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Evaluation of four procedures for preparing mare milk samples for proteomic analysis

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SUMMARY

There is no universal method to prepare physiological fluids for 2-DE proteomic analysis. Furthermore, interspecies differences in milk composition require the formulation of a species-specific sample preparation procedure.

The study was carried out on mare's milk which was prepared for 2-DE in four different methods: the first sample (M1) was defatted, sample M2 was defatted and after casein precipitation, sample M3 was sample M2 after reduction of high molecular proteins and sample M4 was desalted sample M3.

The milk samples prepared in different methods were separated by 1-DE and 2-DE. The obtained gels were analysed qualitatively and quantitatively. Furthermore, selected protein spots were identified by MALDI-TOF MS.

Analysis of 1-DE and 2-DE gel images indicated that the optimal procedure for preparing mare milk samples for 2-DE and identification of proteins by MS is a method based on defatting and precipitation of caseins.

The preparation of mare's milk samples by defatting and then precipitation of caseins is enough to obtain an optimal 2-DE separation for identification of proteins of this body fluid. The method of caseins precipitation should be improved in order to reduce the proportion of these high-abundance protein in milk samples which could increase in the identification of low-abundance proteins in mare's milk.

KEY WORDS*:* mare, milk, proteomic, sample preparation for 2-DE

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INTRODUCTION

In mares, milk is secreted from the second day after foaling. Milk consists of nutritional and regulatory substances, among which, in addition to enzymes, hormones, vitamins, minerals, fatty acids, etc., proteins must be mentioned. Proteomic tools are used to determine the protein composition of biological material. They allowed to identify 379 proteins in human breast milk (Zhang et al. 2017), more than 3100 proteins in cow milk (Zhang et al. 2011, Zhang et al. 2015, Mol et al. 2018, Maity and Ambatipudi 2019), 1307 proteins in goat milk (Verma et al. 2020), 669 proteins in sheep milk (Ha et al. 2015) and 231 proteins in pig milk Ogawa et al. 2014). The low-abundance proteins present in cow's milk are mainly involved in the synthesis and secretion of milk components and in the process of growth and development of the newborn Medeńska and Dratwa-Chałupnik 2021). The available literature has no data on the protein profile of mare milk.

Depending on the proteomic tools used, a key step in proteomic analyses is the preparation of biological material for the appropriate analyses. Considering the different types of samples (cells, tissues, body fluids), there is no homogeneous procedure for preparing biological material for this type of analysis (Dratwa-Chałupnik et al. 2016, Lepczyński et al. 2017). The aim of the presented work was to select the optimal procedure for preparing mare milk samples for proteomic analysis using two-dimensional electrophoresis (2-DE) combined with mass spectrometry (MS). Therefore, based on literature data (Golinelli et al. 2011, Zhang et al. 2011, Zhang et al. 2017), a milk sample preparation methodology was devised in the following four steps: defatting, casein precipitation, reduction of high molecular weight proteins and sample desalting. At each of the stages, sample proteins were separated by 1-DE and 2-DE. In this research work, it was hypothesized that the comparative analysis of 1-DE and 2-DE gels of each stage of the analysis would indicate the optimal procedure for preparing mare milk samples for proteomic analysis using 2-DE and MS.

MATERIALS AND METHODS

Animals

The experiment was carried out on milk of 6 nine-year-old mares of the noble Polish half-bred breed from the horse farm Nowielice z.o.o. The mares were bred in the station-pasture system. During autumn and winter they were fed with oats and sow, while during spring and summer they additionally had access to fresh grass. For the presented methodological study, milk samples collected by hand milking into a clean glass beaker on the 7th day of lactation were used. The sample was cleaned of environmental contaminants using a strainer, portioned into 50 ml phalcones and placed at -20°C until the samples were transported to the laboratory. In the laboratory, samples were placed at -80°C until starting the proteomic analysis.

Sample preparation

In order to select the optimal procedure for preparing mare milk samples for analysis using 2-DE and MS, four sample methods were prepared, which resulted in four milk samples: M1 – defatted, M2: defatted and caseins reduced, M3: M2 + high molecular weight proteins reduced, M4: M3 + desalted. Each samples were precipitated with acetone at -20°C and incubated for 2h. After incubation, the samples were centrifuged at 4°C, 13000g, for 45 min. Finally, the resulting pellet was dissolved in lysis buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 0,2% w/v 3 to 10 carrier ampholytes, 100 mM - 1,4-Dithiothretiol).

1. Defatting

Defatted by centrifugation at 4°C, 4500g for 30 min. The fat layer on the surface was removed mechanically.

2. Precipitation of caseins

Precipitation samples with 30% acetic acid to reduce caseins. Acetic acid was added until a pH of 4,6 was obtained. After precipitation, the samples were centrifuged at 4°C, 3380g for 15 min.

3. Removal of high molecular weight proteins

To remove high molecular weight proteins, Amicon® Ultra-15 filter columns (Sigma-Aldrich) with a cut-off point of 100 kDa were used according to the with the producer's instructions.

4. Desalting

Sample desalting was carried out using the ReadyPrepTM 2-D Cleanup Kit (Bio-Rad) according to the with the producer's instructions.

