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LACTOFERRIN PREPARATIONS ELECTROPHORESIS IN AN ADAPTED STADIER-TYPE APPARATUS FOR MILK PROTEINS ANALYSIS

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Abstract. Lactoferrin preparations used as bioactive additives in the production of food products contain from 3% to 15% of various milk proteins. An important issue is the analysis of the fractional composition of these protein impurities in lactoferrin preparations. To obtain a complete characterization of the composition and state of the protein impurities, it is advisable to use three electrophoretic systems. Two of them (native and in the presence of urea) are successfully used in an adapted Stadier-type apparatus. However, the third important system – disc electrophoresis in the presence of sodium dodecyl sulfate (SDS) has been tested on other devices (in particular, the «Bio Rad» apparatus). This complicates the analysis and comparison of results. Therefore, the purpose of our study was to establish the possibility of using an adapted Stadier-type apparatus for electrophoretic analysis of lactoferrin preparations in the presence of SDS. In the work we used skimmed milk with an acidity of 18^0T, as well as three lactoferrin preparations obtained from PJSC «Ternopil Dairy Plant». The fractional composition of proteins in lactoferrin preparations was determined using three electrophoresis systems (electrophoresis in the presence of urea for the identification of caseins, express electrophoresis in native conditions for the identification of whey proteins, and disc electrophoresis in the presence of SDS). Disc electrophoresis in the presence of SDS was performed in parallel in an adapted Stadier-type apparatus and «Bio Rad» apparatus. As a result, it was found that the adapted Stadier-type apparatus can be used for the analysis of milk protein impurities in these three electrophoresis systems. Comparative analysis of three lactoferrin preparations on both apparatuses for electrophoresis showed identical qualitative composition and close quantitative composition of impurity fractions and lactoferrin, namely, in the adapted Stadier-type apparatus in the Lf_3 preparation, the lactoferrin content was 85±2.5; β-lactoglobulin – 5±0.7; serum albumin – 7±0.6; α_{s1}-casein – 2±0.4. For the apparatus of the «Bio Rad» company, the fraction content was as follows: lactoferrin – 84±1.9; β-lactoglobulin – 5±0.4; serum albumin – 5±0.6; α_{s1}-casein – 3±0.5. This indicates the possibility of comparing the results obtained on both apparatuses.

Key words: lactoferrin preparations, protein fractions, electrophoresis

Introduction. Formulation of the problem

Numerous studies have confirmed that one of the most important bioactive components of milk is lactoferrin [1, 2]. Currently, lactoferrin is isolated on an industrial scale for baby food, for nutrition of athletes, the elderly, and for dietary nutrition [3]. Industrial preparations of lactoferrin can contain from 3% to 15% of other milk proteins. The question of the influence of these proteins on various types of biological action of lactoferrin has not been finally established [4, 5]. In this regard, an important characteristic of lactoferrin preparations is not only the percentage of protein impurities, but also their detailed fractional composition. It depends on the raw materials used, as well as the method of lactoferrin isolation. One of the most common methods for analyzing protein fractions of lactoferrin preparations is disc electrophoresis in polyacrylamide gel (PAG) in one of the variants of the Laemmli system [6]. In this case, various electrophoresis devices are used. Recent studies have shown that the use of the Laemmli system alone is not sufficient for the effective separation and identification of even the main fractions of milk proteins that may be present in lactoferrin preparations [4, 7]. Two electrophoretic systems have been tested on the adapted for the analysis of milk proteins Stadier-type apparatus, which allow for the effective separation of all casein fractions or the main fractions of whey proteins [8]. Considering current data on the differences in the composition of protein fractions of lactoferrin preparations, it would be advisable to analyze them in all three electrophoretic systems using the same type of apparatus to obtain a complete picture.

Analysis of recent research and publications

Lactoferrin is a natural bioactive protein of mammalian milk. Since its discovery in 1937 and to the present time, it has been studied in detail and new types of positive effects on the body have been identified [1]. Since biological action is the main function of lactoferrin, it can be classified as a parapharmaceutical. The most important types of biological action and areas of research on lactoferrin are shown in Fig. 1.

