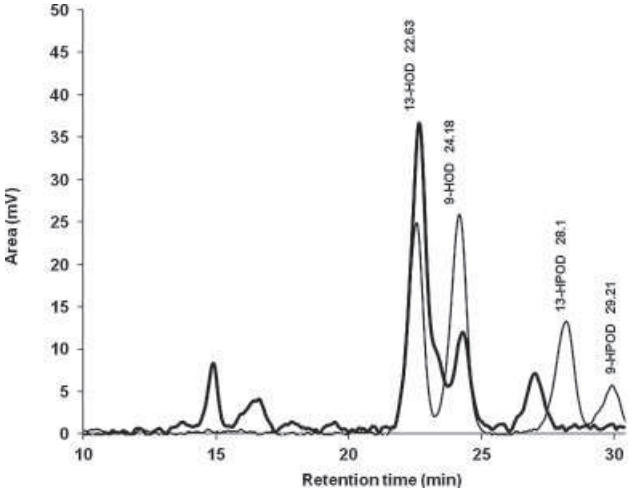

Figure 2. Chromatogram of 13(OOH) *cis*-9,*trans*-11 18:2 and 9(OOH) *trans*-10,*cis*-12 18:2 (13HPOD and 9HPOD), and their respective HOAs (13HOD and 9HOD) obtained from soybeans heated at 110°C and stored under ambient conditions for 6 months (thick line) compared to a mixture of four commercial standards (thin line).

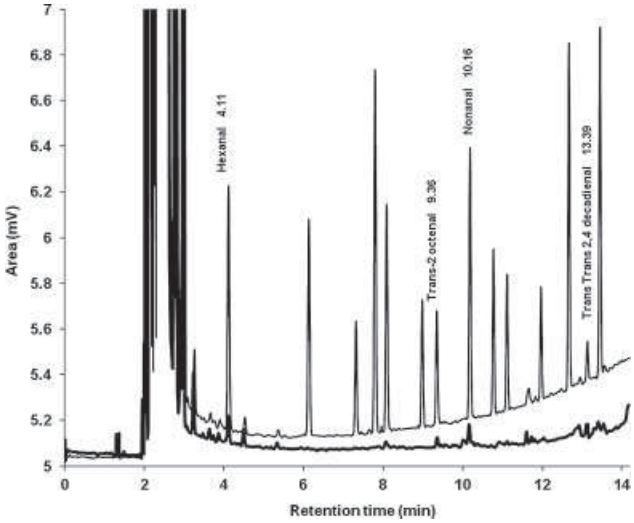

Figure 4. Chromatogram of aldehydes obtained from soybeans heated at 110°C and stored under ambient conditions for 6 months (thick line) compared to a solution of 13 commercial standards (thin line).

For aldehydes, among the 13 standards used for identification of peaks, only four were detected in all the soybeans (example in Fig. 4): hexanal (47% of total aldehydes, on average), nonanal (24%), *trans*-2 octenal (18%) and *trans*-2,*trans*-4 decadienal (12%). Total aldehyde content of soybeans heated to 150°C was greater compared with other treatments (Fig. 5).

Storage duration and its interaction with heating temperature had no effect on any parameter measured in incubated flasks, so the tables only indicate the average values over all storage durations and the significance of heating temperature. Fermentation parameters after 6 h incubation were not affected by heating (Table 2).

Almost the same values were observed for the three heating temperatures for FA profiles after 6-h incubation (Table 3), including *trans* 18:1 and CLA isomers (Table 4), except *cis*-12 18:1 and *trans*-16 18:1 which were significantly decreased with soybeans heated at 150°C.

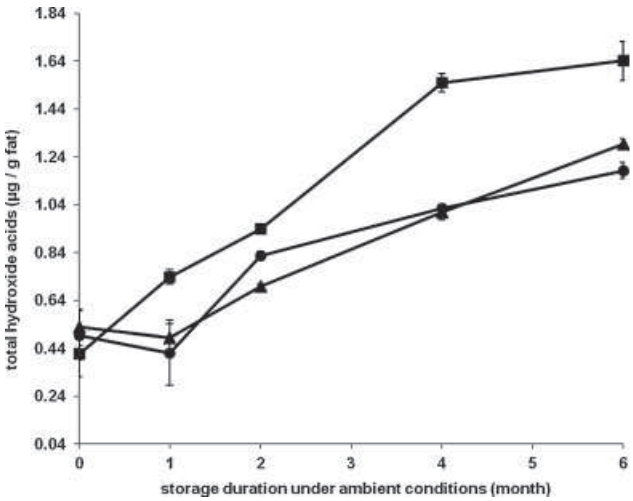

Figure 3. Effect of heating temperature and storage duration under ambient conditions on total HOA (sum of 13(OH) *cis*-9,*trans*-11 18:2 and 9(OH) *trans*-10,*cis*-12 18:2) of soybeans (●: no heating; ■: heating at 110°C; and ▲: heating at 150°C).