One-dimensional electrophoresis

Samples including 10µg of protein were mixed with Laemmili buffer (30% v/v 0,5 M Tris pH 6,8; 10% w/v SDS, 50% v/v glycerol, 20% v/v β-mercaptoethanol, bromophenol blue). In the next step, samples were incubated for 15 min at 60°C and separated on a 12% polyacrylamide gel for 30 min at 40V and then for 90 min at 100V using Mini-PROTEAN® Tetra Cell (Bio-Rad). After the 1-DE, gels were stained using the modified Bradford method with Protein Assay Dye Reagent Concentrate (Bio-Rad) mixed v/v 1:20 with distilled water.

Two-dimensional electrophoresis

Isoelectric focusing was performed in two stages: combined rehydration (passive - 6h, 20°C; active - 12h, 20°C, 50V) on linear 7 cm pH range 4-7 ReadyStrip IPG strips (Bio-Rad) using Protean i12® IEF Cell (Bio-Rad) with 120 µg of mare's milk protein and isoelectric focusing performed in a Protean i12® IEF Cell (Bio-Rad) according to the following procedure: 50V for 100Vh, 250V for 150Vh, 500V for 500Vh, 1000V for 1000Vh, 2h with a linear voltage increase from 1000V to 5000V, after which 5000V for 90000Vh. Following step was strips equilibrated for 15 min in a base buffer (6 M urea; 0,5 M Tris/HCl pH 6,8; 2% w/v SDS, 30% w/v glycerol) with 1% DTT added. Over 15 min, the strips were transferred to base buffer with 2,5% iodoacetamide for 20 min. 2-DE was carried out at 40V for 1h and electrophoresis at 100V for 90 min on 12% polyacrylamide gels in Tris-glycine buffer (25 mM Tris-HCl, 192 mM glycine; 0,1% SDS) using Mini-PROTEAN® Tetra Cell (Bio-Rad). In the end of 2-DE, the gels were stained using the modified Bradford method with Protein Assay Dye Reagent Concentrate (Bio-Rad) mixed v/v 1:20 with distilled water.

Analysis of the images obtained

The stained gels scanned using a GS-800TM Calibrated Densitometer (Bio-Rad). Using PDQuest Advanced Analysis Software 8.0.1 advanced (Bio-Rad), the obtained 2D images were processed and comparative quantitative and qualitative analysis was performed.

Mass spectrometry

Stained spots cut out, then decolourised with 25 mM NH_4HCO_3 in 5% v/v acetonitrile, then dehydrated with 100% acetonitrile and vacuum dried (Concentrator 5301, Eppendorf). The dried spots were incubated with trypsin overnight at 37ºC (20µl/spot 12,5µg trypsin/ml in 25 mM NH_4HCO_3; Promega). After incubation, the obtained peptides were extracted with 100% acetonitrile. In the next step, 1µl of sample and 1µl of matrix solution containing 5 mg/ml CHCA;

0,1% v/v TFA, 50% v/v acetonitrile were mixed on a MALDI-MSP AnchorChipTM 600/96 plate (Bruker Daltonics). A calibrant (Peptide Calibration Standard II) was also applied, which was a mixture of peptides in the mass range 700 - 3200. Microflex™ MALDI TOF MS (Bruker Daltonics) was used to calibrate the mass spectrum. The obtained spectra were processed through MASCOT search tool and compared to mammalian protein databases (Swiss-Prot , NCBI).

RESULTS

The main aim of the study was to create a procedure for the preparation mare's milk samples to obtain optimal protein separations using 2-DE with the highest number of protein spots. For this purpose, 1-DE and 2-DE separations were made on milk samples from the same mare prepared according to different methodologies: sample M1 - defatted milk; sample M2 - defatted milk and caseins reduced; sample M3 - defatted milk, caseins and high molecular proteins reduced; sample M4 - defatted milk, caseins and high molecular proteins reduced, desalted.

The 1-DE gel image shows that the M3 and M4 samples from high molecular weight proteins reduction resulted in the number of proteins with molecular weights between 25 kDa to 50 kDa and above 75 kDa, at the same time showing bands with proteins with molecular weights of 10, 15, 20 and 25 kDa, which were poorly visible in M1 and M2 samples (Fig. 1). The 10-15 kDa range includes α-lactalbumin and β-lactoglobulin.

