Fatty acid composition and regiodistribution in mare's milk triacylglycerols at different lactation stages

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ORIGINAL PAPER

Fatty acid composition and regiodistribution in mare's milk triacylglycerols at different lactation stages

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Abstract The composition and regiodistribution of fatty acids influence the physical and the nutritional properties of milk fat. The aim of this study was to determine the fatty acid composition and the triacylglycerols' (TAGs) structure of mare's milk collected on days 20, 40, and 150 postpartum. Differences in the composition of fatty acids occurred between lactation times. A higher proportion of saturated fatty acids, mainly capric ($C_{10:0}$) and lauric ($C_{12:0}$) acids, were detected on day 20 in lactation milk TAGs and long-chain unsaturated fatty acids detected on days 40 and 150 lactation milk TAGs. Mare's milk had interesting nutritional properties due to a high content in essential fatty acids (23.72%). Despite variations in the regiodistribution between lactation periods, fatty acids were generally distributed within the TAGs' molecules of mare's milk in a highly specific fashion: from 71.84% to 79.50% saturated fatty acids were mainly esterified in the *sn*-2 position while unsaturated fatty acids were always preferentially esterified in the *sn*-1 and *sn*-3 positions. These results suggest some degree of non-randomness in TAG's biosynthesis. The fatty acid composition, in particular the level of unsaturation and chain length appeared to be important selectivity factors in the distribution of fatty acids in TAGs' molecules.

不同泌乳期马乳三酰甘油的脂肪酸组成和区域分布

摘要 脂肪酸的组成和区域分布影响乳脂肪的物理和营养特性。本文研究了泌乳期分别是 20 天、40 天和 150 天马乳的脂肪酸组成和三酰甘油的结构。不同泌乳期马乳三酰甘油脂肪酸的组成存在差异。泌乳期 20 天马乳三酰甘油中含有较高含量的饱和脂肪酸，主要是十碳酸（C10:0）和十二碳酸（C12:0）。泌乳期 40 天和 150 天马乳三酰甘油中含有高含量的长链不饱和脂肪酸。由于马乳含有较高含量的必需脂肪酸（23.72%），因此有较好的营养特性。尽管不同泌乳期马乳脂肪酸的区域分布存在差异，但一般都分布在三酰甘油分子内部，而且是以高度立体专一的形式分布。71.84% 到 79.50% 的饱和脂肪酸主要在 sn-2 位发生酯化，然而不饱和脂肪酸经常优先选择在 sn-1 和 sn-3 位发生酯化。这些结果表明三酰甘油的合成在一定程度上并不是随意的。脂肪酸组成，特别是不饱和脂肪酸和长链脂肪酸组成是三酰甘油分子脂肪酸分布的很重要的选择性因素。

Keywords Mare's milk · Triacylglycerols (TAGs) · Stereospecific analysis · Regiodistribution

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关键词 马乳 · 三酰甘油 · 立体专一性分析 · 区域分布

1 Introduction

Dietary fat is a very important factor influencing human health; not only a reduction in fat intake would improve the quality of health but recommendations should focus on the quality of dietary lipids (Jensen 1999). For example, changes in the ratio of saturated (SFAs) to unsaturated fatty acids (UFAs) in favor of the latter is very important (Orlandi et al. 2003), particularly in the prevention of hyper-cholesterol and cardiovascular disease (Koletzko et al. 1998; Kubow 1996). The essential dietary fatty acid content is of interest as these fatty acids exert important biological functions; they are precursors of the long-chain polyunsaturated fatty acids (LC-PUFAs) which are indispensable structural components of all cellular membrane. Linoleic acid ($C_{18:2}$) is the precursor of prostaglandins and tromboxans which exert different influences on the circulatory system. Prostaglandins such as PGI are derived from linolenic acid ($C_{18:3}$) which have vasodilatory effects, tromboxans, such as TXA, with vasoconstrictive effects, and docosahexaenoic acid (DHA; $C_{22:6\Delta4}$) (Orlandi et al. 2003).

Triacylglycerols (TAGs) represent the most abundant lipid fraction and are the main source of fatty acids in dietary fat. The position at which each fatty acid is esterified to the glycerol backbone has important nutritional implications because it influences their bioavailability and incorporation; the *sn*-1,3 regiospecificity of pancreatic lipase induces preferential absorption of fatty acids in the *sn*-2 position because 2-monoacylglycerols require lower concentrations of bile salts to achieve emulsification into micellar form. However, the incorporation of free fatty acids into mixed micelles is variable; absorption of long-chain saturated fatty acids occurs less readily in comparison to shorter chain or more highly unsaturated fatty acids (Kubow 1996; Mu and Høy 2004). The intra-molecular structure of dietary TAGs also influences the metabolism of chylomicrons because the 2-monoacylglycerols provide the basic structure for the re-synthesis of chylomicron TAGs in the enterocytes (Bracco 1994; Mu and Høy 2004).

In this paper, particular attention has been attributed to mare's milk, as studies have shown that this milk is more similar to human milk in comparison to cow's milk (Pellizzola et al. 2006), and has been recommended for human nutrition and in pediatric diets for children who are allergic to cow's milk (Businco et al. 2000).

The lipid content of mare's milk has been analyzed by several authors (Csapo et al. 1995; Parodi 1983; Pikul and Wójtowsk 2008; Schweigert and Gottwald 1999). These studies have reported that the fat content is very low ranging from 0% to 7.9% (Csapo et al. 1995) but can be influenced by environmental and physiological conditions (Doreau et al. 1990; Doreau 1994). TAGs comprise 80% of the total fat composition of mare's milk (Malacarne et al. 2002) and 98% of the total lipids in both human and cow's milk (Jensen 1999, 2002). While the TAGs structure of human (Martin et al. 1993; Parodi 1982) and cow's (Blasi et al. 2008; Parodi 1982) milk has been exhaustively reviewed, published data on TAGs structure of mare's milk are very poor (Jamsranjav and Grigoreva 1973) with only one study dealing with this topic (Parodi 1982).

The aim of this study was to apply the techniques developed for regiodistribution analysis of TAGs to provide further data about the inter- and the intra-molecular

distribution of fatty acids in mare's milk TAGs and to check whether structural changes occurred during lactation.

