# Phospholipid fingerprints of milk from humans and different animal species determined by $^{31}$P NMR: importance for human health

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## Introduction

Phospholipids (PLs) are best known for being essential components of cell membrane bilayers, but they also play key biological roles in signalling pathways, inflammatory processes, and in lipid digestion, absorption and transport [1]. PLs are either provided directly from the diet or via *de novo* synthesis [1], and are increasingly considered as nutrients with putative health benefits. Among dietary sources, milk contains PLs as important functional bioactive compounds that are likely to have a positive effect on term and preterm infants, and also on adult health. However, few studies have analyzed milk PLs for specific species with respect to health benefits for humans. An efficient method for the determination of PL classes with minimal sample preparation is $^{31}$P NMR spectroscopy. While this method has previously been applied to measure PL profiles in milk [2], we set up a protocol optimized for measuring PL classes over the dynamic range (0.2 to 2% of total lipids) present in different milk sources (human, cow, mare and camel). Our final objective was to compare the PL profile of human milk with that of three different animal species with regards to potential health benefits of cow, mare and camel milk.

## Methods

Mature human milk samples (HM, n = 22, from 22 different mothers) were collected at the Neonatology Department of the Marseille University Hospital. Camel (CaM) and mare (MM) milk specimens (n = 8, from 8 different animals of each species) were obtained from the IRA breeding center in Medenine, Tunisia, and from the Haras Haflinger breeding center in Bourgogne, France, respectively. Thermally treated cow milk (CoM) samples (n=15, each sample representing pooled milk from 4 cows) were provided by the Milk and Egg Research Center in Rennes, France. Total milk lipids were extracted according to the modified Folch's method previously published [3]. Briefly, 4 ml milk was mixed with 20 volumes of chloroform/methanol 2:1. Then, 20% of a 0.9% NaCl/2% acetic acid solution at pH 3 was added before centrifugation for 20 min at 2000 rpm. The lower organic phase containing lipids was separated and evaporated under nitrogen. The dried lipids were weighed using a high-precision balance, and were stored at -20°C until analysis. For $^{31}$P NMR analysis, the extracted milk lipids were redissolved in a ternary solvent system adapted from a previously published protocol [4], consisting of a mixture of $CDCl_3$, $CH_3OH$, and a 5 mM CsCDTA solution in $H_2O$ (100:40:20). This preparation generates a small aqueous phase on top of the organic phase containing the PLs. Spectra were obtained on an Avance 400 spectrometer (Bruker) using 5-mm tubes, and were referenced to methylene diphosphonate (external chemical-shift and quantitation standard). The acquisition parameters used were: interpulse delay = 9s, spectral width = 4194 Hz, pulse angle = 90° (9.5 µs), 32K data points, $^{1}$H decoupling (Waltz 16), 4096 transients. Spectra were integrated using deconvolution (MDCON software, Bruker) as described previously [5]. Non-parametric statistical tests were used for determining significance of differences between groups.

## Results

**Figure 1** High-resolution $^{31}$P NMR spectra of milk extracts: **A:** cow; **B:** camel; **C:** mare; **D:** human.

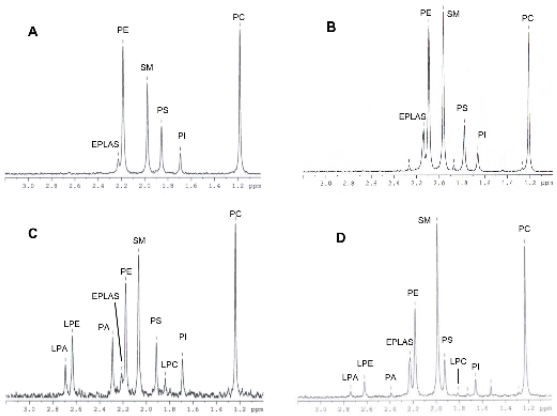
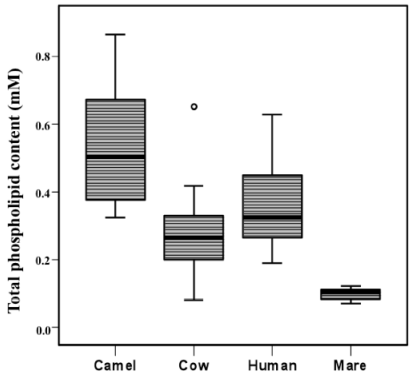



