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Effects of heating process of soybeans on ruminal production of conjugated linoleic acids and *trans*-octadecenoic acids *in situ*.

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ABSTRACT

The effects of two thermal treatments of soybeans, i.e. roasting (150°C dry heat) and extrusion (140-150°C), on conjugated linoleic acids (CLA) and *trans*-octadecenoic acids (*trans*-C18:1) productions obtained throughout ruminal C18:2 biohydrogenation in cows were examined. Nylon bags containing raw, roasted or extruded soybeans were incubated in the rumen of dry fistulated cows, during 2, 4, 8, 16 or 24 hours. After incubation of 2-4 h, significantly greater amounts of linoleic acid (C18:2) remained in bags containing extruded and roasted soybeans than in those with raw soybeans, reflecting a lower biohydrogenation of C18:2 in both case. Furthermore, significant and marked accumulations of CLA and *trans*-C18:1 at a lesser extend were noticed in bags containing extruded soybeans compared to those with raw or roasted soybeans. By calculations of the efficiencies of the three reactions, an inhibition of the C18:2 isomerisation was evidenced with extruded and roasted soybeans, as well as an inhibition of the two reduction steps in presence of extruded soybeans. Consequently, the thermal treatment and the nature of heating process of fat are efficient ways to modulate the CLA and *trans*-C18:1 ruminal productions.

Keywords : conjugated linoleic acids, *trans*-octadecenoic acids, soybeans, heating process, biohydrogenation.

RÉSUMÉ :

Effets du traitement thermique des graines de soja sur la production ruminale d'acides linoléiques conjugués et d'acides *trans*-octadécénoïques *in situ*.

Cette étude se propose d'examiner les effets de deux types de traitement thermique des graines de soja (chauffage à sec à 150 °C et extrusion à 140-150 °C) sur la production ruminale d'acides linoléiques conjugués (CLA) et d'acides *trans*-octadécénoïques (*trans*-C18:1) issus de la biohydrogénation de l'acide linoléique (C18:2) chez la vache. Des sachets de nylon contenant des graines de soja crues, chauffées ou extrudées ont été mis à incuber dans le rumen de vaches taries fistulées, pendant 2, 4, 8, 16 ou 24 heures. Après 2 à 4 h d'incubation, des teneurs significativement plus élevées d'acide linoléique ont été retrouvées dans les sachets contenant les graines extrudées ou chauffées que dans ceux contenant les graines crues, ce qui traduit une moindre biohydrogénation avec les graines traitées. De plus, la production des intermédiaires (CLA et *trans*-C18:1 à un moindre degré) à partir des graines extrudées a été significativement accrue par rapport à celle obtenue avec des graines chauffées ou crues. En calculant les efficacités respectives des 3 réactions, on a pu constater une inhibition de l'isomérisation de C18:2 lors de l'utilisation de graines chauffées ou extrudées, ainsi qu'une inhibition des 2 étapes de réduction en présence des graines extrudées. Le traitement thermique et le type de procédé de chauffage des matières grasses sont donc des facteurs efficaces de modulation des productions ruminales de CLA et de *trans*-C18:1.

Mots-clés : acides linoléiques conjugués, acides *trans*-octadécénoïques, graines de soja, traitement thermique, biohydrogénation.

Introduction

Conjugated linoleic acids (CLA) are isomers of linoleic acid (C18:2). Among them, *cis*Δ9*trans*Δ11-CLA and *trans*Δ10*cis*Δ12-CLA could have beneficial dietetic properties for human, mainly against cancer [12], which are still controversed [15]. They can be found in many human foodstuffs but dairy products present the highest concentrations. In dairy cow, the CLA are synthesised in the rumen during biohydrogenation (BH) by isomerisation of C18:2, and in the mammary gland by desaturation of vaccenic acid (*trans*Δ11-C18:1), also formed during C18:2 BH (figure 1) [7].