Lactoferrin is synthesized in the mammary gland and its content in cow's milk ranges from 20 to 200 mg/l. In colostrum, its content can reach 5000 mg/l. At present, the structure and properties of lactoferrin have been studied in sufficient detail [2]. The primary structure has been established and it includes 689 amino acid residues and the exact molecular weight is 76110 Da [9]. An important property of lactoferrin, which distinguishes it from the main milk proteins (caseins and most whey proteins), is the high value of its isoelectric point - 8.81 and, accordingly, the positive charge of the molecule in fresh milk. In terms of chemical composition, lactoferrin is both a glycoprotein and a metalloprotein. Five asparagine (Asn) residues have been found in its molecule, to which 59 different oligosaccharide groups can be attached [10]. In general, the carbohydrate content in lactoferrin can be more than 11%. By the location of the oligosaccharide group near the Asn_{281} residue, two variants of lactoferrin A and B are distinguished.

Many biological functions of lactoferrin are associated with its ability to attach trivalent iron ions [11]. Sites have been identified where each lactoferrin molecule can bind two Fe^{3+} ions [12]. In reality, in milk, the degree of saturation of lactoferrin with iron ions ranges from 15 to 40%. Lactoferrin molecules with a high degree of saturation with iron ions are resistant to proteolysis by digestive enzymes, which is typical for many bioactive proteins and peptides [13]. The function of lactoferrin as a carrier of iron ions is discussed. This is due to the high stability of lactoferrin complexes with iron ions at neutral and acidic pH values. Another protein that carries iron ions, transferrin, releases them into the environment already at pH values of 5.5, and lactoferrin at 3.5. This indicates that lactoferrin has the inherent property of absorbing iron ions [14].

Based on numerous studies, the bactericidal effect of lactoferrin and individual peptides formed from it during proteolysis by digestive enzymes has been proven [11, 13]. Several mechanisms of bactericidal action have been identified. First of all, it is the binding of iron ions, which are necessary for the development of bacteria that cause foodborne infections and toxicoinfections. It should be noted that the normal microflora of the digestive tract, in particular bifidobacteria, does not suffer [11]. Also, lactoferrin can attach to lipopolysaccharides of cell membranes of gram-negative bacteria. This leads to the death of bacteria due to changes in the value of membrane potential and membrane permeability. In addition, the attachment of lactoferrin to bacterial membranes activates the action of lysozyme and promotes the destruction of cell walls of gram-negative and gram-positive bacteria [15]. The antiviral effect of lactoferrin is associated with its competition for binding sites with cell membranes [16].

Immunomodulatory and anticancer effects are under active research [17]. Lactoferrin is able to stimulate lymphocyte proliferation and increase macrophage activity. In the presence of lactoferrin, the development of many types of cancer cells is inhibited. This may be due to the ability of lactoferrin to bind iron ions, which cause mutations in nucleic acid molecules [18]. Another explanation for the anticancer effect is the ability of lactoferrin to stimulate apoptosis of cancer cells [19].

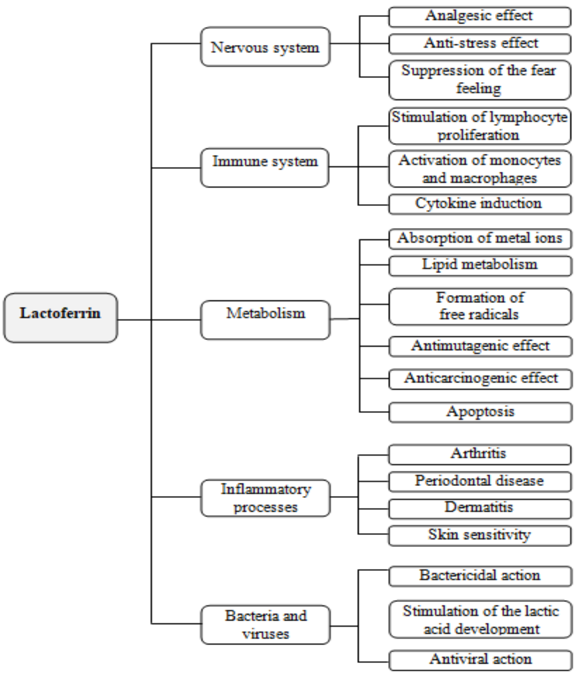


Fig. 1. Main directions of studying the biological action of lactoferrin [2, 13]

An important direction of the biological action of lactoferrin is the ability to inhibit the differentiation of adipose tissue cells - adipocytes [20]. This opens up new ways for the regulation of lipid metabolism and control of human body weight.

