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Positional Distribution of Conjugated Linoleic Acid in Triacylglycerol of *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae was cultivated in the presence of *cis*-9,*trans*-11 or *trans*-10,*cis*-12 isomers of free conjugated linoleic acid (CLA), and the effects of the isomers on the regioisomerisms of triacylglycerol (TAG) of the yeast were elucidated. Both isomers constituted about 34% of all fatty acids and increased drastically the number of different TAG species. Nearly all of the species contained CLA in at least one *sn*-position. In the most abundant species analyzed (20% of total species), the *cis*-9,*trans*-11 isomer appeared in combination with monounsaturated fatty acids (C16:1, C:18:1) whereas *trans*-10,*cis*-12 isomer was most frequently present with a medium chain fatty acid (C10:0 or C12:0) in the *sn*-2 position and C16:0 in one of the end positions (14% of total species). With either isomer, the amount of TAG species in which CLA encompassed all *sn*-positions was ca. 4%. Thus, *S. cerevisiae* can be used to produce edible single cell oil characterized by very heterogeneous distribution of CLA among the different TAG species.

KEYWORDS: Conjugated linoleic acid; CLA; *Saccharomyces cerevisiae*; yeast; triacylglycerol; TAG; positional distribution

INTRODUCTION

The beneficial health effects of conjugated isomers of linoleic acid (CLA) have become the focus of an increasing amount of interest. Fat reduction and lean mass increase by dietary *trans*-10,*cis*-12 CLA have been reported in several animal and human studies (*1*), and similar results have also recently been documented for the eukaryotic yeast, *Saccharomyces cerevisiae* (*2*). Evidence also links the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers of CLA with anticarcinogenic (*3*), antidiabetic, antiinflammatory, and antiatherosclerotic properties (*4*, *5*).

CLA is an inherent, natural component of many foods occurring primarily in dairy products and meat from ruminants. The predominant isomer in milk fat and ruminant tissue fats is the *cis*-9,*trans*-11 isomer (80−90%) with minor proportions of the *trans*-10,*cis*-12 isomer and other geometric isomers (*6*, *7*). In dairy products, the CLA content is not constant but it is influenced, for example, by the diets of the cows (*8*, *9*). Thus, the estimated average daily intake of CLA is variable and the reported values range between 0.05 and 0.44 g/day depending on the person's gender, age, and country of residence (*10*, *11*). For vegetarians and individuals who do not consume milk products as part of their normal daily diet, these values may be expected to be much lower. Because of the fluctuating amounts of CLA in food and the dietary restrictions, the intake of CLA isomers via the normal diet is probably not sufficient for the amounts of the naturally occurring CLA isomers to achieve their beneficial health effects.

Supplementing the foods with additional CLA is possible. However, many biological effects of CLA are due to the separate actions of the isomers (*12*); therefore, there is a need for food products containing isomerically pure CLA. Moreover, as a food additive, safety aspects concerning the origin of CLA or its manufacturing process may pose regulatory challenges. Therefore, a major effort has recently been focused to produce food-grade CLA, which may be used in a variety of food applications. One of the research lines is based on the use of food-grade microorganisms to convert linoleic acid into CLA of high isomeric purity (*13*−*15*). Recently, this microbiological isomerization process has been applied directly to linoleic acid rich food materials to obtain "food-borne" *cis*-9,*trans*-11 CLA with approximately 80% isomeric purity (*16*). Even though the current microbiological methods for CLA production represent, in a sense, natural processing, they all include an intrinsic drawback. The microorganisms known up to date to produce CLA are only capable of carrying out the isomerization from free linoleic acid. As a result, by using such methods for the production of CLA-enriched foods, a concomitant increase in the free fatty acid (FFA) content will occur leading to a decrease in palatability and oxidation tolerance.

A pivotal issue is thus the esterification of free CLA in complex food matrices. Such esterification without separation of the fatty acids from the food matrix would be a demanding task by conventional chemical methods, and on the other hand, the use of solvent extractions would severely compromise the concept of CLA enrichment by natural means. On the other hand, we have shown that the yeast *Saccharomyces cerevisiae*,

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capable of taking up free CLA from its environment, incorporates it into its neutral lipids (*2*). Consequently, it may be suggested that a yeast cultivation stage could be added to the preceding microbiological isomerization as a possible means for the esterification of the free CLA formed. The CLA-containing oil would then represent single cell oil with fatty acid composition and regioisomerism typical for the yeast. Therefore, elucidation of the composition of the single cell oil of *S. cerevisiae* when cultivated in the presence of free CLA is necessary for the evaluation of the suitability of the yeast-based esterification process. As part of this evaluation, *S. cerevisiae* was cultivated in the presence of free CLA isomers and the positional distribution of CLA in major fractions of the yeast triacylglycerol (TAG) was determined.

