A promising $^{31}$P NMR-multivariate analysis approach for the identification of milk phosphorylated metabolites and for rapid authentication of milk samples

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A B S T R A C T

A fast and reliable method for the identification of milk from different mammalians was developed by using $^{31}$P NMR metabolite profile of milk serum coupled to multivariate analysis (PCA and classification models UNEQ, SIMCA and K-NN). Ten milk samples from six different mammalians, relevant to human nutrition (human, cow, donkey, mare, goat, sheep), were analyzed and eight monophosphorylated components were identified and quantified: phosphocreatine (PCr), glycerophosphorylcholine (GPC), glycerophosphorylethanolamine (GPE), N-acetylglycosamine-1-phosphate (NACGlu-1P), lactose-1-phosphate (Lac-1P), galactose-1-phosphate (Gal-1P), phosphorylcholine (PC), glucose-6-phosphate (Glu-6P). PCA showed interesting clustering based on the animal genus. K-NN can be successfully used to discriminate between donkey and cow samples while UNEQ class-modelling resulted more suitable for compliance verification. Results confirm the natural variability of milk samples among different species. These data highlight the great potentials of NMR-multivariate analysis combined method in the rapid analysis of phosphorylated milk serum metabolites for milk origin assessment and milk adulteration detection.

1. Introduction

Milk is a very complex biological fluid containing a wide variety of bioactive compounds and the most important food source of dietary phosphorus for humans and animals [1]. Identification of milk metabolites is therefore important both in animal science, for a better understanding of mammary gland physiology, and in food and dairy sciences for the assessment of milk composition and quality. Milk is also well suited for diagnostic purposes [2,3], as it can be collected routinely and noninvasively. Metabolite profiles variability naturally occurs in milks from different mammalians and in distinct lactation stages to satisfy the nutritional requirements of the neonates of different species. Changes in milk compositions are also closely connected with breeds, diet and age. Seasonal variations and milk processing must also be considered in lactating dairy animals [1]. Along with originating from several cell types or metabolisms in the organisms, metabolites in milk also reflect the metabolic activity of the mammary gland, which can markedly vary the metabolite profile especially during inflammatory mastitis [4]. Metabolic diseases [2,5], microbial secretions [6,7] and enzymatic reactions [8] may also affect milk composition and yields.

Over the past two decades, nuclear magnetic resonance (NMR) has emerged as one of the principal analytical techniques used in metabolomics [9,10] and food analysis [11]. In particular, $^{31}$P NMR spectroscopy turned out to be a useful tool to qualitatively and quantitatively determine phosphorylated compounds in milk samples and several studies have been devoted to the analysis of milk casein fractions [12-17] and to the identification and quantitation of phospholipids in human and animal milk samples and in infant formulas [18-21]. A $^{31}$P NMR fingerprint of phospholipids from the milk of different species was also reported [22]. On the contrary, in the recent past less attention has been addressed to the identification of phosphorous containing small molecules, although the pioneering $^{31}$P NMR studies on raw milk and milk fractions of various mammalians allowed to identify, along with inorganic phosphate (Pi) and serylphosphate residues of casein (Ser-P), a number of water soluble phosphorylated metabolites [23,24]. Among them, glycerophosphorylcholine (GPC), glycerophosphorylethanolamine (GPE) and phosphorylcholine (PC) are part of the phospholipid pathway that is active in many body tissues, including mammary tissue.

