Reprod. Nutr. Dev. 44 (2004) 459–464

DOI: 10.1051/rnd:2004048

Brief communication

Complete stereospecific determination of conjugated linoleic acids in triacylglycerol of milk-fat

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(Received 18 March 2004; accepted 4 June 2004)

Abstract – We analyzed two kinds of dairy fat differing in their contents of *cis*9,*trans*11-conjugated linoleic acid (*cis*9,*trans*11-CLA or rumenic acid), and determined the positional distribution of this CLA-isomer within the three *sn*- positions of the triacylglycerol. In the high rumenic acid fat (HR), the CLA-isomers amounted to 2.1% of total fatty acids, and 0.8% in the low rumenic acid fat (LR). Over 90% of the total CLA-isomers were in the form of rumenic acid, with an identical isomeric CLA distribution in both fats. The two fats differed mainly with regards to their contents in palmitic acid, α-linolenic acid, and isomers of *trans*-C18:1. Conversely, our stereospecific determination indicates that the positional distribution of rumenic acid is preserved among both types of fat, and is more specific for the *sn*-3 position of the triacylglycerol (54 to 64% of the total rumenic acid). Such a positional distribution is believed to be nutritionally relevant.

conjugated linoleic acid / milk-fat / positional distribution

1. INTRODUCTION

Conjugated linoleic acids (CLA) have been the focus of much attention in the past decade because of their pleiotropic health effects such as anti-obesity, anti-atherosclerosis, anti-cancer, and immune system modulation [1]. In nutrition, we are primarily interested in the main CLA-isomer found in foodstuffs, the so-called rumenic acid or the *cis*9,*trans*11-CLA, which has a specific effect on health [1]. A very recent study using structured synthetic triacylglycerol (TAG) acylated with [^{14}C]-rumenic acid showed a better absorption and oxidation when this fatty acid was initially present at the external position (*sn*-1/3) than in the central one (*sn*-2) [2]. Conversely, rumenic acid given in the 2-TAG form exibits a greater incorporation into rat carcasses [2]. This stereospecific factor could therefore be very important when dealing with the bio-availability of rumenic acid, and further with biological efficiency.

In the human diet, milk-fat represents the major source of CLA [3]. To date, the location of rumenic acid among the three *sn* positions of milk-fat TAG is still unknown. Here we report the first data on the stereospecific characterization of CLA in two milk-fats differing in their rumenic acid contents.

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2. MATERIALS AND METHODS

2.1. Material

Several kinds of dairy fat were screened: seven Comté-cheese fats ripened for different times, six fractions of milk fat fractionated by dry fractionation, and one commercial butter. Two fats differing in their rumenic acid content, i.e. butter fat (low rumenic acid content, LR) and a Comté-cheese fat (high rumenic acid content, HR), were selected to elicit a complete stereospecific analysis. The fatty acid analysis was performed as detailed infra.

2.2. Methods

The chromatographic analysis of total fatty acids, *cis* fatty acids, *trans* fatty acids, and conjugated fatty acids was performed on the fatty acid methyl esters which were generated using the derivatization technique appropriate for dairy fat [4]. When necessary, a combination of both reversed-phase HPLC (Nucleosil C18 column, 250 mm length, 10 µm i.d. and 5 µm particle size, Shandon, Cergy-Pontoise, France, and detected with a Waters 410 differential refractometer, Waters, Saint-Quentin-en-Yvelines, using acetonitrile as the mobile phase) and long-size gas-chromatography capillary column (BPX70, 120 m length, 0.32 mm i.d., 0.25 µm film thickness, SGE, Les Ulis, France) [5, 6] was used. The isomeric determination of 18 C chain-length fatty acids was done by comparison with published data [5]. The CLA contents and identification were further ascertained by gas chromatography/mass spectrometry of Diels-Alder adducts, as described in [7].