MATERIALS AND METHODS

Yeast Strain and Culture Conditions. Wild-type baker's yeast strain *S. cerevisiae* B-72021 was provided by the Technical Research Centre of Finland. Cultivation conditions were the same as described previously (*2*). Yeast was cultivated in wort broth (15 g/L malt extract, 12.75 g/L maltose, 3.1 g/L glycerol, 2.75 g/L dextrin, 1.0 g/L ammoniumchloride, and 0.75 g/L peptone, pH 5.0, Merck, Darmstadt, Germany) for 17 h at 30 °C by shaking at 270 rpm. Of this culture, 1 mL was inoculated into 50 mL of the wort broth supplemented with *cis*-9,*trans*-11 (>98% purity) or *trans*-10,*cis*-12 CLA isomer (>98% purity) (Mattreya Inc., Pleasant Gap, United States). The fatty acids were dissolved in ethanol prior to adding them into the medium. The control cells were cultivated in wort broth without fatty acid supplementation. The yeast was cultivated into the late stationary phase for 24 h as mentioned above. The cultivations were performed in duplicate.

Extraction of Yeast Lipids. The cells were harvested by centrifugation, washed with tap water, and freeze-dried. The total lipids were extracted by direct saponification and methylated as described later. For the separation of lipid classes, the lipids were extracted from 50 mg of the dry cell mass by 5.7 mL of dichloromethane/methanol (2:1) for 18 h at ambient temperature by shaking at 240 rpm. The supernatant containing the lipids was evaporated under nitrogen atmosphere.

Separation of Lipid Classes. Thin-layer chromatography was used for the separation of the lipid classes. The lipid sample extracted from the 60 mg portion of the dry cell mass was dissolved in 200 μL of dichloromethane/methanol (100:1). The sample was supplemented with a standard mixture (heptadecanoic acid, triheptadecanoin, L-α-phosphatidylcholine (dipentadecanyl), and 1,3-dipentadecanoin (Sigma Chemical, St. Louis, MO) and applied to a silica plate (Kieselgel 60, Merck). The plate was left to develop with petroleum ether/diethyl ether/acetic acid (80:30:1), and the spots containing TAG, diacylglycerols (DAG), FFAs, and polar lipids (PLs) were visualized after spraying with 0.01% rhodamine 6G under UV light, scraped off, and used for fatty acid analysis.

Determination of Fatty Acid Composition. To determine the concentrations of individual fatty acids in different lipid classes, the fatty acids were converted into methyl esters and analyzed by gas chromatography as described by Suutari et al. (*17*). In this procedure, the fatty acids were saponified with 3.7 M NaOH in 49% MeOH at 100 °C for 30 min and then methylated with 3.3 M HCl in 48% MeOH at 80 °C for 10 min. The methyl esters were extracted in hexane/methyl-*tert*-butyl ether solution (1:1), and the extract was washed with aqueous alkali. Analysis of the fatty acid methyl esters was performed by a Hewlett-Packard model 6890 gas chromatograph equipped with HP-FFAP column (25 m, 0.2 mm i.d., 0.33 μm film thickness; Agilent Technologies, Palo Alto, CA) and a flame ionization detector. The temperature in the column was raised from 70 to 200 °C at a rate of 25 °C/min. Peak areas were measured using a Hewlett-Packard model 3396A integrator. The average fatty acid chain length was calculated as [10 (%C10:0) + 12 (%C12:0) + 14 (%C14:0) + 16 (%C16:0) + 16 (%C16:1) + 18 (%C18:0) + 18 (%C18:1) + 18 (%CLA)]/100.

Molecular Weight Distribution of TAG. TAGs were extracted from the silica gel by 6 mL of dichloromethane/methanol (2:1) by shaking at room temperature for 2 h. The solvent was evaporated into dryness, and the extraction was repeated by shaking for 1 h. The TAG sample was dissolved in hexane. The molecular weight distribution of TAG was determined by ammonia negative ion chemical ionization with a triple quadrupole tandem mass spectrometer (TSQ-700, Finnigan MAT, San Jose, CA) by applying the direct inlet method on a heated rhenium wire loop as described by Laakso and Kallio (*18*).