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[2], and also occur in milk as products of hydrolysis of phosphatidylcholine and phosphatidylethanolamine, phospholipids present in milk as constituents of the milk fat globule membranes. The importance of monitoring changes in GPC and PC levels have been reported in a wide range of research. For instance, hypoxia, which is present in many cancers, is known to cause, in a human prostate cancer model, increased GPC and PC levels as an energy shuttle within the cell through the creatine kinase expression [25]. On the contrary, a “GPC to PC switch” in cultured mammalian cells under “slow acidosis” (pH: 7.3–6.5) [26] was attributed to the activation of phospholipids breakdown as an alternative energy source for the cells, as glycolysis was hampered due to the low pH. GPC is also known for its neuroprotection effect on age-related oxidative damage [27], and GPE showed a direct scavenging effect on superoxide anion in human neutrophils (PMN) and in a cell free system [28]. Moreover, GPC and GPE were found to be effective as membrane stabilizers in antioxidant therapies [29]. As far as milk is concerned, the PC/GPC ratio can be a prognostic biomarker of inflammatory states in breastfeeding women, particularly during the first few days after the delivery [3], metabolic diseases in lactating dairy cows [2] or an indicator of microbial degradation and milk spoilage [30,31]. Phosphorylated sugars are key intermediates in the glycosylation process of membrane proteins and in milk oligosaccharides metabolism. Glucose-6-phosphate (Glu-6P), a central metabolite in lactose synthesis, glycolysis and pentose phosphate pathway, has been suggested as a potential indicator of dairy cow’s oxidative stress in early lactation [32]. Decreasing concentrations of N-Acetylgalactosamine-1-phosphate (NacGal-1P) and Galactose-1-phosphate (Gal-1P) in UHT milk has been observed on storage [33]. Altered concentrations of Gal-1P, glucose-6-phosphate (Glu-6P), phosphocreatine (PCr) and N-Acetylgalactosamine derivatives were also detected in milk from dairy cows experiencing negative energy balance conditions [34,35] while PCr was found to decrease in milk samples from heated-stressed dairy cows [5]. PCr is a high-energy product behaving both as short-term energy reserve and as an energy shuttle within the cell through the creatine kinase/PCr/creatinine system [36]. Creatine is secreted by the mammary gland during lactation and has been shown to be essential for normal brain development and brain function [37]. Differences in milk PCr concentrations among mammals have been correlated to the mammary gland metabolic activity and to specific neonate requirements during the suckling period [38].

These minor phosphorylated compounds have been quantitatively determined by means of 31P NMR analysis in concentrated cow’s milk [12,23,24,30,31] and, more recently, buffalo’s milk [18]. Interestingly, differences were observed in the 31P NMR profiles of phosphorylated metabolites in milk from different mammals [24], suggesting that, together with disease diagnosis and safety food assessment, 31P NMR spectroscopy of milk can be successfully employed for rapid authentication of milk samples.

The aim of this work was to develop a simple, fast and reliable method for the identification of milk from different species by the 31P NMR spectra of their serum, coupled to multivariate analysis, which could routinely be used in laboratory analysis, without milk manipulation, and the use of toxic solvents [39], for origin assessment, medical diagnosis and quality control of milk samples.

Milk relevant to human nutrition (human, donkey, cow) and dairy production (cow, goat, sheep), were investigated. Mare’s milk was also analyzed for a comparison with donkey’s samples. Within each species, samples from specimens of different breeds, ages, and period of lactation have been examined.

2. Materials and methods

This study was conducted in accordance with the Declaration of Helsinki, and with ethical approval from the Medical Ethics Committee of University of Messina.

A written informed consent was obtained from healthy volunteer women.

The animal care and use procedures were approved by the University of Messina Animal Committee and performed in accordance with the EU Directive 2010/63/EU for animal experiment.

Informed consent from animal owners was provided.

2.1. Materials

Sodium cholate, ethylenediaminetetraacetic acid (EDTA), hexamethylenephosphoramidate (HMPA), glycerophosphorylcholine (GPC), phosphocholine (PC), phosphorylethanolamine (PE), galactose-1-phosphate (Gal-1P), N-acetylgalactosamine-1-phosphate (NacGal-1P), glucose-6-phosphate (Glu-6P), Phosphocreatine (PCr) and D2O (δ 4.7 ppm) from Sigma Aldrich (St Louis, MO).

Animal milk samples were obtained from animals by farms of Messina, Palermo and Agrigento areas, Italy. Also commercial fresh milks (2 cows and 1 goat) from local food market were analyzed.

The study was carried out on 10 breastfeeding women (aged 28–32 years, at 90–180 days postpartum); 8 cows (pezzata rossa breed, aged 3–7, mean weight 400 ± 50 kg, in lactation from 90 to 200 days), fed on mixed rations of concentrates (30%) and fresh forage (70%); 10 cross-breeds mares, (aged 5–12 years, mean weight 430 ± 60 kg, in lactation from 30 to 60 days), fed on fresh forage and oats; 10 donkeys (3 sarda and 7 ragusana breeds, aged 3–7, 370 ± 40 kg, in lactation from 40 to 90 days) fed on hay, corn and oat; 10 goats (2 tibetana, 2 siriana, 6 girgentana breeds, aged 3–10, mean weight 20 ± 5 kg, in lactation from 75 to 120 days) fed on fresh grass and 10 sheep (Valle del Belice breed, aged 3–4 years, mean weight 30 ± 6 kg, in lactation from 60 to 120 days) fed on fresh grass.