2 Materials and methods

2.1 Samples and reagents

The experiment was carried out on milk obtained from five multiparous Italian Saddle mares. All the experimental animals were reared in the same environmental conditions and fed the same diet based on perennial ryegrass, lucerne, and lesser amounts of concentrate. Milk samples were collected separately for each subject on days 20, 40, and 150 postpartum. The samples were immediately lyophilized and stored at −20 °C for subsequent analysis.

Phospholipase A_2 (E.C. 3.1.1.4) from honey bee venom (1625 units · mg^{-1} protein) was obtained from Sigma–Aldrich (St. Louis, MO, USA). A Supelco (Bellefonte, PA, USA) standard solution containing a mixture of 37 fatty acid methyl esters was used for the identification of peaks and to calculate the molecular weight correction factors of the individual fatty acid peak areas. All solvents and reagents were of analytical grade and were purchased from Sigma–Aldrich.

2.2 Total lipid extraction

Total lipids were extracted according to the method of Folch et al. (1957). Briefly, 8 g of the lyophilized milk was homogenized with 180 mL of chloroform/methanol (2:1, *v/v*) and stirred overnight at room temperature. The homogenate was then filtered through Whatman GF/C filters (Whatman Ltd., Maidstone, England) and the solvent removed. The extracted lipid was washed with 200 mL of chloroform/methanol (1:1, *v/v*) followed by 90 mL of KCl (0.88%) solution. Once the phases separated, the aqueous layer was removed. The organic layer was dried and evaporated under vacuum, and the required lipid fraction was obtained.

2.3 Isolation and purification of triacylglycerols

For the separation of the lipid classes, preparative thin layer chromatography (TLC) was performed on 20×20 cm silica gel G plates (Merck, Darmstadt, Germany) with hexane/diethyl ether/formic acid (40:10:1 by vol) and chloroform/methanol/water (13:6:1 by vol) as solvent system to separate, respectively, the neutral and the polar lipid fractions. The TAGs (Rf=0.9) were visualized with 2′,7′-dichlorofluorescein, scraped off, extracted with diethyl ether/hexane (1:1, *v/v*), and used for subsequent stereospecific analysis.

2.4 Stereospecific analysis of triacylglycerols

The analysis procedure was reported in detail by Christie (1982) and required the preparation of partial glycerides α,β-diacylglycerols (DAGs) and α,α′-DAGs by Grignard degradation of TAGs (40 mg) using ethyl magnesium bromide (170 μL, 3 mol.L) in dry diethyl ether (2 mL) (Fig. 1). α,β-DAGs and α,α′-DAGs were

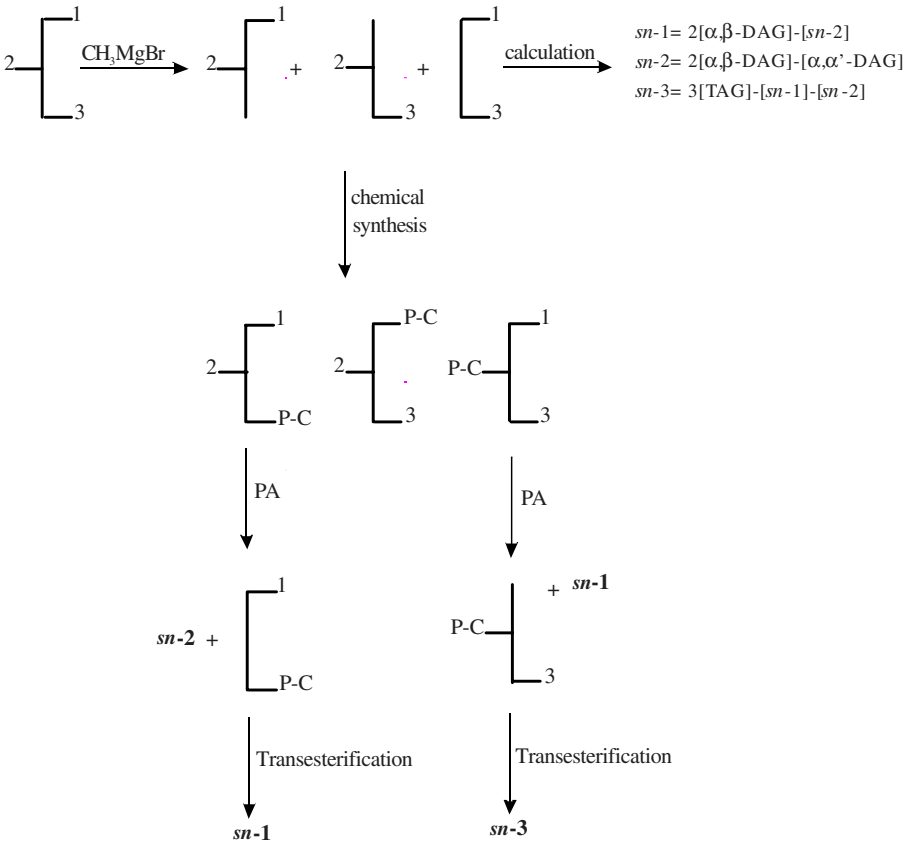


Fig. 1 Stereospecific analysis of triacylglycerols according to Christie (1982) and Brockerhoff (1967) methods. 1,2,3-Triacylglycerols were hydrolyzed by means of the Grignard reagent to α,β-DAGs and α,α′-DAGs, and converted to a phosphatidylcholines (*P-C*). The 1,2-diacyl-*sn*-glycero-3-phosphatidylcholines and 1,3-diacyl-*sn*-glycero-2-phosphatidylcholines were purified and resolved with the aid of the stereospecific enzyme phospholipase A_2 (*PA*)

separated by preparative TLC on boric acid (5%, *w/w*) pre-impregnated silica gel G layers in order to minimize isomerization. Then, DAGs were visualized with aqueous Rhodamine 6G (0.01%, *w/v*) and recovered from the TLC with diethyl ether.

