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**RESEARCH ARTICLE**

# Comparison of major lipid components in human and donkey milk: new perspectives for a hypoallergenic diet in humans

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**Abstract**

Recently, donkey milk has been indicated as a nutraceutical food thanks to some bioactive compounds interesting in the human diet; these substances are the lipids, which are characterized by the ability in conditioning indirectly or directly the intestinal environment and immunity, taking part in the prevention and treatment of some pathologies. With the aim to compare some nutritional properties of human and donkey milk, the triacylglycerol (TAG) composition and the positional isomers of donkey milk samples were analyzed by HPLC-APCI-MS on reversed phase and silver ion columns. The technique allowed the identification of 72 TAGs in the samples analyzed. Similarities and differences among the TAGs fraction of human and donkey milk can be easily determined by HPLC analysis of the lipid fraction. Donkey milk presents TAGs with partition number (PN) values starting from 30 up to 50. In human milk, the short-chain fatty acids (FAs) are not well-represented and the PN values range between 36 and 52. Other significant differences are among TAGs containing polyunsaturated fatty acids (PUFA). In fact, donkey milk presents a larger number and amount of ω3 and ω6 FAs than human milk, which contains only significant amount of ω6 FA (linoleic). Both donkey and human milk present the saturated FA preferably on the sn-2 position. Data testify a certain degree of similarity of these products in relation to their digestibility and confirm the increasing interest toward donkey milk as an alternative food for hypoallergenic diet in humans.

**Keywords:** Human milk, donkey milk, triacylglycerols, fatty acids, nutraceutical properties



## Introduction

Breast-feeding is considered the safest feeding method for the newborn mammals.[1] For humans, in the last years, there has been a growing decline in the use of this nutritional source and, consequently, an increase of the allergic reactions on immunological basis to cow's milk protein allergy (CMPA), which represents the most frequent type of alimentary allergy in the infants with an incidence that varies between 0.3% and 7.0%.[2] In these subjects, the CMPA condition becomes a serious problem of alimentary emergency from the first infancy to the schooling years and it could develop toward a multiple alimentary intolerance in 10% of the cases.[2]

The rational treatment of the alimentary allergies in humans requires the substitution in the diet of the injurious food with other hypoallergenic formulas. In clinical practice, a widespread alternative to these hypoallergenic formulas are the vegetable proteins, such as soy, and the animal proteins (goat milk, mare–donkey milk, lamb/chicken meat-based formulas).[3,4] Nevertheless, soy milk, which is the most diffuse substitute, is unfortunately often characterized by low palatability ("cooked taste") due to the high temperatures applied during the technological treatments and by risks of cross-reactivity in highly sensitive patients. Moreover, different proteins of the common animal species can cause allergic reactions (approximately 25–30%) in babies affected by CMPA.[5] On the contrary, it has been demonstrated that donkey milk can be safely used also in cases of intolerance to milk of ruminant species.[6] Last but not least is the reticence of the public opinion on

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the use of genetically modified soy. Moreover, efficacy of donkey milk feeding is also related to the consistent probability for infants with CMPA to develop a multiple alimentary intolerance.[7] Salimei et al. (2006)[7] reported about some trials carried out in patients with diagnosis of CMPA or multiple alimentary intolerance, based on the use of donkey milk[2] showed, in the treated patients, growth trends similar to those obtained with other formulas, total disappearance of the symptomatology and, after some months of treatment (15–20 months), no adverse reaction of allergic type when cow milk was reintroduced in the infant's diet.[3] For these reasons, donkey milk has been indicated for its peculiar composition as promising alternative protein source in the CMPA and multiple alimentary intolerance treatment. Moreover, recently, donkey milk has been suggested as nutraceutical food thanks to some bioactive compounds interesting not only in the diet of the infants but also in patients with osteogenesis processes, premature senescence, coronary diseases or in the atherosclerosis therapy, and hypocholesterolemic diets.[8,9] These substances are the lipids capable of conditioning indirectly or directly the intestinal environment and immunity,[10] taking part in the prevention and cures of some pathologies.[11,12] In fact, recent studies[13] on the immunomodulatory properties of fatty acids (FAs) and antioxidant, including α-tocopherol, of certain nutrients, have shown that food not only is a source of dietary antigens causing sensitization but also may contain protective factors that through the regulation of the immune function may counteract with the oxidative stress and may diminish the inflammatory response in allergic disease.

The comparison between human and donkey milk has focused mainly on the protein fraction,[9,14] some components of the lipid profile,[5] and mineral composition[16,17] in order to elucidate similarities and differences on the nutritional quality and tolerable properties of both milk. Nevertheless, a direct comparison of some component of the lipid fraction, specifically intact triacylglycerols (TAGs) and FAs, between the human and donkey milk has never been studied. Lipids, in addition to being an important source of energy, play several important biological roles[18]; the identification of the TAGs, the major component of the lipid fraction, is important in order to elucidate the physical properties, the sensorial characteristics, and the nutritional value of milk.[19,20] It has been also demonstrated that TAG digestion is dependent on the FA position on the glycerol bone. Human milk presents palmitic acid preferentially on the sn-2 position in the TAG molecule, and it is assumed to be absorbed as sn-2 monoacylglycerol rather than as free FA (FFA).[21,22] On the contrary, infant formula, mainly composed of vegetable oil and cow milk fat, although characterized by palmitic acid as the major components in analogy with human milk composition, presents this FA predominately esterified in the sn-1,3 position of the TAG.[23–25] Therefore, during the lipid digestion in the new born which occurs in the stomach, for the specific activity of the pancreatic lipase toward the primary ethereal bonds, the digestion of the human milk permits a greater absorption of the palmitic acid like 2-monoacylglyceride[26]; although, during the digestion of the cow's milk, the palmitic acid frees from the glycerol, binds itself to the calcium present in the intestinal lumen, and precipitates as calcium soaps that are not soluble at body temperature; therefore, it is excreted through the feces with a consequent 2-fold nutritional damage for the infants: the loss of palmitic acid and of calcium from the body.[27] Therefore, it is important to study the positional isometry of TAG that occurs in human and donkey milk, for a more detailed nutritional evaluation. Data about the human milk can be found from the literature,[28] but few data on the qualitative–quantitative TAG composition in lactating donkeys[29] can be found and, in authors' knowledge, no data about TAG positional isometry in donkey milk can be found.

Moreover, numerous methods have been set up for the analysis of the TAGs in milk[20,30–43] but it was not possible to find in the literature a direct comparison of the TAGs fraction of these milk kinds determined by the same methodology.