2.2. Milk sample preparation

Milk samples (3–4 ml) were collected into sterile tubes, stored at 277 K and analyzed within 24 h after milking.

In order to acquire 31P NMR spectra milk samples were prepared as follows: 0.9 ml of milk was added to 0.2 ml of a sodium cholate solution (with 5 mM EDTA) in D2O to reach a final sodium cholate concentration of 220 mM and then centrifuged at 13,000 g for 10 min. To 0.5 ml of supernatant, 10 μl of HMPA solution (0.1 M) were added (HMPA final concentration 2 mM) and the solution transferred to NMR tubes (5 mm diameter). Prior to the 31P(1)H NMR analysis samples were bath sonicated for 4–6 min (Model 8891, Cole-Parmer). The pH of all raw milk samples investigated was in the 6.5–7.0 range.

2.3. NMR spectroscopy and quantification of phosphorylated compounds

High-resolution 31P NMR spectra were recorded on a Varian VNMRS-500 spectrometer, operating at 202.45 MHz. Broadband proton irradiation (waltz 16) was applied to eliminate 1H-31P NMR coupling.

 Routinely 256 transients, with 1 s delay time, 90° pulse width, were collected for a total acquisition time of about 11 min for each sample. The probe temperature was 298 ± 1 K. All measurements were performed with D2O as the internal field/frequency lock. Spectra were processed, using the MestReNova 12.0.2 software, with 2 Hz apodization, manually phased and base line corrected. Chemical shift assignments were referenced relative to hexamethylenephosphoramidate (HMPA) at δ 29.90 ppm and were attributed with the help of standards or literature data. Concentrations were calculated from the spectra by comparing the peak integrals of phosphorylated metabolites resonances with HMPA (2 mM) peak area.

2.4. Multivariate analysis

The whole data analysis was performed in the Matlab environment (The MathWorks, Inc., Natick, MA, USA, Version 2020a) using the PLSToolbox package (Eigenvector Research, Inc. Manson, Washington).
Dataset (Table S1) contains sixty samples from milks and eight measured variables, corresponding to the concentrations (mM) deriving from the integration of $^{31}\text{P}$ NMR signals relative to seven phosphorylated metabolites (PCr, GPC, GPE, NAcGlu-1P, Gal-1P, PC and Glu-6P), and to $\varphi_1$ chemical shift ($\delta$, ppm). Each sample is labeled with a class variable corresponding to the species from which it has been sampled. Six classes of the same numerosity are then listed: cow (C), donkey (D), mare horse (M), sheep (S), woman (W), and goat (G).

Dataset has been submitted to PCA (Principal Component Analysis) to explore variability and to check for similarities among samples and variable. Kaiser’s rule was adopted to assess the significance of the principal components. In addition, a subset containing only the data of donkeys and cows was selected for classification purposes. To this aim, two linear, UNEQ [40] and SIMCA [41], and one non-linear, K-NN (K-Nearest Neighbors) [42], algorithms were tested. For the classification methods, two variables were eliminated, PC and Glu-6P, since in all samples of cow milks concentration values of PC and Glu-6P were below LOQ, so listed as 0 in the dataset. The resulting subset (containing twenty samples) was divided into a training set containing fourteen samples of donkey and cow, are reported in Table 2.

Multivariate analysis

Metabolite concentrations (mM) calculated by $^{31}\text{P}$ NMR analyses of milk serum spectra and $\varphi_1$ chemical shift ($\delta$, ppm) (Table S1) were used in the dataset for multivariate analysis. The Lac-1P variable was eliminated because its resonance showed overlapping with $\varphi_1$ signals in cow’s, goat’s and some mare’s samples. For the same reason, in sample G5 the fields of NAcGlu-1P and Lac-1P were filled with the average class value for those variables. A value equal to 0 was inserted when metabolites’ resonances were absent or could not be integrated ($<0.1 \text{ mM}$). Results obtained from PCA analysis are reported in Fig. 3.

The results of the classification models (UNEQ, SIMCA and K-NN), tested on a subset (Table S2) of twenty data belonging just to the classes of donkey and cow, are reported in Table 2.

4. Discussion

4.1. $^{31}\text{P}$ NMR analysis of milk serum

Compared to other analytical tools, NMR is not a very sensitive technique and it should not be used to safely determine low analyte concentrations. In addition, a major drawback in quantitative $^{31}\text{P}$ NMR analysis is the long $T_1$ relaxation of phosphorous nuclei that makes fully relaxed experiments, desirable for accurate signal integration, extremely time-consuming.

