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ORIGINAL ARTICLE

A comparative study of the composition of triacylglycerol molecular species in equine and human milks

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Abstract The composition of triacylglycerols (TAGs) is a very useful parameter to understand several properties of creams and anhydrous milk fat products such as nutritional value, technological applications, and textural properties. In this paper, the molecular species of TAGs of equine milk were identified using a combination of non-aqueous reversed phase high-performance liquid chromatography, electrospray ionization mass spectrometry and high temperature gas chromatography and were then compared to the TAGs composition of human milk. The data showed that equine milk contained a complex mixture of different TAGs molecular species resulting from the widest range of its fatty acids as compared to human milk. In particular, the largest differences in TAGs composition between equine and human milk concerned TAGs containing medium-chain fatty acids; for C_8, C_{10}, and C_{12}. In terms of carbon number (CN), TAGs with CN36–CN46 were the most abundant in equine milk with a maximum concentration at CN44 (15.3%), while TAGs with CN44–CN52 were the predominant in human milk with a maximum of CN52 (30%). For both milks studied, the experimentally determined distributions of TAGs molecular species deviated from those predicted by the random hypothesis. TAGs with CN44 corresponding to a fatty acids carbon number distribution of $C_8/C_{18}/C_{18}$, $C_{12}/C_{14}/C_{18}$, and $C_{10}/C_{16}/C_{18}$ were apparently preferentially synthesized in equine milk. Whereas, in human milk, mixed TAGs with CN52 containing $C_{16}/C_{18}/C_{18}$ fatty acids were much higher than would be expected from the random calculation.

马乳和人乳中三酰基甘油的分子组成

摘要 奶油和无水乳脂肪产品的性质取决于三酰基甘油(TAGs)的分子组成，同时TAGs的分子组成也决定了乳脂肪营养价值、加工特性和质构特性。本文采用非水反相液相色谱法 (NARP–HPLC) 、电喷雾离子质谱(ESI–MS3)和高温气相色谱(GC)法分析了马奶和人奶中TAGs的组成 与人奶相比，马奶TAGs分子中脂肪酸组成非常复杂，两者在脂肪酸组成上显著的不同，

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特别是在中链脂肪酸C8、C10和C12上差异较大。用CN表示碳原子数，马奶TAGs中CN36–CN46最为丰富，CN44的最大浓度达到15.3%，而在人奶TAGs中CN44–CN52占主导地位，CN52最大浓度达到30%。根据脂肪酸随机分布假说来预测TAGs分子中脂肪酸的分布，马奶中具有CN44的TAGs所对应的脂肪酸碳原子分布主要为C8/C18/C18、C12/C14/C18和C10/C16/C18。而在人奶中，含有C16/C18/C18脂肪酸分布的CN52在TAGs中所占的比例比随机计算值要高。

Keywords Equine milk · Human milk · Triacylglycerols · NARP–HPLC · GC · ESI–MS/MS

关键词 马奶 · 人奶 · 三酰基甘油 · 非水反相液相色谱法 · 气相色谱 · 电喷雾离子质谱

Abbreviations

APCI	Atmospheric pressure chemical ionization
Ca	$C_{10:0}$
CN	Carbon number
Co	$C_{10:1}$
Cy	$C_{8:0}$
DB	Double bond
DG	Diacylglycerol
ECN	Equivalent carbon number
ESI	Electrospray ionization
FA	Fatty acid
FID	Flame ionization detector
GC	Gas chromatography
L	$C_{18:2}$
La	$C_{12:0}$
LCT	Long chain triacylglycerol
Ln	$C_{18:3}$
M	$C_{14:0}$
MCT	Medium chain triacylglycerol
Mo	$C_{14:1}$
MS	Mass spectrometry
MUFA	Monounsaturated fatty acid
MW	Molecular weight
NARP–HPLC	Non-aqueous reversed phase high performance liquid chromatography
O	$C_{18:1}$
P	$C_{16:0}$
Po	$C_{16:1}$
PUFA	Polyunsaturated fatty acid
S	$C_{18:0}$
SFA	Saturated fatty acid
TAG	Triacylglycerol

1 Introduction

Recently, research interest and capital investment have increased in the use of equine milk for feeding young infants affected by severe IgE-mediated bovine

milk allergy, as several studies have shown that the chemical composition of equine milk and in particular its protein content are close to that of human milk (Businco et al. 2000). However, the literature on the composition of triacylglycerols (TAGs) from equine milk is very limited with relatively few published papers dealing with this topic (Breckenridge and Kuksis 1967; Haddad et al. 2011; Parodi 1982).

Analysis of TAGs constitutes a challenge for the analyst due to the large number of TAGs molecular species that can be derived from the common, naturally occurring fatty acyl groups found in biological sources. Consequently, more effort has focused on the separation and determination of molecular species of TAGs than to any other single-lipid class (Lipp 1995).

Knowledge of the composition of human milk lipids, in terms of TAGs, has been fundamental in order to improve the quality of infant-fed formula, as most clinical studies have shown that human milk fat is better absorbed, particularly in premature and newborn infants (Straarup et al. 2006; Tomarelli et al. 1968) due to its TAGs structure. Hence, various researcher groups have employed complementary techniques to separate human milk TAGs prior to the actual analysis. Data from Dotson et al. (1992) obtained using high-performance liquid chromatography (HPLC) separation of the TAGs and determination of the fatty acids in the fractions by gas chromatography (GC), identified 27 different TAGs molecular species in which LPP (15.03%), PPO (13.64%), SSL (8.46%), and LSO (6.55%) were the most abundant. Winter et al. (1993) also utilized HPLC separation of milk TAGs from German women, but with a silver ion column and light-scattering detector. Further fractionation and analyses resulted in the determination of 170 TAGs; 22 species were found in quantities of 1% or more, the predominant species being: POO (11.8%), POL (10%), and PPO (4.4%). Currie and Kallio (1993) analyzed milk TAGs by ammonia negative ion tandem mass spectrometry which resulted in the identification of 59 TAGs. In the earlier studies of Morera et al. (2000, 2003), using HPLC with light-scattering detector and atmospheric pressure chemical ionization mass spectrometry (APCI–MS), 43 TAGs molecular species were identified in milk from Spanish women which were mainly composed of POO (27.24%) and POL (16.65%).

To characterize and compare the composition of TAGs molecular species of equine and human milks, a prefractionation technique followed by molecular species identification by means of electrospray ionization mass spectrometry (ESI–MS^3) and gas chromatographic quantification were employed. Results were then compared with the values calculated on the basis of the random distribution of fatty acids in the TAGs molecules.

2 Materials and methods

2.1 Samples and reagents

Eleven milk samples were obtained from healthy multiparous Italian Saddle mares. Samples of mature human milk (produced from day 15 after delivery) were collected at the University Hospital of Marche, Italy from eight Italian women volunteers with no pregnancy complications. The diets of the donors were unrestricted omnivorous

diets and the women were 29±3.5 years old. Milk samples were collected separately for each subject by manual expression at two different times of the day (morning and afternoon). All samples were immediately lyophilized and stored at −20 °C for subsequent analysis.