The aim of this study was to compare the lipid composition, TAGs, and FAs of human and donkey milk in order to consider these compounds as new factors in the potential dietary intervention for the human diseases on immunological basis.

## Materials and methods

### Abbreviations and nomenclature

TAGs are defined by means of three abbreviations corresponding to the fatty acyl residues linked to the glycerol backbone. The FAs found in TAGs of studied milk samples are listed in Table 1 with their abbreviations, systematic and common names, carbon numbers (CN), and double bond numbers (DB).

### Milk samples

*Donkey milk*: The samples were collected from three pluriparous, healthy lactating donkeys of Ragusana breed at the same month of lactation (fourth month). Ragusana breed is an authochthonous breed of Sicily region (Southern Italy). The donkeys, reared at an experimental private farm located in the typical breeding area for this breed (Ragusa, south-eastern part of Sicily), were monitored by the Department of Morphology, Biochemistry, Physiology and Animal Production of the University of Messina (Italy) in the context of a research project supported by the Ministry of the Education, University and Research (MIUR-2003). The experiment on the animals followed the Italian Legislative Decree 116/92/D.Lgs.,[44] according to specific European Commission Council Directive 86/609/EEC regulating the use of animals for experimental and other scientific purposes in the European Union.[45]

*Human milk*: The samples were offered by three healthy volunteers Sicilian women at the same month of lactation (fourth month).

Table 1. Systematic and common names of fatty acids found in triacylglycerols (TAGs) of studied milk samples listed with their abbreviations, systematic names, common names, carbon numbers (CN), and double bond numbers (DB).

| Abbreviation | Systematic name | Common name | CN:DB |
|---|---|---|---|
| Bu | Butanoic acid | Butyric acid | 4:0 |
| Cp | Hexanoic acid | Caproic acid | 6:0 |
| E | Heptanoic acid | Enanthic acid | 7:0 |
| C | Octanoic acid | Caprylic acid | 8:0 |
| Ca | Decanoic acid | Capric acid | 10:0 |
| Co | *cis*-9-Decanoic acid | Caproleic acid | 10:1 |
| La | Dodecanoic acid | Lauric acid | 12:0 |
| Lo | *cis*-9-Dodecanoic acid | Lauroleic acid/Linderic acid | 12:1 |
| $C_{13}$ | Tridecanoic acid | — | 13:0 |
| T | *cis*-9-Tridecanoic acid | — | 13:1 |
| M | Tetradecanoic acid | Myristic acid | 14:0 |
| Mo | *cis*-9-Tetradecanoic acid | Myristoleic acid | 14:1 |
| Pd | Pentadecanoic acid | — | 15:0 |
| Pa | *cis*-9-Pentadecanoic acid | — | 15:1 |
| P | Hexadecanoic acid | Palmitic acid | 16:0 |
| Po | *cis*-9-Hexadecanoic acid | Palmitoleic acid | 16:1 |
| Pn | *cis*-9,12-Hexadecanoic acid | Palmitolenic acid | 16:2 |
| Hn | *cis*-6,9,12-Hexadecanoic acid | Hiragonic | 16:3 |
| Ed | Heptadecanoic acid | Margaric acid | 17:0 |
| Eo | *cis*-9-Heptadecanoic acid | — | 17:1 |
| S | Octadecanoic acid | Stearic acid | 18:0 |
| O | *cis*-9-Octadecanoic acid | Oleic acid | 18:1 |
| L | *cis*-9,12-Octadecadienoic acid | Linoleic acid | 18:2 |
| Ln | *cis*-6,9,12-Octadecatrienoic acid | Linolenic acid | 18:3 |
| A | Eicosanoic acid | Arachic acid | 20.0 |
| G | Eicosaenoic acid | Gadoleic acid | 20:1 |
| Es | *cis*-11,14-Eicosadienoic acid | — | 20:2 |
| Et | *cis*-6,9,12-Eicosatrienoic acid | — | 20:3 |
| Ao | *cis*-6,9,12,15-Eicosatetraenoic acid | Arachidonic acid | 20:4 |



## Preparation of milk samples

Each milk sample was frozen and kept at –18°C until analysis. No other sample treatment after the sample collection was used.

## Lipid extraction from the whole milk

The lipid fraction was extracted from whole milk samples (frozen at –20°C until the extraction) following the method described by Romeu-Nadal et al.[46] with methanol and dichloromethane. The extraction procedure was as follows: 3 mL of milk was pipetted into the centrifuge tubes. Twenty-seven milliliters of a dichloromethane–methanol solution (2:1 v/v) was added to each tube. The mixture was shaken mechanically for 15 min and centrifuged at 2500 rpm for 8 min. Approximately 8 mL of distilled water was added into each tube and, after shaking for a further 15 min, the sample was, again centrifuged at 2500 rpm for 8 min. As much of the upper aqueous fraction as possible was removed with a pipette. The organic layer was washed with 8 mL of a saturated solution of the sodium chloride, then mixed mechanically for 15 min, and centrifuged for 8 min at 2500 rpm. Again, the upper aqueous fraction was removed with a pipette. The organic fraction was transferred to a separating funnel and filtered through a filter paper containing anhydrous sodium sulfate. The extract was concentrated by removing dichloromethane in a rotatory evaporator and finally dried under a gentle stream of nitrogen. About 10 mg of extracted lipids were solved in 1 mL of acetone prior to RP-HPLC analysis.

## RP-HPLC-APCI-MS

HPLC-MS analyses were performed on a Shimadzu HPLC system equipped with a SCL-10Avp controller, two LC-10ADvp pumps, a DGU-14A degasser, a LCMS 2010A mass spectrometer with an APCI interface (Shimadzu, Milan, Italy).

The analytical columns used were two Discovery® HS C18 (25 cm × 4.6 mm ID, 5 µm particle size) columns (Supelco, Bellefonte, PA) connected in series.

The chromatographic separation was carried out using a mobile phase of 2-propanol and acetonitrile with a gradient elution. The initial gradient consisted of 70% of acetonitrile, then the concentration of acetonitrile decreased linearly according to the following gradient: from 0 to 30 min from 70% to 60%, from 30 to 50 min from 60% to 45%, from 50 to 75 min from 45% to 30% with a hold at 30% of acetonitrile until the end of the analysis (85 min).

The flow rate was 1 mL/min and the volume of injected sample was 20 µL.

The following MS conditions were used: nebulizer gas: $N_2$; nebulizer gas flow rate: 2 mL/min; APCI mode: positive; APCI temperature: 400°C; probe voltage: 3 kV; CDL temperature: 230°C; CDL voltage: –34 V; block temperature: 230°C; Q array: scan; Detector gain: 1.4 kV.