The stereospecific analysis was carried out based on the multi-step method developed by Itabashi et al. [8], as slightly modified by us [9]. The analysis was performed on short-chain and long-chain TAG of both fats after we had fractionated TAG by thin-layer chromatography (20 × 20 plates, 0.25 µm of silica gel thickness, SDS, Peypin, France), as required for dairy fat [10]. For both the short-chain and the long-chain TAG, representative acylglycerols (diacyl-1,3-*sn*-glycerol, 1,2(2,3)-diacyl-*rac*-glycerol, 2-monoacyl-*sn*-glycerol, 1(3)-monoacyl-*rac*-glycerol) were generated from the original TAG by partial Grignard degradation. Dinitrophenylurethane (DNPU) derivatives of the 1,2(2,3)-diacyl-*rac*-glycerol were then resolved by chiral-phase HPLC cooled at 0 °C (YMC-Pack A-K03 column, 250 mm length, YMC Inc., Kyoto, Japan, using isocratic *n*-hexane/1,2-dichloroethane/ethanol, 40:10:1, vol/vol/vol as the mobile phase) into 1,2-diacyl-*sn*-glycerol DNPU and 2,3-diacyl-*sn*-glycerol DNPU derivatives, prior to GLC analysis of their constituve fatty acid moieties. For the determination of the *sn*-2 position, a time-course study indicated that the porcine pancreatic lipase method (described in [11]) was unreliable for this assay and that it was necessary to cross-check the results with the species generated by the Grignard method.

The accuracy of the stereospecific determination can be determined by the following: the fatty acid composition of a lipid species obtained at step *n* of the analysis can be reconstituted with that obtained from the lipid species of step *n+1*. Thus (1,2-diacyl-*sn*-glycerol + 2,3-diacyl-*sn*-glycerol)/2 should give the same fatty acid composition as the original 1,2(2,3)-diacyl-*rac*-glycerol from which it is derived.

Homogenous TAG composed of synthetic 9,11-positional isomers of CLA (83% pure, CLA isomeric distribution: *cis*9,*trans*11-, 92.5%, *cis*9,*cis*11-, 5.03%, *trans*9,*trans*11-CLA, 2.46% and synthesized according to [12]), was produced essentially as described elsewhere [13, 14].

3. RESULTS AND DISCUSSION

The butter (LR) and Comté-cheese (HR) fats differed mainly with regards to their palmitic acid contents (28.6% vs. 38% in the HR fat and in the LR fat, respectively)

Table I. Positional distribution of the main fatty acids in the triacylglycerol of a low rumenic acid dairy-fat (LR) and in a high rumenic acid dairy-fat (HR).

Fatty acids	Type of fat	TAG	*sn*-1	*sn*-2	*sn*-3
C8:0	LR	0.1	–	0.1	0.1
	HR	–	–	–	0.1
C10:0	LR	0.6	–	0.6	1.3
	HR	0.4	–	0.2	0.9
C12:0	LR	1.9	2.3	2.8	0.6
	HR	0.7	–	1.8	0.5
C14:0	LR	9.0	2.5	19.6	5.0
	HR	9.2	3.1	14.7	9.9
C14:1	LR	1.1	0.5	1.5	1.5
	HR	1.1	0.3	1.1	1.9
C15:0	LR	1.2	1.0	1.7	0.9
	HR	1.3	1.4	1.6	0.9
C16:0	LR	38.0	41.8	46.1	26.0
	HR	29.6	36.9	37.3	14.8
C16:1	LR	2.1	1.1	2.6	2.5
	HR	1.9	1.7	1.6	2.4
C18:0	LR	11.6	18.0	5.8	11.2
	HR	10.6	15.0	10.2	6.7
C18:1[a]	LR	30.3	30.9	16.5	43.6
	HR	38.3	36.8	26.2	51.8
C18:2n-6	LR	2.6	1.0	1.5	5.4
	HR	3.2	2.6	2.5	4.4
C18:3n-3	LR	0.6	0.8	0.4	0.5
	HR	1.3	1.3	1.4	1.1
CLA[b,c]	LR	0.8	0.8	0.3	1.2
	HR	2.1	1.2	1.0	4.0