The CLA amount could be enhanced in milk by different ways. A previous study [14] investigated the effects of pH, and the C18:2 and linolenic acid (C18:3) concentrations on the production of CLA and vaccenic acid in the rumen: great quantities of C18:2 and a pH near neutrality enhance CLA and *trans*Δ11-C18:1 productions in rumen. In cow diets, a usual source of C18:2 is soybean, which can be distributed in a raw form or in a processed form (roasted, extruded...). Soybeans extrusion [3, 4, 13] and roasting [3, 5], have both been shown to enhance CLA in milk, compared with raw soybeans. But as these studies did not investigate ruminal BH reactions, the mechanisms of action of roasting and extrusion on CLA production are not fully understood.

As the *in situ* method has been validated for studying the impact of physical treatment of fat sources [6], different forms of soybeans (raw, roasted and extruded) were incubated in nylon bags in the rumen of cows receiving a forage enriched ration, in order to examine the effect of soybean processing on the reactions of ruminal C18:2 BH and its consequences on the ruminal production of CLA and *trans*-octadecenoic acids (*trans*-C18:1).

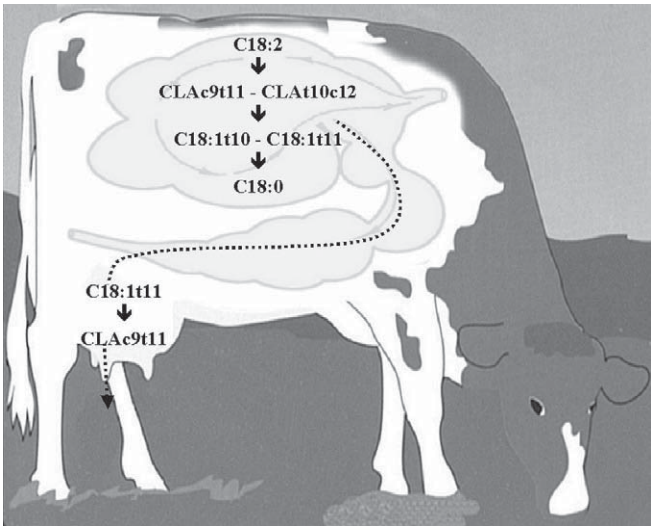

FIGURE 1: Ruminal biohydrogenation of linoleic acid and mammary desaturation of vaccenic acid.

Materials and methods

ANIMALS AND DIETS

Four fistulated dry Holstein cows were fed with a ration composed of 84.2% of orchard grass hay, 14.0% of soybeans extruded at 140-150˚C, and 1.8% of a mineral complement (Dry matter basis). They were adapted for three weeks [1] to this diet before beginning incubations in order to have competent bacteria for C18:2 BH.

PREPARATION OF BAGS

Heat-sealed nylon bags (11×6 cm; mean pore size 45 µm; Blutex, Tissage Tissus Techniques, Combles, France) were filled with 3 g of soybeans and 1 g of orchard grass hay. The nutrient composition of each bag is shown in Table I. Hay was added in bags in order to facilitate the dispersion of fat for avoiding the formation of fat agglomerates, to limit the diffusion of fatty acids out of the bag and to facilitate the settling of cellulolytic bacteria, since fatty acids adsorb onto feed particles. Hay, raw and extruded (140-150˚C) commercial soybeans were ground with a mill (0.5 mm diameter screen). Roasted soybeans were obtained by exposing ground raw soybeans at 150˚C dry heat for 10 min. Then hay and ground soybeans (raw, roasted and extruded) were strained through a metal sieve (0.063 mm mesh), in order to eliminate particles with a size under the pore size of nylon bags.

IN SITU EXPERIMENT

During five consecutive days, two nylon bags with raw soybeans, two with roasted soybeans and two with extruded soybeans were placed in the rumen of the fistulated cows after the morning feeding, and were incubated for 2, 4, 8, 16 or 24 hours in independent bags. After removal from the rumen, bags were washed, frozen at -18˚C during 24 h, and machine-washed (2×5 min) in cold water. Then they were freeze-dried (Virtis Freezemobile 25; Virtis, Gardiner, NY), weighed, ground, homogenized in a ball mill (Dangoumau, distributed by Prolabo, Nogent-sur-Marne, France) and kept at -18˚C until analysis.