Mass spectra were acquired by scanning over the range of 215–1100 $m/z$ with an interval of 0.2 sec. Data acquisition and evaluation was performed by using a Shimadzu LCMS-Solution software (Shimadzu, Milan, Italy).

### Prep-RP-HPLC-APCI-MS

Shimadzu HPLC system equipped with a SCL-10Avp controller, two LC-10ADvp pumps, a DGU-14A degasser, a LC-10ADvp UV–vis detector, a SIL 10ADvp autosampler, a CTO 10Avp HPLC-oven.

Column: Restek Ultra C18 25 cm × 4.6 mm ID, 5 µm particle size

Mobile phase: the same gradient described above

Flow rate: 1 mL/min, the volume of injected sample: 20 µL.

Detector: UV–vis at 210 nm.

### $Ag^+$-HPLC-APCI-MS

HPLC-MS analyses were performed on the same Shimadzu HPLC system as used for RP-HPLC analyses.

Column: Nucleosil 5S, 250 mm × 4.6 mm ID, 5 µm particle size (Alltech Italia Srl., Sedriano, Italy). The $Ag^+$ column was prepared according to the method described by Christie[47] by flushing the column with a 1.2 M silver nitrate aqueous solution.

Mobile phase: *n*-Hexane–acetonitrile (99.5:0.5) in isocratic mode.

Flow rate: 1 mL/min; injection volume 20 µL.

The MS conditions were the same as described for RP-HPLC-APCI-MS.

### FAMEs methylation

Prior to the GC analysis, 10 mg of extracted lipids was treated in screw-capped Pyrex tubes by adding 1 mL of sodium methoxide (0.5% w/v in methanol) and heated to 95°C in a water bath for 5 min. After cooling to 25°C, samples were esterified with 1 mL of boron trifluoride–methanol ($BF_3$/MeOH) reagent (also in water bath at 95°C) for 15 min. Once the tubes were cooled to 25°C, the FAMEs were isolated by adding 1 mL of *n*-hexane. The mixture was shaken and after 2 min the top layer formed with *n*-hexane was transferred into the vial for the GC injection.

### FAMEs GC analyses

A Shimadzu GC-2010 system (Shimadzu, Milan, Italy) was used. The split/splitless injector was held at a temperature of 250°C, and a split ratio of 1:20 was applied. Carrier gas, He, at a constant linear velocity of 30.1 cm/sec and an initial head pressure of 99.8 kPa was applied. The analyses were carried out on a Omegawax 250 column, 30 m × 0.25 mm i.d. × 0.25 µm film thickness (Supelco). The temperature was programmed as follows: from 50°C (3 min) to 280°C (5 min) at 3°C/min. The flame ionization detector (FID) temperature was set at 300°C (sampling rate 80 msec); FID gas flows: $H_2$: 50 mL/min; makeup gas ($N_2$): 50 mL/min; air: 400 mL/min. The data were collected by using GC solution software (Shimadzu, Milan, Italy).

Each TAG was calculated as percentage of the relative peak area and expressed as mean value of the three individual donkeys and of the three individual human milk samples.

## Results and discussion

### Milk TAG composition

The analytical technique used allowed to separate numerous TAGs in the samples analyzed.

The chromatographic behavior of TAGs in RP-HPLC is in function of their partition number (PN) value: retention increases with increasing PN. When the same PN value occurs, TAGs with higher unsaturation number elute before the saturated ones.[48] In some cases, the PN groups overlapped, in fact TAGs containing unsaturated or short-chain FA elute before TAGs with lower PN values that contain long and saturated chain FA. For example, OLLn and PoOLn with PN = 42 contain 6 and 5 double bonds, respectively. These TAGs elute before than CPP, a TAG with PN = 40 but totally saturated. This behavior was already described by Dotson et al.[32] in human milk analysis, by Mottram and Evershed[48] who analyzed cow milk by the same technique, and by Dugo et al.[49] in donkey milk analyses. In our case, fractionation and off-line coupling with $Ag^+$-HPLC rendered the identification easier. Peak identification was performed by a combination of the MS spectra, characterized by the presence of a pseudomolecular ion ($[M+H]^+$) and the diacylglycerol ion (DG, $[DG]^+$) generated by the loss of one FA, PN values, and $Ag^+$ chromatographic behavior.[49] Using the off-line multidimensional approach, nearly 80 TAGs were identified in both samples analyzed, as reported in Table 2. In the table, the percent areas of the chromatographic peaks are also reported.

The semiquantitative determination was performed without the use of correction factors, assuming that the response factor for TAGs containing long- and medium-chain FAs is similar. Even though this is only an approximation, considering the great difficulty in calculating correction factors for the high number of TAGs in milk samples, it can be used for comparison of different samples analyzed under the same conditions. This procedure was already reported for TAGs analysis in other natural matrices[50] and the method used has been described before for the qualitative and quantitative determination of donkey milk TAGs.[49] In the previous work, the corresponding CV% values were reported. It has to be mentioned that this quantitative data should be considered only indicative, due to the fact that only few samples of both kinds were analyzed. However, this is enough to obtain a reliable comparison of the two milk kinds.

Table 2. Average composition of triacylglycerols (TAGs) determined in the human and donkey milk (expressed as area %, average of three different human and three donkey milk samples) analyzed by RP-HPLC-APCI-MS.

