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Original article

Effects of preconditioning and extrusion of linseed on the ruminal biohydrogenation of fatty acids. 2. In vitro and in situ studies

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Abstract – The extent and/or intermediates of ruminal biohydrogenation (BH) of fatty acids (FA) were investigated in vitro and in situ using a raw, pre-conditioned or extruded blend of linseed and wheat bran (70:30). The duration of in vitro incubations were 2, 4, 8, 16 and 24 h, with 5 replicates. In situ studies used 3 dry ruminally fistulated Holstein cows in a 3 × 3 Latin square design, with 3 weeks adaptation to the linseed form. The diet contained 20% (DM basis) of the linseed based blend. The duration of in situ incubations were 2, 4, 8, 16, 24 and 48 h. BH was much slower in situ than in vitro, resulting in a much lower effective disappearance of C18:2 and C18:3. Moreover, the in situ technique suggested that the technological pre-treatment of linseed did not affect C18:2 and C18:3 rate of BH, whereas reduced rates were observed in vitro. After 8 h of in vitro incubation and onwards, proportions of *cis*-9,*trans*-11C18:2 were the highest with extruded linseed. The BH of FA from linseed resulted in the appearance of great proportions of *trans*-10+11 to *trans*-16C18:1 intermediates. Extrusion increased the proportions of *trans*-10+11C18:1 both in vitro and in situ and proportions or *trans*-C18:1 were higher in situ than in vitro. Compared to previous in vivo results with the same material, the in situ method provided poor estimates of BH rates and intermediates.

biohydrogenation / linseed / preconditioning / extrusion / in vitro / in situ

Résumé – Effets du préconditionnement et de l'extrusion de la graine de lin sur la biohydrogénation ruminale des acides gras. 2. Études in vitro et in situ. L'importance et/ou les intermédiaires de la biohydrogénation ruminale (BH) des acides gras ont été étudiés in vitro et in situ sur un mélange lin/son (70:30) cru, préconditionné ou extrudé. Les incubations in vitro duraient 2, 4, 8, 16 et 24 heures, avec 5 répétitions. Les études in situ ont été réalisées sur 3 vaches Holstein taries équipées d'une canule ruminale, avec un protocole en carré latin 3 × 3, chaque période comprenant 3 semaines d'adaptation à la forme de lin testée. La ration contenait 20 % (par rapport à la matière sèche) de mélange à base de lin. Les incubations in situ duraient 2, 4, 8, 16, 24 et 48 heures. La BH

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a été beaucoup plus lente in situ qu'in vitro, si bien que la disparition effective de C18:2 et C18:3 y était beaucoup plus faible. De plus, la technique in situ a suggéré que le pré-traitement technologique n'affecte pas la vitesse de BH de C18:2 et C18:3, alors qu'une vitesse réduite a été montrée in vitro. Après 8 heures d'incubation, les proportions les plus élevées de C18:2*cis*-9,*trans*-11 étaient observées avec le lin extrudé. La BH des AG du lin a conduit à l'apparition de proportions importantes de C18:1*trans*-10+11 à *trans*-16. L'extrusion a accru les proportions de C18:1*trans*10+11 in vitro et in situ, et les proportions de C18:1*trans* étaient plus élevées in situ qu'in vitro. Par comparaison à des résultats obtenus in vivo avec les mêmes formes de lin, la méthode in situ n'a pas permis d'obtenir de bonnes estimations de la vitesse et des intermédiaires de biohydrogenation.

biohydrogénation / lin / préconditionnement / extrusion / in vitro / in situ

1. INTRODUCTION

Adding polyunsaturated fatty acids (PUFA) in the diet of ruminants can lead to increased contents of PUFA and biohydrogenation (BH) intermediates in tissues [29] or in milk fat [6]. Among PUFA, omega-3 fatty acids (FA) are in insufficient amounts in most human diets [28] and addition of linseed fat, rich in C18:3, to the diet of ruminants can be a way to improve the omega-3 content of human food. Moreover, through its high C18:2 and C18:3 content, linseed also increases the concentration in ruminant products of *cis*-9, *trans*-11C18:2, which has been shown to have interesting dietetic properties in humans [23]. *Cis*-9,*trans*-11C18:2 is an intermediate of C18:2 BH, whereas both C18:2 and C18:3 BH produce *trans*-11C18:1 [14], which can be desaturated to *cis*-9, *trans*-11C18:2 in human [1] and ruminant tissues.