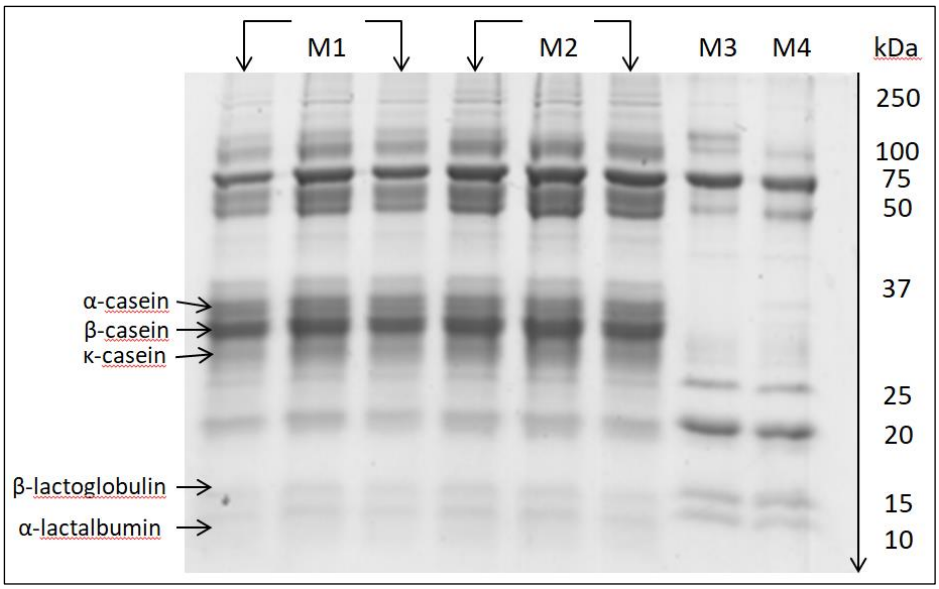

Fig. 1. 1-DE protein profiles of all mare milk sample preparation methods (M1 – defatting; M2 - defatting and casein reduction; M3 - defatting, casein and high-molecular-weight proteins reduction; M4 - defatting, casein and high-molecular-weight proteins reduction and desalting).

Following the analyses carried out, the characteristic bands for the individual protein fractions present in the mare's milk were marked on the gel image after 2-DE separation (Fig. 2).

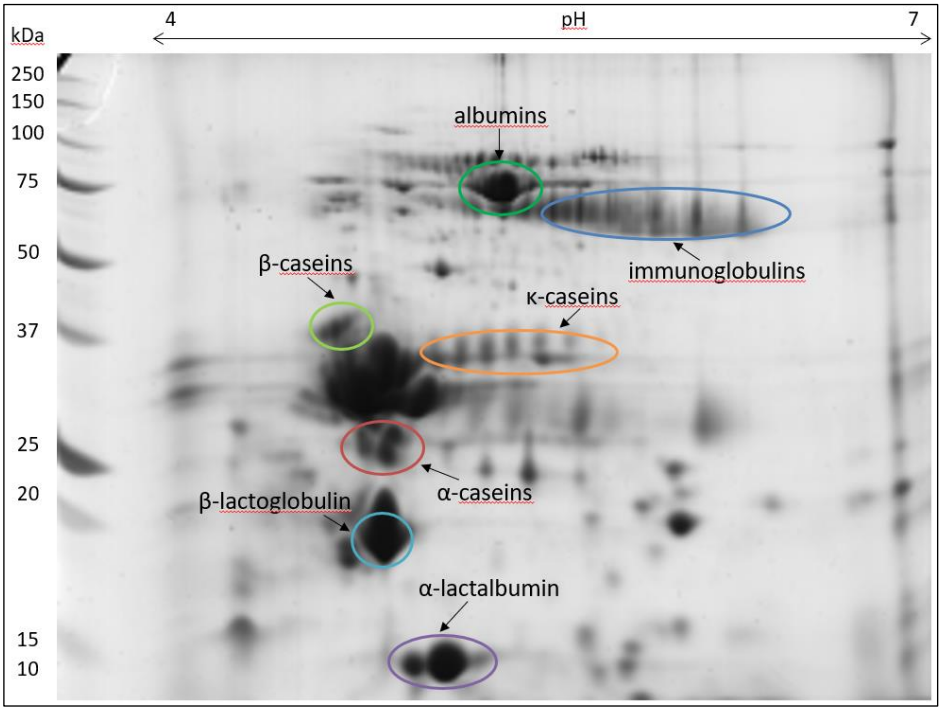

Fig. 2. 2D gel image of a mare's milk sample defatted and reduced casein (M2) stained using the modified Bradford method. Proteins (120 µg) were separated on 7cm IPG with a pH gradient of 4-7 for the first dimension, followed by 12% SDS-PAGE. Protein spots representing the protein fraction of the mare's milk were marked with different colours.

Quantitative and qualitative analysis of 2-DE gels using PDQuest Advanced Analysis Software 8.0.1 advanced (Bio-Rad) showed that 473 protein spots were present in the defatted milk sample (M1), 566 protein spots were present in the defatted sample with reduced caseins (M2), 323 protein spots were present in the defatted sample with reduced caseins and high molecular weight proteins (M3), and 348 protein spots were present in the defatted sample with reduced caseins and high molecular weight proteins and desalted (Fig. 3). As a result, it was shown that the 303 protein spots present on the 2-DE gel of sample M2 were not present on the other 2-DE gels (Fig. 3).

The 2-DE gel image of sample M1 showed streaks (Fig. 3). Furthermore, comparative analysis showed that there were 93 less protein spots on the gel image of sample M1 than on the gel image of sample M2. The M3 sample showed a significant reduction in the number of protein spots in the range of molecular masses from 25 kDa to 50 kDa. The results of the comparative analysis showed that there are 239 less protein spots on the 2-DE gel of sample M3 than on the gel of sample M2. Additional step of preparing the milk samples by removing salt resulted in more spots being

visualised compared to sample M3. However, compared to the 2-DE gel of sample M2, there were 209 less protein spots on the 2-DE gel image of sample M4.