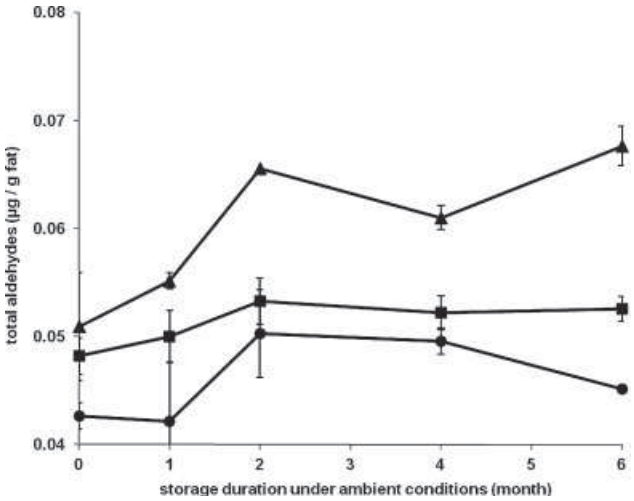

Figure 5. Effect of heating temperature and storage duration under ambient conditions on total aldehydes assayed in soybeans (●: no heating; ■: heating at 110°C; and ▲: heating at 150°C).

Table 2. Effects of temperature of heating of ground soybeans on fermentation parameters after 6-h incubation with ruminal content

		Incubated flasks				
			Heated soybeans			
	Control flasks[a)]	Unheated soybeans	110°C	150°C	SEM	*p*
pH	7.13	6.75	6.74	6.74	0.01	0.92
NH_3 (mg/L)	64.38	126.60	130.07	128.43	2.95	0.71
VFA total (mM)	39.17	92.68	93.31	92.74	1.01	0.89
Acetate (%)[b)]	70.62	67.06	67.09	67.00	0.08	0.72
Propionate (%)[b)]	13.99	16.61	16.58	16.61	0.06	0.95
Butyrate (%)[b)]	11.20	12.10	12.08	12.15	0.12	0.91
Isobutyrate (%)[b)]	1.32	1.04	1.05	1.05	0.01	0.53
Valerate (%)[b)]	0.99	1.46	1.45	1.46	0.01	0.80
Isovalerate (%)[b)]	1.88	1.74	1.75	1.74	0.02	0.82

[a)] Control flasks, non-incubated flasks, not included in the statistical analysis.
[b)] Molar percentage.

Table 3. Fatty acid profile (% of total fatty acids) after 6-h incubation in ruminal cultures with unheated or 110°C or 150°C heated soybeans

		Heated soybeans			
	Unheated Soybeans	110°C	150°C	SEM	*p*
12:0	0.17	0.17	0.17	0.00	0.79
13:0	0.06	0.06	0.06	0.00	0.84
13:0*anteiso*	0.02	0.02	0.02	0.00	0.43
13:0*iso*	0.11	0.11	0.11	0.00	0.72
14:0	1.05	1.04	1.04	0.01	0.46
14:0*iso*	0.24	0.23	0.23	0.00	0.89
15:0	0.70	0.69	0.69	0.01	0.77
15:0*anteiso*	0.85	0.82	0.84	0.02	0.50
15:0*iso*	0.59	0.58	0.58	0.01	0.72
16:0	15.65	15.62	15.57	0.08	0.78
16:1	0.06	0.06	0.06	0.00	0.80
17:0	0.41	0.41	0.41	0.00	0.73
17:0*anteiso*	0.45	0.45	0.44	0.01	0.82
17:0*iso*	0.21	0.20	0.20	0.00	0.44
18:0	39.13	38.98	38.64	0.25	0.35
18:1 *cis*-9	6.45	6.48	6.58	0.08	0.42
18:1 *cis*-11	0.36	0.37	0.34	0.01	0.30
18:1 *cis*-12	0.31[a]	0.31[ab]	0.30[b]	0.00	0.05
18:1 *cis*-15	0.05	0.05	0.04	0.00	0.60
18:1 *trans*[a)]	8.54	8.46	8.40	0.15	0.79
18:2 *cis*-9,*cis*-12	9.41	9.56	10.01	0.27	0.27
CLA[a)]	0.40	0.40	0.47	0.04	0.40
18:2 *trans*-11,*cis*-15	0.15	0.12	0.14	0.01	0.46
18:3 *cis*-9,*cis*-12,*cis*-15	2.14	2.17	2.25	0.06	0.45
CLnA	0.50	0.50	0.50	0.00	0.98