Extrusion, often preceded by preconditioning for adjustment of moisture content [5], is a common treatment of oilseeds and the effect of this technological treatment on the ruminal metabolism of dietary fat or tissue or milk FA profile have been studied, but conflicting results have been reported. Chouinard et al. [8] and Clinquart et al. [9] reported any lack of effect on milk or tissue PUFA, although Enjalbert et al. [11] reported a faster BH in vitro and in situ whereas Reddy et al. [25] observed a reduced in vitro BH of extruded oil seeds.

The extent and intermediates of BH can be studied in vivo, using ruminal [18] or duodenal [30] contents, or in vitro and in situ via the measurement of disappearance of unsaturated FA [2, 11, 13], but the results can strongly depend on experimental conditions. The in situ method has not been extensively used for the measurement of ruminal lipid metabolism [2, 8, 11, 24], but BH is known to be slower in situ than in vitro [11]. In situ BH can be hastened by adaptation of donor cows to dietary lipids and mixing the fat source with a fibre source [2]. In one of our former studies, the in situ and in vitro evaluation of BH resulted in the same hierarchy when comparing raw and extruded oil seeds [11].

The objective of the work was to compare raw, pre-conditioned (35 °C) and extruded (50 °C pre-conditioned and 120 °C during extrusion) linseed, ground prior to in vitro and in situ incubation, on the kinetics and extent of BH and on the proportions of *cis*-9, *trans*-11C18:2 and *trans*-C18:1 isomers.

2. MATERIALS AND METHODS

2.1. Treatments of linseed

The investigated linseed was a commercial blend of 70% linseed and 30% wheat bran and linseed will designate this blend. Three forms of linseed were compared: raw linseed crushed through a 3 mm screen (RL), linseed crushed through a 6 mm

Table I. Chemical composition of substrates.

Ingredients	RL[1]	CL[1]	EL[1]	Peas
DM (%)	92.3	89.3	93.5	87.1
	(% of DM)			
CP	20.8	21.7	21.0	24.0
NDF	30.8	33.1	32.5	12.8
ADF	14.5	13.6	13.2	6.4
Total C18[2]	25.1	24.3	25.9	1.4
	(% of total C18)			
C18:0	3.5	3.3	3.5	4.3
C18:1	18.0	16.6	8.1	31.9
C18:2	19.1	19.8	19.2	54.5
C18:3	58.1	59.1	57.9	8.8

[1] RL: raw linseed; CL: pre-conditioned linseed; EL: extruded linseed.
[2] Fatty acids with 18 carbon atoms.

screen and pre-conditioned at 35 °C (CL), linseed crushed through a 3 mm screen, pre-conditioned at 50 °C and extruded at 120 °C (EL). The chemical composition and fatty acid profile of linseed are presented in Table I.

2.2. In vitro experiment

Linseed was crushed through a 0.5 mm screen and 400 mg of substrate (1 of the 3 linseed forms) in addition to 1.2 g of ground peas (Tab. I), 60 mL of rumen fluid and 60 mL of buffer solution were added to each Erlenmeyer flask. Furthermore, 2.5 g of straw, incubated overnight in the rumen of the donor cow were added to each flask because BH takes place on lipids adsorbed onto food particles [15]. The donor cow was a ruminally cannulated dry Holstein cow receiving a diet based on corn silage, hay and concentrates. Incubation times were 2, 4, 8, 16 and 24 h, with 5 replicates for each linseed form and incubation time. For each incubation time, a flask without added linseed was incubated (control). Details including buffer solution, instruments and procedure have been described previously [11].