Chemical synthesis of α,β-phosphatidylcholines was performed by the use of a mixture of chloroform/pyridine/phosphorous oxychloride (9.5:9.5:1, by vol, 0.65 mL) at 4 °C as described by Christie (1982). The 1,2-diacyl-*sn*-glycero-3-phosphatidylcholine, after being isolated and purified, was subjected to hydrolysis with 100 μL of phospholipase A_2 preparation (2.5 mg of Bee venom was dissolved in 0.5 mL Tris buffer solution at pH 8.9 containing 4 mmol.L^{-1} of calcium chloride) to obtain the *sn*-1-lysophosphatidylcholine and free fatty acids released from the *sn*-2 position. Analysis of the *sn*-3 position was performed according to Brockerhoff (1967) and as recently applied by Haddad et al. (2010). The α,α′-DAGs obtained by Grignard degradation were converted to 1,3-diacyl-*sn*-glycero-2-phosphatidylcholines from which phospholipase A_2 released the fatty acids in position *sn*-1 (Fig. 1). The remaining lysophosphatidylcholine was analyzed to yield the fatty acids of position

sn-3 of the original TAGs. The success of this analysis step was verified by adding *sn*-1 and *sn*-3 and comparing results with α,α′-DAGs composition. Lysophosphatidylcholine (Rf=0.22) and free fatty acids (Rf=0.86) were visualized with a molybdenum blue reagent and 2′,7′-dichlorofluorescein, respectively, and were recovered and extracted from the adsorbent for subsequent gas chromatography analysis.

Additionally, for a reliable and accurate determination of fatty acids regiodistribution, we have also investigated the fatty acid composition of partial glycerides obtained by the Grignard chemical deacylation to provide a check for all the measured positions by means of the following formulas:

$$sn{-}1(\%\text{mol}) = 2[\alpha,\beta - \text{DAGs}] - [sn{-}2] \quad (1)$$

$$sn{-}2(\%\,\text{mol}) = 2[\alpha,\beta - \text{DAGs}] - [\alpha,\alpha'{-}\text{DAGs}] \quad (2)$$

$$sn{-}3(\%\,\text{mol}) = 3[\text{TAGs}] - [sn{-}1] - [sn{-}2] \quad (3)$$

where [TAGs]=total percentage of fatty acid in intact TAGs, [α,α′-DAGs]=percent composition of fatty acid in α,α′-DAGs, and [α,β-DAGs]=percent composition of fatty acid in α,β-DAGs.

The inter-molecular distribution was obtained by normalizing to 100 the intra-molecular distribution in *sn*-1, *sn*-2, and *sn*-3 positions of each fatty acid applying the following formula:

$$\%\,sn{-}x = ([sn{-}x]/3[\text{TAGs}])100,$$

where [*sn*-*x*]=intra-molecular % of fatty acid esterified in *sn*-*x* position and [TAGs]= total percent composition of fatty acid in intact TAGs.

2.5 Fatty acid analyses

Fatty acid methyl esters were prepared by sodium methoxide-catalyzed transesterification as described by Christie (1982), while free fatty acids were methylated by treatment with an ethereal solution of diazomethane. Analysis of fatty acid methyl esters was carried out on a Carlo Erba Mega II series MFC 800 (Fisons Instruments, Milan, Italy) gas chromatograph equipped with a fused silica capillary column (SP-{2330}, 60 m×0.25 mm i.d., 0.2 μm film thickness; Supelco, Bellefonte, PA, USA). Helium was used as carrier gas at a linear velocity of 25cm.s^{-1}. The temperature program was 120 °C for 1 min and was then increased at a rate of 3 °C min^{-1} to the final temperature of 220 °C and a final hold time of 15 min. Injector and flame ionization detector temperatures were 250 °C. The injector was operated in split mode (split ratio 1:50). Fatty acid methyl esters' identification was confirmed by gas chromatography coupled to mass spectrometry (GC/MS) analysis using a Varian 3900 gas chromatograph (Palo Alto, CA, U.S.A.) equipped with a fused silica capillary column factor (30 m×0.25 mm i.d., 0.25 μm film thickness, Folsom, CA, USA) and coupled to a Saturn 2100 ion trap mass spectrometer. The temperature program of the gas chromatograph was the same as described above. The transfer line was set at 300 °C. Data were processed with a Varian MS-Workstation 6 software.

2.6 Statistical analysis

Mare's milk fatty acid composition was compared within the lactation stages by analysis of variance for P set at 0.01 and 0.05.

3 Results

3.1 Lipid composition

The average fat content in the milk of the experimental mare's milk was 2.10% (±0.17%), 2.05% (±0.10%), and 0.83% (±0.30%), respectively, on days 20, 40, and 150. The fatty acid composition of total TAGs, after the isolation of the neutral lipids of the studied milk by TLC, was determined by GC and GC/MS (Table 1). The main fatty acids in mare's milk TAGs were found to be caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), palmitic ($C_{16:0}$), oleic ($C_{18:1}$), linoleic ($C_{18:2}$), and linolenic ($C_{18:3}$) acids which constituted approximately 89.75% of the total fatty acid composition.

To compare the fatty acid composition relative to the considered lactation times, the percent fatty acid content grouped as saturated (SFAs), monounsaturated (MUFAs), and polyunsaturated (PUFAs) was reported in Table 1.

Overall, the saturated fatty acids constituted 56.56%; the proportion of medium chains ($C_{8:0}$–$C_{14:0}$) was 38.28% with $C_{10:0}$ (13.01%) and $C_{12:0}$ (10.99%) as the dominant ones, and the amount of long chains ($C_{15:0}$–$C_{20:0}$) was 18.28% primarily due to palmitic acid (16.72%).

The unsaturated fatty acid fraction made up approximately 42.85%, and was particularly rich in oleic (11.09%), linoleic (10.17%), and linolenic (13.55%) acids.

On average, the composition of fatty acids varied with lactation time: out of the 24 identified fatty acids, 17 showed statistically meaningful differences in their composition. Fatty acids that showed a highly significant variation at $P<0.01$ were, in particular, $C_{10:1}$, $C_{11:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:1}$, $C_{17:0}$, $C_{17:1}$, and $C_{20:4}$ (Table 1). Those which varied significantly at $P<0.05$ were $C_{10:0}$, $C_{12:0}$, $C_{12:1\Delta9}$, $C_{14:1}$, $C_{18:0}$, $C_{18:1}$, $C_{18:2}$, and $C_{18:3}$. No statistically significant differences in the composition of only seven fatty acids were observed between lactation periods, namely $C_{8:0}$, $C_{12:1\Delta7}$, iso$C_{15:0}$, $C_{16:0}$, $C_{20:0}$, $C_{20:1}$, and $C_{20:2}$.