The primary structure of lactoferrin in cow's milk is almost 70% identical to the primary structure of lactoferrin in human milk [2]. Therefore, it is a valuable functional ingredient for human food products. First of all, this applies to baby food products in the postnatal period, nutrition for athletes, the elderly, and patients with disorders of the immune and digestive systems [1, 3]. Highly purified lactoferrin is used in pharmaceuticals and cosmetics [4].

The main sources of lactoferrin are skim milk, as well as various types of whey. Depending on the raw materials used and the method of isolation, lactoferrin preparations can contain from 3% to 15% of other milk proteins. These are primarily whey proteins – β-lactoglobulin (β-lg), α-lactalbumin (α-la), blood serum albumin (BSA), immunoglobulins (Ig) and lactoperoxidase (LP). There also may be casein fractions (α_{s1}-casein, β-casein, α_{s2}-casein and χ-casein), which are able to form complexes with lactoferrin due to electrostatic interactions [4, 5]. The listed proteins can affect the biological activity of lactoferrin. Therefore, the issue of analyzing protein fractions of impurities in lactoferrin preparations remains relevant. For the analysis of industrial lactoferrin preparations, modified Laemmli disc electrophoretic systems in polyacrylamide gel (PAG) in the presence of sodium dodecyl sulfate (SDS) on Bio Rad devices are most often used [6, 22]. Also, for a more complete analysis of some minor fractions of the preparations, it is advisable to use other systems of electrophoresis in PAG, namely, electrophoresis in the presence of urea and native electrophoresis. These systems were developed for the analysis of caseins and whey proteins on an adapted Stadier-type apparatus for milk protein electrophoresis [8]. When comparing and contrasting the results of the analysis of lactoferrin preparations in different electrophoretic systems and on different apparatuses, certain difficulties arise. Therefore, it may be advisable to compare the results of disc electrophoresis in the presence of SDS on both apparatuses and further use of the adapted Stadier-type apparatus for the analysis of lactoferrin preparations in all three systems.

The purpose and objectives of the study.

The purpose of this study was to establish the possibility of using an adapted Stadier-type apparatus for electrophoretic analysis of lactoferrin preparations in the presence of sodium dodecyl sulfate.

To achieve the goal, the following research objectives were formulated:

- to analyze proteins that can be transferred from milk into lactoferrin preparations and lactoferrin preparations on an adapted Stadier-type apparatus for milk protein electrophoresis;
- to analyze lactoferrin preparations by disc electrophoresis in the presence of SDS on a «Bio Rad» apparatus;
- to compare the results of the quantitative composition of protein fractions of lactoferrin preparations obtained on an adapted Stadier-type apparatus and a «Bio Rad» apparatus.

Materials and methods.

Skim milk with an acidity of 18ºT, as well as three lactoferrin preparations (Lf_1, Lf_2, Lf_3) provided for the study by PJSC «Ternopil Dairy Plant», were used in the work. Total control casein and control whey were isolated from skim milk by centrifugation (5000 rpm, 10 min) in an OPN-8 centrifuge after adjusting the pH value to 4.6 by adding 1N hydrochloric acid.

Electrophoresis of casein complex and milk whey proteins, as well as lactoferrin preparations, was performed in an adapted Stadier-type apparatus for milk protein electrophoresis, manufactured in the Biochemistry Laboratory of the Department of Food Biotechnology and Chemistry of Ternopil Ivan Puluj National Technical University (Fig. 2). The parameters of the apparatus were described earlier [8].