The contents of the two bags with the same form of soybeans and incubated for the same period in the same cow were mixed, so that there were four replicates by form of soybeans and each period of incubation. For each form of soybeans, a zero-hour bag without incubation was treated with a similar procedure than bags removed from the rumen, and used for analysis of the initial profile of fatty acids.

ANALYSIS OF FATTY ACIDS

The fatty acids of bag contents were extracted and methylated *in situ* with the procedure of PARK and GOINS [11], except that the solution of 14 % of borontrifluoride in methanol was replaced by a solution of methanol-acetylchloride (10:1). The nonadecanoic acid (C19:0) was used as an internal standard. The fatty acid methyl esters were then quantified by gas chromatography (Agilent 6890N, equipped with a model 7683 auto injector, Network GC System, Palo Alto, California, USA), using the method described by ENJALBERT *et al.* [6]. The column was a fused silica capil-

	Treatment of soybeans		
	Raw	**Roasted**	**Extruded**
Crude Protein	340.1	343.4	338.0
Crude Fat	165.1	171.5	171.3
NDF*	323.1	336.0	278.0
ADF*	165.9	176.3	148.5
C16:0	14.8	15.1	16.8
C18:0	5.6	5.8	6.7
cis9-C18:1	24.2	23.3	20.1
C18:2	41.9	39.7	36.4
C18:3	5.9	6.3	6.8

* NDF: Neutral Detergent Fibre; ADF: Acid Detergent Fibre.

TABLE I. – Nutrient composition (g / kg of Dry matter) and fatty acids profile (% / total fatty acids) of bags contents according to the physical treatment of soybeans.

lary (CPSil88, 100 m x .25 mm ID, 0.20 µm film thickness; Chrompack-Varian, Middleburg, Netherlands). Peaks were identified and quantified by comparison with commercial standards (Sigma, St. Louis, MO).

The fatty acid contents were expressed as percentages of fatty acids with eighteen carbons (C18). The assayed CLA isomers were the *cis*Δ9*trans*Δ11-CLA and the *trans*Δ10*cis*Δ12-CLA and were regrouped under the term of CLA. The assayed *trans*-C18:1 isomers were the *trans*Δ10-C18:1 + *trans*Δ11-C18:1, the major *trans*-C18:1 acids produced during C18:2 BH. They were regrouped under the term of *trans*-C18:1.

CALCULATIONS AND STATISTICAL ANALYSIS

The efficiency of a reaction (E) was estimated by the amount of substrate disappeared during the period of incubation (t) divided by the total amount of substrate available for the reaction considered. Biohydrogenation of C18:2 is divided in three reactions [8]: a first reaction is the isomerisation of C18:2 into CLA, a second reaction reduces CLA to *trans*-C18:1, mainly *trans*Δ11-C18:1 under neutral pH conditions [7], and a third reaction reduces *trans*-C18:1 to stearic acid (C18:0) (figure 1).

The efficiency of the first reaction, called reaction 1, was calculated as following:

$$E1 = ([C18:2]i - [C18:2]t) / [C18:2]i$$

where [C18:2]i and [C18:2]t represented the C18:2 concentrations at the beginning and at the end of the considered incubation period (t), respectively.

For the second reaction, called reaction 2, the efficiency was calculated as following:

$$E2 = ([C18:2]i - [C18:2]t - [CLA]t) / ([C18:2]i - [C18:2]t)$$

where [CLA]t represented the CLA concentration of at the end of the considered incubation period (t), and [C18:2]i - [C18:2]t the CLA produced by the first reaction during this period.