| | TAG | PN[a] | CN[b] | DB[c] | Human | Donkey |
|---|---|---|---|---|---|---|
| 1 | CCaLa | 30 | 30 | 0 | — | 0.53 |
| 2 | CaCaLa | 32 | 32 | 0 | — | 0.77 |
| 3 | CaLaLn | 34 | 40 | 3 | — | 0.93 |
| 4 | CaCO | 34 | 36 | 1 | — | 0.89 |
| 5 | CaCaM | 34 | 34 | 0 | — | 0.93 |
| 6 | CaCP | 34 | 34 | 0 | — | 0.42 |
| 7 | COLn | 36 | 44 | 4 | — | 1.32 |
| 8 | CaMM | 38 | 38 | 0 | 1.11 | 1.45 |
| 9 | CaCaS | 38 | 38 | 0 | — | * |
| 10 | COL | 38 | 44 | 3 | — | 2.74 |
| 11 | CaOLn | 38 | 46 | 4 | — | * |
| 12 | COPo | 38 | 42 | 2 | — | 0.77 |
| 13 | BuPO | 36 | 38 | 1 | 0.41 | - |
| 14 | CaLaO | 38 | 40 | 1 | 0.95 | 3.67 |
| 15 | CaPLn | 38 | 44 | 3 | — | * |
| 16 | CaLaP | 38 | 38 | 0 | — | 3.42 |
| 17 | LaMM | 40 | 40 | 0 | — | * |
| 18 | CaOL | 40 | 46 | 3 | — | 3.72 |
| 19 | LaOLn | 40 | 48 | 4 | — | * |
| 20 | PLnLn | 40 | 52 | 6 | — | * |
| 21 | COO | 40 | 44 | 2 | — | 1.14 |
| 22 | LaLaM | 38 | 38 | 0 | 0.24 | — |
| 23 | CpOP | 38 | 40 | 1 | 0.62 | — |
| 24 | CPoS | 40 | 42 | 1 | * | — |
| 25 | PoPoMo | 40 | 46 | 3 | 0.26 | |
| 26 | LaLaO | 40 | 42 | 1 | 2.34 | — |
| 27 | CPO | 40 | 42 | 1 | 0.91 | 2.92 |
| 28 | OLLn | 42 | 54 | 6 | — | 1.37 |
| 29 | CaMP | 40 | 40 | 0 | — | 2.11 |
| 30 | PoOLn | 42 | 52 | 5 | — | * |
| 31 | CPP | 40 | 40 | 0 | — | 1.78 |
| 32 | LaLO | 42 | 48 | 3 | 2.11 | 1.82 |
| 33 | PLLn | 42 | 52 | 5 | — | * |
| 34 | CaOO | 42 | 46 | 2 | 1.71 | 3.37 |
| 35 | PPoLn | 42 | 50 | 4 | — | * |
| 36 | LaPL | 42 | 46 | 2 | 0.99 | 0.97 |
| 37 | LaOM | 42 | 44 | 1 | 2.89 | — |
| 38 | CaPO | 42 | 44 | 1 | 3.00 | 4.65 |
| 39 | OLL | 44 | 54 | 5 | — | 1.02 |
| 40 | PoOL | 44 | 52 | 4 | — | 3.07 |
| 41 | OOLn | 44 | 54 | 5 | — | * |
| 42 | CaPP | 42 | 42 | 0 | 1.27 | 3.23 |
| 43 | PLL | 44 | 52 | 4 | * | * |
| 44 | POLn | 44 | 52 | 4 | — | 7.38 |
| 45 | LaPM | 42 | 42 | 0 | 2.21 | — |
| 46 | LMM | 42 | 46 | 2 | * | — |
| 47 | PMM | 44 | 44 | 0 | * | — |
| 48 | MOL | 44 | 50 | 3 | 3.61 | — |
| 49 | EsPoPo | 44 | 52 | 4 | * | — |
| 50 | PPoPo | 44 | 48 | 2 | * | — |
| 51 | LaOO | 44 | 48 | 2 | 3.13 | — |

Table 2. continued on next page

Table 2. Continued.

| | TAG | PN[a] | CN[b] | DB[c] | Human | Donkey |
|---|---|---|---|---|---|---|
| 52 | MPL | 44 | 48 | 2 | 1.16 | 4.67 |
| 53 | LnPP | 44 | 50 | 3 | — | * |
| 54 | LaPP | 44 | 44 | 0 | — | * |
| 55 | LaOP* | 44 | 46 | 1 | 6.60 | * |
| 56 | OOL | 46 | 54 | 4 | 3.80 | 2.50 |
| 57 | PoOO | 46 | 52 | 3 | 2.25 | 3.08 |
| 58 | POL | 46 | 52 | 3 | 9.06 | 4.91 |
| 59 | MOO | 46 | 50 | 2 | 5.00 | - |
| 60 | PPoO | 46 | 50 | 2 | 1.01 | 4.69 |
| 61 | PPL | 46 | 50 | 2 | 6.73 | 1.96 |
| 62 | MOP | 46 | 48 | 1 | — | 2.93 |
| 63 | OOO | 48 | 54 | 3 | 5.86 | 3.09 |
| 64 | MPP | 46 | 46 | 0 | * | * |
| 65 | POO | 48 | 52 | 2 | 14.12 | 7.57 |
| 66 | PPO | 48 | 50 | 1 | 8.03 | 5.23 |
| 67 | PPP | 48 | 48 | 0 | — | 0.70 |
| 68 | PdEdEd | 50 | 49 | 0 | 4.14 | — |
| 69 | SOO | 50 | 54 | 2 | * | 0.54 |
| 70 | POS | 50 | 52 | 1 | 5.09 | 1.30 |
| 71 | PPS | 50 | 50 | 0 | 1.12 | 3.35 |
| 72 | PSS | 52 | 52 | 0 | 0.41 | — |

[a]PN = Partition numbers.
[b]CN = Carbon numbers.
[c]BD = Double bond numbers.
* Chromatographic co-elution with the compound just above; the area % values refer to the sum of these compounds.

Human milk was analyzed by RP-HPLC-APCI-MS, and its lipidic fraction was separated in <40 peaks, partially or totally resolved in <95 min. The chromatogram relative to one analysis of human milk sample is reported in Figure 1. The PN value ranged from 36 to 52 (Table 2). As it is possible to observe, the chromatogram (Figure 1) presents better resolution for later eluting components, therefore their identification and quantitative analysis were simpler than the early eluting ones, which were poorly separated. The MS spectra of these peaks were characterized by the presence of numerous DG ions, and therefore the identification was not always possible. To overcome this limit, the bidimensional approach was extremely useful. In fact, fractions obtained by RP-HPLC were subjected to $Ag^+$ chromatography, obtaining a complete separation. The spectra relative to these TAGs were more easily interpreted and the identification resulted possibility for most of those components not identifiable by the monodimensional approach.

The lipid fraction of donkey milk was analyzed under the same conditions of human milk.[49] The technique allowed the separation of 50 peaks in <80 min; PN values ranging from 30 to 50 (Table 2). The multidimensional approach was again necessary and helpful for the identification of components otherwise not sufficiently resolved by the monodimensional analysis.