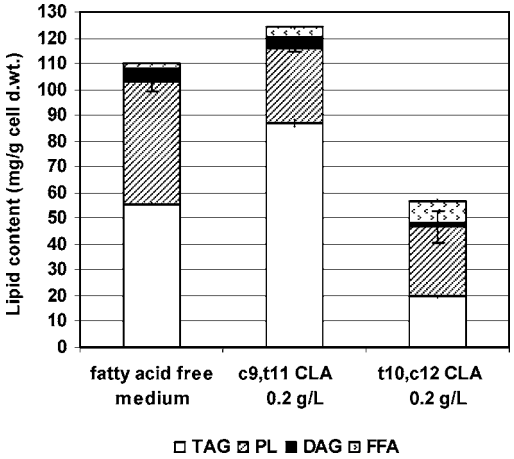

Figure 1. Main lipid classes (mg/g cell dry wt) of *S. cerevisiae* grown with *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA isomers (0.2 g/L growth medium). Data are averages ± SD ($n = 4$).

Regioisomerism of the Selected Molecular Weight Species. The most abundant molecular weight fractions (acyl carbon number:number of double bonds, ACN:DB) were selected for the analysis. From TAGs of control cells were analyzed fractions 48:2 (ACN:DB), 50:2, 50:3, and 52:3 of the cells grown with *cis*-9,*trans*-11 CLA fractions 50:3, 50:4, 52:3, 52:4, and 52:5 and of the cells grown with *trans*-10,*cis*-12 CLA fractions 44:2, 46:2, 50:3, 50:4, 52:4, and 52:5. *sn*-Regioisomers were identified and quantified with MS-MS (TSQ-700, Finnigan MAT) by collision-induced dissociation of the deprotonated parent ions [M − H]$^-$ described by Kallio and Currie (*19*). In this method, primary (*sn*-1/3) vs secondary (*sn*-2) positions of fatty acids could be determined. For the conversion of [RCOO]$^-$ signals to FA proportions, quantitative correction factors were determined using reference TAGs. The results were calculated by the TAGS-100 program (Nutrifen Ltd., Turku, Finland). The analyses were repeated four times each.

RESULTS

S. cerevisiae was cultivated in the presence of 0.2 mg/mL of either *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA and thereafter analyzed for lipid content. The fatty acid composition of TAG fraction, isolated from total lipids, and the major molecular mass fractions were then determined. The *sn*-regioisomerism was analyzed in the most abundant ACN:DB fractions in order to obtain as comprehensive a view as possible of the positioning of CLA isomers in those TAG fractions.

Lipid Class Composition of the Yeast. Cultivation of the yeast in the presence of the CLA isomers changed the lipid class composition from that of the yeast cultivated in the absence of CLA. The *cis*-9,*trans*-11 isomer increased the TAG fraction by 57% (from 55.5 to 86.9 mg/g cell dry wt) (**Figure 1**). Instead, the *trans*-10,*cis*-12 isomer reduced the TAG fraction by 63% (from 55.5 to 20.7 mg/g cell dry wt), and as a result, the total cellular lipid content was reduced markedly. Both isomers slightly reduced the content of PL and increased that of FFA. These results do not essentially differ from the earlier observations on the effects of the CLA isomers on the yeast lipid class composition (*2*).

Fatty Acid Composition of TAG. In the control cells, the major fatty acids in TAG were C16:1, C18:1, and C16:0, which together encompassed over 90% of total fatty acids (**Table 1**).

Table 1. Fatty Acid Composition of TAG (%) in the Cells of *S. cerevisiae* Grown with CLA Isomers (0.2 g/L) or without Fatty Acid Supplementation[a]

	control cells	cells grown with *c*9,*t*11-CLA	cells grown with *t*10,*c*12-CLA
C10:0	0.1 ± 0.1	1.0 ± 0.2	2.1 ± 0.6
C12:0	0.6 ± 0.2	1.3 ± 0.3	3.1 ± 0.3
C14:0	1.6 ± 0.5	1.5 ± 0.2	3.1 ± 0.3
C16:0	13.4 ± 0.1	9.3 ± 0.3	23.0 ± 0.2
C16:1	43.4 ± 4.1	22.2 ± 0.8	17.1 ± 1.7
C18:0	5.8 ± 0.7	3.4 ± 0.2	5.1 ± 0.5
C18:1	35.4 ± 3.4	13.2 ± 0.1	6.0 ± 1.3
*c*9,*t*11-C18:2	ND	48.0 ± 1.9	ND
*t*10,*c*12-C18:2	ND	ND	39.8 ± 1.5
average chain length	16.8 ± 0.1	17.1 ± 0.1	16.6 ± 0.0

[a] Data are averages of triplicate samples ± SD.