As a consequence of these variations, the proportion of saturated fatty acids was observed to decrease with stage of lactation from 61.09% to 54.43% with a simultaneous progressive increase in the percentage of unsaturated fatty acids from 38.75% to 45.43%.

The ratios of unsaturated to saturated fatty acids as well as the ratios of essential fatty acids in mare's milk TAGs were compared between the lactation stages. On day 20 lactation milk TAGs, the UFAs/SFAs ratio was about 0.63 and increased to reach a mean value of 0.8 on days 40 and 150 of lactation.

On day 20 lactation milk TAGs, the $C_{18:2}/C_{18:3}$ ratio was 0.92, while on day 150 lactation milk TAGs, it was reduced to 0.85.

Table 1 Total fatty acid composition (% mol mean values±standard deviation, n=5) of mare's milk triacylglycerols

Fatty acids (% mol)	Lactation days			
	20	40	150	P value
$C_{8:0}$	6.75±1.39	6.10±0.91	7.23±0.15	ns
$C_{10:0}$	15.38±0.62	11.9±2.46	11.77±0.76	*
$C_{10:1}$	2.30±0.08	1.81±0.53	2.93±0.29	**
$C_{11:0}$	0.00±0.00	0.00±0.00	0.28±0.02	**
$C_{12:0}$	12.56±0.57	9.86±2.33	10.56±0.79	*
$C_{12:1\Delta7}$	0.19±0.02	0.24±0.19	0.32±0.04	ns
$C_{12:1\Delta9}$	0.14±0.03	0.10±0.14	0.27±0.04	*
$C_{14:0}$	8.74±0.52	7.56±0.64	6.17±0.36	**
$C_{14:1\Delta9c}$	0.59±0.05	0.60±0.06	0.64±0.04	*
$C_{15:0}$	0.23±0.04	0.24±0.01	0.47±0.02	**
$IsoC_{15:0}$	0.00±0.00	0.36±0.01	0.00±0.00	ns
$C_{16:0}$	16.04±0.58	17.09±2.00	17.05±0.16	ns
$C_{16:1\Delta7}$	0.14±0.02	0.26±0.04	0.19±0.02	**
$C_{16:1\Delta9c}$	3.20±0.09	4.48±0.15	3.11±0.09	**
$C_{17:0}$	0.15±0.02	0.18±0.02	0.32±0.01	**
$C_{17:1\Delta10c}$	0.17±0.03	0.25±0.05	0.49±0.02	**
$C_{18:0}$	1.13±0.17	0.89±0.20	0.58±0.04	*
$C_{18:1\Delta9c+\Delta11c}$	9.23±0.74	11.89±2.07	12.16±3.68	*
$C_{18:2\Delta9c,12c}$	10.66±0.40	8.36±1.84	11.51±1.11	*
$C_{20:0}$	0.11±0.09	0.00±0.00	0.00±0.00	ns
$C_{18:3\Delta9c,12c,15c}$	11.58±0.31	15.60±2.96	13.49±3.24	*
$C_{20:1\Delta11c}$	0.10±0.01	0.17±0.14	0.16±0.02	ns
$C_{20:2\ \Delta11c,14c}$	0.17±0.03	0.18±0.05	0.22±0.09	ns
$C_{20:4\Delta5c,8c,11c,14c}$	0.28±0.02	0.37±0.20	0.00±0.00	**
SFAs	61.09±1.26	54.18±3.42	54.43±0.02	
UFAs	38.75±0.76	44.31±3.21	45.49±1.90	
MUFAs	16.06±0.50	19.80±1.19	20.27±0.45	
PUFAs	22.69±0.68	24.51 ±2.17	25.22±1.45	
UFAs/SFAs	0.63	0.82	0.84	
$C_{18:2}/C_{18:3}$	0.92	0.54	0.85	

SFAs saturated fatty acids, *UFAs* unsaturated fatty acids, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids, *ns* not significant

*P<0.05; **P<0.01

3.2 Regiodistribution of fatty acids in mare's milk triacylglycerols

Under our experimental conditions, the three *sn*-positions were directly measured. The method presented is reliable for the detection of fatty acids down to a level of around 0.5%. Below this threshold, results must be interpreted with care.

Table 2 Intra-molecular distribution (% mol mean values±standard deviation, n=5) of fatty acids in mare's milk triacylglycerols