The efficiency of the third reaction, called reaction 3, was calculated as following:

$$E3 = ([C18:2]i - [C18:2]t - [CLA]t - [trans\text{-}C18:1]t) / ([C18:2]i - [C18:2]t - [CLA]t)$$

where [*trans*-C18:1]t represented the *trans*-C18:1 concentration at the end of the considered incubation period (t) and [C18:2]i - [C18:2]t - [CLA]t the *trans*-C18:1 produced by the second reaction during this period. We assumed that *trans*Δ10-C18:1 and *trans*Δ11-C18:1 were only formed throughout the C18:2 BH, because the initial amount of C18:3 was low compared to that of C18:2, and because C18:3 produces many other *trans*-C18:1during its BH, like *trans*Δ15-C18:1 [9], *trans*Δ13-C18:1 and *trans*Δ14-C18:1[10].

The percentages of each fatty acid and the efficiency of each reaction of C18:2 BH, were compared at each period of incubation between physical treatments of soybeans using the general linear model of SYSTAT, followed by the Tukey's pair wise comparison test. Differences were considered significant at $P < 0.05$.

Results

Figures 2, 3, 4 and 5 present the evolution of each intermediate of C18:2 BH according to the physical form of soybeans and the duration of incubation. The C18:2 percentages (figure 2) gradually decreased according to the incubation time whatever the physical treatment of soybeans. However, the percentage of C18:2 was significantly higher with the roasted and extruded soybeans than with the raw soybeans, for 2 and 4 h of incubation ($P < 0.05$). Roasted and extruded soybeans resulted in similar C18:2 percentages. For the other durations of incubation, differences were not significant.

Except for 2 h of incubation, the percentage of CLA (figure 3) was about 2 at 6 times higher with extruded soybeans than with the two other forms ($P < 0.05$), which gave similar results. Moreover, the CLA production with extruded soybeans reached a threshold for 16 and 24 h of incubation, whereas the lower CLA production with raw and roasted soybeans remained nearly constant.

In all cases (figure 4), the *trans*-C18:1 production markedly increased with the incubation duration. Physical treatment of soybeans did not influence the *trans*-C18:1 production for a short incubation period (2 h), but the percentage of *trans*-C18:1tended ($P = 0.056$) to be higher with extruded soybeans than with roasted and raw soybeans for an incubation of 4 h. This difference became significant ($P < 0.05$) for 8 h of incubation, but for more longer incubations (16 and 24 h) no significant difference could be obtained.

The C18:0 formation (figure 5) was low during the 2 h of incubation, and thereafter gradually increased with the duration of incubation, particularly when soybeans were raw. Indeed the percentage of C18:0 tended to be higher in the bags with raw soybeans than with the other two forms, for 2 ($P = 0.107$) and 4 ($P = 0.120$) h of incubation. This difference became significant ($P < 0.05$) for 16 h of incubation. For 24 h of incubation, the percentage of C18:0 was significantly ($P < 0.05$) lower with the extruded soybeans than with the roasted and raw soybeans, which did not differ any more.

Figures 6, 7 and 8 present the evolution of the efficiencies of the three reactions of C18:2 BH, according to the physical form of soybeans and the period of incubation.

The efficiency of the reaction 1 (figure 6) increased according to the incubation duration and its evolution was quite linear when roasted soybeans were used. By contrast, this parameter was rapidly enhanced between 2 and 8 h of incubation with raw and extruded soybeans. It was significantly greater in bags containing raw soybeans than in bags containing extruded or roasted soybeans, for 2 and 4 h of incubation ($P < 0.05$). For 8 h of incubation, this difference was only a tendency ($P = 0.158$). For 16 and 24 h of incubation, efficiencies did not significantly differ.

The CLA reduction efficiency (figure 7) remained nearly constant according to the incubation duration for the roasted and the raw soybeans, and did not significantly differ between these two forms. In the case of extruded soybeans, the reaction 2 efficiency markedly decreased between 2 and 8 h of incubation, slowly increased for 16 h of incubation and thereafter was constant. It was globally lower than reaction 2