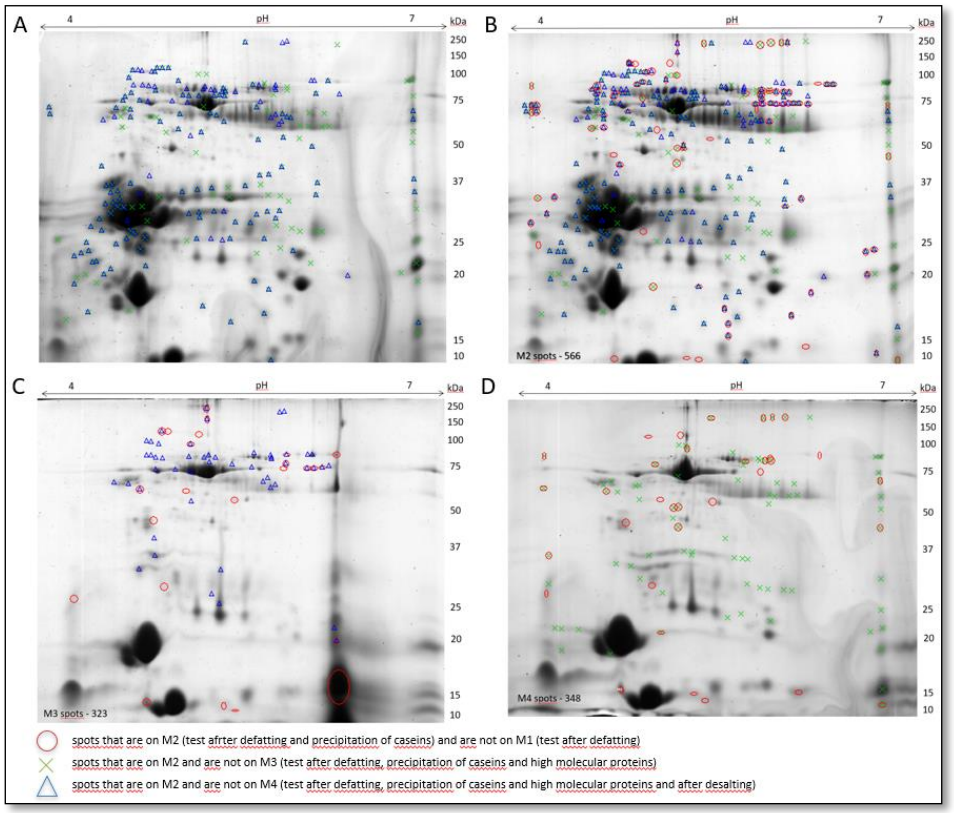

Fig. 3. Images of 2-DE gels of mare milk samples (M1-M4) after analysis with PDQuest software. Different figures show protein spots that were not present on the 2-DE gels of samples M1, M3 and M4. The 2-DE gels show mare's milk proteins stained using the modified Bradford method (120µg of proteins, 4-7L IPG strips, 12% SDS-PAGE). A) sample M1 – defatted milk; B) sample M2 – defatted milk and after casein precipitation; C) sample M3 – defatted milk, after casein precipitation and with reduced amount of high molecular proteins; D) sample M4 – defatted milk, after casein precipitation and with reduced amount of high molecular proteins and desalted.

From the 2-DE gel, 23 protein spots characteristic of the protein fractions highlighted in Fig. 2 were selectively cut out. Among the 10 identified proteins, marked in Fig. 4, serum albumin, beta actin and beta-lactoglobulin-1 were present on all 2-DE gels (Tab. 1).