**Figure 2**




The mean concentrations of total lipids varied considerably between species: 60.4 ± 29.5 mg/mL in HM, 45.6 ± 18.7 mg/mL in CaM, 37.1 ± 6.1 mg/mL in CoM, and 6.8 ± 2 mg/mL in MM, in agreement with values found in the literature. Moreover, total PL concentrations were characterized by significantly different values (Fig. 2; horizontal bars represent medians; boxes represent 25th–75th percentiles, and whiskers indicate 5th-95th percentiles. One outlier was identified and is indicated by a small circle). These data were in accordance with the PL content quantified using GC analysis with an internal standard (data not shown). Among the milk species analyzed, MM showed the lowest absolute total PL value (Table 1) and the least dispersed distribution pattern for PL content within samples (Fig. 2). PLs represented 0.2 to 1.8 % of total milk lipid content, and this proportion was significantly higher in MM than in the other species measured (approximately twice as high as in HM; Table 1).

Six out of twelve identified PL classes and subclasses, i.e. phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylethanolamine (PE) phosphatidylserine (PS), phosphatidylinositol (PI) and ethanolamine plasmalogen (EPLAS) were consistently detectable and quantifiable in milk of all species, and their concentrations varied greatly between species. PC concentrations were similar in CoM, CaM and HM, but significantly lower in MM. CaM exhibited the highest levels of SM, PE and PS. Concentrations of PC, SM and PE were rather balanced only in MM.

**Table 1** Concentrations of the most prevalent PL classes in milk from different species. Medians [1st ; 99th percentile] and the total PL content as % of total lipids (bottom line) are given.

| µg/ml | human | cow | camel | mare |
|---|---|---|---|---|
| EPLAS | 27.3 [17 ; 54.9] | 7.1 [0.5 ; 30.7] | 24 [13.4 ; 66.3] | 2.4 [1.2 ; 8.5] |
| PE | 41.5 [26.1 ; 102.8] | 61.8 [19.2 ; 143] | 125 [68.3 ; 182.7] | 15.3 [8.4 ; 23.4] |
| SM | 78.3 [49.9 ; 132.9] | 46.1 [11.9 ; 98.9] | 117.5 [58.3 ; 174.6] | 18 [12.9 ; 22.9] |
| PS | 22.1 [11.3 ; 44.7] | 21.3 [2.5 ; 56.5] | 50 [1.6 : 66.8] | 5.8 [1.8 ; 15.1] |
| PI | 11.1 [2.2 ; 20.7] | 7.6 [0.5 ; 26.7] | 20 [10.3 ; 31.9] | 5.8 [3.7 ; 8.2] |
| PC | 60.3 [32.1 ; 124.2] | 60.3 [18.2 ; 128] | 78.4 [42.2 ; 123.8] | 17.3 [11.3 ; 21.1] |
| total PL | 250.3 [152.9 ; 473.6] | 204 [63 ; 483.7] | 393.4 [257 ; 660.3] | 77.8 [52.6 ; 87.9] |
| PL % of total lipids | 0.56 [0.17 ; 1.07] | 0.55 [0.18 ; 1.27] | 0.87 [0.49 ; 1.79] | 1.08 [0.76 ; 1.73 |

## Discussion

The determination of milk PL fingerprints from humans and different animal species by high-resolution $^{31}$P NMR spectroscopy revealed that human and mare milk provide a broader variety of PL classes than do cow and camel milk, and that human and camel milk are specifically richer in sphingomyelin and plasmalogens compared to the other species studied. This finding is of particular practical interest since it demonstrates that, for instance, camel milk readily available in arid countries can potentially replace human milk with respect to plasmalogens and SM in term newborns. However, premature infants will fail to achieve the same amount of these PLs due to the lower milk volumes ingested by premature infants per day (about one fifth of term newborns). As a consequence of its high amount of total PLs, camel milk also appears as a promising dietary source of PI, PS, SM, PE and plasmalogens for adults, notably in view of their cardioprotective, hepatoprotective and brain protective effects [6].

## References

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