[a)] 18:1 *trans* and CLA are the sums of the respective assayed isomers.
[a,b]Means in the same row with different superscripts differ significantly ($p < 0.05$).

Nevertheless, some significant effects of heating temperature were observed on unsaturated FA disappearance: *cis*-9,*cis*-12 18:2 and *cis*-9,*cis*-12,*cis*-15 18:3 percentages of disappearance were significantly ($p < 0.05$) decreased with heating, from

Table 4. Percentages of *trans* 18:1 and CLA isomers (% of total fatty acids) after 6-h incubation in ruminal cultures with soybeans unheated or heated at 110°C or 150°C

		Heated soybeans			
	Unheated soybeans	110°C	150°C	SEM	*p*
trans 18:1 isomers					
18:1 *trans*-4	0.05	0.05	0.05	0.00	0.30
18:1 *trans*-5	0.04	0.04	0.04	0.00	0.08
18:1 *trans*-6,7,8	0.33	0.32	0.32	0.00	0.69
18:1 *trans*-9	0.21	0.21	0.21	0.00	0.75
18:1 *trans*-10	0.46	0.47	0.45	0.01	0.24
18:1 *trans*-11	5.17	5.08	5.06	0.13	0.82
18:1 *trans*-12	0.53	0.54	0.51	0.01	0.34
18:1 *trans*-13,14	0.69	0.71	0.71	0.01	0.57
18:1 *trans*-15	0.62	0.61	0.63	0.01	0.59
18:1 *trans*-16[a)]	0.43[a]	0.43[ab]	0.42[b]	0.00	0.01
CLA isomers					
CLA *trans*-10,*cis*-12	0.04	0.04	0.04	0.01	0.98
CLA *cis*-9,*cis*-11	0.03	0.04	0.04	0.00	0.25
CLA *cis*-9,*trans*-11	0.27	0.26	0.33	0.03	0.22
CLA *trans*-9,*trans*-11	0.06	0.06	0.07	0.01	0.85
trans-10[b)]	0.51	0.52	0.50	0.01	0.62
trans-11[b)]	5.50	5.40	5.45	0.15	0.90

[a)] Coeluted with *cis*-14 18:1.
[b)] *trans*-11 and *trans*-10 are the sums of the respective assayed *trans* 18:1 and CLA isomers.
[a,b]Means in the same row with different superscripts differ significantly ($p < 0.05$).

Table 5. Percentage disappearance of unsaturated fatty acids after 6-h incubation in ruminal cultures with soybeans unheated or heated at 110°C or 150°C

	Unheated soybeans	Heated soybeans			
		110°C	150°C	SEM	p
18:1 *cis*-9	47.19	50.83	50.29	1.31	0.11
18:2 *cis*-9,*cis*-12	69.71[a]	66.55[ab]	64.51[b]	1.13	0.01
18:3 *cis*-9,*cis*-12,*cis*-15	73.02[a]	64.24[b]	62.88[b]	1.14	0.00

[a,b]Means in the same row with different superscripts differ significantly ($p < 0.05$).

70 to 65% for *cis*-9,*cis*-12 18:2 and from 76 to 63% for *cis*-9,*cis*-12,*cis*-15 18:3 (Table 5).

4 Discussion

4.1 Oxidation products and C18 fatty acid proportions of soybeans as affected by heating temperature and storage duration

Soybeans were heated at 110 or 150°C or unheated and kept for 0, 1, 2, 4, or 6 months under ambient conditions in order to obtain different levels of lipid oxidation, in terms of concentration in seeds and nature of oxidation products.