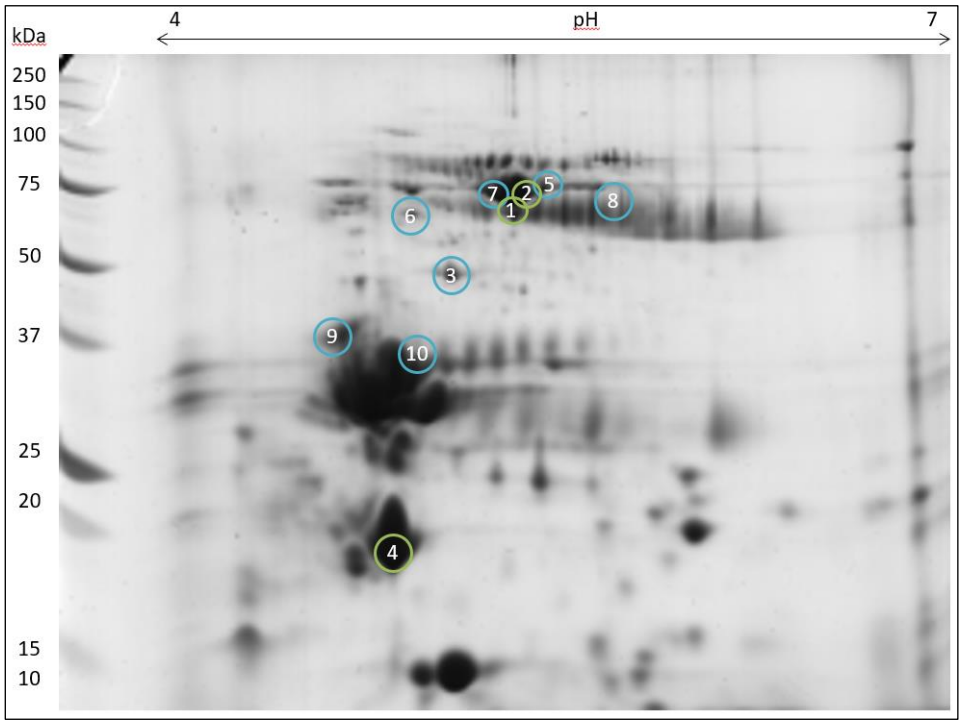

Fig. 4. Gel after 2-DE separation with marked spots that were identified by MS. The spot numbers correspond to the numbers of Tab. 1. The green colour represents proteins present on all gels. Blue indicates proteins present only on the 2-DE gel of sample M2.

Table 1

Protein spots identified using MALDI-TOF mass spectrometer and MASCOT search tool using UniProt and NCBI databases.

No.	Protein name	Acession number	Mass values searched/ matched	Sequence coverage (%)/ mascot score	Calculated pI	Taxonomy
1	Serum albumin	XP_008524663	23/9	22/81	5,78	*Equus przewalskii*
2	Serum albumin	XP_008524663	22/10	23/97	5,78	*Equus przewalskii*
3	Beta actin, partial	AGH58109	22/11	55/130	5,56	*Microtus levis*
4	Beta-lactoglobulin-1 precursor	NP_001075962	17/7	52/90	4,95	*Equus caballus*
5	Serum albumin	XP_008524663	22/12	26/133	5,78	*Equus przewalskii*
6	Vitamin D-binding protein	XP_001489400	19/9	32/106	5,46	*Equus caballus*
7	Serum albumin	XP_008524663	28/14	33/142	5,78	*Equus przewalskii*
8	Immunoglobulin G heavy chain, partial	AAG01011	18/7	27/73	5,70	*Equus przewalskii*
9	Alpha S2 – casein precursa	NP_001164238	11/5	19/63	6,34	*Equus caballus*
10	Interleukin-24 isoform x1	XP_008508951	13/5	25/73	9,35	*Equus przewalskii*

DISCUSSION

The using of proteomic analyses enable the determination of the protein composition of a different biological material (tissues, organs, body fluids). Various research tools are used for proteomic analyses of body fluids. To determine the plasma/serum proteome, 1-DE and 2-DE in combination with MS/MS, MALDI-TOF MS, nanoLC LTQ Orbitrap MS or gel and liquid chromatography/quadrupole have been used (Berman et al. 2009, Sandra et al. 2010, Kalra et al. 2013, Lepczyński et al. 2017). Proteomic analyses have been used to determine the protein profile of ruminant milk using 1-DE in combination with LC-MS/MS (Zhang et al. 2015), 2-DE in combination with LC-MS/MS (Zhang et al. 2011) or MALDI-TOF MS (Di Girolamo et al. 2014), affinity chromatography and nLC-Chip-QTOF-MS (Le Parc et al. 2014) and 2-DE/ELISA (Hernández-Castellano et al. 2016).

Depending on the research equipment used and the species, an individual procedure of sample preparation for proteomic analyses should be prepared. To identify proteins, the most commonly used proteomic tools are 2-DE combined with MS (Łuczak et al. 2009, Rocanda et al. 2012). This method involves successively isolating and purifying proteins, separating them by 2-DE, and then identifying the separated proteins by MS (Bohemer et al. 2008, Mao et al. 2016, Sabha et al. 2020). The precision of the carrying out of each of the mentioned steps determines the quality and reliability of the obtained results of proteomic analyses. The most important step in proteomic analyses using 2-DE, is the preparation of the biological material before appropriate analyses. Therefore, due to the lack of reports on the proteomic analyses of mare milk in the present study, we carried out an optimal procedure to prepare mare milk samples for proteomic analyses using 2-DE combined with MALDI-TOF MS. Based on the available literature describing the preparation of milk samples for proteomic analyses (Mier et al. 2008, Golinelli et al. 2011, Zhang et al. 2011, Zhang et al. 2017), four procedures were prepared mare milk samples for 2-DE separation, differing in the number of following stages. The first procedure was limited to defatting the milk samples, the second to defatting and casein reduction, the third procedure respectively to defatting, casein reduction and removal of high molecular weight proteins, and the fourth to defatting, casein reduction, removal of high molecular weight proteins and desalting to remove interfering salts.

Sample defatting is a necessary process to enable proper 2-DE (Berkelman 2008, Bathla et al. 2019). During fat centrifugation, lactose is also removed from the sample, as well as some of the caseins that build micelles, whose pore structure has the ability to bind lactose (Bernatowicz and Reklewska 2003). After removal of the lipids, the resulting image of the 2-DE gels is not satisfactory as it shows streaking (M1).

Caseins are proteins with a molecular weight from 23 kDa to 43 kDa which, in addition to lactose, are also bound with calcium and phosphates. Therefore, the additional reduction of caseins by precipitation with 30% acetic acid improved the 2-DE gel image (M2) highlighting more separated proteins. Lactose and other compounds interfering with 2-DE separation process were also removed along with some caseins. However, the 2-DE gel image after casein precipitation (sample M2) shows no significant change in the number of caseins compared to the gel image of sample M1. It seems that the precipitation with acetic acid to pH 4,6 suggested in the publication (Mier et al. 2008) is not enough.