$^{31}\text{P}$ NMR spectra of phosphorylated components extracted from foodstuff may be broad and unsuitable for quantitative analysis, as they tend to self-aggregate in both polar and apolar solvents. Detergents, as sodium cholate, normally possess one polar and one apolar moiety, and, as phospholipids, are able to produce very small micelles consisting of...
only a very few molecules yielding highly resolved $^{31}$P NMR spectra [44]. Moreover, the pH and temperature dependence of $^{31}$P chemical shifts when using sodium cholate as detergent has been reported [45].

To set up a rapid and efficient milk analysis protocol, preliminary experiments were performed to establish the best experimental conditions for the preparation of the NMR samples and the NMR acquisition.

Fig. 2. Representative $^{31}$P NMR (202.45 MHz, cholate/D$_2$O, 298.15 K) spectra of milk serum from a) cow; b) donkey; c) mare; d) goat; e) sheep; f) human.

**Table 1**

Concentration ranges (mM) of phosphorylated metabolites in milk samples.

|        | PCr | GPC | GPE | NAcGlu-1P | Lac-1P | Gal-1P | PC | Gla-6P |
|--------|-----|-----|-----|-----------|--------|--------|----|--------|
| cow    | 0–0.14 | 1.01–1.28 | 0.17–0.32 | 0.57–2.52 | n.d. (overlap)$^a$ | 0.15–0.47 | n.d. | n.d.   |
| donkey | 0.36–1.04 | 0.57–1.38 | 0.13–0.36 | 1.62–4.29 | 0.56–1.55 | 1.05–2.15 | 0.0–0.56 | 0.0–0.16 |
| mare   | 0.37–0.76 | 0.39–0.81 | 0.16–0.50 | 1.43–2.48 | 0.49–0.90$^b$ | 0.21–0.92 | 0.49–1.52 | n.d.   |
| sheep  | n.d.  | 0.4–1.08 | 0–0.27 | n.d. | n.d. | n.d. | 0–0.34 | n.d.   |
| human  | n.d.  | 0.29–0.69 | 0–0.20 | n.d. | n.d. | n.d. | 0–1.23 | n.d.   |
| goat   | 0.35–0.81 | 0.21–0.85 | 0.1–0.33 | 0.15–0.61$^b$ | n.d. (overlap)$^a$ | 0.15–0.62$^b$ | 0.6–1.49 | 0–0.35 |

$^a$ Signals overlapping with P$_i$ resonance.

$^b$ Referred to a number of samples < 10 because of the overlapping with P$_i$ resonance.

Fig. 3. a) Score plot (PC1 vs. PC2) of the milk dataset. Assigned classes are reported with the first letter (e.g., W for Women); b) Loading plot (PC1 vs. PC2).
parameters. To this end, 0.9 mL of cow’s milk was mixed with 0.2 mL of either D2O or a solution of detergent (TritonX-100 or sodium cholate)/EDTA in D2O (D2O was used as internal field/frequency lock) [46]. EDTA was added to complex divalent cations that are known to broaden 31P NMR resonances [47], because of their strong association with phosphate anions. In all samples (Fig. S1), the low intensity observed for the resonances relative to the serine-phosphate residues (Ser-P, 2.5–4.0 ppm) of casein indicates that an efficient separation of this protein from milk serum has occurred. In the cholate/EDTA/D2O solvent system, the signal relative to inorganic phosphate (Pi) appeared sharper and, because of the slightly higher pH, shifted towards lower fields, thus allowing to determine the presence of a major number of resonances relative to sugar-1-phosphate derivatives in the region 1.5–2.5 ppm. According to these observations, the sodium cholate solution was selected as the solvent of choice for the preparation of milk samples.

In addition, when compared to HMPA standard, no significant variations were found in the metabolites’ peak integrals at different relaxation delay times (1, 10 or 60 s) [30], indicating that all the phosphorous nuclei in the sample have similar relaxation times. The shorter delays could therefore be used in the acquisition of the spectra for the determination of metabolites’ concentration, greatly reducing the experimental times.

In our analyses we decided to investigate the 31P NMR spectral region (from −5 to 5 ppm) displaying the resonances of monophosphorylated metabolites. However, no detectable resonances were found in the remaining spectral regions of the spectra (δ spanning from −10 to 40 ppm).

In a typical experiment, individual 31P NMR spectra were recorded after the addition of each standard in the milk sample. The presence or absence of target phosphorylated metabolites was derived from a change in peak intensities or the appearance of new resonances. Final addition of PE slightly affected the chemical shifts of pH sensitive sugar phosphates, Pi and PC.