To better compare the composition of TAG fraction of these two milk kinds, Figure 2 reports the graph relative to the TAGs groups based on DB for each milk kind. As it is possible to observe, donkey milk is characterized by numerous TAGs with high unsaturation degree. Human milk presents a distribution similar to donkey milk for TAGs content with BD = 3; other TAGs showed quantitative differences. Human milk TAG fraction is characterized by the presence of 9 TAGs with DB = 0 representing about 12%, 12 TAGs with DB = 1 that count about 12%, 11 TAGs with DB = 2 that represent about 10%, 6 TAGs with DB = 3 and 3 with DB = 4 that represent 7% and 2%, respectively. The mostly represented TAGs in human milk are: POO (14.1%), POL (9.1%), PPO (8.0%), LaOP (6.6%), POS (5.1%), MOO (5.0%) (Table 2). The TAG fraction of donkey milk lipid (Figure 2) is characterized by the presence of 16 saturated (DB = 0) TAGs that count for <14%, 8 TAGs with DB = 1 that represent 8%, 9 TAGs with DB = 2 that represent about 10%, 9 TAGs with DB = 3 that represent about 9%, 7 TAGs with DB = 4 that represent about 7%, 4 TAGs with DB = 5 and 2 TAGs with DB = 6 that represent 4% and 2%, respectively. The mostly represented TAGs in donkey milk are: POLn (7.4%), POO (7.6%), PPO (5.23%), POL (4.9%), PPoO (4.69%), CaPO (4.65%) (Table 2). Results underline some qualitative–quantitative similarities in the FA typology that form the donkey's milk TAGs in comparison with those of human's milk; in fact, the mostly represented TAGs in human and donkey milk were POO, PPO, and POL, confirming that the palmitic and oleic acids were the most abundant FAs in both milk. Moreover, TAGs in human and donkey milk are very dissimilar from those of cow milk; in the latter, the main components are BuPO, BuPP and BuMP, and TAGs containing polyunsaturated FAs (PUFA) (linoleic and linolenic acids) are not present.[51,52]

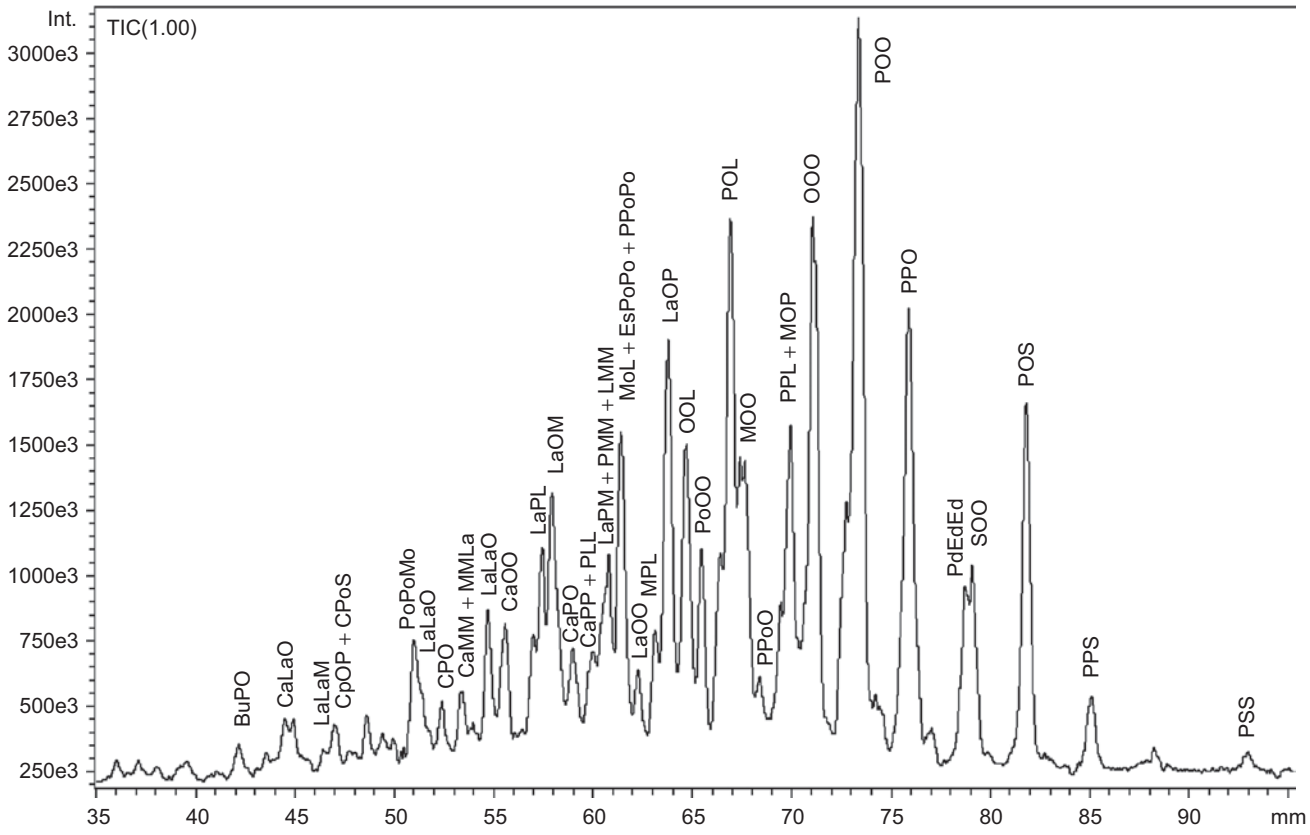


Figure 1. RP-HPLC-APCI/MS chromatogram of human milk.

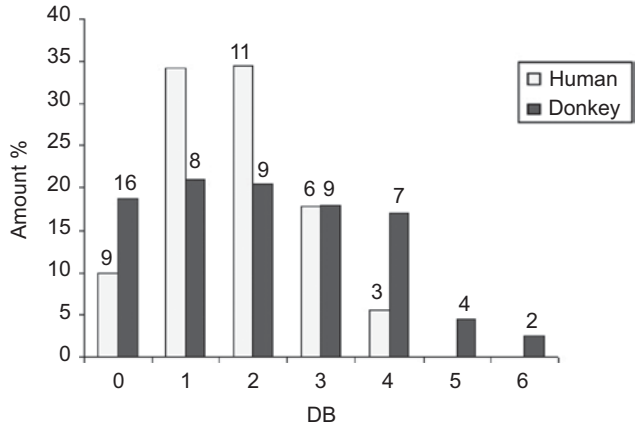


Figure 2. Qualitative distribution of triacylglycerols (TAGs) in function of double bond numbers (DB), determined in human and donkey milk. The numbers above the bars represent the number of TAGs of each group.