2.3. In situ experiment

Three dry Holstein cows, fitted with a ruminal cannula, were utilised. The diet was based on hay, soybean meal and 1 of the 3 forms of linseed, which provided 86.1% of all dietary 18 carbon FA. Cows were housed in individual tie stalls, meals were at 0800 and 1800h and water was available ad libitum. The experiment was organised as a 3 × 3 Latin square with 3 periods of 3 weeks. Nylon bags (11 × 6 cm, mean pore size 45 μm) were filled with 1.5 g of one linseed form crushed through a 0.5 mm screen and 1.5 g of hay [2]. Because previous comparisons showed that BH is slower in situ than in vitro [11], a 48 h incubation was completed, so the bags were incubated for 2, 4, 8, 16, 24 and 48 h. For each incubation time, 2 replicates of each linseed form were incubated in each cow receiving the same linseed form in the diet. Once removed from the rumen, the bags were rinsed in cold water until the colour of water returned clear and then they were frozen at –18 °C.

2.4. Analytical procedures

The contents of the in vitro incubation flasks and of the nylon bags were freeze-dried (Vitris Freezemobile 25; Vitris Gardiner, NY) and subsequently ground in a ball mill (Dangoumau, distributed by Prolabo, Nogent-sur-Marne, France).

Fatty acids were extracted from freeze-dried samples and converted to methyl esters in one step, using sodium methoxide followed by boron trifluoride as described by Park and Goins [22]. One part of FA methyl esters of each sample was fractionated by argentation TLC (plates 20 × 20 cm, Silica gel 60, Merk KGaA, Germany) as described by Le Doux et al. [17],

in order to obtain an exact quantification of *trans*-13 to *trans*-15C18:1.

Total and *trans*-C18:1 FA were analysed by GLC (Agilent 6890N, equipped with a model 7683 auto injector, Network GC System, Palo alto, California, USA) as previously described [3].

2.5. Calculations and statistical analysis

For the in vitro experiment, FA that did not originate from tested linseed were excluded by subtracting from the FA of flasks with added fat, the FA in the control flask incubated on the same day and during the same incubation period. The estimation of ruminal BH of C18:2 and C18:3 in incubated flasks or bags was based on disappearance. Assuming no immediate disappearance of PUFA and the potential of all initial PUFA to be hydrogenated, the kinetic parameters of the BH were estimated based on the model described by Enjalbert et al. [11]:

$$P = P0e^{-c(t-1)}$$

where P = proportion of PUFA relative to total C18 after t hours of incubation, P0 = initial proportion of PUFA, c = rate of BH, l = lag time before BH begins.

Lag time and rate of BH for RL, CL and EL were compared using the following model:

$$P = P0e^{(-(cr\ Dr + cc\ Dc + ce\ De)(t-(lr\ Dr + lc\ Dc + le\ De)))}$$

where Dr is coded 1 for RL and 0 for other diets, Dc is coded 1 for CL and 0 for other diets, De is coded 1 for EL and 0 for other diets, so that cr, cc and ce are the rates of BH for RL, CL and EL, respectively and lr, lc and le are the lag times for RL, CL and EL, respectively. Data were computed with the nonlinear regression procedure of SYSTAT (Version 9; SPSS Inc., 1998, Chicago IL). Lag times were considered different from zero if their confidence interval did not include zero. The differences between forms of linseed were computed as function parameters and were considered significant when their confidence intervals did not include zero.

Effective BH was calculated from the model described by McDonald [20] when rumen action begins after a lag time, as:

$$EBH = ce^{-(c+k)l}/(c + k)$$

where c and l are as before, k represents the outflow rate of rumen contents. Because the main aim of ruminal BH estimation is the prediction of effects of adding dietary FA on the milk FA profile, k was calculated according to the equation proposed by the NRC [21] for a lactating cow eating 20 kg DM of a 40% concentrate diet, i.e. 6.3% h^{-1}. The differences between forms of linseed were computed as function parameters and compared in the same manner as for BH rates or lag times.

Within each investigation method and at each incubation time for in vitro and in situ studies, proportions of each FA were compared between different linseed forms using the general linear model of SYSTAT (version 9, SPPS Inc., 1998 Chicago). The model used for the in vitro study was:

$$Y_{ij} = \mu + L_i + D_j + \varepsilon_{ij}$$

where Y are the individual values for dependent variables, μ is the overall mean, L is the effect of the form of linseed and D is the effect of incubation day. The model used for the in situ study was:

$$Y_{ijk} = \mu + L_i + C_j + P_k + \varepsilon_{ijk}$$

where C is the cow effect and P is the period effect. The Tukey pairwise comparison test was used to compare the different forms of linseed.