Interestingly, phosphorous containing molecules showed similar 31P NMR profiles in individual animal species, almost independently from breed, age and lactation, supporting that fingerprints of milk from different mammals can be easily determined by 31P NMR spectroscopy (Fig. 2).

Although treated with an alkaline cholate solution, the pH-dependent chemical shift of Pi reflected the known variability of original pH in milks from different mammals. As an example, the pH of untreated donkey’s milk (pH 6.8) and cow’s milk (pH 6.6) milks proportionally turned to higher pH (pH 7.2 and 7.0 respectively). A shifting of Pi resonance to lower fields (higher ppm values) corresponds to a pH increasing in milk samples. PCr can be detected by 31P NMR in the milk from donkeys, mares and goats and it is also present in some cow’s milk samples, regardless of age, race and feeding. GPC and GPE are always present, even if GPE resonance could not be integrated in some human and sheep samples (<0.1 mM). Detection of low concentration of PC was sometimes hampered by the overlapping with casein Ser-P as well as PE, which could not be determined under our experimental conditions.

In more details, cow’s milk is characterized by the presence of GPC, GPE, NAcGlu-1P and Gal-1P. In the experimental conditions the Pi resonance always overlaps with the signal of Lac-1P. PCr was detected in four out of ten cow’s milk samples while Glu-6P was never detected. Not significant differences were found between raw milks and commercial fresh milks (C1 and C7, Table S1).

Equine milks showed a higher abundance of sugar-1-phosphate derivatives. As reported for rabbit’s milk [24], the larger amount of these metabolites can be ascribable to the low activity of alkaline phosphatase enzymes in equine milk (35–350 times less than cow’s milk) [48]. Apart from Glu-6P, which was detected only in one donkey’s sample, all the remaining metabolites were always present in donkey’s and mare’s milks. However, in half of the mare’s samples the Pi resonances overlapped with the signals from Lac-1P. In any case, quantitative determination of Lac-1P can be carried out by increasing the solution pH (addition of NaOH) as shown in Fig. S2.

In line with the lower fat content of donkey’s milk, clearer NMR samples were obtained during serum separation. On the contrary, samples deriving from goats’ milks resulted less transparent and residual resonances, ascribable to phospholipids, appeared in the −1–0 ppm region [49]. Except for Lac-1P resonances, overlapping with Pi, all the other metabolites were detected in goat’s milk. In particular, Glu-6P was present in four samples. Commercial sample (G1, Table S1) displayed similar characteristics of just milked ones. Although milks from sheep and woman displayed similar 31P NMR spectra, which only allowed the identification of GPC, GPE and PC, more attention should be paid in the separation of serum in sheep’s milk samples in order to avoid the extraction of casein precipitates and floating cream. Nevertheless, the samples of sheep’s milk serum always appeared yellowish when compared to the other milk samples, while human milk samples looked almost transparent all the time. PC was detected only in two samples of sheep’s milk serum, while it was almost ubiquitous in human milk (eight samples out of ten). Phosphorylated sugars, that probably are metabolic intermediates of biochemical pathways, were not detected in human and sheep milk. Because of the errors associated to peak integration and the low concentration of some of these metabolites in diluted milk samples, only concentrations greater than 0.1 mM were determined. The range of concentrations (Table 1) determined for these metabolites is in line with literature data, thus confirming the viability of our methodology to detect phosphorylated metabolites in milk serum without pre-concentration of milk samples.

### 4.2. Multivariate analysis

In PCA analysis, only two principal components appear to be significant according to the Kaiser’s rule, with an explained variance of 45.19% and 23.99%, respectively. Score plot (PC1 vs. PC2) is given in Fig. 3a, showing that classes are fairly well discriminated in this space. Evaluation of further PCs did not improve the interpretation of the dataset variability (Table S3).

PC1 explains the genus, having positive values for equines (donkeys and mares), values around 0 for cows, slightly negative values for ovines (sheep and goats), and negative values (<1) for women. More in detail, sheep and women are almost superimposed, goats’ class is more spread along PC1 with values comprised between −2 and +2. PC2 splits mares (positive values) from donkeys (negative values), cows have negative values, goats have positive values, sheep and women around 0. According to the loading plot reported in Fig. 3b, variables responsible of PC1 are PCr, GPE, Ga1-1P, Pi and NAcGlu-1P, whereas those responsible
for PC2 are PC, Glu-6P and GPC. Moreover, since samples belonging to the same class are close to each other and seem to form groups, score plot reported in Fig. 3a seems to be promising in the development of an NMR-based classification method for compliance verification and milk adulteration detection. In these cases, class-modeling models should be preferred rather than discriminant approaches.