[a] See isomeric distribution in Figure 1.
[b] % isomeric distribution determined by GLC (in LR and HR, respectively): *cis*9,*trans*11+*cis*7,*trans*9; 94.15 and 91.93, *trans*8,*cis*10; 0.0 and 0.7, *trans*,9*cis*11+*cis*10,*cis*12; 0.0 and 0.81, *trans*11,*cis*13; 0.25 and 0.0, *trans*10,*cis*12; 0.76 and 0.0, *cis*7,*cis*9; 0.0 and 5.64, *cis*8,*cis*10; 2.39 and 0.35, *trans*11,*trans*13; 0.51 and 0.58, other *trans*,*trans*; 2.04 and 2.
[c] % isomeric distribution determined by GC/MS of Diels-Alder adducts (in LR and HR, respectively): iso 7,9; 2.79 and 1.34, iso 8,10; 0.0 and 0.74; iso 9,11; 95.47 and 89.62, iso 10,12; 0.52 and 0.89, iso 11,13; 1.22 and 6.84, iso 12,14; 0.0 and 0.57.

(Tab. I). Other discernible differences were found for quantitatively minor fatty acids, especially C18:3n-3 (1.3% vs. 0.60% in HR- and LR-fats, respectively) (Tab. I), and *trans* isomers of C18:1 (7.26% vs. 3.27% in HR- and LR-fats, respectively). For the latter, *trans* vaccenic acid (*trans*-C18:1Δ11) was the dominating isomer, but it is present in a much greater proportion in the HR-fat than in the LR-fat (66.9% vs. 29.7% of total *trans*, respectively) (Fig. 1). In addition, the CLA content, e.g. the precursor of *trans*-vaccenic acid along the biohydrogenation process, was also markedly higher in the HR-fat than in the LR-fat (2.1% vs. 0.8%, respectively). Six different positional isomers of CLA in the HR-fat and four in the LR-fat were detected and quantified by GC/MS of MTAD derivatives (Tab. I, footnote). Rumenic acid was the dominating

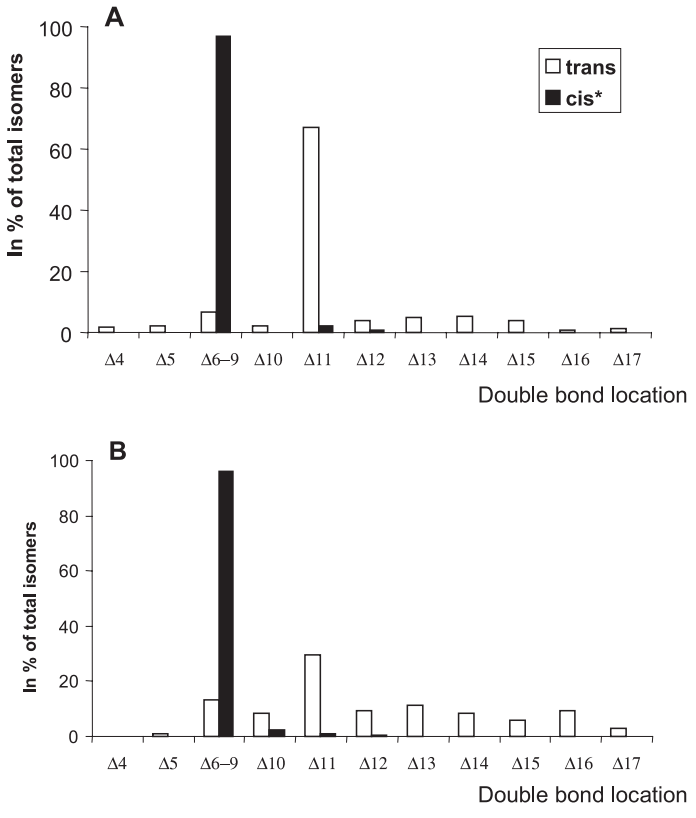

Figure 1. *Cis* and *trans* positional isomers of octadeca-monenoic acids in the high rumenic acid (panel **A**) and low rumenic acid (panel **B**) dairy-fats. The symbol Δ denotes the position of the double bond numbered from the carboxyl end. The asterisk indicates values only for the Δ9 *cis* isomer.

isomer in both fats, with a similar proportion in total CLA (≥ 90%).