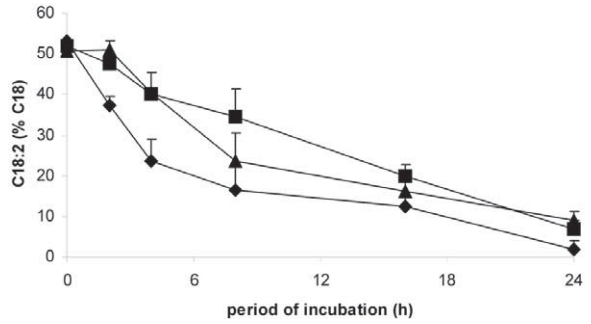

FIGURE 2: Evolution of linoleic acid (C18:2) percentages reported to the C18 quantities according to the duration of incubation and the physical treatment of soybeans: raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

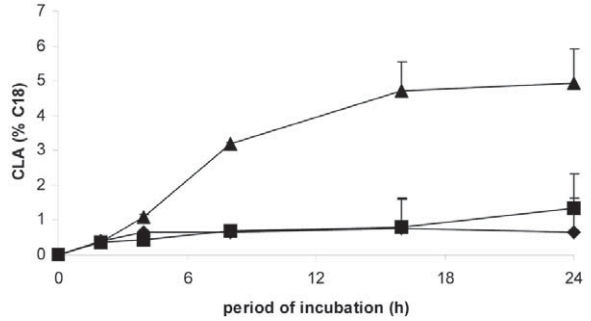

FIGURE 3: Evolution of conjugated linoleic acids (CLA) percentages reported to the C18 quantities according to the duration of incubation and the physical treatment of soybeans: raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

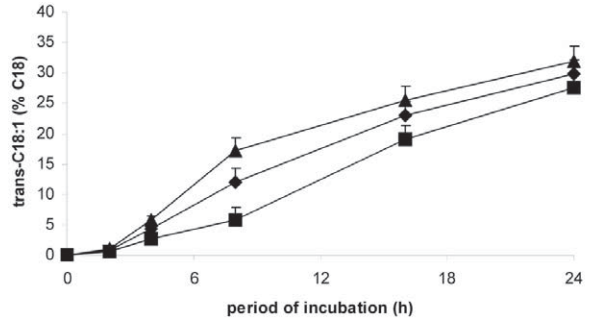

FIGURE 4: Evolution of *trans*-octadecenoic acid (*trans*-C18:1) percentages reported to the C18 quantities according to the duration of incubation and the physical treatment of soybeans: raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

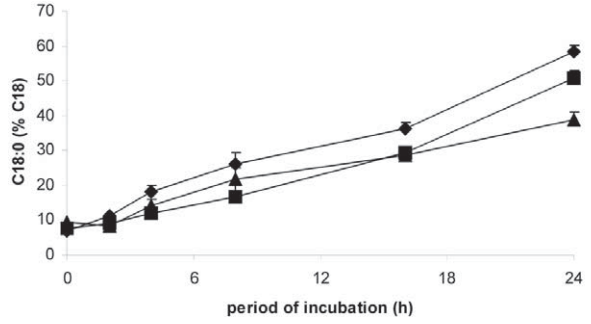

FIGURE 5: Evolution of stearic acid (C18:0) percentages reported to the C18 quantities according to the duration of incubation and the physical treatment of soybeans: raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

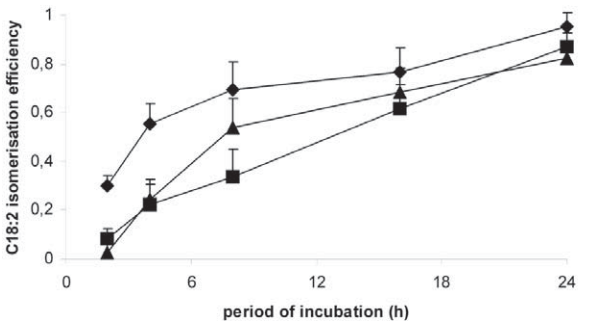

FIGURE 6: .Evolution of the efficiency of the reaction 1 (isomerisation of linoleic acid into conjugated lonoleic acids) according to the duration of incubation and the physical treatment of soybeans : raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