Lactation day	20			40			150		
FA (% mol)	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3	*sn*-1	*sn*-2	*sn*-3
$C_{8:0}$	3.94±0.65	8.43±1.31	7.57±0.68	3.55±0.12	9.35±0.08	5.38±1.10	2.17±0.04	14.53±0.73	3.44±1.02
$C_{10:0}$	12.44±0.80	17.40±2.74	15.98±0.88	5.97±1.33	16.38±0.91	13.03±2.48	5.86±0.30	19.33±0.39	6.05±0.37
$C_{10:1}$	1.22±0.55	2.80±0.44	2.90±0.28	0.65±0.29	1.27±0.12	3.54±0.34	0.43±0.18	0.75±0.19	0.00±0.00
$C_{11:0}$	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.69±0.07	0.00±0.00
$C_{12:0}$	17.39±1.12	17.51±0.68	2.72±0.44	8.40±1.02	9.07±0.26	12.01±0.98	8.08±0.10	13.42±0.22	8.22±0.07
$C_{12:1\Delta7}$	0.19±0.03	0.11±0.10	0.27±0.00	0.00±0.00	0.07±0.10	0.64±0.00	0.00±0.00	0.10±0.02	0.00±0.00
$C_{12:1\Delta9}$	0.13±0.04	0.07±0.00	0.22±0.00	0.00±0.00	0.14±0.20	0.15±0.00	0.00±0.00	0.03±0.04	0.00±0.00
$C_{14:0}$	10.95±0.99	12.09±1.20	3.16±1.83	6.88±0.05	9.51±0.33	6.29±0.14	4.46±0.29	9.21±0.49	4.19±0.21
$C_{14:1\Delta9c}$	0.65±0.13	0.56±0.17	0.56±0.56	0.26 ±0.11	0.54±0.02	0.55±0.29	0.31±0.05	0.30±0.01	0.26±0.13
$C_{15:0}$	0.24±0.04	0.37±0.04	0.08±0.08	0.20±0.05	0.44±0.01	0.09±0.03	0.39±0.01	0.51±0.01	0.32±0.24
$IsoC_{15:0}$	0.00±0.00	0.00±0.00	0.00±0.00	0.05±0.07	0.05±0.01	1.02±0.13	0.00±0.00	0.00±0.00	0.00±0.00
$C_{16:0}$	15.85±3.11	22.35±2.84	9.92±2.90	16.73 ±1.76	26.30±6.06	8.27±6.06	15.26±0.17	16.64±0.49	16.06±0.27
$C_{16:1\Delta7c}$	0.23±0.06	0.08±0.07	0.12±0.12	0.33±0.02	0.22±0.03	0.24±0.07	0.18±0.02	0.13±0.03	0.20±0.29
$C_{16:1\Delta9c}$	3.19±0.24	2.79±0.23	3.63±0.52	4.23±0.06	4.30±0.12	4.95±0.34	3.33±0.07	2.29±0.06	2.68±0.20
$C_{17:0}$	0.21±0.03	0.13±0.03	0.11±0.02	0.28±0.02	0.20±0.03	0.05±0.04	0.31±0.06	0.29±0.00	0.47±0.05
$C_{17:1\Delta10c}$	0.26±0.13	0.12±0.02	0.30±0.07	0.26±0.00	0.29±0.15	0.22±0.18	0.66±0.02	0.30±0.00	0.53±0.24
$C_{18:0}$	1.43±0.12	1.22±0.16	0.78 ±0.13	2.52±0.51	0.54±0.09	0.00 ±0.00	1.39±0.13	0.33±0.06	0.00±0.00
$C_{18:1\ \Delta9c\ +\ \Delta11c}$	9.13±1.17	3.20±0.60	15.55±5.35	18.20±2.41	5.33±0.29	12.32±3.43	21.27±1.15	5.34±0.26	21.49±1.02
$C_{18:2\Delta9c,\ 12c}$	9.21±0.74	5.39±0.76	17.63±2.75	9.16±0.05	5.65±0.32	10.42±0.33	14.16±0.62	6.79±0.38	13.23±3.93
$C_{18:3\Delta9c,12c,15c}$	12.95±1.28	5.23±0.87	16.74±0.36	21.65±1.70	8.64±0.63	16.71±0.05	21.18±0.62	8.83±0.14	22.27±2.52
$C_{20:1\Delta11c}$	0.06±0.05	0.00±0.00	0.24±0.08	0.00±0.00	0.00±0.00	0.52±0.08	0.22±0.00	0.09±0.13	0.00±0.00
$C_{20:2\Delta11c,14c}$	0.08±0.07	0.00 ±0.00	0.44±0.06	0.19±0.02	0.00±0.00	0.37±0.00	0.23±0.09	0.10±0.04	0.28±0.08

$C_{20:4\Delta 5c,8c,11c,14c}$	0.13±0.11	0.00±0.00	0.71±0.00	0.39±0.00	0.00±0.00	0.74±0.00	0.00±0.00	0.00±0.00	0.00±0.00
SFAs	62.45±3.61	79.50±5.19	40.32±6.89	44.58±0.06	71.84±1.55	46.14±1.60	37.92±0.07	74.95±1.10	38.75±1.38
UFAs	37.43±3.68	20.35±5.11	59.31±6.87	55.32±0.06	26.45±1.10	51.37±2.10	61.97±0.30	25.05 ±1.13	60.94±2.07
MUFAs	15.06±1.65	9.73±1.25	23.79±3.69	23.93±1.69	12.16±0.96	23.13±0.30	26.40±1.04	9.33±0.61	25.16±0.56
PUFAs	22.37±1.28	10.62±4.04	35.52±3.19	31.39±1.43	14.29±2.27	28.24±0.05	35.57±1.85	15.72±1.33	35.78±1.50

FA fatty acid, *SFAs* saturated fatty acids, *UFAs* unsaturated fatty acids, *MUFAs* monounsaturated fatty acids, *PUFAs* polyunsaturated fatty acids

The mean value and the standard deviation, expressed as percent mole, and time changes of the intra-molecular distribution of fatty acids in mare's milk TAGs are given in Table 2. At the first stage of lactation, the *sn*-1 position was esterified predominantly by $C_{12:0}$ (17.39%), $C_{16:0}$ (15.85%), $C_{18:3}$ (12.95%), and $C_{10:0}$ (12.44%). The *sn*-2 position was esterified primarily with $C_{16:0}$ (22.35%), $C_{12:0}$ (17.51%), $C_{10:0}$ (17.40%), and $C_{14:0}$ (12.09%). The *sn*-3 position was principally composed of $C_{18:2}$ (17.63%), $C_{18:3}$ (16.74%), $C_{10:0}$ (15.98%), and $C_{18:1}$ (15.55%). With the lengthening of the lactation time, variations in the regiodistribution of fatty acids were observed. In comparison with the intra-molecular distribution seen on day 20 of lactation, the *sn*-1 and *sn*-3 positions appeared to be the main sites in which changes had occurred. The *sn*-1 position was composed of $C_{18:3}$ (21.65% and 21.18%), $C_{18:1}$ (18.20% and 21.27%), $C_{16:0}$ (16.73% and 15.26%), and $C_{18:2}$ (9.16% and 14.16%) on days 40 and 150 lactation milk TAGs, respectively. The *sn*-3 position was principally esterified by $C_{18:3}$ (16.71%), $C_{10:0}$ (13.03%), $C_{18:1}$ (12.32%), and $C_{12:0}$ (12.01%) on day 40 lactation milk TAGs, whereas on day 150 lactation milk TAGs, it was occupied mainly by $C_{18:3}$ (22.27%), $C_{18:1}$ (21.49%), $C_{16:0}$ (16.06%), and $C_{18:2}$ (13.23%).