A Supelco (Bellefonte, PA, USA) standard solution containing a mixture of 37 fatty acids methyl esters was used for the identification of peaks and for the calculation of the molecular weight correction factors of the individual fatty acid peak areas. The response factors of TAGs were determined using standard solutions of TAGs with carbon number (CN) ranging from 30 to 54. TAGs standards as well as solvents and reagents were of analytical grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2 Isolation and purification of triacylglycerols

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, UK) 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.

For the separation of the lipid classes and the purification of the TAGs fraction, preparative thin layer chromatography was performed on Silica gel G plates (20×20 cm, 0.25 mm thickness Merck, Darmstadt, Germany) as described by Haddad et al. (2011).

2.3 NARP–HPLC analysis of triacylglycerols

The NARP–HPLC system used for TAGs separation was a Varian Prostar 330 HPLC system (Palo Alto, CA, USA) equipped with a ternary gradient pump and a photodiode array detector. Two columns (Rstek Ultra C18, 250×4.6 mm i.d., 5 μm particle size, Bellefonte, PA, USA) and a guard column (4×2 mm) were used in series thermostated at 30 °C. The best resolution was observed under the improved conditions reported by Dugo et al. (2004). A gradient of acetone/acetonitrile (65:35, *v*/*v*) in isocratic mode with a flow rate of 1 $mL.min^{-1}$ for 60 min and an injection volume of 20 μL were applied. The acquisition wavelength was 210 nm.

2.4 ESI–MS^3 analysis of triacylglycerols

TAGs ammonium adducts were prepared as described by McAnoy et al. (2005). Each TAG fraction collected by preparative NARP–HPLC was diluted in 0.5 mL of chloroform/methanol (1:1, *v*/*v*) and 25 μL aqueous NH_4OAc (10 mM) was then added. Pure TAGs standards were prepared in an identical solvent system at a concentration of 10 ppm. ESI–MS analysis was carried out using a Thermo-Finnigan LCQDuo mass spectrometer (Thermo Finnigan, San Jose, CA, USA) equipped with

an electrospray ionization source. The fractions were introduced into the ESI source directly via a drawn microcapillary at a rate of 1 $mL.min^{-1}$. The mass spectrometer was operated in positive ESI mode. Typical experimental conditions were as follows; source voltage, 2.53 kV; sheath gas flow rate, 49.66; aux gas flow rate, 9.13; capillary temperature, 235 °C; capillary voltage, 20.27 V; source fragmentation collision energy, 10 V, maximum ion inject time, 200 ms.

2.5 GC and GC–MS analysis of fatty acid methyl esters

Methyl esters of the fatty acids contained in total milk TAGs as well as in the TAGs fractions (which were separated and isolated by preparative NARP–HPLC) were prepared by sodium methoxide-catalyzed transesterification as described by Christie (1982). 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 the carrier gas at a linear velocity of 25 $cm.s^{-1}$. 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 (FID) temperatures were 250 °C. The injector was operated in split mode (split ratio 50:1). 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, USA) equipped with a fused silica capillary column (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 using the Varian MS–Workstation 6 software package.

2.6 GC analysis of triacylglycerols

Characterization of the composition of total milk TAGs as well as of TAGs fractions (which were separated and isolated by preparative NARP–HPLC) according to the total CN and double bond (DB) was carried out on a CP-9002 gas chromatograph (Chrompack, Middelburg, Netherland) equipped with an Rtx-65TG column (30 m×0.25 mm i.d., 0.1 μm film thickness, Restek, Bellefonte, PA, USA) of fused silica coated with a 65% diphenyl and 35% dimethyl polysiloxane. Helium was used as carrier gas at a linear velocity of 35 $cm.s^{-1}$. The temperature program was 240 °C for 5 min and was then increased at a rate of 4 $°C.min^{-1}$ to the final temperature of 350 °C and a final hold time of 25 min. Injector and FID temperatures were 360 °C. The injector was operated in split mode (split ratio 50:1).

2.7 Determination of fatty acids distribution

The proportions of the determined TAGs species made up of fatty acids ranging from C_6 to C_{22} obtained in this study were compared with the values calculated on the

basis of the random distribution of these fatty acids in the TAGs molecules according to Bailey (1951). The formulas used for calculation were as follows:

$$\%\ \text{TAG aaa} = (A \times A \times A) \times 1/10,000$$
$$\%\ \text{TAG aab} = (A \times A \times B) \times 3/10,000$$
$$\%\ \text{TAG abc} = (A \times B \times C) \times 6 = 10,000$$

where A, B, and C are the molar percentages of the fatty acids a, b and c.

3 Results and discussion

3.1 Identification of triacylglycerols by NARP–HPLC–ESI–MS^3

Total TAGs were separated according to the equivalent carbon number (ECN), defined as the total CN in the fatty acid acyl chains minus twice the number of DB per molecule (ECN=CN−2 DB), through the application of NARP–HPLC technique. Chromatograms showing these separations are illustrated in Fig. 1. The TAGs fractions that were obtained by NARP–HPLC were recovered separately and