The stereospecific determination gave good results for most of the fatty acids with a fatty acyl chain ≥ 12 carbons, but a great loss of CLA was shown both at the Grignard degradation step and at the DNPU derivatization step, thus impeding a reliable positional determination. A similar difficulty was reported by others in their study of punicic acid, an 18C fatty acid of *Trichosanthes kirilowii* seed oil with 3 conjugated double bonds (*cis*9,*trans*11,*cis*13-18:3) [15]. It was thus necessary to determine the extent of such losses at each step of the stereospecific process in order to achieve a better analysis. For this, homogenous TAG made up of synthetic 9,11-positional isomers of CLA were subjected to the whole stereospecific procedure and the intermediary products were analyzed at each step. According to the acylglycerol species, the waste of CLA ranged between 7.5 and 18.9% at the Grignard degradation steps, and cumulated up to 23.4% at the DNPU derivatization step (e.g. including both the Grignard degradation step and the DNPU derivatization). Interestingly, no significant isomerization of either isomer occurred at any step. We used these data to offset the loss of CLA that occurred during

the stereospecific analysis of the milk fats (Tab. I). Our finding indicated that the location of CLA on the glycerol moiety was highly asymetrical, and that over 50% of CLA (primarily as the *cis*9,*trans*11-CLA) was located mainly in the *sn*-3 position of the milk fat TAG. Together with the *sn*-1 position, over two-thirds of CLA was located at the external position of milk fat TAG. This was in agreement with what was found by others ([2] found 74% CLA) and thus may constitute a common feature of dairy fat.

These findings have interesting implications concerning digestion. Once milk-fat is ingested, the preferential *sn*-3 location of rumenic acid renders it very suitable to hydrolysis by both the gastric (*sn*-3 specific) and the pancreatic lipases (*sn*-1 and *sn*-3 specific) operating in the intestinal tract. One may predict from its location that CLA as a free acid is the preferred form arising upon hydrolysis, and that it has a faster release and also a faster uptake by the enterocyte into that form. This could be taken into account when dealing with nutritional studies examining in particular the effect of CLA and rumenic acid. Because of the *sn*-3 regiospecificity of the gastric lipase, one should also consider that rumenic acid acylated in equal amounts in both the *sn*-1 and *sn*-3 positions of synthetic TAG [2] might not be nutritionally equivalent to a preferrential location in the *sn*-3 position as occurs in natural dairy fat.

The differences in the rumenic acid content in our two milk-fats are mainly due to the animal's diet [16]. Conversely, this dietary factor does not substantially modify the location of rumenic acid within TAG (Tab. I). On the contrary, the CLA positional distribution in TAG (*sn*-2 vs. *sn*-1/3) of ruminant (lamb) muscle or subcutaneous adipose tissue has been reported to be modified by this dietary factor [17]. Thus, due to its specific rumenic acid distribution within TAG, dairy-fat might not be nutritionally equivalent to other ruminant products in terms of rumenic acid availability and activity.

From a metabolic point of view, since TAG synthesis in the mammary gland cells proceeds via the glycerol-phosphate pathway [18], the preferred *sn*-3 positioning of CLA indicates that this fatty acid is not a favoured substrate for the acyl-transferases acting at the first steps of TAG synthesis (synthesis of the 1,2-diacyl-*sn*-phosphate intermediate from *sn*-3 glycerol-phosphate), but is processed last.

In conclusion, our study confirmed that rumenic acid is preferentially localized in the external position of the TAG of dairy fat, and that it is predominantly acylated in the *sn*-3 position. Such a positioning is highly favorable for a complete release in the free form, which is likely relevant in terms of bio-availability and biological activity.

ACKNOWLEDGEMENTS

This work was funded by a research grant 727-2000 from ARILAIT/CERIN/ministère de la Recherche, France. Karine Valeille is a recipient of an ARILAIT/ministère de la recherche fellowship.

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