Inter-molecular fatty acid composition data reported in Fig. 2 were useful to better understand the specificity of distribution of each fatty acid in the three glycerol backbone positions and to better understand the nutritional properties of the considered fat. According to the mathematical rules of randomness, a fatty acid will not repeat itself in a TAG until it exceeds 33.33% (continuous baseline in Fig. 2) of total fatty acids.

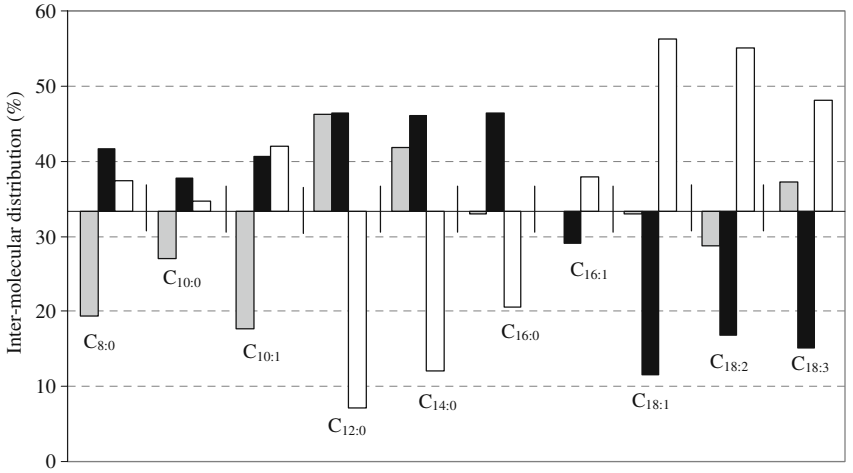
Caprylic ($C_{8:0}$), capric ($C_{10:0}$), lauric ($C_{12:0}$), myristic ($C_{14:0}$), and palmitic ($C_{16:0}$), acids were preferably esterified in the *sn*-2 position, except on day 40 lactation milk TAGs where there was a little more $C_{12:0}$ at the *sn*-3 position than at the *sn*-2 position. Approximately 75% of saturated fatty acids were acylated in the *sn*-2 position with substantial amounts also being found in position *sn*-1 on day 20 lactation milk TAGs. Palmitoleic acid ($C_{16:1}$) was almost evenly distributed between the three *sn*-positions. However, C_{18} unsaturated fatty acids showed selectivity for the *sn*-1/3 positions; on average, only 10.59% of $C_{18:1}$, 21.9% of $C_{18:2}$, and 19% of $C_{18:3}$ were located in the *sn*-2 position, with the remaining being preferentially distributed on the *sn*-3 position at the beginning of the lactation period with a tendency for the *sn*-1/3 positions afterwards.

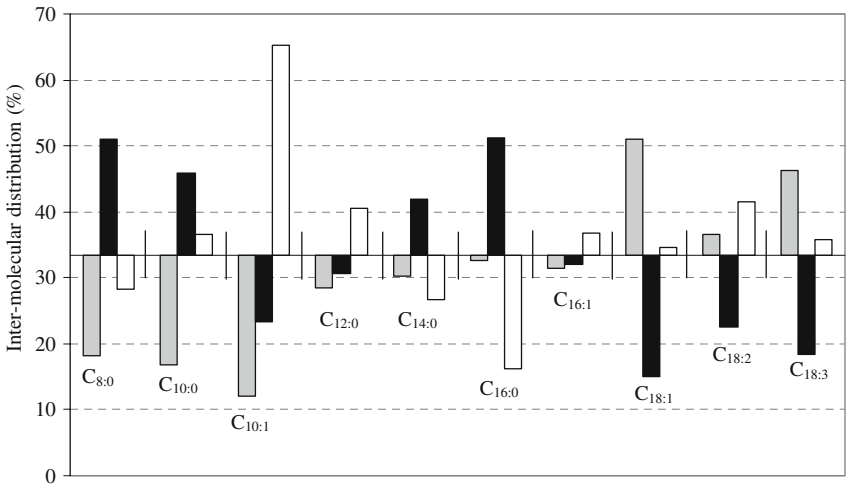
4 Discussion

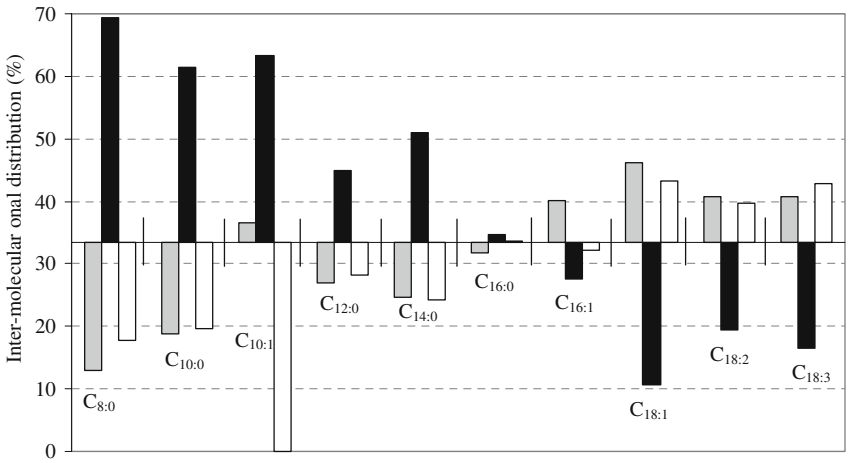
4.1 Lipid composition

The average lipid content was very low in mare's milk in comparison to that of human (2.04–4.87%) and cows (2–5%) (Jensen 1999, 2002), and had reduced to half of its initial value during the lactation period. These results agree with data in the

Fig. 2 Inter-molecular distribution (% mol mean values±standard deviation, $n=5$) of fatty acids in the *sn*-1 position (*gray bar*), *sn*-2 position (*black bar*) and *sn*-3 position (*empty bar*) of mare's milk triacylglycerols at lactation day 20 (*top*), lactation day 40 (*middle*), and lactation day 150 (*bottom*). On the basis of a random regiodistribution, 33.33% (1/3) represents the proportion of each fatty acid expected