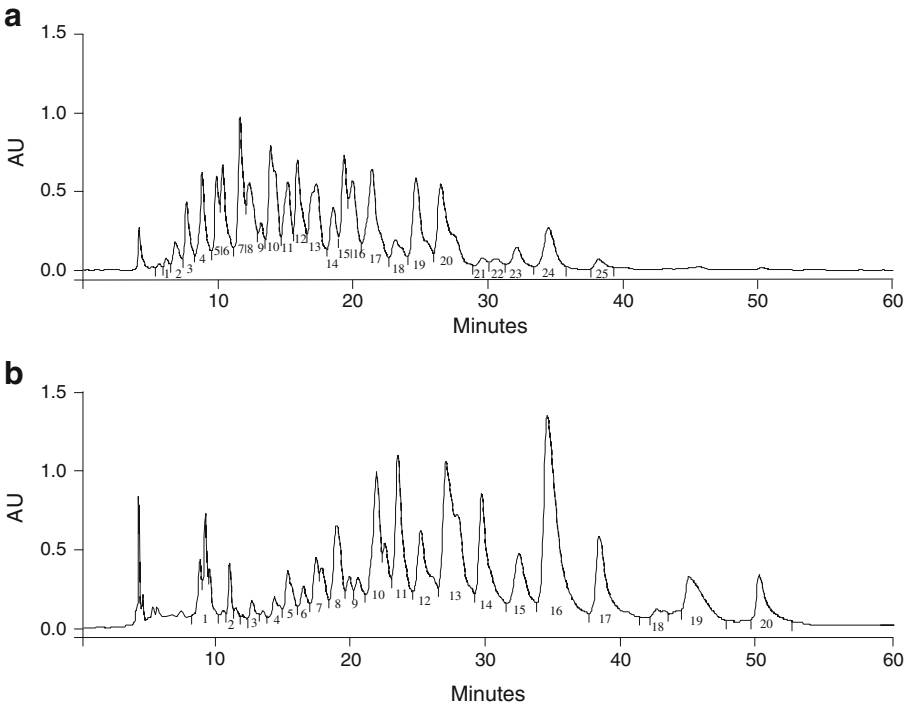

Fig. 1 NARP–HPLC–UV separation of equine (**a**) and human (**b**) milk triacylglycerols using two columns in series (Restek Ultra C18, 250 mm×4.6 mm i.d., 5 μm) at 30 °C with a mobile phase of acetone/acetonitrile (65:35, v/v^{-1}) 1, fraction 1; 2, fraction 2; 3 fraction 3; 4, fraction 4; 5, fraction 5; 6, fraction 6; 7, fraction 7; 8, fraction 8; 9, fraction 9; 10, fraction 10; 11, fraction 11; 12, fraction 12; 13, fraction 13; 14, fraction 14; 15, fraction 15; 16, fraction 16; 17, fraction 17; 18, fraction 18; 19, fraction 19; 20, fraction 20; 21, fraction 21; 22, fraction 22; 23, fraction 23; 24, fraction 24; 25, fraction 25

their constituent fatty acids were determined by gas chromatography. Data recorded from fraction 10 of human milk TAGs are used as an example to explain how TAGs molecular species were identified. More than three fatty acids were detected in the same chromatographic peak (Table 1) indicating that TAGs had co-eluted. This was not surprising as it has previously been documented (Dorschel 2002; Mottram and Evershed 2001) that components with the same ECN value, known as "critical pairs", are linked to problems in their separation by HPLC. Additionally, co-elution probably occurred as long chain saturated components caused the effective ECN to be increased as previously reported (Dorschel 2002; Mottram and Evershed 2001).

All the TAGs fractions previously separated by NARP–HPLC were subjected to ESI–MS^3 analysis. Figure 2 shows the ESI–MS/MS spectrum of fraction 10 from human milk TAGs. The main ions obtained in the mass spectrum corresponded to ammoniated TAGs. Ions corresponding to $[M+Na]^+$ and $[M+H]^+$ were observed at low levels. The $[M+NH_4]^+$ ions in Fig. 2 revealed the complexity of the TAG fraction examined which included a mixture of species with ECN42. In this collision spectrum, the loss of neutral carboxylic acid (RCOOH) and NH_3 from the TAGs ammonium adducts was observed as previously reported (Marzilli et al. 2003). So, for each $[M+NH_4]^+$ molecular adduct, three distinct diacyl product ions could be determined. All the resulting diacyl fragment ions $[M+NH_4\text{-}NH_3\text{-}RCOOH]^+$ were then selected and collided. Additional spectral data generated by MS^3 experiments (Fig. 2) shown the same types of ions as described by Marzilli et al. (2003): $[RCO]^+$, $[RCO\text{-}H_2O]^+$, and $[RCO+74]^+$ were obtained and fatty acid composition of each diacyl product ion was determined. This latter information was very useful to ascertain the identities of the TAGs, in particular when more than one TAG were identified from the diacyl fragment ion. The identified fatty acyl substituent $[RCO]^+$ was then assembled for each diacyl $[DG]^+$ mass and the TAGs molecular species were determined by combining the acyl chains present for each $[M+NH_4]^+$ such that the total CN and DB were consistent with the molecular weight of the TAG.

Table 1 shows the results of ESI–MS^3 analysis of fraction 10 from human milk. First, all possible TAGs molecular species were theoretically calculated based on possible combinations of its fatty acids data. Secondly, the $[M+NH_4]^+$ and $[M+Na]^+$ results were used to calculate the molecular weight (MW) of individual TAGs species present in this fraction. Then, the total CN and DB were determined using expected fatty acyl groups. For example, ion mass at m/z792.5 corresponded to TAGs with a MW of 774.6 containing 46 CN and two DB, which indicated the possible presence of CaSL, CaOO, LaPL, LaPoO, MML, and MPoPo molecular species. The three expected diacyl product ions $[DG]^+$ were calculated for each TAG probably present and then compared with the diacyl fragment ions effectively present as identified by ESI–MS/MS. The diacyl ions obtained in fraction 10 (Table 1) revealed the absence of the fragment at m/z491.4 that would be derived from the loss of SL diacyl product if CaSL TAG type was present. In addition, the MS^3 collision of the diacyl fragments ions at m/z 575.5, m/z 521.4, and m/z 547.3 gave rise to P, L, O; O, La, M; and M, L, S, respectively, while Po was not detected (Table 1). Consequently, the presence of CaSL, LaPoO, and MPoPo TAGs was excluded and only CaOO, LaPL, and MML were identified as the possible TAGs species corresponding to the mass ion at m/z792.5.

Table 1 Possible TAGs molecular species present in fraction 10 of human milk consistent with fatty acid composition (% mol ± standard deviation) and results of ESI–MS^3 analysis

FA[a]	%mol±SD	$[M+NH_4]^{+b}$ (m/z)	$[M+Na]^{+c}$ (m/z)	MW[d]	Possible TAGs [e]	$[DG]^{+f}$ (m/z)	$[DG]^{+g}$ (m/z)	$[RCO]^{+h}$ (m/z)	TAGs[i] (ECN)
Ca	5.61±0.122	766.4	771.6	748.6	CaPO	467/577/493	467.5	155 (Ca), 183 (La), 211 (M), 239 (P)	LaMO (42)
					CaPoS	465/577/495	493.4	265 (O), 155 (Ca) 237 (Po), 265 (O), 183 (La), 211 (M)	CaPO (42)
La	18.97±0.386				LaMO	467/549/521	495.4	183 (La), 239 (P) 211 (M), 155 (Ca), 267 (S)	
					LaPPo	495/549/493	519.4	263 (L), 183 (La)	
M	8.9±0.025				MMPo	495/521/521	521.4	265 (O), 183 (La), 211 (M)	
		792.5	797.6	774.6	CaSL	495/603/491	523.3	183 (La), 211 (M), 239 (P), 267 (S), 265 (O)	CaOO (42)
P	7.24±0.079				CaOO	493/603/493	547.3	211 (M), 263 (L), 267 (S)	LaPL (42)
					LaPL	495/575/519	549.4	211 (M), 265 (O), 183 (La)	MML(42)
Po	2.37±0.05				LaPoO	493/575/521	575.4	239 (P), 263 (L), 265 (O)	
					MML	495/547/547	577.5	239 (P), 265 (O), 267 (S)	
S	1.16±0.110				MPoPo	521/547/521	601.4	265 (O), 263 (L)	
		818.4	823.6	800.6	LaSLn	523/601/517	603.5	265 (O)	LaOL (42)
O	22.45±1.231				LaOL	521/601/519			
					MPLn	523/573/545			
L	29.74±2.525				MPoL	521/573/547			
					PoPoPo	547			
Ln	3.56±0.202		849.6	826.6	MOLn	549/599/545			MLL (42)
					MLL	547/599/547			
					PPoLn	549/571/573			
					PoPoL	547/573/573			