Differences between treatments were declared significant at $P < 0.05$.

Table II. Lag time, rate of biohydrogenation and effective biohydrogenation of polyunsaturated fatty acids from three linseed forms incubated in vitro (mean ± SE) (n = 25).

Treatment	RL[1]	CL[1]	EL[1]
C18:2			
Lag time (h)	1.0[c] ± 0.3	0.4 ± 0.5	0.8[c] ± 0.4
Rate of biohydrogenation (% h^{-1})	23.8[a] ± 3.3	13.7[b] ± 1.7	18.2[ab] ± 2.4
R^2		0.89	
Effective biohydrogenation (%)	59.3 ± 5.9	63.3 ± 4.6	61.2 ± 5.1
C18:3			
Lag time (h)	0.4 ± 0.5	0.3 ± 0.5	0.3 ± 0.5
Rate of biohydrogenation (% h^{-1})	23.2[a] ± 3.8	13.0[b] ± 1.6	18.2[ab] ± 2.7
R^2		0.87	
Effective biohydrogenation (%)	69.9 ± 8.4	63.3 ± 4.8	69.6 ± 6.9

[1] RL: raw linseed, CL: pre-conditioned linseed, EL: extruded linseed.
[a, b] Means in the same row with different superscripts differ.
[c] Lag time significantly different from zero.

3. RESULTS

3.1. In vitro incubation

BH kinetics and FA proportions at different incubation times are presented in Tables II and III, respectively. Rate of BH was significantly lower with CL than RL for both PUFA and EL resulted in intermediate values. Lag times of BH were under 1 h and were not affected by the treatments. Effective BH was near 60% for C18:2 and somewhat higher for C18:3, but did not depend significantly on linseed treatment.

Over all incubation times, the proportion of C18:0 was higher with RL than with CL or EL and the proportions of C18:2 and C18:3 remained the highest for CL and intermediate for EL (Tab. III). The C18:0 proportion in incubations with RL was significantly higher from 8 h of incubation. Differences in the C18:2 proportion between CL and RL were significant at almost all incubation times, whereas CL and EL differed significantly after 8 h and EL and RL differed significantly after 8 and 16 h of incubation only. Similarly, from 2 to 16 h of incubation, CL resulted in significantly higher proportions of C18:3 than RL or EL and at 24 h of incubation EL and CL both resulted in significantly higher proportions of C18:3 than RL.

The most abundant BH intermediates were *trans*-10+11C18:1, followed by *cis*-9,*trans*-11C18:2, *trans*-13+14C18:1, *trans*-15C18:1 and *trans*-16C18:1. *Trans*-5C18:1 was below the detection limit. The peak of BH intermediates was reached after 8 h of incubation. The extrusion of linseed resulted in significantly higher proportions of *cis*-9,*trans*-11C18:2 and *trans*-10+11C18:1 compared to RL from 8 h of incubation. Proportions of *trans*-13+14C18:1 and *trans*-15C18:1 were significantly higher with RL than CL at 4 and 8 h of incubation and EL resulted in intermediate values. From 16 h of incubation, RL and CL resulted in similar proportions of all *trans*-C18:1.

3.2. In situ incubation

In situ kinetics of BH of PUFA were characterised by low BH rates (Tab. IV).

Table III. Effect of linseed processing on the profile of fatty acids with 18 carbons during in vitro incubation (n = 5).