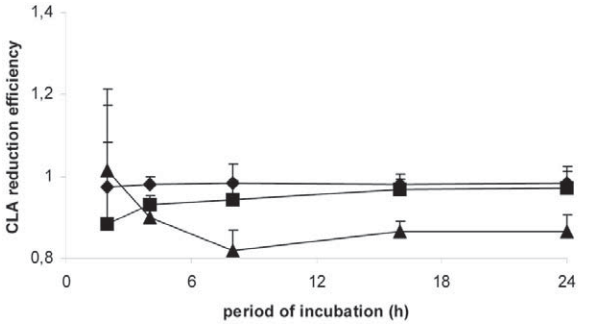

FIGURE 7: Evolution of the efficiency of the reaction 2 (reduction of conjugated linoleic acids to *trans*-octadecenoic acids) according to the duration of incubation and the physical treatment of soybeans: raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

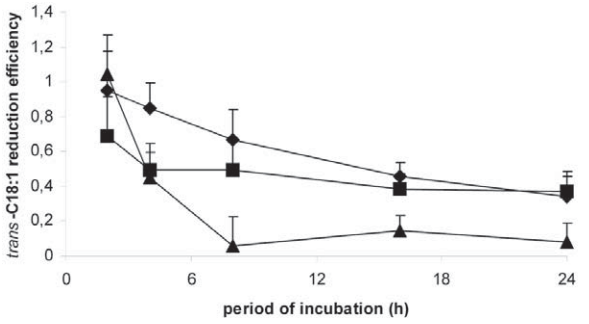

FIGURE 8: Evolution of the efficiency of the reaction 3 (reduction of *trans*-octadecenoic acids to stearic acid) according to the duration of incubation and the physical treatment of soybeans: raw soybeans (◆), roasted soybeans (■) and extruded soybeans (▲).

efficiencies observed with roasted and raw soybeans : except for 2 h of incubation, the efficiency of the reaction 2 was lower for the extruded form than for the raw form (4 h, P = 0.051; 8 h, P = 0.094; 16 h, P = 0.022; 24 h, P = 0.151), and except for 2 and 4 h of incubation, it was lower for the extruded form than for the roasted form (8 h, P = 0.219; 16 h, P = 0.039; 24 h, P = 0.167).

As shown in the figure 8, the efficiency of the reaction 3 (*trans*-C18:1 reduction) progressively declined according to the incubation duration. For roasted soybeans, a slight decrease of this parameter was observed between 2 and 4 h of incubation and thereafter the reaction efficiency was nearly constant. The efficiency obtained with raw soybeans declined

more progressively with the incubation duration. By contrast, when extruded soybeans were used, the efficiency of the third reaction markedly declined between 2 and 8 h of incubation, and then became nearly stable. Except for 2 and 4 h of incubation, for which there was no difference between treatment, the efficiency of the reaction 3 (Figure 7) tended to be lower for the extruded form than for the raw (8 h, $P = 0.074$; 16 h, $P = 0.064$; 24 h, $P = 0.231$) or the roasted (8 h, $P = 0.222$; 16 h, $P = 0.157$; 24 h, $P = 0.140$) form, both presenting no difference.

Discussion

The disappearance of C18:2 was lowered by roasting or extrusion. REDDY *et al.* [13] obtained similar results in their *in vitro* ruminal incubations: BH of C18:2 was higher for raw soybeans than for extruded and roasted soybeans. *In vivo*, DHIMAN *et al.* [5] and CHOUINARD *et al.* [3] also noticed that cows receiving roasted soybeans exported more C18:2 in milk than cows receiving raw soybeans. But *in vivo* literature data about extruded soybeans are conflicting: CHOUINARD *et al.* [3] obtained less C18:2 in the milk of cows receiving extruded soybeans than in the milk of cows receiving raw soybeans, whereas opposite results would be expected from the present study. This difference between experiments could be due to the extrusion temperature. Indeed, CHOUINARD *et al.* [3] observed a small increase of the proportion of C18:2 in milk fat when the extrusion temperature rose from 120 to 140˚C. Our commercial soybeans were extruded between 140 and 150˚C, which could explain the high C18:2 percentage in the incubated bags with extruded soybeans, similar to that obtained with the 150˚C roasted soybeans.