Table 1 (continued)

FA[a]	%mol±SD	$[M+NH_4]^{+b}$ (m/z)	$[M+Na]^{+c}$ (m/z)	MW[d]	Possible TAGs [e]	$[DG]^{+f}$ (m/z)	$[DG]^{+g}$ (m/z)	$[RCO]^{+h}$ (m/z)	TAGs[i] (ECN)
			875.6	852.6	PoLL	573/599/573			PoLL (42)
					PoOLn	575/599/571			
					PLLn	575/597/573			

Underlined $[RCO]^+$ ions are present at lower levels and are estimated based on their relative abundances

[a] *FA* Fatty acid; Ca: $C_{10:0}$, La: $C_{12:0}$, M: $C_{14:0}$, P: $C_{16:0}$, Po: $C_{16:1}$, S: $C_{18:0}$, O: $C_{18:1}$, L: $C_{18:2}$, Ln: $C_{18:3}$

[b] $[M+NH_4]^+$: ammoniated TAG ions

[c] $[M+Na]^+$: TAG sodium adduct ions

[d] MW: molecular weight determined based on $[M+NH_4]^+$ and $[M+Na]^+$ results

[e] Possible TAGs: determined using expected fatty acyl groups according to the identified MW

[f] $[DG]^+$: expected diacyl product ions calculated for each possible TAG and given in the following order: 1,2-$[DG]^+$/2,3-$[DG]^+$ / 1,3-$[DG]^+$

[g] $[DG]^+$: diacyl fragment ions resulting from ESI-MS/MS analyses

[h] $[RCO]^+$: fatty acyl substituent resulting from the MS^3 analyses of the $[DG]^+$ ions

[i] Effective TAGs molecular species present in the fraction

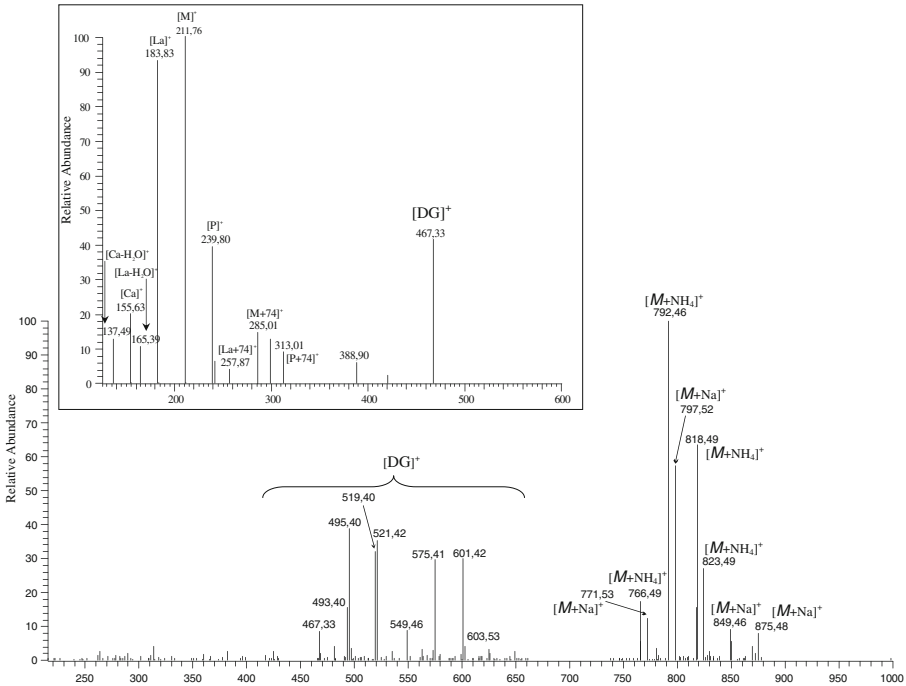

Fig. 2 Electrospray ionization tandem MS/MS (ESI–MS/MS) spectrum of ammoniated triacylglycerols of fraction 10 obtained by NARP–HPLC separation of human milk. *Inset* shows the MS^3 spectrum of the diacyl fragment $[DG]^+$ ion with *m/z* 467.33. $[M+NH_4]^+$, ammoniated TAG ions; $[M+Na]^+$, TAG sodium adduct ions; $[DG]^+$, diacyl fragment ion; Ca, $C_{10:0}$; La, $C_{12:0}$; M, $C_{14:0}$; P, $C_{16:0}$

Based on ESI–MS^3 data, 180 and 98 TAGs molecular species were identified respectively in equine and human milks (Table 2), even when the MW observed could be comprised of other different species possibly present in milk fat of both species as several minor TAGs including some odd number TAGs and very long chain polyunsaturated fatty acids were not studied.

For peak assignment and quantification, the TAGs were separated according to their CN and their DB by GC analysis (Fig. 3); molecular species previously identified by ESI–MS^3 in each fraction were correlated with those in the chromatogram of the whole milk fat by their retention time as shown in Fig. 3. The best separation of TAGs was achieved by GC because it avoids the problem of the solubility of saturated TAGs in HPLC solvents (Moya et al. 2000) and even when a complete chromatographic separation between critical pairs of TAGs was not achieved, the proportions of $[DG]^+$ and $[RCO]^+$ ions were investigated to estimate the major TAGs in order of abundances when it was possible, because this was related to the number of TAGs that had co-eluted in the analyzed fraction.

3.2 Triacylglycerols composition determined by GC

The TAGs molecular species identified in human and equine milk constituting 99.51% and 99.66%, respectively, of the total composition, were expressed as molar percentage grouped according to their CN and DB and are reported in Table 2 and Fig. 4.

Table 2 Triacylglycerols molecular species composition (% mol±standard deviation) in human and equine milk determined by GC