Inter-molecular distribution (%)
60
50
40
30
20
10
0
C8:0
C10:0
C10:1
C12:0
C14:0
C16:0
C16:1
C18:1
C18:2
C18:3


Inter-molecular distribution (%)
70
60
50
40
30
20
10
0
C8:0
C10:0
C10:1
C12:0
C14:0
C16:0
C16:1
C18:1
C18:2
C18:3


Inter-molecular onal distribution (%)
70
60
50
40
30
20
10
0
C8:0
C10:0
C10:1
C12:0
C14:0
C16:0
C16:1
C18:1
C18:2
C18:3

literature considering that, although variations from one trial to another, the fat content in mare's milk is an inverse function of the lactation stage (Pikul and Wójtowsk 2008).

Results of fatty acid composition of these analyses were in agreement with previous findings. Exceptions were palmitic acid ($C_{16:0}$) and oleic acid ($C_{18:1}$) which were lower and caprylic acid ($C_{8:0}$) which was higher than in the literature data (Csapo et al. 1995; Malacarne et al. 2002; Pikul and Wójtowsk 2008).

The UFAs/SFAs ratios were considerably lower than the results reported by Orlandi et al. (2003) and Pikul and Wójtowsk (2008) for Haflinger and Wielkopolska mares milk respectively, which can mainly be attributed to the twice lower percentage of oleic acid ($C_{18:1}$) found in our study.

The proportion of $C_{18:2}$ and $C_{18:3}$ varied significantly ($P<0.05$) in the milk obtained between lactation stages. A nonlinear decrease in the $C_{18:2}/C_{18:3}$ ratios was observed between the lactation periods, and these ratios ranged from 0.92 (day 20 postpartum) to 0.85 (day 150 postpartum). These ratios were considerably lower than those reported in the abovementioned experiments as a result of the lower content of $C_{18:3}$ and the higher percent of $C_{18:2}$ recorded by authors.

The stage of lactation was the unique variation factor taken into account in this study despite the role of region, season, species, and feeding on the fatty acid composition. Earlier studies (Orlandi et al. 2003; Pikul and Wójtowsk 2008) showed that the lactation period did not show any significant effect on the percentage rate of some fatty acids such as palmitic acid. Other authors (Csapo et al. 1995; Doreau 1994) have identified the physiological stage as the most important factor influencing mare's milk fatty acid composition and deduced that it was strongly related to the seasonal changes on feeding quality. According to Doreau (1994), the milk from mares fed on a diet rich in forage compared with milk from mares fed on a diet rich in concentrate had higher fat, protein, and $C_{18:3}$ and lower $C_{18:2}$ concentration.

Conjugated linoleic acid (CLA) was not detected in milk samples, in accordance with recent investigations (Jahreis et al. 1999; Pikul and Wójtowsk 2008), where only traces (0.09%) were observed. This can be explained by a dietary origin of CLA in monogastric milk (Hoffman et al. 1998), whereas in ruminant milk CLA originates from biohydrogenation (Jahreis et al. 1999).

Malacarne et al. (2002), who studied the chemical composition specificity of mare's milk, suggested that it could be more suitable than cow's milk for human consumers. When comparing the fatty acid composition of mare's milk fat with human and cow's, it can be stated that on average the saturated fatty acid content was similar in mare's and human (Malacarne et al. 2002) milk (56.5% versus 54.2%) but lower in mare's than in cow's milk (62.6%). Mare's milk had the highest content in medium-chain saturated fatty acids ($C_{8:0}$–$C_{14:0}$) (38.28%) and the lowest in long-chain saturated fatty acids ($C_{15:0}$–$C_{20:0}$) (18.28%) compared to human (14.17% and 28.18%, respectively) (Jensen 1999; Malacarne et al. 2002) and cow's milk (21.16% and 36.75% respectively) (Jensen 2002).

The unsaturated fatty acids made up on average 42.85% and 45.20% of total fatty acids in mare's and human (Malacarne et al. 2002) milk fat, respectively, while cow's milk fat was found to be less unsaturated (27.34%) (Malacarne et al. 2002). The unsaturated fraction exhibited an interesting profile in mare's milk fat, which was found to be more polyunsaturated than human and cow's milk fat since it

contained only 0.33 and 0.43 times as much oleic acid as human and cow's milk fat, respectively. It is also characterized by a higher percentage of essential fatty acids $C_{18:2}$ (10.17%) and $C_{18:3}$ (13.55%) than in human (6.40% and 1.70%, respectively) and in cow's (2.9% and 1.1% respectively) milk fat (Malacarne et al. 2002).

Linoleic ($C_{18:2}$) and linolenic ($C_{18:3}$) acids are required in human nutrition because they cannot be produced by the human body and could be extremely important for formula-fed infants as conversion of $C_{18:2}$ and $C_{18:3}$ acids by desaturation and chain elongation in long-chain polyunsaturated fatty acids (Pikul and Wójtowsk 2008) by microsomes from infant liver is possible. In this way, the low content (around 0.32%) of arachidonic acid (AA; $C_{20:4\Delta5}$) especially the absence of eicosapentaenoic acid (EPA; $C_{20:\Delta5}$) and docosahexaenoic acid (DHA; $C_{22:6\Delta4}$) observed in the studied mare's milk samples (Table 1) might not be a problem for child nutrition.

It should be noted, however, that this conversion occurred at considerably lower rates and generally is limited. Thus, the observed low level of these fatty acids as well as the low conversion rate of $C_{18:2}$ and $C_{18:3}$ acids to EPA and DHA need to be taken into consideration in cases when mare's milk is offered to children (Pikul and Wójtowsk 2008).