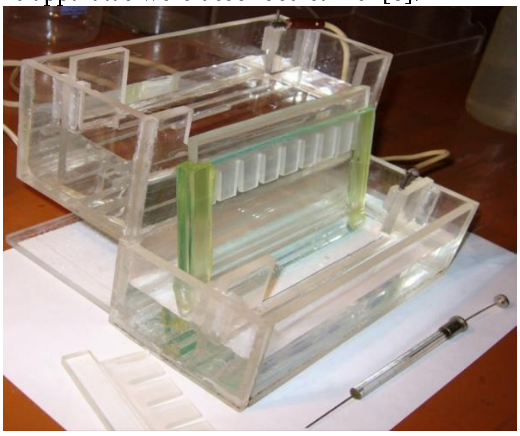


Fig. 2. Adapted Stadier-type apparatus for milk proteins electrophoresis

For the analysis of whey proteins, an express electrophoresis system was used under native conditions at constant current – 3 mA [21]. Casein complex proteins were analyzed in a homogeneous gel electrophoretic system in the presence of urea at constant current – 37 mA [8]. Disc electrophoresis of milk proteins and lactoferrin preparations in the presence of SDS was performed according to the modified Laemmli method at constant current – 50 mA [22]. In the first two cases, prephoresis was performed at 15 mA for 10 min.

Disc electrophoresis in the presence of SDS of lactoferrin preparations was performed in the scientific laboratory of the Department of Dairy Science and Quality Management of the University of Warmia and Mazury (Olsztyn, Poland) according to the method [22]. Electrophoretic analysis was performed on a «Bio Rad» (Italy) apparatus using ready-made PAG plates and a set of marker proteins (from 10,000 to 250,000 Da) from «Bio Rad Laboratories» (USA).

Quantitative processing of gel plates obtained on both apparatuses was performed using the imread graphic image reading function in the Matlab system.

The concentration of proteins in the samples was determined by absorption at λ=280 nm on a spectrophotometer SF-46. The absorption coefficient ($\boldsymbol{D^{1\%}_{1\,cm}}$): was used: 12.3 for whey proteins; 8.2 for total casein and 9.91 for lactoferrin [9].

Electrophoretic analyses were performed in quadruplicates. Mathematical and statistical processing of the results was carried out using the Microsoft Office Excel 2007 software packages.

Results of the research and their discussion

To obtain a complete picture of the fractional composition of proteins in lactoferrin preparations, it is necessary to use three systems of electrophoresis in PAG. Two of them were developed on an adapted apparatus for the analysis of milk proteins. This is, first of all, a homogeneous gel system in the presence of urea, which allows the effective separation of all fractions of casein complex proteins. The results of the separation of total casein in this system are shown in Fig. 3.1. In particular, the electrophoregram clearly shows two main fractions - α_{s1}-casein (α_{s1}-CN) and β-casein (β-CN), which are most often found in lactoferrin preparations. The second important system of express electrophoresis under native conditions allows to establish the main fractions of whey proteins that can be included in the lactoferrin preparation - β-lactoglobulin, α-lactalbumin, serum albumin, immunoglobulins. Also, this system allows confirming the native state of these fractions [7]. The results of the analysis of whey proteins are shown in Fig. 3.2.

For the simultaneous analysis of all impurity proteins in lactoferrin preparations and the determination of the degree of its purification, variants of the Laemmli disc electrophoresis system in the presence of SDS are usually used [4, 6]. In this system, first of all, lactoferrin itself can be identified, as well as β-lactoglobulin, α-lactalbumin and immunoglobulins. That is, proteins that differ significantly in molecular mass values [2]. The bands of casein fractions that have similar molecular masses overlap and form one blurred band. Also, lactoperoxidase, which is close in molecular mass value to lactoferrin, can form a common large band with it.

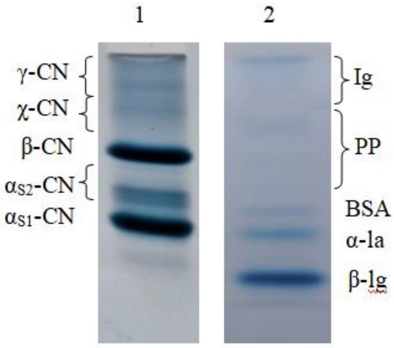


Fig. 3. Electrophoresis of total casein in the presence of urea (1), whey proteins in the native express system (2), obtained in an adapted apparatus for the analysis of milk proteins

For the analysis of lactoferrin preparations in the adapted apparatus, we selected a variant of the Laemmli system [22], which was also used in the same study for comparison during electrophoresis in the apparatus of the «Bio Rad» company. The results of disc electrophoresis in our apparatus of three samples of lactoferrin Lf_1, Lf_2, Lf_3, as well as total casein and whey proteins are shown in Fig. 4.