	Human milk			Equine milk		
CN:DB	Triacylglycerols	Mean	SD	Triacylglycerols	Mean	SD
26:0				CyCyCa	0.118	0.038
26:1				CyCyCo	0.034	0.025
28:0				CyCaCa/CyCyLa	0.649	0.054
28:1				CyCaCo	0.130	0.028
30:0				CyCaLa	1.657	0.509
				CaCaCa	0.344	0.111
30:1				CyCoLa/CaCaCo	0.583	0.138
30:2				CaCoCo	0.029	0.012
32:0				CyCaM	1.539	0.627
				CyLaLa	1.213	0.341
				CyCyP	0.339	0.086
32:1				CaCoLa	0.821	0.120
				CyCoM/CyCaMo	0.248	0.037
				CyCyPo	0.244	0.053
32:2				CoCoLa	0.280	0.115
32:3				CCyLn	0.036	0.017
34:0	CaLaLa	0.124	0.026	**CaLaLa**/**CyCaP**/CyLaM/CaCaM	3.367	1.266
34:1				CoLaLa/CyCoP/CyCaPo/CoCaM	1.846	0.131
34:2				**CyCyL**/CaCoMo/CoCoM	0.168	0.170
34:3				CyCyLn	0.261	0.141
36:0	CyLaP/CaLaM/CaCaP/LaLaLa	0.324	0.183	CyLaP/**CaLaM**/**CaCaP**/CyMM	3.489	1.296
36:1				**CyCaO**/CoCaP/CaCaPo	2.579	0.174
36:2	CyCaL	0.126	0.090	**CyCaL**/CoCaPo/CyCoO	1.551	0.370
36:3	CyCaLn	0.093	0.097	CyCaLn	0.489	0.291
36:4				CyCoLn	0.133	0.037
38:0	**LaLaM**/CaMM/CaLaP/CaCaS	1.075	0.588	LaLaM/CaMM/**CaLaP**/CaCaS/**CyMP**	3.333	1.183
38:1	CyLaO/CaCaO	0.117	0.005	CyLaO/**CaCaO**/CyMPo/CoLaP	3.115	0.187
38:2				**CaCaL**/CyLaL/CaCoO	2.214	0.593
38:3				**CaCaLn**/**CyLaLn**/CaCoL	1.184	0.411
40:0	**LaLaP**/**LaMM**/CaMP	1.753	0.809	**LaLaP**/LaMM/**CaMP**/CyPP/CaLaS	2.848	0.926
40:1	**CaLaO**/LaLaPo	0.501	0.058	**CaLaO**/CyMO/CyPPo/CaMPo	3.416	0.395
40:2	CaLaL	0.084	0.045	**CaLaL**/CoMPo/CoLaO	0.934	0.072
	CyML	0.036	0.012	CyML	1.333	0.551
40:3				**CaLaLn**/CyMLn/CoLaL	1.205	0.470

Table 2 (continued)

	Human milk			Equine milk		
CN:DB	Triacylglycerols	Mean	SD	Triacylglycerols	Mean	SD
40:4				CoLaLn	1.363	1.280
42:0	**LaMP**/CaMS/CaPP/LaLaS/MMM	2.402	0.801	**LaMP**/CaMS/**CaPP**/MMM	1.842	0.702
42:1	**LaLaO**/CaMO	1.742	0.174	LaLaO/CaMO	1.430	0.566
	CyPO/**LaMPo**	0.494	0.170	**CyPO**/LaMPo/CaPPo	2.914	0.448
42:2	**LaLaL**/CaML	0.294	0.154	**LaLaL**/CaML/CyPL	4.202	1.132
42:3				CyPLn/LaLaLn/**CaMLn**/CoPL	2.254	0.669
42:4				CyPoLn	0.326	0.167
44:0	**LaPP**/MMP/LaMS	3.120	0.438	**LaPP**/MMP/CaPS	0.775	0.372
44:1	**LaMO**/CaPO	5.162	0.108	**LaMO**/**CaPO**/MMPo	3.495	0.615
	LaPPo	0.176	0.034	LaPPo	0.148	0.018
44:2	**CaPL**/**LaML**/CaPoO/CyOO	1.142	0.196	**CaPL**/**LaML**/CoPO/CyOO/CaPoO	4.525	0.916
44:3				**CaPLn**/LaMLn/CyOL/CaPoL	4.263	0.176
44:4				**CyOLn**/CyLL/CoPLn/**CaPoLn**	1.383	0.561
44:5				CyLLn	0.751	0.197
46:0	LaPS	2.305	0.352	LaPS/**MPP**	0.265	0.182
46:1	**LaPO**/MMO/MPPo	8.078	0.351	**LaPO**/MMO/MPPo	2.033	0.395
46:2	**LaPL**/CaOO/MML/LaPoO	2.401	0.424	**LaPL**/CaOO/MML/LaPoO	2.931	0.469
46:3	CaOL/LaPoL	0.239	0.133	CaOL/LaPoL/**LaPLn**	2.998	0.634
46:4	LaPoLn	0.130	0.030	LaPoLn/CyLL/CaOLn	1.596	0.680
46:5				CaLLn	0.417	0.078
48:0	MPS	1.909	0.542	MPS	0.417	0.338
48:1	**MPO**/PPPo/LaSO	6.088	0.707	**MPO**/PPPo	1.175	0.495
48:2	**LaOO**/MPoO	2.221	0.315	LaOO/MPoO	0.636	0.210
	MPL/LaSL	1.845	0.121	MPL	1.238	0.138
48:3	LaOL	1.197	0.278	LaOL/**MPLn**/LaSLn	1.112	0.501
48:4	LaLL	0.389	0.149	LaLL/**LaOLn**/MPoLn	0.502	0.378
48:5	LaLLn	0.415	0.256	LaLLn	0.610	0.618
50:0	PPS	1.178	0.528	PPS	0.154	0.113
50:1	**PPO**/MSO	9.790	1.963	**PPO**/MSO	0.801	0.229
50:2	**MOO**/**PPL**/MSL/PPoO	7.449	2.009	MOO/**PPL**/PPoO	2.193	0.445
50:3	MOL/PPoL	1.498	0.433	MOL/PPoL/**PPLn**	0.701	0.325
50:4	MOLn/PPoLn	0.170	0.047	**MOLn**/PPoLn/MLL	0.613	0.511
50:5	MLLn/MoOLn	0.293	0.015			
52:0	PSS	0.618	0.182	PSS	0.005	0.001
52:1	PSO	3.059	0.563	PSO	0.264	0.173
52:2	PSL/PoSO	2.460	0.414			

Table 2 (continued)

	Human milk			Equine milk		
CN:DB	Triacylglycerols	Mean	SD	Triacylglycerols	Mean	SD
	POO	13.587	4.260	POO	1.112	0.529
52:3	**POL**/PoOO	3.043	1.006	**POL**/PSLn	0.316	0.058
52:4	**PLL**/PoOL/POLn	7.067	1.765	PLL/PoOL/**POLn**	2.794	0.376
52:5	PoLL	0.214	0.125	PoLL/**PLLn**/PoOLn	2.219	0.520
54:0	SSS	0.004	0.003	SSS	0.047	0.061
54:1	SOS	0.057	0.077	SOS	0.069	0.073
54:2	SOO	0.624	0.238	SOO	0.065	0.010
54:3	OOO/SOL	1.019	0.655	OOO/SOL	0.206	0.155
54:4	OLO/SLL/SOLn	0.411	0.155	OLO/SLL/SOLn	0.248	0.122
54:5	OLL	0.368	0.161	OLL/OOLn/SLLn	0.481	0.072
54:6	OLLn/LLL	0.621	0.452	OLLn/LLL	0.038	0.019
54:7				OLnLn/LLLn	0.009	0.007
SATAGs		14.812	0.829		22.401	0.446
MUTAGs		35.264	0.552		25.345	0.248
DUTAGs		32.269	1.101		23.411	0.343
PUTAGs		17.167	0.464		28.508	0.421

CN carbon number, *DB* double bond, *SATAGs* saturated TAGs, *MUTAGs* monunsaturated TAGs, *DUTAGs* diunsaturated TAGs, *PUTAGs* polyunsaturated TAGs, *Cy* $C_{8:0}$, *Ca* $C_{10:0}$, *Co* $C_{10:1}$, *La* $C_{12:0}$, *M* $C_{14:0}$, *Mo* $C_{14:1}$, *P* $C_{16:0}$, *Po* $C_{16:1}$, *S* $C_{18:0}$, *O* $C_{18:1}$, *L* $C_{18:2}$, *Ln* $C_{18:3}$