Finally, the use of filter columns with a cut-off point of 100 kDa significantly reduced the proportion of caseins in the milk samples, which is clearly visible in the 2-D gel image of the sample

marked M3. Removal of caseins from the milk sample despite their low molecular weight was possible because caseins form structures called micelles whose molecular weight exceeds 100 kDa (Bernatowicz and Reklewska 2003).

The primary aim of using the columns mentioned above in the procedure was to reduce the proportion of high molecular weight proteins in the samples especially immunoglobulins, which cover low molecular weight proteins on the 2-D gel (Lepczyński et al. 2017). The use of filter columns reduced the proportion of immunoglobulins except for the light and heavy chains of IgG, which have a molecular weights of 25 and 50 kDa, respectively. Unfortunately, the use of filter columns resulted in the loss of 243 protein spots compared to the M2 sample. The removed of high molecular weight proteins contributed to an increase in the proportion of low molecular weight proteins in milk samples M3 and M4 as also seen in 1-DE separations (Fig. 1).

Golinelli et al. (2011) to identify low molecular weight proteins in colostrum and cow milk samples removed high molecular weight proteins by using an albumin and IgG removal kit and using a Vivaspin 500 ultrafiltration membrane with a cut-off point of 100 kDa. The used of these methods have enable the identification in colostrum of IGHG1 (immunoglobulin heavy constant gamma 1) and previously uncharacterised C1 (complement component 1), f158 and VI1a. Furthermore, the presence of CREBL2 (cAMP-responsive element-binding protein-like 2) and vitamin D-binding protein were demonstrated for the first time in mature cow milk. In the present research, the application of a method limited to defatting and reducing of caseins in a mare milk sample also provided to identify vitamin D-binding proteins.

Body fluids are rich in electrolytes and other ionic compounds that interfere with the first stage of 2-DE, which is isoelectric focusing (Dratwa-Chałupnik et al. 2016, Lepczyński et al. 2017). Authors (Golinelli et al. 2011) suggested that milk samples should be desalted before the proteomic analysis. In our research, the ReadyPrepTM 2-D Cleanup Kit (Bio-Rad) was used to desalt the milk sample. The use of this step in the procedure highlighted 25 more spots compared to the M3 sample, but 218 protein spots were lost compared to the M2 sample.

The results have shown that the most effective method of sample preparation for proteomic analysis based on 2-DE separation is the M2 procedure. This procedure provided the largest amount of proteins separated on gel. However, the method of casein precipitation needs to be improved to become more effective. Possibly using trichloroacetic acid for caseins precipitation, as suggested by Golinelli et al. (2011), would be a better method.

Furthermore, in the presented research, selected protein spots were cut from the 2-DE gel of the M2 sample and identified by MALDI-TOF MS. The identified proteins included serum albumin, alpha S2-casein, beta-lactoglobulin-1, beta actin, interleukin-24 isoform x1, immunoglobulin G heavy chain and vitamin D-binding protein.

Mechanical defatting of milk samples used in the present work allowed the identification of low-abundance proteins such as α_{S2}-casein and β-actin which are component of the MFGM. These proteins play an important role in lipid secretion and also protect fat globules from adhering to the cell membrane (Fong et al. 2007). These proteins have been identified in the MFGM of cow's milk (Fong et al. 2007).

Serum albumin from lactating mare's blood was also present in the mare's milk (Lönnerdal 2003). This globular protein is water-soluble and contains 11 different binding domains in its structure,

which enables it to transport fatty acids, glucose, copper and hormones, and promotes their distribution and metabolism (Sun et al. 2019).

Furthermore, in the mare milk samples were identified proteins that play an important role in neonatal immunity: IgG, interleukin-24 and β-lactoglobulin. IgG, from the blood of the lactating mare, stimulates the immune system of the new-born through opsonisation of antigens. This provides the binding of antigens to phagocytes, lymphocytes, platelets, mast cells and basophils (Kenzig 2009). IgG also activates the complement system, which participates in the destruction of pathogens and influences the immune response during the neutralisation of toxins and viruses (Schroeder and Cavacini 2010, Król and Brodziak 2015). The interleukin-24 isoform x1 identified in mare milk is a cytokine signalling molecule of the immune system. It controls cell survival and proliferation by inducing rapid activation of specific transcription factors (STAT1, STAT3) and is important in injured healing (Kragastrup et al. 2008). β-lactoglobulin is helpful to the neonatal during viral infections by inhibiting the adhesion of pathogenic microorganisms to the mucosa of the infant's gastrointestinal tract (Golinelli et al. 2011). It is also responsible for binding vitamin D (Pieszka et al. 2016), which has been identified in mare milk.

CONCLUSIONS

The preparation of mare's milk samples including only two steps: defatting and then precipitation of caseins is enough to obtain an optimal 2-DE separation for the further identification of proteins of this body fluid.

It is known that the more steps involved in preparing the sample for proteomic analysis, the increased the risk of protein loss relative to the pool of proteins present in the milk sample directly collected from the mare.