## Milk FA composition

Figure 3 represents the comparative graph of the average composition of FAs determined in the two milk samples. The CV% of FAMEs analyses was always lower than 8.7% (the values were dependent on the FA concentration, giving higher values for those FAs at very low levels); however, in most of the cases it was <3%. The data obtained are in agreement with Collomb et al.[25]

The composition of donkey milk lipid fraction greatly resembles that of human milk (Figure 3). The most abundant FAs in both milk were: among the saturated FA, palmitic acid (24% in human milk and 26% in donkey milk), among the monounsaturated FA, oleic acid (38% in human milk and 32% in donkey milk), and among the polyunsaturated FA, linoleic acid (10% in human milk and 8% in donkey milk). The main differences among the FAs are given by the presence of higher amounts of short-chain FAs, C6–C10, and a considerable higher level of linolenic acid in donkey milk (8.1%) than the human milk (0.7%). Donkey milk in fact is characterized by large amounts of PUFAs, for the absence in the small intestine of donkey of isomerization and hydrogenation process of the FAs prior to absorption and esterification in blood.[29] This composition renders donkey's milk a good source of essential FAs (ω6 and ω3), counteracting with the oxidative stress and, therefore, diminishing the inflammatory response in allergic disease, may regulate the immune function.[7] Moreover, the low presence of short-chain FA renders it adequate (better tolerance) for infant diet.[11,15,16]

## Milk TAG positional isometry

The FA distribution along the glycerol backbone (positions named sn-1, sn-2, and sn-3) was determined by the combination of $Ag^+$-HPLC with mass spectrometric detection on fractions of TAGs separated by RP-HPLC. The direct analysis of real complex mixtures for the determination of the positional isomer distribution of TAG species would be impossible using the monodimensional approach (RP- or $Ag^+$-HPLC), even coupled

Table 3. Triacylglycerol (TAG) positional isomers detected in human milk.

| TAG | PN[a] | CN[b] | DB[c] | [DG]$^{+d}$ | [DG]$^{+}$ | [DG]$^{+}$ | % |
|---|---|---|---|---|---|---|---|
| LaLO | 42 | 48 | 3 | LaO 522 | LaL 520 | OL 602 | 23.2 |
| LOLa | 42 | 48 | 3 | LaO 522 | OL 602 | LaL 520 | 7.6 |
| LLaO | 42 | 48 | 3 | LaO 522 | LaL 520 | OL 602 | 69.1 |
| MLO | 44 | 50 | 3 | MO 550 | OL 602 | ML 548 | 18.8 |
| LOM | 44 | 50 | 3 | MO 550 | OL 602 | ML 548 | 6.4 |
| LMO | 44 | 50 | 3 | MO 550 | ML 548 | OL 602 | 74.7 |
| LLP | 44 | 52 | 4 | PL 576 | LL 600 | | 16.5 |
| LPL | 44 | 52 | 4 | PL 576 | LL 600 | | 83.4 |
| PLO + POL | 46 | 52 | 3 | PL 576 | PO 578 | OL 602 | 12.4 |
| LPO | 46 | 52 | 3 | PL 576 | PO 578 | OL 602 | 87.6 |
| POO | 48 | 52 | 2 | PO 578 | OO 604 | | 9.4 |
| OPO | 48 | 52 | 2 | PO 578 | OO 604 | | 90.2 |
| POP | 48 | 50 | 1 | PO 578 | PP 552 | | 6.0 |
| PPO | 48 | 50 | 1 | PO 578 | PP 552 | | 94.0 |

In the table are also reported the parameters used for identification of the components and the ratio (%) of the isomers of each TAG.
[a]PN = Partition numbers.
[b]CN = Carbon numbers.
[c]BD = Double bond numbers.
[d][DG]$^{+}$ = Diacylglycerol ion.

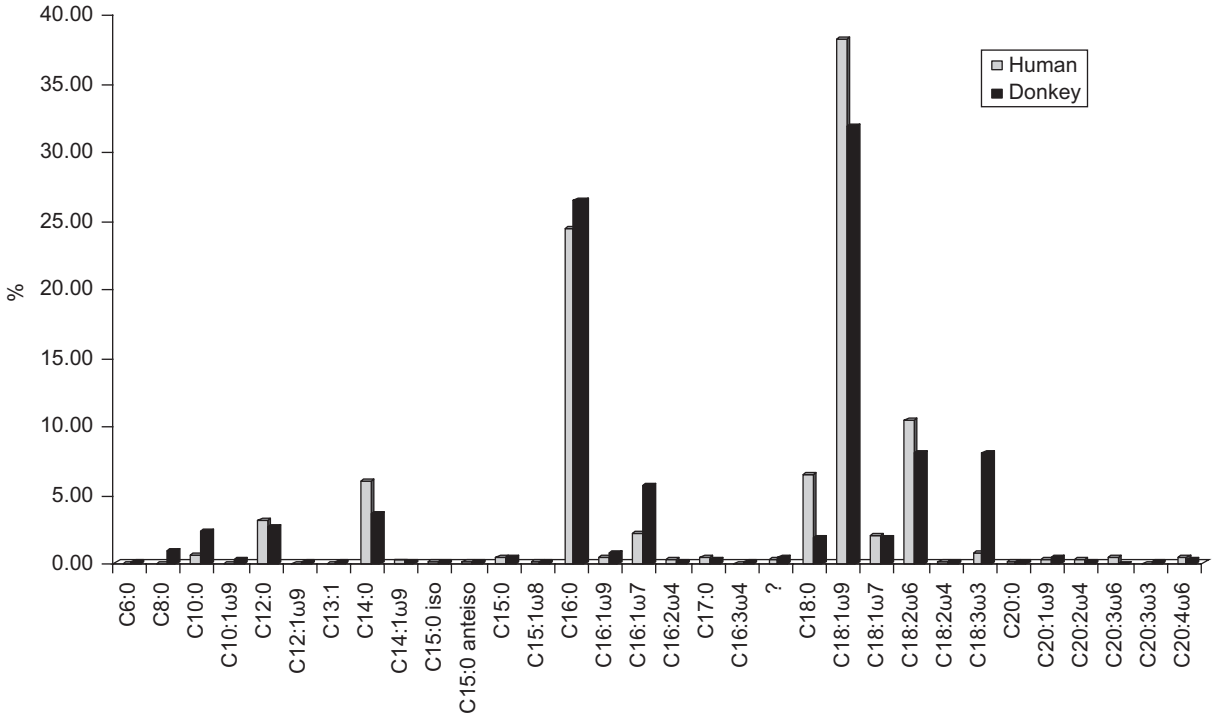


Figure 3. Fatty acid distribution in human and donkey milk, determined by GC-FID.

to APCI-MS detection, due to peak overlapping between different TAG species or between TAG isomers and other TAG species.