No TAG polymers were found in any of the different treated soybeans. The HPLC analysis of peroxides revealed only HAO, but saponification required before analysis is known to induce a partial reduction of hydroperoxides to their respective HOA [21], so we cannot be sure that the samples did not contain any hydroperoxide. Compared to the production of HOA, the production of aldehydes was very low, probably because the primary oxidation products are relatively stable at room temperature [23]. As expected with heating, fat rich in *cis*-9,*cis*-12 18:2, the major HOA obtained were 13HOA and 9HOA resulting from 13(OOH) *cis*-9,*trans*-11 18:2 and 9(OOH) *trans*-10,*cis*-12 18:2 respectively [24], and the major aldehydes obtained were hexanal, which represented nearly half of detected components, followed by nonanal, *trans*-2 octenal, and *trans*-2,*trans*-4 decadienal, which is in agreement with the literature [25]. As expected, HOA and aldehyde contents were less abundant in unheated soybeans than in heated soybeans, suggesting an increase of oxidation products in heated soybeans that could explain a slight decrease of PUFA content compared to unheated soybeans (Table 1), as reported previously [1, 26]. Thus, HOA were most abundant in soybeans heated at 110°C (Fig. 3) as opposed to aldehydes, which were most abundant with 150°C heating (Fig. 5). Probably the reaction of lipid oxidation reached its terminal phase for 150°C heated soybeans whereas it still was in the propagation phase for 110°C heated soybeans. The increase of HOA with storage duration of ground soybeans is in agreement with the increase of peroxide values observed by Rao and Artz [27] during storage of their extruded mixture of soybean oil and corn starch.

4.2 Effect of heating and time of storage of soybeans on fermentation parameters and biohydrogenation of polyunsaturated fatty acids

Heating and storage duration of soybeans did not alter ruminal fermentation. Nevertheless, the decrease of pH and increase of NH_3 and VFA contents in incubated flasks compared to non-incubated control flasks confirmed the effectiveness of our in vitro cultures. In vitro, Vazquez-Anon and Jenkins [28] also observed no significant effect of addition of oxidized oil in in vitro ruminal cultures on ruminal pH, VFA and NH_3 content, despite a high level of oxidation (215 mEq of O_2/kg of oil). Similarly, Gonthier et al. [29] did not observe changes in fermentation parameters in cows receiving diets containing heat-treated flaxseed (micronization for 90 s at 115°C or extrusion for 43 s at 155°C) compared to raw flaxseed.

No significant difference in FA profile after incubation was observed except for *cis*-12 18:1 and *trans*-16 18:1 percentages. The same effect of heating temperature on *trans*-16 18:1 was previously observed with oil heated at 150°C [1] but the reason is not yet clear. So, contrary to some other studies with extruded oilseeds [5, 12] showing an increase of *trans*-11 isomers, or with heated oils [1] showing an increase of *trans*-10 isomers, the present study showed no effect of heating of soybeans on BH intermediates of *cis*-9,*cis*-12 18:2. Differences of results among studies could relate to the heating process, free oil or oil liberated during extrusion possibly having a different behavior than oil inside ground seeds. Consistent with this hypothesis, roasted soybeans have been shown *in sacco* to be less efficient than extruded ones to increase BH intermediates [6].

As already shown in studies with 130 to 160°C roasted oilseeds [8, 9] or with heated oil [1, 30], heating offered a partial protection of PUFA from BH in this study. With extruded oilseeds, some protection of PUFA was sometimes observed [6, 7] but not always [11, 12]. Differences between studies using extruded oilseeds could be linked to the temperature of extrusion: the studies of Troegeler-Meynadier et al. [6] and Reddy et al. [7] used high extrusion temperatures (140–150°C), compared to those of Neves et al. [11] and Doreau et al. [12] who used oilseeds extruded at 120°C. Such an effect of temperature is in agreement with the increase of PUFA protection from BH with extrusion temperature (120 to 140°C) observed in situ in dairy cows [31]. However, few or no indications about extrusion conditions were reported in any of the published studies, and some other parameters could affect ruminal BH, like preconditioning [32], percent moisture or retention time in

the extruder [2]. Nevertheless, when extrusion and roasting were compared, roasting always offered a more efficient protection against BH than extrusion even with high extrusion temperature [6, 7], as demonstrated by a higher decrease of *cis*-9,*cis*-12 18:2 isomerization efficiency with roasted than with extruded oilseeds [6]. Such differences in PUFA protection from BH between extrusion and roasting could be linked to differences in terms of quantity or nature of oxidation products generated during these two kinds of processes. Privé et al. [1] showed that isomerization efficiency of *cis*-9,*cis*-12 18:2 was partly linked to peroxide values. However, as far as we are aware, no data have been published comparing oxidation products from roasting and extrusion.