The CLA isomers supplemented in the growth medium were incorporated in yeast TAG with high abundance. The *cis*-9,*trans*-11 isomer reached the proportion of nearly 50% of TAG fatty acids, and the proportion of the *trans*-10,*cis*-12 isomer was almost 40%. With the incorporation of CLA, the proportions of monounsaturated fatty acids were decreased reflecting repression of the endogenous desaturation system of the yeast. This phenomenon was even more emphasized with the *trans*-10,*cis*-12 isomer, which has been reported to inhibit desaturase action and the underlying gene expression also in mammalian studies (*20*, *21*). In the yeast grown with *trans*-10,*cis*-12 CLA, the proportion of medium chain fatty acids (MCFA) increased reaching 5.2% (C10:0 + C12:0) of all TAG fatty acids. In control cells and in the cells grown with *cis*-9,*trans*-11 CLA, the corresponding values were 0.7 and 2.3%, respectively.

Distribution of TAG Species. TAGs of *S. cerevisiae* consist mainly of three fatty acids when cultivated in a fatty acid-free medium. Therefore, the number of TAG species can be expected to be rather limited. In those cells, five major TAG species encompassed 42% of TAGs (**Figure 2A**). Supplementation of the cultivation medium by CLA that is not present naturally in the yeast increased the number of possible species dramatically, and as a consequence, the relative amounts of each different TAG species were also reduced. In the presence of *cis*-9,*trans*-11 CLA, even the most abundant TAG species were below 6% of all TAGs and 42% of TAGs consisted of 17 TAG species (**Figure 2B**). With *trans*-10,*cis*-12 CLA, the 42% proportion of all TAGs was even more diverse consisting of 27 TAG species (**Figure 2C**).

Regioisomerism of TAG. When the yeast was grown without the CLA supplementation, the most abundant TAGs were C16:1-C18:1-C16:1 (10.8%), C16:1-C18:1-C16:0 (9.3%), and C16:1-C16:1-C18:1 (8.6%) (**Figure 2A**). Instead, almost all of the TAG species in the CLA-fed cells contained at least one molecule of CLA; thus, TAGs similar to that of control cells, so-called "natural TAGs", were in the minority. In the cells grown with *cis*-9,*trans*-11 CLA, the most abundant TAG was C16:1-C16:1-*c*9,*t*11C18:2 (5.3%) (**Figure 2B**). Overall, in the largest TAG fractions, the *cis*-9,*trans*-11 isomer was present in at least one position. Besides the *cis*-9,*trans*-11 CLA isomer, TAGs consisted mainly of monounsaturated fatty acids, C16:1 and C18:1. The proportion of TAGs, which contained this isomer in all *sn*-positions, was relatively high, 4.7%. Also, the *trans*-10,*cis*-12 isomer was abundantly incorporated in TAGs. However, the other fatty acids of TAGs differed from those observed with the *cis*-9,*trans*-11 isomer. With the *trans*-10,*cis*-12 isomer, two of the most abundant TAG species did not contain monounsaturated fatty acids but, instead, had a MCFA in the *sn*-2 position. In those cells, the largest TAG species were C16:0-C10:0-*t*10,*c*12C18:2 (8.6%) and C16:0-C12:0-*t*10,*c*12C18:2 (5.1%), followed by a species where this isomer was in every *sn*-position (4.1%) (**Figure 2C**).

The fact that the *trans*-10,*cis*-12 isomer occurred frequently in combination with MCFA can raise a question whether the reduction in total TAGs with this isomer could be explained by the abundance of fatty acids with lower molecular weights. However, **Table 1** shows that the average chain lengths remained practically the same as in the control. Interestingly, with the *cis*-9,*trans*-11 isomer, the average chain length was only slightly higher even though this isomer most frequently occurred in combinations with C16:1 or C18:1.

Preference of *sn*-Positions. Among the TAGs analyzed, TAGs with only one CLA molecule in either the *sn*-1 or the *sn*-3 position were the most abundant, independent of which of the two isomers was used (**Table 2**). The second in abundance was a TAG with the CLA isomers in both *sn*-1 and *sn*-3 positions. Instead, acylation of the CLA isomers in the *sn*-2 position appeared to be less favored, especially in the case of *trans*-10,*cis*-12 CLA.