The positional isomers were separated by Ag$^{+}$-HPLC, where the retention behavior depends on the number and configuration of double bonds present in TAG molecule[53,54] and the identification was confirmed by diglyceride ion ratios derived from APCI-MS detection. In fact, the ratio of the fragment intensities changes in relation to the position occupied by the three FAs.[50,55,56] For example, with respect to bifunctional TAGs, in the case of symmetric isomers (i.e. sn-OPO), the relative abundance of the diglyceride ions (i.e. [OP]$^{+}$ and [OO]$^{+}$) are 100 and about 30, respectively. In the case of asymmetric isomers (i.e. sn-POO), the relative abundance of the diglyceride ions [OP]$^{+}$ and [OO]$^{+}$ are 100 and about 50, respectively. With respect to the TAGs with three different FAs, the less intense is the DG ion with FA missing from the sn-2 position, due to the fact that the loss of the FA in sn-2 position is the most hindered. With respect to chromatographic behavior, TAGs with the highest number of unsaturations on the sn-2 position elute before those with DBs on the external position. Figure 4 illustrates the Ag$^{+}$ analyses of one fraction separated from donkey milk TAGs.

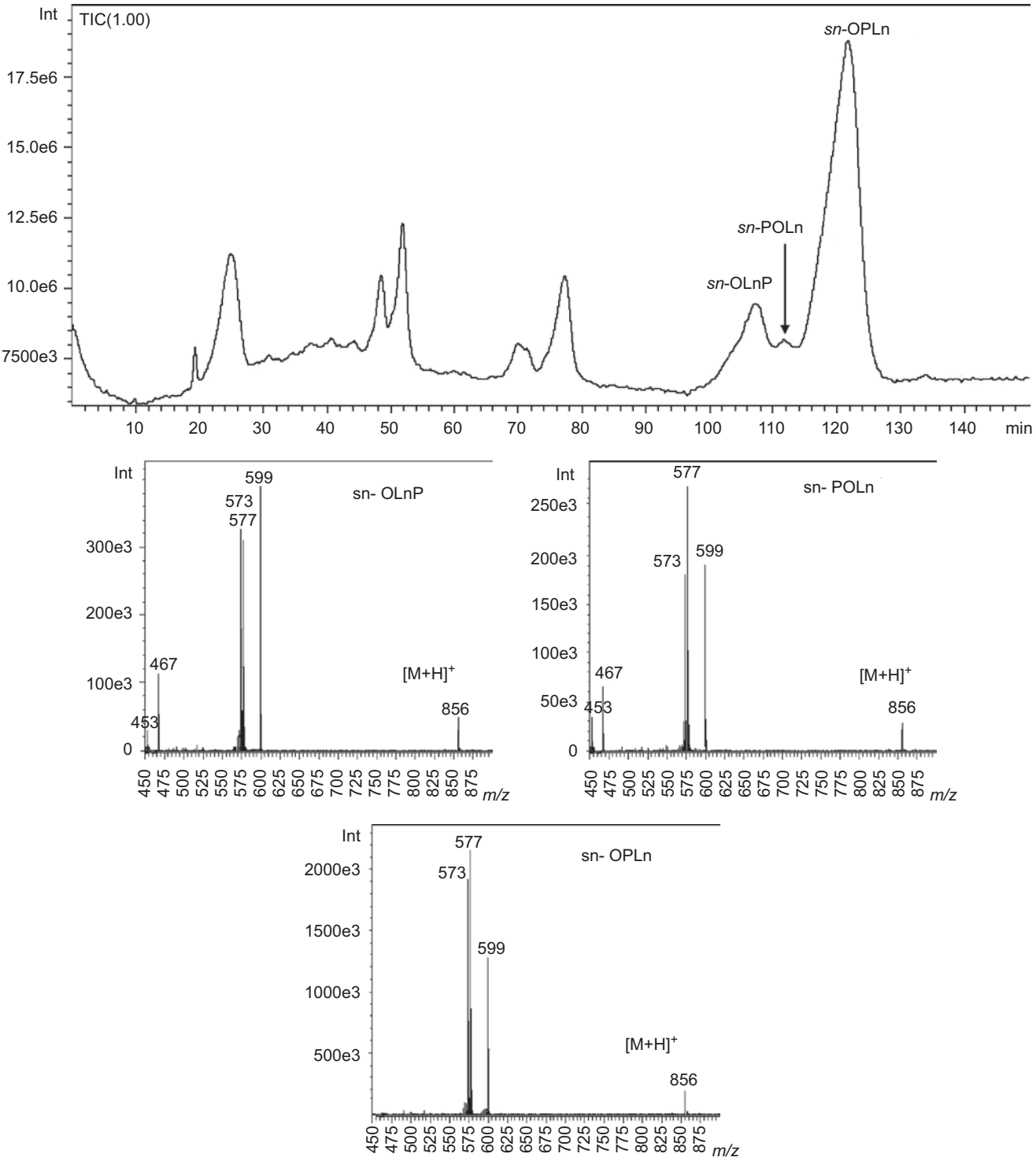


Figure 4. $Ag^+$-HPLC-APCI/MS chromatogram of fraction no. 18 of donkey milk with MS spectra of the three isomers of POLn.

Separation and APCI mass spectra of the three isomers of POLn are shown. As it is possible to observe, the first eluting peak is the OLnP (FA with 3 DB in sn-2 position), the second, least abundant is the POLn (FA with 2 DB in sn-2 position), followed by OPLn (saturated FA in sn-2 position), which is the most abundant of the three isomers. The three isomers were totally co-eluted in RP-HPLC.

The fractionation of TAGs on RP HPLC column and successive separation on $Ag^+$ HPLC column has allowed to determine some positional isomers on the samples analyzed. Results obtained are reported in Tables 3 and 4, respectively, for human and donkey milk. In particular, for six TAGs that represented about 35% of the total TAG fraction of human milk it was possible to obtain information on the distribution of FA in the glycerol backbone.