The order of the abbreviations (e.g., POL) does not mean the binding position of each fatty acid

Triacylglycerols species indicated with bold type font are the most abundant and are estimated based on the relative abundances of the $[DG]^+$ and $[RCO]^+$ ions

First, it is important to highlight that the distribution of TAGs within the CN in human milk reported in this survey is mainly in agreement with the findings of Currie and Kallio (1993) obtained by APCI–MS analysis. According to Currie and Kallio (1993), the major CN species of Australian human milk were, in decreasing order of abundances, CN52 (23%), CN50 (16%), CN48 (16%), CN46 (16%), CN44 (11%), CN42 (7%), CN54 (5%), CN40 (5%), and CN38 (3%). They pointed out that the most abundant TAGs was POO which represented about 10% of all TAGs and constitutes 13.58% of all TAGs in the present study. The amounts of several TAGs are also within the ranges found in the study of Kurvinen et al. (2002), who determined 83% of the total TAGs composition in milk from Finnish women. These later identified as main species CN52 (42.9%), CN50 (15%), CN54 (14.8%), CN48 (7.4%), and CN46 (3%). The list of the most abundant TAGs CN species identified herein showed, however, a different order of relative abundance from the results of Dotson et al. (1992) and Morera et al. (2000, 2003). This discrepancy can be attributed to differences between the experimental designs, e.g., Currie and Kallio (1993) suggested that there is an underestimation of the abundances of the higher MW TAGs by the ammonia negative ion chemical ionization mass spectrometry method. Dotson et al. (1992) assessed the composition of human milk TAGs from

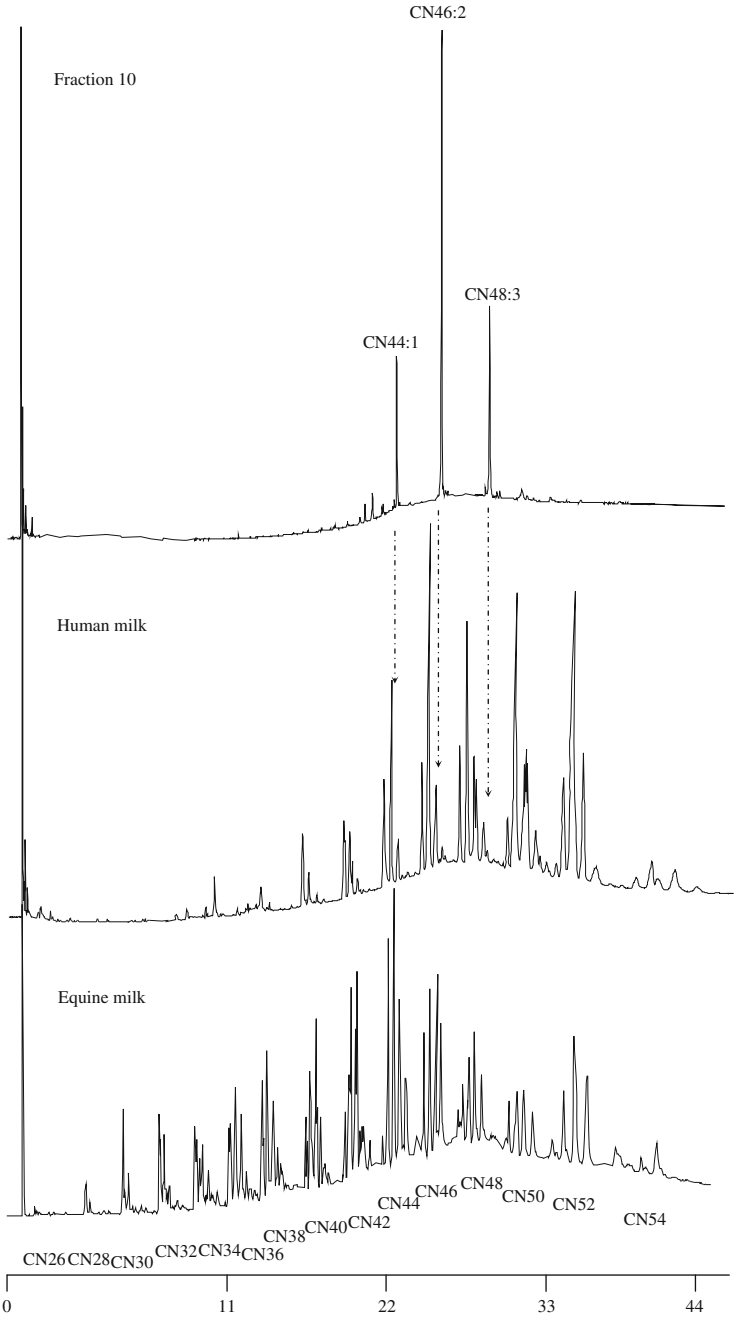

Fig. 3 Correlation of the GC peaks of fraction 10 of human milk TAGs, previously separated by NARP–HPLC and identified by ESI–MS^3, with those in the chromatogram of the whole milk fat through retention time

data obtained on a single HPLC column, although a greater resolution was achieved when using two columns in series. Some natural factors such as randomization in human milk, the physiological stage (Dotson et al. 1992; Morera et al. 2003), and