Incubation time (h)	2				4				8				16				24			
Treatment	RL[1]	CL[1]	EL[1]	SE	RL	CL	EL	SE	RL	CL	EL	SE	RL	CL	EL	SE	RL	CL	EL	SE
											(% of C18)								
C18:0	12.6	12.2	11.5	1.2	26.5	15.2	19.6	2.8	54.3^{a}	43.7^{b}	45.2^{ab}	1.6	62.2^{a}	55.4^{ab}	52.5^{b}	1.6	68.5^{a}	62.1^{ab}	59.3^{b}	1.1
trans-4C18:1	0.25	0.14	0.24	0.06	0.25^{a}	0.10^{b}	0.20^{ab}	0.03	0.34	0.30	0.39	0.03	0.44	0.15	0.24	0.09	0.27	0.30	0.18	0.03
trans-6+7+8C18:1	0.35^{a}	0.21^{b}	0.30^{b}	0.02	0.67^{a}	0.30^{b}	0.49^{a}	0.05	0.89	0.75	0.76	0.05	0.76^{a}	0.63^{b}	0.71^{ab}	0.03	0.59	0.48	0.61	0.04
trans-9C18:1	0.30^{a}	0.14^{b}	0.28^{a}	0.03	0.47^{a}	0.19^{b}	0.40^{ab}	0.05	0.48	0.42	0.40	0.03	0.48	0.43	0.51	0.02	0.54	0.31	0.58	0.07
trans-10+11C18:1	4.15^{a}	2.88^{b}	3.53^{ab}	0.21	6.87^{a}	5.20^{b}	7.32^{a}	0.19	10.53^{b}	11.16^{ab}	12.84^{a}	0.53	8.77^{b}	8.87^{b}	11.03^{a}	0.32	7.56^{b}	7.81^{b}	10.23^{a}	0.43
trans-12C18:1	0.38	0.23	0.20	0.05	0.64^{a}	0.34^{b}	0.55^{ab}	0.04	0.65	0.69	0.48	0.13	0.58	0.65	0.76	0.08	0.24	0.59	0.48	0.12
trans-13+14C18:1	1.01^{a}	0.66^{b}	0.72^{ab}	0.06	2. 06^{a}	1.05^{c}	1.56^{b}	0.12	2.48^{a}	2.14^{b}	2.35^{ab}	0.07	2.22	1.96	2.24	0.07	2.02	1.92	2.04	0.09
trans-15C18:1	0.39	0.24	0.20	0.07	0.68^{a}	0.43^{b}	0.55^{ab}	0.07	1.26^{a}	1.01^{b}	1.11^{ab}	0.06	1.01	0.89	1.00	0.04	1.00	0.91	0.94	0.05
trans-16C18:1	0.41	0.25	0.30	0.05	0.95^{a}	0.49^{c}	0.70^{b}	0.05	1.41	0.97	1.36	0.15	1.19	1.10	1.18	0.04	1.15	1.12	1.08	0.05
cis-9,*trans*-11C18:2	0.41	0.19	0.33	0.07	1.06	0.96	1.29	0.09	0.85^{b}	1.12^{ab}	1.47^{a}	0.09	0.99^{b}	1.29^{ab}	1.62^{a}	0.09	0.95^{b}	1.13^{ab}	1.40^{a}	0.09
C18:2	14.8^{b}	16.1^{a}	15.7^{ab}	0.3	9.84	12.7	10.9	0.80	2.38^{c}	4.82^{a}	3.68^{b}	0.26	2.30^{b}	3.62^{a}	3.07^{a}	0.17	1.39^{b}	2.76^{a}	1.99^{ab}	0.23
C18:3	40.0^{b}	46.8^{a}	42.3^{b}	1.0	26.0^{b}	40.7^{a}	30.8^{b}	1.6	7.53^{b}	15.1^{a}	9.34^{b}	0.79	5.41^{b}	10.1^{a}	8.05^{b}	0.52	3.72^{b}	8.12^{a}	6.56^{a}	0.54

[1] RL: raw linseed; CL: pre-conditioned linseed; EL: extruded linseed.

[a, c] Means in the same row and for the same incubation time with different superscripts differ.

Table IV. Lag time, rate of biohydrogenation and effective biohydrogenation of polyunsaturated fatty acids from three linseed forms incubated in situ (mean ± SE) (n = 36).