DISCUSSION

The efficiency or *S. cerevisiae* as a single cell oil producer is far from that of the truly oleaginous yeast such as *Lipomyces* spp. or some *Candida* spp. On the other hand, as baker's yeast is a traditional food-grade organism, it may have more potential as a source of bioactive specialty oils, such as of CLA-enriched TAGs, rather than as an oil source for more traditional applications. However, achieving a substantial improvement of the nutritional intake of CLA by using the yeast as a vehicle is critically dependent on the amount of the yeast or components thereof, which should be included in the daily diet. Such an estimation can be made by using the data on the cellular TAG content of the yeast and the CLA content in the yeast TAG. By assuming that the current daily intake of CLA is in the range of 0.05−0.44 g and doubling the average of this value (0.25 g) by *cis*-9,*trans*-11 CLA containing TAG, it can be estimated that 6 g of whole yeast cells or 0.5 g of pure yeast TAG should be included in the daily diet. However, in foodstuffs where the use of whole cells or whole cell extracts is possible, the required amount of added yeast would be substantially lower as also the PLs, which constitute some 24−47% of total yeast lipids and contain approximately 33% CLA (*2*). Therefore, it is evident that improving the intake of CLA by administration of the *S. cerevisiae* or components thereof depends largely on how these components can be included into foods with improved nutritional profiles.

The present study has shown that the TAG of *S. cerevisiae* consists of a relatively large number of different TAG species. According to results of the present study, this is primarily due to the incorporation of CLA into each of the *sn*-positions and CLA can occupy even all three *sn*-positions. However, among the many species, TAGs with CLA in the *sn*-1/3 positions dominated and TAGs with CLA in the *sn*-2 position were a minority. These findings are in accord with a recent study, where stereochemically pure TAG was fed to rats and CLA was found to be preferentially absorbed from the *sn*-1/3 positions of TAG (*22*). This report, although only being a single report, supports the view that CLA-containing yeast TAG could be an efficient vehicle for the absorption of CLA from foods.

Overall, studies on the positional distribution of CLA in TAG are scarce and have focused on TAG from animal tissues where CLA has been accumulated naturally or after feeding with CLA-