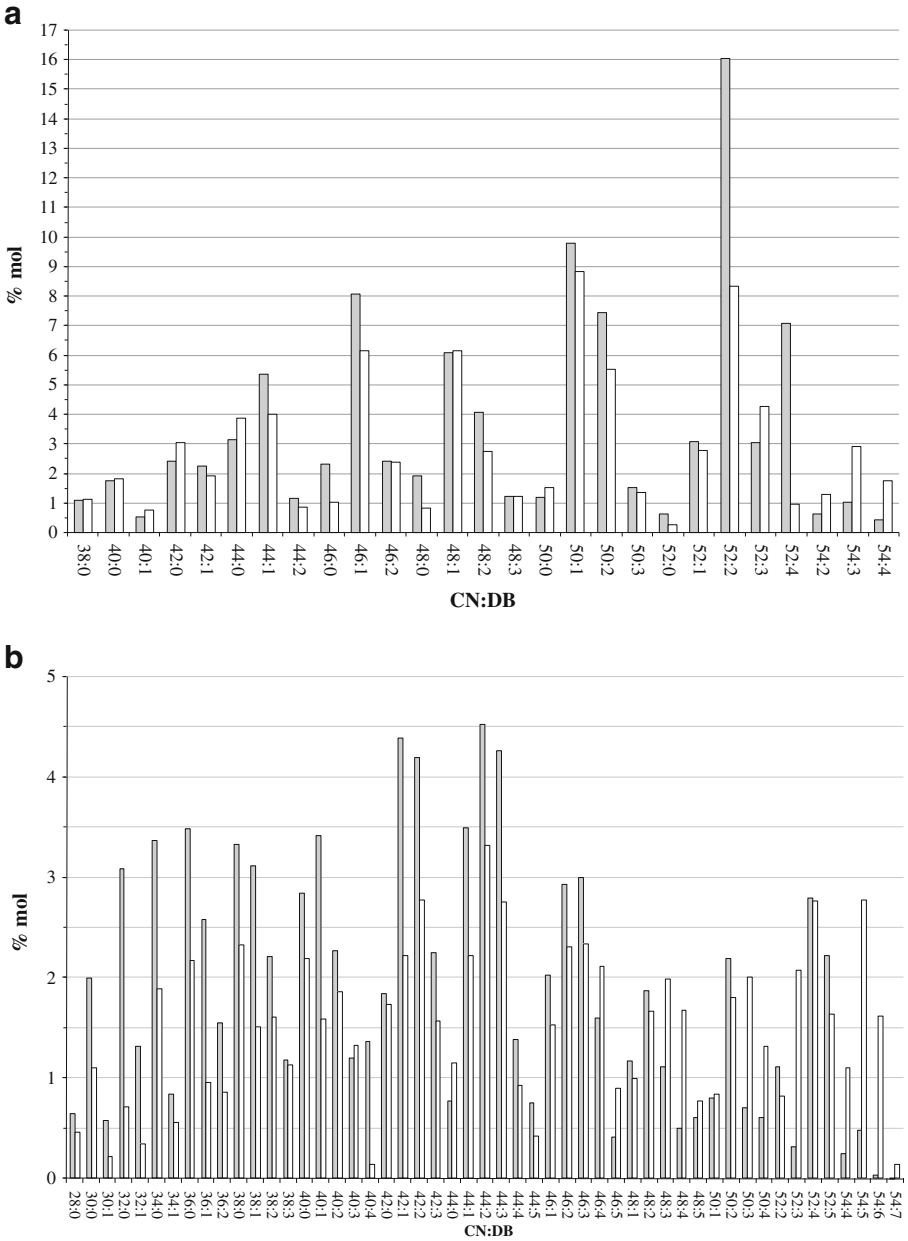

Fig. 4 Experimental (*shaded bar*) and random (*empty bar*) distributions of triacylglycerols in human (**a**) and equine (**b**) milks according to the acyl carbon numbers (CN) and double bonds (DB)

mainly the susceptibility of human milk TAGs to dietary habits, the first external factor which would be expected to affect TAGs structure (Jensen 1999) may account for these differences.

When comparing the structure of human and equine milk TAGs (Table 2 and Fig. 4), a remarkable difference in the location of the minimum and the maximum

acyl carbon and in the composition of TAGs molecular species were evident. The distribution pattern of TAGs according to the number of carbon atoms followed a typical unimodal distribution for each milk; TAGs with CN36–CN46 were the most abundant with a maximum concentration at CN44 (15.3%) in equine milk fat, whereas TAGs with CN44–CN52 were the predominant ones in human milk with a maximum at CN52 (30%). While the TAGs fraction from equine milk contained a considerable amounts of species with CN26–CN38, data were shown from CN34 for human milk (Table 2) because no TAG was identified in the groups of CN22–CN32 due to its lower level in $C_{8:0}$ (0.41%) and $C_{10:0}$ (2.97%) fatty acids and to the absence of short chain ($C_{4:0}$–$C_{6:0}$) fatty acids.

Interestingly, this study revealed that out of the total TAGs identified, 76.7% in equine milk and 84.9% in human milk resulted from the combination of both saturated and unsaturated fatty acids. Monoene and diene TAGs were the most abundant fractions and were present in higher concentrations in human milk (35.2% and 32.2%, respectively) compared to equine milk (25.3% and 23.4%, respectively). Conversely, the level of the saturated and polyene TAGs in human milk (14.8% and 17.16%, respectively) were much lower than the levels in equine milk (22.4% and 28.5%, respectively).

In equine milk, the saturated TAGs fraction was mainly comprised of medium chain saturated fatty acids, which from the nutritional standpoint, undergo preferential intestinal absorption versus long chain fatty acids (Mu and Høy 2004). Carnielli et al. (1996) reported that a large number of very low birth weight infant are fed formulas containing coconut oil or oils with medium-chain TAGs (MCTs) as a preferred source of saturated fat because MCTs promote intestinal absorption of fat and calcium. Recent investigations (Lai and Chen 2000) on a relatively homogeneous group of young children undergoing major gastrointestinal surgery showed a positive effect of medium chain TAGs/long chain TAGs (MCTs/LCTs) mixes with regard to liver function and nitrogen balance when compared to LCTs alone. Fats were better used and protein was spared in this group. The study strongly suggests that parenteral nutrition with lipid infusions containing 50% MCTs in their lipid content is safe in pediatric surgical patients.

Equine milk has considerably higher level of polyunsaturated fatty acids (PUFAs) as compared to bovine milk. Consequently, the percentage of TAGs containing at least one PUFAs in bovine milk is about 1%, whereas in *n*-3 enriched bovine milk it increases to 3.5% (Gastaldi et al. 2011). Although strategies for increasing the content of *n*-3 fatty acids in milk by incorporating *n*-3 fatty acids in the bovine diet has been applied (Gastaldi et al. 2011), the PUFA content still considerably below that of equine milk.

When analyzing the individual TAGs species reported in Table 2, it can be stated that unlike human milk, which contained five major species (LaPO, MPO, PPO, POO, and PLL) which represent approximately 50% of the total TAGs, no really predominant TAGs were synthesized by the equine mammary gland. There were three CN/DB classes of TAGs in equine milk, each of which represented more than 4% of the total TAGs (CN/DB, 42:2; CN/DB, 44:2; and CN/DB, 44:3), six CN/DB classes of TAGs accounted for more than 3% each (CN/DB, 34:0; CN/DB, 36:0; CN/DB, 38:0; CN/DB, 38:1; CN/DB, 40:1; and CN/DB, 44:1), 11 CN/DB classes of TAGs constituted about 2% each and 27.9% of the total composition, whereas 38.4% of TAGs were present in proportions lower than 2%.

Breckenridge and Kuksis (1967) anticipated that equine milk would contain significant amounts of TAGs of types $C_{10}/C_{16}/C_{16}$ and $C_{10}/C_{16}/C_{18}$, which was borne out by the CN42 and CN44 TAGs in its GC elution pattern. Results of this survey demonstrated that TAGs with CN42 and CN44 containing one medium chain fatty acid (C_8–C_{12}) per molecule occurred at highest level, in particular $C_8/C_{16}/C_{18}$, $C_{12}/C_{12}/C_{18}$, $C_{10}/C_{14}/C_{18}$ and $C_{10}/C_{16}/C_{16}$ were the predominant TAGs species in the groups of CN42 and $C_8/C_{18}/C_{18}$, $C_{12}/C_{14}/C_{18}$ and $C_{10}/C_{16}/C_{18}$ were the major TAGs in the groups of CN44.