Treatment			
	RL[1]	CL[1]	EL[1]
C18:2			
Lag time (h)	1.8[b,c] ± 0.7	1.7[b,c] ± 0.6	3.6[a,c] ± 0.6
Rate of biohydrogenation (% h^{-1})	5.9 ± 0.6	6.3 ± 0.7	7.9 ± 0.9
R^2		0.89	
Effective biohydrogenation (%)	38.6 ± 2.6	40.2 ± 2.6	33.3 ± 2.6
C18:3			
Lag time (h)	0.9[b,c] ± 0.7	1.1[b,c] ± 0.6	3.4[a,c] ± 0.6
Rate of biohydrogenation (% h^{-1})	6.8 ± 0.7	7.6 ± 0.8	8.6 ± 0.9
R^2		0.90	
Effective biohydrogenation (%)	45.8[a] ± 3.0	46.9[a] ± 2.9	34.8[b] ± 2.7

[1] RL: raw linseed, CL: pre-conditioned linseed, EL: extruded linseed.
[a, b] Means in the same row with different superscripts differ.
[c] Lag time significantly different from zero.

There was no significant effect of treatments on BH rate of PUFA, which tended to be higher with EL than with RL (P = 0.06 and P = 0.11 for C18:2 and C18:3, respectively). Lag times were significantly different from zero and lag times for both PUFA were longer with EL, resulting in a lower effective BH for C18:3. Over all incubation times, proportions of PUFA were in the same range for all 3 forms of linseed (Tab. V). Compared to RL and EL, CL resulted in significantly higher proportions of *cis*-9,*trans*11C18:2 after 4, 8 and 16 h and after 24 h of incubation, respectively. There was no significant difference between EL and RL.

There were no significant differences between CL and EL on the proportions of *trans*-5C18:1 to *trans*-9C18:1 (Tab. V). At all incubation times and irrespective of treatment, the most abundant isomers were *trans*-10+11C18:1, followed by *trans*-13+14C18:1. Extrusion of linseed resulted in a significantly higher proportion of *trans*-10+11C18:1 after 16 h of incubation. The proportions of *trans*-12C18:1, *trans*-13+14C18:1 and *trans*-16C18:1 were significantly higher with RL after 4 h of incubation. After 48 h, the proportion of *trans*-13+14C18:1 was significantly lower with CL, whereas incubation of RL significantly increased the proportion of *trans*-15C18:1 compared to CL after 8 h.

4. DISCUSSION

4.1. Extent of biohydrogenation of PUFA

Effective BH of PUFA was fairly low both in vitro and in situ. In vivo studies have shown BH extents over 80% for both PUFA [3, 10, 19], which suggests an underestimation of BH extent with the former investigation methods. The slow in vitro FA disappearance could be due to the accumulation of intermediates and end-products of fermentation or BH in the flasks during incubation. Further studies, including continuous cultures, should be needed to establish optimised conditions for the in vitro study of rumen BH.

Table V. Effect of linseed processing on the profile of fatty acids with 18 carbons during in sacco incubation (n = 6).