In the aim of increasing the amount of low-abundance proteins in milk samples, a more efficient method for casein precipitation should be prepared.

The low-abundance mare's milk proteins identified by MALDI-TOF MS were mainly involved in the binding and transport of various molecules and in the regulation of the neonatal immune system.

The identification of the mare's milk proteins and the determination of which biological processes they are involved in will provide additional data on the bioactive milk components involved in milk synthesis and secretion as well as responsible for the proper development of the suckling foal.

REFERENCES

1. Bathla S., Sindhu A., Dubey S.K., Kumar S., Mohanty A.K. (2019). Optimization of whey extraction methods for milk bottom up proteomics. Journal of Pharmaceutical Innovation, 8: 163-164
2. Bereman M., Williams T., Muddiman C. (2009). Development of nanoLC LTQ Orbitrap Mass spectrometric Method for Profiling Glycans Derived from Plasma from Healthy, Benign Tumor Control, and Epithelial Ovarian Cancer Patients. Analytical Chemistry, 81: 1130-1136, doi: 10.1021/ac802262w
3. Berkelman T. (2008). Removal of interfering substances in samples prepared for two-dimensional (2-D) electrophoresis. Methods in Molecular Biology, 424: 24-51, doi: 10.1007/978-1-60327-064-9_5

4. Bernatowicz E., Reklewska B. (2003). Bioactive components of the milk protein fraction (in Polish). Breeding Review, 3: 1-10
5. Boehemer J.L., Bannerman D.D., Shefcheck K., Ward J.L. (2008). Proteomic Analysis of Differentially Expressed Proteins in Bovine Milk During Experimentally Induced *Escherichia coli* Mastitis. International Journal of Dairy Science, 91: 4206-4218, doi: 10.3168/jds.2008-1297
6. Di Girolamo F., Masoti A., Salvatori G., Scapaticci M., Muraca M., Putignani L. (2014). A sensitive and effective proteomic approach to identify she-donkey's and goat's milk adulterations by MALDI-TOF MS fingerprinting. International Journal of Molecular Science, 15: 13697-13719, doi: 10.3390/ijms150813697
7. Dratwa-Chałupnik A., Ożgo M., Lepczyński A., Herosimczyk A., Michałek K. (2016). Excessive amount of lactose in the diet of two-week-old calves induces urinary protein changes. Archives Animal Breeding, 59: 417-422, doi: 10.5194/aab-59-417-2016
8. Fong B.Y., Norris C.S., MacGibbon A.K.H. (2017). Protein and lipid composition of bovine milk-fat-globule membrane. International Dairy Journal, 17: 275-288, doi: 10.1016/j.dairyj.2006.05.004
9. Golinelli L.P., Conte-Junior C.A., Paschoalin V.M.F., Silva J.T. (2011). Proteomic Analysis of Whey from Bovine Colostrum and Mature Milk. Brazilian Archives of Biology Technology, 54: 761-768, doi: 10.1590/S1516-89132011000400016
10. Ha M., Sabherwal M., Duncan E., Stevens S., Stockwell P., McConnell M., Bekhit A., Carne A. (2015). In-Depth Characterization of Sheep (Ovis aries) Milk Whey Proteome and Comparison with Cow (Bos taurus). PLoS ONE, 10(10): e0139774, doi: 10.1371/journal.pone.0139774
11. Hernández-Castellano L., Almeida A., Renaut J., Argüello A., Castro N. (2016). A proteomics study of colostrum and milk from the two major small ruminant dairy breeds from the Canary Islands: A bovine milk comparison perspective. Journal of Dairy Research, 83: 366-374, doi: 10.1017/S0022029916000273
12. Kalra H., Adda C., Liem M., Ang C. Mechler A., Simpson R.J., Hulett M.D., Mathivanan S. (2013). Comparative proteomics evaluation of plasma exosome isolation techniques and assessment of the stability of exosomes in normal human blood plasma. Proteomics, 13: 3354-3364, doi: 10.1002/pmic.201300282
13. Kenzig A.R. (2019). Colostral, Milk, and Serum Immunoglobulin G Concentrations in Quarter Horse Mares and their Foals from Birth to Weaning. Journal of Equine Veterinary Science, 29: 486-487, doi: 10.1016/j.jevs.2009.04.177
14. Kragstrup T.W., Otkjaer K., Holm C., Jørgensen A., Hokland M., Iversen L., Deleuran B. (2008). The expression of IL-20 and IL-24 and their shared receptors are increased in rheumatoid arthritis and spondyloarthropathy. Cytokine, 41: 16-23, doi: 10.1016/j.cyto.2007.10.004
15. Król J., Brodziak A. (2015). Milk proteins with antibacterial properties (in Polish). Problems of Hygiene and Epidemiology, 96: 399-405
16. Le Parc A., Dallas D., Duaut S., Leonil J., Martin P., Barile D. (2014). Characterization of goat milk lactoferrin N-glycans and comparison with the N-glycomes of human and bovine milk. Electrophoresis, 35: 1560-1570, doi: 10.1002/elps.201300619
17. Lepczyński A., Ożgo M., Dratwa-Chałupnik A., Robak P., Pyć A., Zaborski D., Herosimczyk A. (2017). An update on medium- and low-abundant blood plasma proteome of horse. Animal, 12: 1-12, doi: 10.1017/S1751731117001409