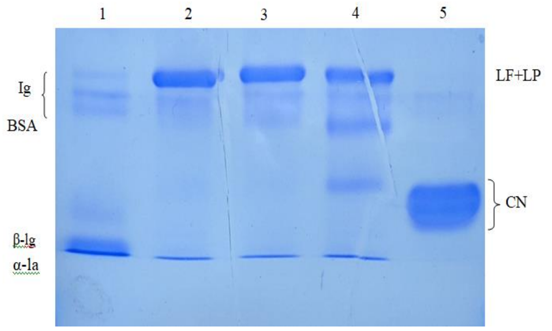


Fig. 4. Electrophoregram of disc electrophoresis in the presence of SDS of whey proteins (1), lactoferrin preparations – Lf_1(2), Lf_2(3), Lf_3(4) and total casein (5), obtained in an adapted apparatus for the analysis of milk proteins

To compare the results, the same three lactoferrin preparations, as well as a set of standard marker proteins, were analyzed in a disc electrophoresis system in the presence of SDS in a «Bio Rad» apparatus. The results are shown in Fig. 5.

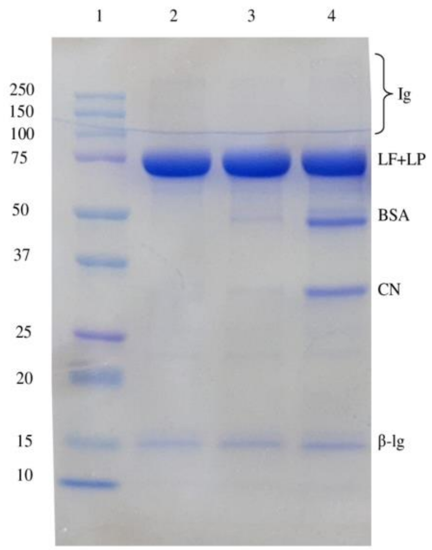


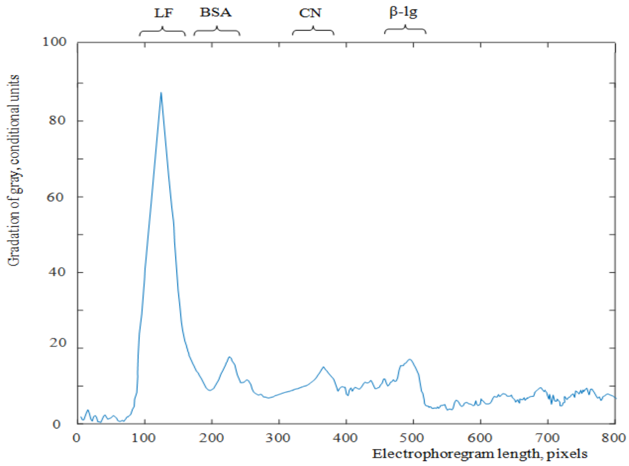
Fig. 5. Electrophoregram of disc electrophoresis in the presence of SDS of a set of marker proteins (×1000 Da) – 1; lactoferrin preparations – Lf_1(2), Lf_2(3) and Lf_3(4), obtained in the apparatus of the company «Bio Rad»

Both electrophoresis (Fig. 4 and Fig. 5) clearly show the lactoferrin fraction, as well as the largest fractions of impurity proteins. They are identified as β-lactoglobulins, α_{s1}-casein and serum albumins. Other unidentified minor fractions in trace amounts are also visible on the electrophoregram.