4.2 Regiodistribution of fatty acids in mare's milk triacylglycerols

There are relatively few research papers describing the TAGs structure of mare's milk, particularly no study has yet focused on the effect of the lactation stage before this survey. Different trends in the distribution of fatty acids have been recorded during lactation in the considered milk TAGs, even if generally 71% to 79% of saturated fatty acids were esterified in the *sn*-2 position and unsaturated fatty acids occupied mainly the *sn*-1/3 positions. This partial conservation of fatty acids regiodistribution could indicate that the maturation of the mammary gland does not affect the general pattern of fatty acids stereospecific acylation in the TAGs' molecular species. These results suggest some degree of non-randomness in the distribution of fatty acids in mare's milk TAGs' molecules. It is likely that the observed modifications in the regiodistribution were linked to the variations in fatty acids composition during the lactation times.

It has also been reported that the regiodistribution of fatty acids among the three *sn*-positions of human (Jensen 1999; Martin et al. 1993; Straarup et al. 2006) and bovine (Blasi et al. 2008; Parodi 1982) milk TAGs is non-random. Data from Canadian and from French women milks showed that the distributions are unique within the three major fatty acids: oleic acid being preferentially distributed in the *sn*-1 position, palmitic acid in *sn*-2 position, and linoleic acid in *sn*-3 position. In cow's milk TAGs, $C_{4:0}$ and $C_{6:0}$ fatty acids were esterified almost exclusively in the *sn*-3 position. $C_{8:0}$, $C_{10:0}$, $C_{12:0}$, and $C_{14:0}$ fatty acids were located preferentially in the *sn*-2 position and sometimes also in the *sn*-3 position; $C_{16:0}$ was almost equally distributed between the *sn*-1 and *sn*-2 positions, and $C_{18:0}$ was esterified selectively in the *sn*-1 position. $C_{18:1}$ usually was distributed roughly equally between the *sn*-1 and *sn*-3 positions.

Parodi (1983) observed variations in regiodistribution of fatty acids with changing TAGs' composition in early postpartum bovine milk. However, with varying TAGs' fatty acid content, their regiodistribution in the three *sn*-positions did not change in a

unique manner. For $C_{18:1}$, $C_{16:0}$, and $C_{14:0}$ acids, the change in content in the *sn*-3 position was more pronounced than in the *sn*-1 and *sn*-2 positions. The change in content of $C_{18:0}$ was most marked in the *sn*-1 position, and changes in $C_{12:0}$ and $C_{10:0}$ were more pronounced in the *sn*-2 position.

Factors which may influence the specific fatty acid distribution pattern in milk TAGs include: acyl-CoA concentrations, activity as well as specificity of acyltransferase, and the biochemical pathways used for TAGs synthesis. Animal studies (bovine and murine models) have demonstrated that the fatty acid specificities of mammary *sn*-glycerol-3-phosphate acyltransferases and 1-acyl-*sn*-glycerol-3-phosphate acyltransferases are related to the fatty acid composition at the *sn*-1 and *sn*-2 positions of milk TAGs (Parodi 1982). It can be assumed that the specificities of other acyltransferases will also vary among animals, and this may explain why a particular fatty acid was preferentially esterified in a given position in mare's milk TAGs.

On the whole, the regiodistribution of fatty acids observed in the present study is in accordance with the finding of other authors (Parodi 1982). There are, however, discrepancies that may be accounted for in part to differences between the experimental design; Parodi (1982) determined the regiodistribution of fatty acids based mainly on pancreatic lipase hydrolysis and on partial glycerides formulas, whereas this study directly analyzed all the three *sn*-positions.

According to Christie (1982) and Turon et al. (2003), the Grignard degradation is the most reliable method in regiospecific analysis of TAGs, and the formed α,β-DAGs and α,α′-DAGs are considered as most representative of the original TAGs composition, despite some unavoidable acyl migration that always occurs during chemical deacylation. However, the conventional method of pancreatic lipase degradation, in which the *sn*-1 and *sn*-3 positions fatty acids are cleaved allowing the determination of fatty acids in the *sn*-2 position, is plagued with problems derived from the non-randomness of enzyme action due to its specificity for short- and medium-chain fatty acids (C_{12} or less) in TAGs (Turon et al. 2003).

Additionally, in view of a proven variability in the fatty acid composition of mare's milk TAGs ascribed to external and internal factors (diet, breed, and physiological stage), we could consequently expect that their regiodistribution on the glycerol backbone to be also affected: if compared to our results, a lower percentage of medium-chain saturated fatty acids, $C_{8:0}$ (2.3%), $C_{10:0}$ (3.6%), $C_{12:0}$ (4.3%), and $C_{14:0}$ (5%) and a greater amount of $C_{18:3}$ (29.8%) were determined in mare's milk samples analyzed by Parodi (1982).

From a nutritional point of view, the proportions of saturated fatty acids in the *sn*-2 position can be of importance as studies assessing the influence of TAGs structure on fat absorption in human milk suggested that after hydrolysis of the *sn*-1 and *sn*-3 positions by lingual and pancreatic lipases, much of the remaining 2-monoacylglycerols of fatty acids, particularly of $C_{14:0}$ and $C_{16:0}$, are more readily absorbed by neonate than free fatty acids (Filer et al. 1969; Mu and Høy 2004; Tomarelli et al. 1968). In addition, mare's milk has a relevant nutritional importance because it represents an important dietary source of essential fatty acids. In particular, $C_{18:2}$ of the ω6 group and $C_{18:3}$ of the ω3 group, both precursors of long-chain polyunsaturated fatty acids, were located in the *sn*-2 position at concentrations (13.50%) higher than reported in human (9.25%) (Martin et al. 1993) and in cow's milk fat (2.10%) (Blasi et al. 2008).

5 Conclusion

Mare's milk TAGs contain a large variety of different fatty acids including a considerable proportion of polyunsaturated fatty acids. This investigation is useful to better understand the way these fatty acids are esterified in the TAGs glycerol backbone and so to predict the physical and nutritional properties of the considered milk fat. Differences in fatty acid composition and regiodistribution were demonstrated in mare's milk obtained on days 20, 40, and 150 postpartum.

The results of this study support the use of mare's milk for human nutrition; in spite of its lower fat content, mare's milk is very interesting for the lower proportion of saturated fatty acids compared to cow's milk (Blasi et al. 2008) and its higher amount of essential fatty acids. Particularly, the percentage of saturated and essential fatty acids in the *sn*-2 position would be favorable for their absorption and incorporation.

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