Incubation time (h)	2				4				8				16				24				48			
Treatment	RL[1]	CL[1]	EL[1]	SE	RL	CL	EL	SE	RL	CL	EL	SE	RL	CL	EL	SE	RL	CL	EL	SE	RL	CL	EL	SE
												(% of C18)												
C18:0	5.55	5.78	5.76	0.22	9.15^{a}	7.31^{ab}	5.45^{b}	0.60	9.13	11.2	10.8	0.67	16.7	16.6	18.0	1.7	33.9	34.0	33.9	3.0	50.6	62.4	56.0	4.8
trans-4C18:1	0.05	0.03	0.03	0.02	0.06	0.08	0.02	0.04	0.08	0.05	0.04	0.03	0.05	0.05	0.04	0.01	0.08^{ab}	0.10^{a}	0.07^{b}	0.01	0.13	0.13	0.12	0.02
trans-5C18:1	0.00	0.01	0.00	0.00	0.00	0.01	0.00	0.01	0.02	0.00	0.01	0.01	0.05	0.05	0.03	0.01	0.06	0.08	0.05	0.01	0.1	0.03	0.07	0.06
trans-6+7+8C18:1	0.04	0.02	0.01	0.01	0.10	0.04	0.00	0.03	0.12	0.06	0.10	0.04	0.26	0.19	0.26	0.06	0.67	0.57	0.38	0.09	0.88	0.55	0.76	0.29
trans-9C18:1	0.02	0.00	0.00	0.01	0.06	0.00	0.01	0.03	0.07	0.04	0.06	0.03	0.22	0.13	0.20	0.04	0.41	0.31	0.44	0.08	0.54	0.36	0.64	0.13
trans-10+11C18:1	1.05	0.48	0.85	0.16	3.69	1.11	0.85	0.80	4.02	2.90	4.28	0.71	8.60^{b}	6.23^{b}	11.39^{a}	1.24	17.48	16.91	19.64	1.77	19.31	13.50	15.78	2.44
trans-12C18:1	0.07	0.04	0.02	0.02	0.20^{a}	0.07^{b}	0.00^{b}	0.03	0.18	0.11	0.15	0.04	0.44	0.28	0.54	0.09	0.79	0.64	0.46	0.15	1.13	0.83	1.24	0.13
trans-13+14C18:1	0.21	0.10	0.13	0.03	0.59^{a}	0.21^{b}	0.10^{b}	0.63	0.63	0.42	0.57	0.09	1.28	0.91	1.50	0.16	2.55	2.18	3.05	0.29	3.88^{a}	3.02^{b}	4.16^{a}	0.21
trans-15C18:1	0.13	0.09	0.08	0.02	0.23	0.15	0.14	0.05	0.33^{a}	0.18^{b}	0.29^{ab}	0.04	0.51	0.67	0.50	0.17	1.02	0.86	1.34	0.20	1.65^{ab}	1.40^{b}	2.06^{a}	0.15
trans-16C18:1	0.05	0.06	0.05	0.01	0.19^{a}	0.10^{b}	0.06^{b}	0.02	0.22	0.20	0.18	0.04	0.37	0.34	0.35	0.05	0.79	0.71	0.90	0.14	1.28	1.29	1.60	0.13
cis-9,*trans*-11C18:2	0.18	0.49	0.46	0.03	0.36^{b}	1.34^{a}	0.27^{b}	0.19	0.45^{b}	2.76^{a}	1.36^{ab}	0.34	1.50^{b}	5.02^{a}	3.30^{ab}	0.60	2.69^{ab}	4.04^{a}	1.49^{b}	0.46	0.30	0.38	0.60	0.13
C18:2	18.2	18.3	18.0	0.2	15.8^{b}	17.0^{ab}	18.3^{a}	0.6	16.2^{a}	14.1^{b}	14.4^{b}	0.9	10.6	9.46	7.68	1.03	1.72	2.40	2.09	0.22	0.57^{b}	0.97^{a}	0.99^{a}	0.08
C18:3	52.1^{b}	54.4^{a}	54.3^{a}	0.6	43.3^{b}	47.7^{ab}	54.2^{a}	2.2	43.2	37.0	40.9	1.9	24.1	22.0	19.2	3.6	4.00	5.61	5.27	0.64	1.40^{b}	2.37^{a}	2.49^{a}	0.23

[1] RL: raw linseed; CL: pre-conditioned linseed; EL: extruded linseed.

[a, c] Means in the same row and for the same incubation time with different superscripts differ.

The current results confirmed the lower extent of BH in situ compared to in vitro, as formerly observed for rapeseed [11], despite the optimisation of the conditions to evaluate in situ BH in terms of dietary adaptation of the animals and supplementation in the bags of straw according to Agazzi et al. [2]. This very low in situ BH suggests that this method cannot provide accurate BH estimates.

Moreover, further research should focus on a more appropriate sample pretreatment for in vitro and in situ BH studies. Indeed, although standardised, grounding might not be the best way to simulate in vivo chewing accurately.

In our experiment, grinding resulted in similar particle size for the different linseed treatments, but could have affected the particular shape of material due to extrusion, so that the effects of extrusion have to be interpreted as effects of high temperature treatment. Both preconditioning and extrusion slowed down in vitro BH of C18:2 and C18:3 but had no significant effect on effective in vitro BH. In situ, CL did not differ from RL, and EL tended to result in higher BH rates, but resulted in a lower effective BH due to a much longer lag time. Conflicting results have been published on the effects of extrusion on in vitro and in situ BH: slower BH of extruded soybeans in vitro [25], slower in situ disappearance of PUFA from extruded soybeans [8], or hastened BH with rapeseed in vitro and in situ [11]. The differences between experiments could be due to differences in PUFA concentrations, or to the FA profile of the fat source. PUFA are suggested to reduce the isomerisation rate of C18:2 [4, 26], so that linseed and soybeans, which have a high proportion of PUFA, could cause an inhibition of BH which is not observed with rapeseed.