4.3 Relationship between nature of lipid oxidation products and protection from biohydrogenation

The decrease of percentages of disappearance of PUFA could not be linked to the difference of amounts of incubated PUFA since these differences were very small (Table 1). Because our heating conditions did not result in TAG polymer production, our experiment could not indicate any relationship between these compounds and protection of PUFA against ruminal BH. As HOA values did not differ between soybeans heated at 150°C and unheated, in contrast to a significant protection of PUFA from BH in those heated at 150°C, HOA would not be major oxidation products affecting the disappearance of PUFA. The oxidation products most probably responsible for the observed effect on PUFA disappearance would be aldehydes. The percentages of disappearance of *cis*-9,*cis*-12 18:2 and of *cis*-9,*cis*-12,*cis*-15 18:3 after incubation were negatively correlated with the initial quantity of aldehydes in the cultures ($r = -0.76$, $p = 0.001$ and $r = -0.71$, $p = 0.003$, respectively). An effect of aldehydes on BH was previously reported by Lee et al. [14], who observed some effects on BH intermediates production, in particular an increase of *trans* 18:1 with *trans*-2 decenal, but showed no effect of aldehydes on PUFA disappearance, except *trans*-2 decenal which increased PUFA disappearance. These authors suggested an antimicrobial action of *trans*-2 decenal as reported by Kubo et al. [15], and aldehyde action increasing with chain length and number of double bonds. A general antimicrobial action was not supported by the results of the present study because fermentation parameters did not depend on oilseed treatment. Nevertheless, specific bacteria could be affected, consistent with a previous study reporting a modification of bacterial ruminal community after addition of heated oil [1]. Moreover, in our study PUFA disappearance was mainly linked to the added quantity of hexanal ($r = -0.74$, $p = 0.002$ and $r = -0.70$, $p = 0.004$ for *cis*-9,*cis*-12 18:2 and of *cis*-9,*cis*-12,*cis*-15 18:3 percentages of disappearance, respectively) and to a lesser extent to the quantity of nonanal ($r = -0.62$, $p = 0.013$ and $r = -0.52$, $p = 0.047$ for *cis*-9,*cis*-12 18:2 and of *cis*-9,*cis*-12,*cis*-15 18:3, respectively). Another possible action of aldehydes would be their capacity to interact with the amine group of proteins during the Maillard reaction [33], which could lead to a diminution of protein degradability of soybeans, and therefore to a slower release of fat. In the present study, no effect on NH_3 production was observed, suggesting no effect on protein degradation. Similarly, Vazquez-Anon and Jenkins [28] did not observe a decrease in NH_3 content in their ruminal cultures with oxidized fat compared with non-oxidized fat, in spite of a decrease of nitrogen digestibility. Besides lipid oxidation products, some soybean secondary metabolites could have played a role in PUFA disappearance, because some of them can affect BH [34] and because their activity can be heat-labile [35]. Soybeans contain several secondary metabolites [35], of which saponins and condensed tannins are known to decrease BH [34, 36], but in soybeans these are heat-stable [35]. Moreover our cultures initially contained 2.50 mg of saponins and 0.23 mg of condensed tannins per 0.16 L flask (assuming a concentration of 500 mg of saponins and 45 mg of condensed tannins per 100 g of soybeans, [35]), i.e., 15.63 and 1.44 mg/L of rumen, respectively. This corresponds to 1.56 g of saponins and 0.14 mg of condensed tannins for a cow, which is far below the 6 g of saponins and the 105 g of condensed tannins per day and per cow tested by Benchaar and Chouinard [36], who observed low or no effect at these doses, and concluded that these products would be unlikely to alter ruminal BH. Moreover, as far as we are aware, the possible effects on BH of other soybean secondary metabolites, including protease inhibitors, have not been studied. As a consequence, an interference of the effects of heating on BH due to secondary metabolites seems very improbable in our experiment.

5 Conclusions

Increasing heating temperature of ground soybeans resulted in a partial protection of PUFA from BH, without modifying ruminal fermentation. This effect would be at least partly due to the generation of aldehydes during heating. Further studies will be necessary to ascertain this effect and propose a mechanism of action.

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