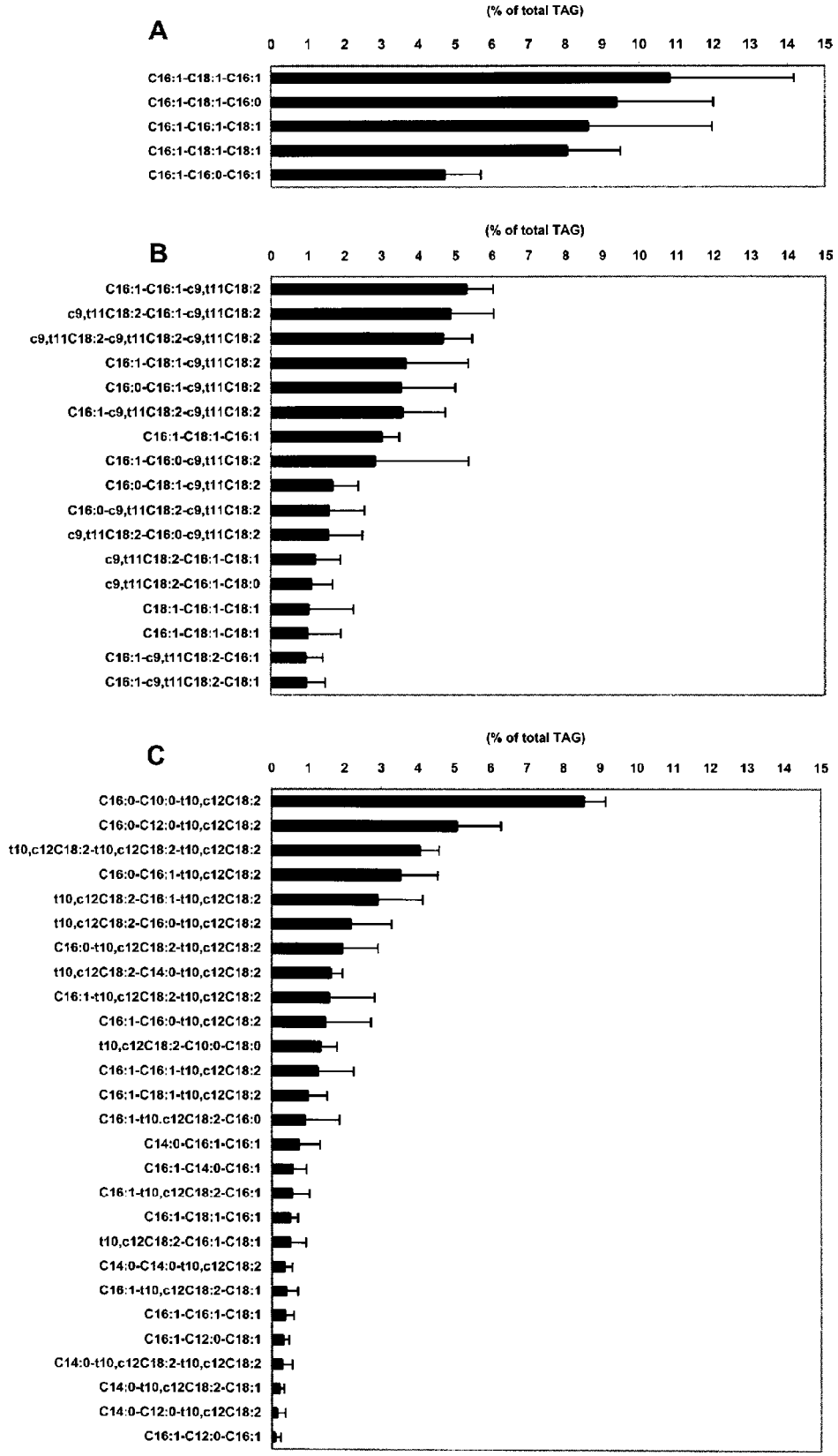

Figure 2. Proportions of the most abundant TAG species in the cells of *S. cerevisiae* grown (**A**) without fatty acid supplementation, (**B**) with *cis*-9,*trans*-11 CLA isomer (0.2 g/L growth medium), and (**C**) with *trans*-10,*cis*-12 CLA isomer (0.2 g/L). The data are averages ± SD ($n = 8$).

containing diets. In animals fed with CLA, the *sn*-2 position was the most abundant (*23*, *24*), while the CLA synthesized by the animals, lamb and cow, was primarily located in the *sn*-1/3 positions (*24*, *25*). However, currently available data do not allow for a final conclusion to be drawn on how the positional distribution of CLA in ingested TAG affects the positioning of CLA in tissue TAG or, more importantly, how this affects the physiological effectiveness of CLA. Therefore, according to currently available data, the yeast TAG, which consists of species with CLA in any *sn*-position and originates from a safe

Table 2. Amounts (mg/g) and Proportions (%) of *cis*-9,*trans*-11 or *trans*-10,*cis*-12 CLA in *sn*-Positions of TAG[a] in *S. cerevisiae* Grown in the Presence of These Isomers (0.2 g/L Growth Medium)

	cells grown with			
	cis-9,*trans*-11 CLA		*trans*-10,*cis*-12 CLA	
sn-position of CLA in TAG molecule	mg/g cell dry wt	%	mg/g cell dry wt	%
sn-1/3	17.8	44.5	4.8	56.0
sn-1 + 3	5.6	14.0	1.4	16.1
sn-1/3 + 2	4.9	12.2	0.8	9.2
sn-1 + 2 + 3	4.6	11.5	1.0	11.7
sn-2	2.5	6.2	0.4	4.9
none	4.7	11.6	0.2	2.1

[a] The analyzed TAG encompassed 42% of total TAG.

and natural source, could prove to be a useful dietary source of CLA. Moreover, it is noteworthy that as shown in the present study, the yeast TAG was composed of an abundance of different species where the *trans*-10,*cis*-12 isomer occurred in combination with MCFA. Structured TAGs consisting of MCFA and long chain fatty acids (LCFA) have been used as dietary lipids for groups suffering from malabsorption, and they are also considered as one of the potential compounds in preventing obesity (*26*, *27*). In addition, previous studies have shown that C10:0 and C12:0 have antimicrobial properties (*28*), which may also bring added value to the use of the yeast TAG.

ABBREVIATIONS USED

ACN, acyl carbon number; CLA, conjugated linoleic acid; DAG, diacylglycerol; DB, double bond; FFA, free fatty acid; LCFA, long chain fatty acid; MCFA, medium chain fatty acid; PL, polar lipid; TAG, triacylglycerol.

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