3.3 Fatty acids distribution in triacylglycerols

In order to compare the experimental and theoretical distributions of fatty acids in TAGs for equine and human milks, the content of TAGs were established based on the fatty acid composition of total TAGs (Table 3) and compared with the experimental values (Fig. 4). A non-random fatty acid distribution profile in the milks TAGs studied was evident. Among the most abundant TAGs in human milk, those with CN46 (13.15%), CN50 (20.37%), and CN52 (30.04%) were present in amounts greater than predicted by the fatty acid distribution hypothesis (Fig. 4a), where the corresponding random values were 10.07%, 17.40%, and 16.62%. Conversely, concentrations of TAGs with CN44 (9.59%) and CN48 (14.06%) were approximately close to the random percentages (9.04% and 12.34%, respectively) while TAGs species with CN54 resulting mainly from association between three long chain C_{18} fatty acids occurred less frequently than the theoretical proportion (3.10% and 6.61%, respectively).

The non-random distribution of fatty acids in human milk has been demonstrated by stereospecific analyses of TAGs and the identities of the fatty acids in the three *sn* positions have been determined (Jensen 1999; Martin et al. 1993; Straarup et al. 2006). Data from milks of Canadian and French women 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 the *sn*-2 position, and linoleic acid in the *sn*-3 position.

As regards equine milk, the discrepancies between the random and experimental values favored the preferential synthesis of CN32–CN46 TAGs in comparison to CN48–CN54 TAGs. The examination of the main CN/DB TAGs classes presented in Fig. 4b revealed that the differences between both distributions were as small as 2.5% on average. However, analysis of TAGs by the CN composition showed a number of great discrepancies; TAGs with CN32 (4.72%), CN34 (5.64%), CN36 (8.24%), CN38 (9.84%), CN40 (11.1%), CN42 (12.96%), CN44 (15.34%), and CN46 (10.24%) have the largest deviation from the calculated values showing a differences in the range of 2.7–4.59%. Interestingly, the concentration of CN48–CN54 TAGs was not as high as expected, in particular the amount of CN54 (1.16%) was much lower than the random value (6.02%).

The present study revealed that equine milk TAGs had minor discrepancies (maximally 5%) between the experimental and the random distribution when compared to human milk TAGs which showed large differences of up to 16% (CN52) between both distributions. This result is probably because, as demonstrated by stereospecific analyses (Haddad et al. 2011), medium chain fatty acids, the typical

Table 3 Fatty acid composition (% mol mean values±standard deviation) of human and equine milk triacylglycerols

Fatty acids	Human milk		Equine milk	
	Mean	SD	Mean	SD
6:0	0.000	0.000	0.702	0.659
8:0	0.411	0.209	8.095	1.759
10:0	2.973	0.794	13.603	1.309
10:1	0.000	0.000	2.789	0.779
11:0	0.000	0.000	0.103	0.146
12:0	8.079	0.519	10.742	1.535
$12{:}1\Delta^{7}$	0.000	0.000	0.205	0.088
$12{:}1\Delta^{9}$	0.000	0.000	0.170	0.072
13:0	0.000	0.000	0.030	0.033
14:0	7.957	0.783	7.183	1.427
$14{:}1\Delta^{9c}$	0.331	0.095	0.524	0.134
15:0	0.318	0.073	0.279	0.159
Iso15:0	0.000	0.000	0.022	0.037
16:0	27.860	1.107	14.725	1.965
$16{:}1\Delta^{7}$	0.327	0.061	0.206	0.075
$16{:}1\Delta^{9}$	2.169	0.338	3.522	0.699
$16{:}1\Delta^{8}$	0.069	0.051	0.051	0.046
17:0	0.295	0.070	0.194	0.105
17:1	0.089	0.036	0.293	0.164
18:0	4.480	0.232	0.888	0.294
$18{:}1\Delta^{9t}$	0.715	0.361	0.000	0.000
$18{:}1\Delta^{9c}+\Delta^{11c}$	31.173	3.133	11.108	1.528
$18{:}2\Delta^{9t,12t}$	0.377	0.419	0.000	0.000
$18{:}2\Delta^{9c,12c}$	10.539	0.286	12.956	6.003
$18{:}3\Delta^{6c,9c,12c}$	0.024	0.025	0.000	0.000
20:0	0.073	0.027	0.046	0.055
$18{:}3\Delta^{9c,12c,15c}$	0.396	0.081	10.484	5.433
$20{:}1\Delta^{11c}$	0.394	0.099	0.122	0.022
$20{:}2\Delta^{11c,14c}$	0.114	0.056	0.163	0.037
$20{:}3\Delta^{8c,11c,14c}$	0.290	0.058	0.000	0.000
$20{:}4\Delta^{5c8c,11c,14c}$	0.452	0.271	0.182	0.158
$22{:}6\Delta^{4c,7c,10c,13c,\ 16c,19c}$	0.071	0.050	0.000	0.000
Others	0.023	0.033	0.615	0.631
SFA	52.447	2.485	56.612	3.477
MUFA	35.267	2.757	18.988	2.361
PUFA	12.264	0.328	23.785	0.757

SFA saturated fatty acids, MUFA monounsaturated fatty acids, PUFA polyunsaturated fatty acids

constituents of equine milk present in the majority of TAGs species, were esterified mainly in the *sn*-2 position with substantial amounts also being found in the *sn*-1 and *sn*-3 positions. Approximately 75% of saturated fatty acids were acylated in the *sn*-2 position. C_{18} unsaturated fatty acids, incorporated into the most unsaturated TAGs species, 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 outer positions.

4 Conclusion

There are relatively few research papers describing the TAGs structure in equine milk, in particular no study has focused on its TAGs molecular species composition before this work. Furthermore, the present study has increased our knowledge about human milk TAGs composition and variability between women; in particular, it is the first in the literature that examined the human milk TAGs composition by means of the ESI–MS technique.

Although not all the TAGs could be quantified in terms of molecular species due to issues of co-elution and the impossibility of distinguishing between regioisomers and minor TAGs species containing *trans* acids, (such as 14:1 or 18:1 which cannot be distinguished from the corresponding *cis* acids, nor $C_{18:3\omega3}$ and $C_{18:3\omega6}$ be separated without silver ion HPLC or an equivalent fractionation method), the combination of NARP–HPLC, ESI–MS^3 and GC was successfully used, leading to the identification and quantification of 180 and 98 TAGs molecular species, respectively, in equine and human milks.

The identities of TAGs reported here could be useful to understand the nutritional value of milk. These findings should encourage further detailed investigation on the nutritional significance of equine milk TAGs for milk-fed infants. In particular, the differences in TAGs molecular species composition between equine milk and human milk should be taken into account when preparing specific equine milk-based formulas for infant nutrition, e.g., physical fractionation of mare's TAGs would be possible to approach human milk composition.

Additionally, our results are very important to understand the technological properties of the equine TAGs fraction since it is responsible for the crystallization behavior and the rheological properties of milk fat.

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