As the ruminal isomerisation of C18:2 needs free fatty acids, soybean fat in triglyceride form has to be firstly hydrolyzed. The delayed utilization of C18:2 obtained with extruded and roasted soybeans could result from a lack of lipolysis compared to raw soybeans. But REDDY *et al.* [13] showed that the fatty acids are quicker released from extruded soybeans (about 6 h for a total lipolysis) than from raw and roasted soybeans (about 12 h for a total lipolysis).

A second hypothesis of the mode of action of heat processing could be a direct action on isomerisation. The inhibition of isomerisation, shown by its lower efficiency with of extruded and roasted soybeans (Figure 5), could be due to substances generated by heating, and mainly at high temperatures. REDDY *et al.* [13] hypothesized that the "protection" of PUFA against BH in roasted soybeans was proportional to the temperature and could be due to the formation of peroxides during heating. Extrusion is a cooking process too, and can also produce peroxides [2]. CHOUINARD *et al.* [3] used 120˚C extruded soybeans in their comparative study with raw soybeans. Possibly, an extrusion at 120˚C did not produce enough peroxides to markedly slow down the BH of C18:2. On the contrary, a higher extrusion temperature, like in our study, could generate more peroxides which could affect C18:2 BH. REDDY *et al.* [13] suggested that the lower disappearance of C18:2 with heated than raw soybeans could be due to the possible peroxide binding to amino groups of proteins, especially of enzymes. Consequently, the peroxided fatty acids would not be isomerised, because rumen bacteria isomerase requires a free amino group to act. However, as fatty acid peroxides did not co-elute with the corresponding fatty acid on a GC assay, they were not included in the quantifications and calculations of the present study, and they did not interfere with the real quantity of C18:2 isomerised into CLA. We can only conclude that a 140-150 ˚C heating of seeds inhibited the isomerisation of C18:2, but more investigations will be necessary to identify the inhibition factor.

The second remarkable phenomenon was the higher percentage of CLA, and of *trans*-C18:1 at a lesser extend, in bags with extruded soybeans compared with bags containing the roasted and raw soybeans. CHOUINARD *et al.* [3] noticed the same evolution in milk. The increased CLA proportion in the milk from cows consuming extruded soybeans compared with cows receiving raw or roasted soybeans was attributed by CHOUINARD *et al.* [3] to a better rate of lipolysis of extruded soybeans, resulting in a greater extent of C18:2 BH and in reduction of the milk C18:2 exportation. They also observed that the proportion of CLA increased with the extrusion temperature. In the present study, with extruded soybeans, the C18:2 disappearance is delayed, leading to a continuous and gradual C18:2 release in rumen instead of a massive delivery of this substrate obtained with raw soybeans. The consequent CLA formation would be favored when the incubation duration was increased. On the other hand, the calculations of the reaction 2 and 3 efficiencies suggested an inhibition of the two reduction reactions with extruded soybeans, due to a substance only contained in extruded soybeans. Consequently, the C18:0 formation was reduced and the CLA and *trans*-C18:1 were accumulated in the rumen. Differences in C18:2 BH between extrusion and roasting could be due to the different heating process. Indeed, the process of extrusion can generate high temperatures, but it also affect the structure of beans by an efficient mechanic treatment, resulting in the rupture of cell membranes. This alteration of cell structure led to a release of cell content which was more susceptible to external factors, possibly oxygen, during heating and storage. During this process, a substance could be synthesised and then could inhibit the C18:2 BH reductases. More investigations are necessary to identify this compound and to understand how it acts on these enzymes.

Conclusion

A high temperature of heating of soybeans led to an inhibition of the isomerisation of C18:2. Extrusion of soybeans led to a higher production of CLA and *trans*-C18:1 during ruminal C18:2 BH than raw or roasted soybeans, because of the inhibition of the sequential reductions of CLA and *trans*-C18:1. As a consequence, thermal treatments of fat are efficient modulators of C18:2 BH, and the nature of heating process are important to consider in order to increase the ruminal production of CLA and *trans*-C18:1.

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