In vivo, preconditioning the same linseed/wheat bran mix also decreased the extent of BH compared to RL, but extrusion did not [3]. The authors suggested that this effect could be partly due to a difference in particle size, which was higher for CL. However, in the present experiment, all linseed forms were finely crushed before in vitro and in situ incubation, so that particle size cannot explain the slower BH observed in vitro with CL.

4.2. Intermediates of biohydrogenation

From 8 h of incubation in vitro, proportions of *cis*-9,*trans*-11C18:2 were higher with EL than with RL. This is consistent with the observations of Chouinard et al. [7] who reported higher proportions of *cis*-9,*trans*-11C18:2 in milk fat after dietary addition of extruded soybeans compared to raw soybeans. The in situ method did not show a significant effect of extrusion on *cis*-9,*trans*-11C18:2. The in vitro and in situ methods also led to different estimates of preconditioning effects: preconditioning resulted in the highest proportions of *cis*-9,*trans*-11C18:2 in situ but not in vitro. This suggests an interaction between treatment of seeds and method of investigation. FA can only be hydrogenated when leaving the cells, so that with the in situ method PUFA remain in the bags and free BH intermediates and C18:0 can leave the bags or be removed during their washing. This could explain high proportions of PUFA and low proportions of C18:0 in the bag residues. BH intermediates remaining in the bags after washing could also be due to their presence in bacteria [27], which are not completely removed by washing. These effects possibly explain the huge differences between FA profiles observed in situ and profiles observed in vitro in this experiment, or in ruminal and duodenal contents with the same material [3], and suggest that the in situ method cannot accurately predict the proportions of BH intermediates in digesta. Hence, further discussion on the effect of technological pre-treatment of linseed on

the accumulation of BH intermediates will be focussed on the results of the in vitro incubation only. Preconditioning did not significantly affect the proportions of *trans*-C18:1. On the contrary, compared with RL, EL resulted in higher proportions of *trans*-10+11C18:1 from 8 h of in vitro incubation. Since linseed treatments provoked differences in the in vitro accumulation of specific BH intermediates, it is suggested that heat treatment not only affects the rate of PUFA disappearance, but also the predominance of specific BH pathways. Increased *trans*-11C18:1 proportions in digestive content or milk fat in response to extrusion has been mentioned in vivo with soybeans [8] and in vitro with canola [11]. This effect has not been observed in duodenal contents of dry cows fed linseed [3], but was observed in the milk of dairy cattle [12]. Similarly, Jenkins and Adams [16] observed much higher differences in vitro than in vivo when measuring the BH of unsaturated FA between linoleic acid and linoleamide. This suggests that the in vitro method could amplify BH differences between fat sources compared to studies based on duodenal FA profiles and could be a good tool for speculations on the milk FA profile. Nevertheless, caution is needed since the profile of *trans*-C18:1 was different in vitro from the profile observed in the duodenum with the same linseed sources [3], or the duodenal profile observed by Loor et al. [19] after dietary addition of linseed oil. *Trans*-13+14C18:1 represented less than 15% of total *trans*-C18:1 in the present in vitro experiment vs. about 20% in the duodenum [3]. This was consistent with the suggestion that rumen production of *trans*-13+14C18:1 is high in vivo [18].

5. CONCLUSIONS

In situ BH was very slow, resulting in a low effective BH and high proportions of BH intermediates, which makes the in situ method doubtful as an accurate predictor of in vivo BH. In vitro effective BH of PUFA was higher than in situ, but lower than previously reported in vivo, suggesting that further studies are necessary to optimise conditions of in vitro studies, including pre-treatment of feedstuffs. Extrusion did not affect in vitro effective BH, but increased the proportions of *cis*-9,*trans*-11C18:2 and *trans*-C18:1 intermediates of BH. Preconditioning slowed down BH rate without important modification of the effective BH or the proportions of its intermediates.

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