Donkey milk has been recently revalued for its nutritional properties and proposed as an effective alternative food for infants with cow milk allergy [50]. Blending cow milk with more expensive donkey milk could represent a fraudulent practice that may result hazardous to the health of these sensitive patients. Some methods have been recently described to detect possible donkey milk adulteration [51–53]. In this light, three classification algorithms were tested on a subset (Table S2) of twenty data belonging just to the classes of donkey and cow, namely UNEQ, SIMCA and K-NN. Even if the latter is a non-linear discriminant classification algorithm, it was used for its peculiarity of introducing a “grey zone” of unassigned samples when the value of K is set to be even. The results of the classification models are summarized in Table 2.

K-NN resulted very powerful in discriminating donkey and cow milks, with only one error in the training and no errors in the test set. All the three mixtures (Table S2) were assigned to the grey zone, as for the aim of the classification. Both UNEQ and SIMCA showed high efficiency in the test set, with values of 100 % and 90.3 %, respectively. Performances on the calibration set are similar, but if UNEQ performed 100 % in sensitivity and 56.1 % in specificity, SIMCA obtained exactly opposite results. Considering that sensitivity can be seen as the experimental measure of the confidence level, whereas specificity can be measured only in association to another class, UNEQ should be preferred over SIMCA. By the way, UNEQ predicted the sample 75:25 (D:C) to be a donkey milk, the other two mixtures to be assigned to both classes, whereas SIMCA predicted these samples to be unassigned. The mentioned class-modeling algorithms (UNEQ and SIMCA) were also tested to not trained classes by simply assigning data of such classes to class 1 (donkey) in the validation set (n = 40). In this way, samples predicted to be into class 1 are regarded as a wrong assignation. Results show that only one sample was predicted to class 1 using the SIMCA model (2.5 % of errors) and two for the UNEQ model (5 % of errors); K-NN was not tested since it is not a class-modeling algorithm.

Yet, from the data obtained, novel NMR/chemometric models look very promising for the compliance verification of such an important commercial product.

5. Conclusions

Our results reveal the great potentials of the combined $^{31}$P NMR/chemometrics approach in the analysis of a very complex biological fluid such as milk, for the assessment of milk origin through the detection/analysis of only a few milk serum phosphorylated metabolites.

A rapid $^{31}$P NMR analysis protocol of milk serum, requiring minimum sample manipulation and short acquisition times, was developed and the phosphorylated metabolite profiles from different mammalians were compared highlighting differences in samples from different species. Multivariate analysis resulted to be a powerful tool for the interpretation of data coming from NMR analysis. PCA showed interesting grouping based on the peculiarity of the genus while classification models were proposed for compliance verification among commercially valuable donkey’s and cow’s milk samples. Performances of the classification algorithms are encouraging for building reliable and robust models of wide applicability. Our methodology can be proposed as a valuable tool for confirming the presence of phosphorous-containing metabolic compounds in a very complex system as milk, to characterize interrelations between each other, and the effect of influencing factors as different management systems, farms, lactation times, and also to monitor specific traits related to the state of health and handling. In addition, the $^{31}$P NMR/multivariate statistical analysis protocol shows promising potentials in food and dairy sciences and technologies for verifying the authenticity of milk commercial formula from different brands and/or for the design of functional food through biotechnological strategies, improving nutritive and dietary values of milk for the enhancement of human health.

6. Author contributions

All authors have made substantial contributions to each step of experimental procedure and manuscript preparation. Alida Ferlazzo and Anna Notti conceived the idea for the project and designed the experiments. Anna Notti and Giuseppe Bruschetta performed the experiments. Anna Notti, Giuseppe Bruschetta and Gabriele Lando analyzed data. Gabriele Lando performed multivariate analysis. Alida Ferlazzo, Anna Notti and Giuseppe Bruschetta wrote the manuscript. All authors oversaw all stages of the present study and drafted and revised this manuscript.

6. Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101087.

Author declaration template

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

We further confirm that the order of authors listed in the manuscript has been approved by all of us.

We confirm that we have given due consideration to the protection of intellectual property associated with this work and that there are no impediments to publication, including the timing of publication, with respect to intellectual property. In so doing we confirm that we have followed the regulations of our institutions concerning intellectual property.

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