The trend of the positional isometry of TAGs in human milk showed that the saturated long-chain FA are mainly

Table 4. Triacylglycerols (TAG) positional isomers detected in donkey milk, parameters used for identification of the components, and ratios (%) of the isomers of each TAG.

| TAG | PN[a] | CN[b] | DB[c] | $[DG]^{+d}$ | $[DG]^{+}$ | $[DG]^{+}$ | % |
|---|---|---|---|---|---|---|---|
| CLnP | 36 | 42 | 3 | LnC 462 | CP 440 | PLn 574 | 10.3 |
| LnPC + LnCP | 36 | 42 | 3 | CP 440 | PLn 574 | LnC 462 | 89.7 |
| CaLnP | 38 | 44 | 3 | CaP 468 | CaLn 490 | PLn 574 | 10.7 |
| LnCaP + LnPCa | 38 | 44 | 3 | CaP 468 | CaLn 490 | PLn 574 | 89.3 |
| CLP | 38 | 42 | 2 | CL 464 | CP 440 | PL 576 | 27.9 |
| LCP + LPC | 38 | 42 | 2 | CP 440 | CL 464 | PL 576 | 72.1 |
| LLnM | 40 | 50 | 5 | MLn 546 | ML 548 | LLn 598 | 28.5 |
| LMLn | 40 | 50 | 5 | MLn 546 | ML 548 | LLn 598 | 71.5 |
| LaLnO | 40 | 48 | 4 | LaO 522 | LaLn 518 | OLn 600 | 24.0 |
| LaOLn | 40 | 48 | 4 | LaO 522 | LaLn 518 | OLn 600 | 14.3 |
| LnLaO | 40 | 48 | 4 | LaO 522 | LaLn 518 | OLn 600 | 61.8 |
| CaLO + CaOL | 40 | 46 | 3 | CaL 492 | CaO 494 | OL 602 | 64.5 |
| LCaO | 40 | 46 | 3 | CaO 494 | CaL 492 | OL 602 | 35.5 |
| CaLP | 40 | 44 | 2 | CaL 492 | CaP 468 | PL 576 | 31.6 |
| LCaP + LPCa | 40 | 44 | 2 | CaP 468 | CaL 492 | PL 576 | 68.4 |
| COP | 40 | 42 | 1 | CO 466 | CP 440 | PO 578 | 18.2 |
| OCP + OPC | 40 | 42 | 1 | CP 440 | CO 466 | PO 578 | 81.8 |
| OLnPo | 42 | 52 | 5 | PoLn 572 | PoO 576 | OLn 600 | 40.2 |
| LnPoO + LnOPo | 42 | 52 | 5 | PoO 576 | PoLn 572 | OLn 600 | 59.8 |
| CaOP | 42 | 44 | 1 | CaO 494 | PO 578 | CaP 468 | 16.0 |
| OCaP | 42 | 44 | 1 | CaP 468 | CaO 494 | PO 578 | 83.9 |
| OLnO | 44 | 54 | 5 | OLn 600 | OO 604 | | 67.1 |
| OOLn | 44 | 54 | 5 | OLn 600 | OO 604 | | 32.9 |
| OLnP | 44 | 52 | 4 | OLn 600 | PLn 574 | PO 578 | 18.7 |
| LnOP | 44 | 52 | 4 | PO 578 | OLn 600 | PLn 574 | 4.6 |
| LnPO | 44 | 52 | 4 | PO 578 | PLn 574 | OLn 600 | 76.6 |
| PLPo | 44 | 50 | 3 | PPo 550 | PoL 574 | PL 576 | 32.8 |
| LPPo | 44 | 50 | 3 | PPo 550 | PoL 574 | PL 576 | 67.2 |
| OPoP + PoOP | 46 | 50 | 2 | PoP 550 | PoO 576 | PO 578 | 27.3 |
| OPPo | 46 | 50 | 2 | PoP 550 | PO 578 | PoO 576 | 72.6 |
| PLP | 46 | 50 | 2 | PL 576 | PP 552 | | 4.8 |
| PPL | 46 | 50 | 2 | PL 576 | PP 552 | | 95.1 |
| MOP | 46 | 48 | 1 | MO 550 | MP 524 | PO 578 | 8.4 |
| OMP | 46 | 48 | 1 | MO 550 | MP 524 | PO 578 | 91.5 |
| POO | 48 | 52 | 2 | PO 578 | OO 604 | | 16.6 |
| OPO | 48 | 52 | 2 | PO 578 | OO 604 | | 83.4 |
| POP | 48 | 50 | 1 | PO 578 | PP 552 | | 4.4 |
| PPO | 48 | 50 | 1 | PO 578 | PP 552 | | 95.6 |
| POS | 50 | 52 | 1 | SP 580 | PO 578 | SO 606 | 32.5 |
| OPS + OSP | 50 | 52 | 1 | SP 580 | PO 578 | SO 606 | 67.4 |

[a]PN = Partition numbers.
[b]CN = Carbon numbers.
[c]BD = Double bond numbers.
[d]$[DG]^{+}$ = Diacylglycerol ion.

esterified in the sn-2 position of the TAG. This was in accordance with the literature data.[28,57–59] The same trend was observed in the donkey milk samples analyzed as reported in Table 4. In this milk for 19 TAGs that represented about 45% of the total TAG fraction, it was possible to obtain information on the positional isometry. The sn-1,3 positional isomers of saturated FA in both human and donkey milk occur rarely.

Not for all the TAGs identified, it was possible to determine the sn positional isometry. This is probably due to the very low levels of the isomers with the unsaturated FAs in sn-2, which are difficult to detect by this technique relatively to the complexity of this fraction. This assumption is supported by the MS detection. In fact, in most of the cases, if only one isomer is detectable, the spectrum is characteristic of the sn-2 positional isomers of saturated FA.

## Conclusions

The results of TAG fraction have given a further contribution for the characterization of the composition of donkey milk and for its nutritional evaluation.

In fact, the methodology here applied can be useful for a direct comparison of the lipid fraction of different matrices and their characterization. The combination of the RP-HPLC separation, based on the elution in function of the PN value, and the APCI-MS, that generates pseudomolecular ion and diacylglycerols ions, is a valuable tool for the identification and quantitation of large number of TAGs in the samples analyzed for their differentiation.

Moreover, data underline similar aspects also in the lipid composition between donkey and human milk, with particular regards to the TAG fraction and FA profile.

Nevertheless, the main differences are given by the presence in donkey milk of minor components at low PN values and TAGs with high unsaturation degree, due to the larger amount of PUFA in this milk.

The most significant result of this study is given by the positional isometry determined in donkey milk. In fact, we have already discussed the influence on the nutritional value of saturated FA on the sn-2 position. In both milk samples, ~80% of TAGs present the saturated FA on the sn-2 position.

In conclusion, donkey milk has been indicated as a valuable and safe source for the nutrition of cow milk-intolerant infants as well as an interesting nutraceutical food for older people, therefore, as a brand new functional food. Moreover, the results here discussed confirm that it represents a natural source of lipids with high similarity to human milk and therefore high nutritional value in the regulation of the immune–inflammatory system. In this context, the production of "donkey milk" could represent, similar to happening in some Italian regions, an economic justification for breeding endangered domestic Equidae breeds, considering that the Regulation (EC) No 853/2004, lays down specific hygienic rules for food on animal origin, including donkey's raw milk.

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## Declaration of interest

The authors declare they do not have any conflict of interest.

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