18. Lönnerdal B. (2003). Nutritional and physiologic significance of human milk proteins. American Journal of Clinical Nutrition, 77: 1537-1543, doi: 10.1093/ajcn/77.6.1537S
19. Łuczak M., Figlerowicz M., Wojtaszek P. (2009). Methodological aspects of proteomic analyses using two-way electrophoresis and mass spectrometry methods (in Polish). Biotechnology, 2: 1-6
20. Maity S., Ambatipudi K. (2019). Quantitative proteomics of milk whey reveals breed and season specific variation in protein abundance in Holstein Friesian cow and Murrah buffalo. Research in Veterinary Science, 125: 244-252, doi: 10.1016/j.rvsc.2019.07.001
21. Mao Y., Hopkins D.L., Zhang Y., Zhu L., Dong P., Liang R., Dai J., Wang X., Luo X. (2016). Beef quality with different intramuscular fat content and proteomic analysis using isobaric tag for relative and absolute quantitation of differentially expressed protein. Meat Science, 188: 96-102, doi: 10.1016/j.meatsci.2016.03.028
22. Medeńska W., Dratwa-Chałupnik A. (2021). Cow's whey Proteins Involved in Mammary Gland Development and Milk Components Synthesis. Archives of Veterinary Science, 3: 2-7
23. Mier M.P., Ibañez R., Ortiz I. (2008). Influence of proces variables on the production of bovine milk casein by electrodialysis with bipolar membranes. Biochemical Engineering Journal, 40: 304-311, doi: 10.1016/j.bej.2007.12.023
24. Mol P., Kannegundla U., Dey G., Gopalakrishnan L., Dammalli M., Kumar M., Patil A.H., Basavaraju M., Rao A., Ramesha K.P., Prasad T.S.K. (2018). Bovine milk comparative proteome analysis from early, mid, and late lactation in the cattle breed, Malnad Gidda (Bos indicus). OMICS, 22: 223-235, doi: 10.1089/omi.2017.0162
25. Ogawa S., Tsukahara T., Nishibayashi R., Nakatani M., Okutani M., Nakanishi N., Ushida K., Inoue R. (2014). Shotgun proteomic analysis of porcine colostrum and mature milk. Animal Science Journal, 85: 440-448, doi: 10.1111/asj.12165
26. Pieszka M., Łuczyński J., Zamachowska M., Augustyn R., Długosz B., Hędrzak M. (2016). Is mare milk an appropriate food for people? – A review. Annals of Animal Science, 16: 33-51, doi: 10.1515/aoas-2015-0041
27. Rocanda P., Piras C., Soggiu A., Turk R., Urbani A., Bonizzi L. (2012). Farm animal milk proteomics. Journal of Proteomics, 72: 4259-4274, doi: 10.1016/j.jprot.2012.05.028
28. Sabha B.H., Masood A., Analnazi I.O., Alfadda A.A., Almehdar H.A., Benabdelkamel H., Redwan E.M. (2020). Comparative Analysis of Milk Fat Globular Membrane (MFGM) Proteome between Saudi Arabia *Camelus dromedary* Safra and Wadha Breeds. Molecules, 25: 1-17, doi: 10.3390/molecules25092146
29. Sandra K., Pereira A., Vanhoenacker G., David F., Sandra P. (2010). Comprehensive blood plasma lipidomics by liquid chromatography/quadrupole time-of-flight mass spectrometry. Journal of Chromatography, 1217: 4087-4099, doi: 10.1016/j.chroma.2010.02.039
30. Schroeder H.W., Cavacini L. (2010). Structure and function of immunoglobulins. Journal of Allergy and Clinical Immunology, 125: 41-52, doi: 10.1016/j.jaci.2009.09.046
31. Sun X., Ferguson H.N., Hagerman A.E. (2019) Conformation and Aggregation of Human Serum Albumin in the Presence of Green Tea Polyphenol (EGCg) and/or Palmitic Acid. Biomolecules, 9(705): 1-14, doi: 10.3390/biom9110705

32. Verma M., Dige M.S., Gautam D., De S., Rout P.K. (2020). Functional milk proteome analysis of genetically diverse goats from different agro climatic regions. Journal of Proteomics, 227(15): 1-25, doi: 10.1016/j.jprot.2020.103916
33. Zhang L., Boeren S., Hageman A.J., Hooijdonk T., Vervoort J., Hettinga K. (2015). Bovine Milk Proteome in the First 9 Days: Protein Interactions in Maturation of the Immune and Digestive System of the Newborn. PLoS ONE, 10: e0116710, doi: 10.1371/journal.pone.0116710
34. Zhang L., van Dijk A.D.J., Hettinga K. (2017). An interactomics overview of the human and bovine milk proteome over lactation. Proteome Science, 15: 1-14, doi: 10.1186/s12953-016-0110-0
35. Zhang L., Wang J., Yang Y., Bu D., Li S., Zhou L. (2011). Comparative Proteomic Analysis of Changes in the Bovine Whey Proteome during the Transition from Colostrum to Milk. Asian-Australasian Journal of Animal Sciences, 24: 272-278, doi: 10.5713/ajas.2011.10122

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