For quantitative evaluation, electrophoresis was obtained in four replicates and densitometry was performed. For comparison, Fig. 6 shows the densitograms of the lactoferrin Lf_3 preparation after disc electrophoresis in the presence of SDS in an adapted milk protein apparatus (Fig. 6.1) and a «Bio Rad» apparatus (Fig. 6.2). More impurities were detected in this preparation, which allows a better comparison of the separation efficiency on different apparatuses.

Based on the densitograms, the content of lactoferrin and the main fractions of impurities was calculated. The results of the calculations are shown in Table 2. The results of quantitative analysis of similar lactoferrin preparations separated on both electrophoresis apparatuses showed a high coincidence of the values of the relative content of protein fractions. The differences are practically within the error limits. Thus, the possibility of adapted apparatus using for qualitative and quantitative electrophoretic analysis of lactoferrin preparations and other milk proteins in the disc electrophoresis system in the presence of SDS has been demonstrated for the first time.

1



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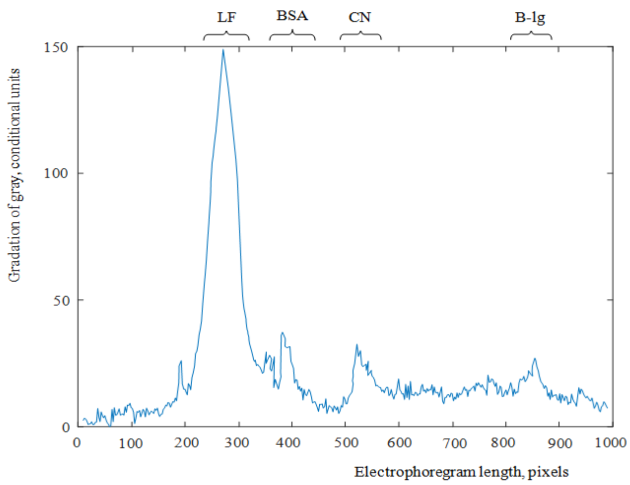

Fig. 6. Densitograms of electrophoresis of the Lf_3 preparation obtained by disc electrophoresis in the presence of SDS on an adapted Stadier-type apparatus (1) and an apparatus from the «Bio Rad» company (2)

Table 2 – The relative content of protein fractions in lactoferrin preparations, calculated based on the results of electrophoresis obtained in an adapted Stadier-type apparatus for the analysis of milk proteins and the apparatus of the «Bio Rad» company (M±m, n=4)

Lactoferrin preparation	Relative content (%) of protein fractions			
	lactoferrin	β-lactoglobulin	serum albumin	α_{s1}-casein
Results obtained on an adapted apparatus for milk protein electrophoresis				
Lf_1	91±3,1	4±0,6	traces	traces
Lf_2	89±2,9	4±0,7	2±0,4	1±0,3
Lf_3	85±2,5	5±0,7	7±0,6	2±0,4
Results obtained in the «Bio Rad» apparatus				
Lf_1	93±2,5	3±0,4	traces	traces
Lf_2	90±2,3	3±0,3	1±0,3	1±0,2
Lf_3	84±1,9	5±0,4	5±0,6	3±0,5

*Note. Traces – content less than 1%.

Conclusion

1. An adapted Stadier-type apparatus for the analysis of milk proteins can be used for the effective study of the main fractions of milk proteins in three systems of electrophoresis in PAG. These are the following systems: in the presence of urea – for the identification of all casein fractions; express electrophoresis under native conditions for the analysis of the main whey proteins – β-lactoglobulin, α-lactalbumin, serum albumin and immunoglobulins; disc electrophoresis in the presence of sodium dodecyl sulfate for the simultaneous analysis of all the main milk proteins, as well as lactoferrin.

2. Comparative qualitative and quantitative analysis of three lactoferrin preparations on an adapted Stadier-type apparatus and an electrophoresis apparatus from the «Bio Rad» company showed the same composition of protein fractions of impurities (β-lactoglobulin, α-lactalbumin, serum albumin) and similar values of the relative content of impurity fractions and lactoferrin itself. This indicates the possibility of comparing the results obtained on both apparatuses.

3. The adapted apparatus is more accessible and cheaper to manufacture and use in factory laboratories. At the same time, the adapted apparatus allows for electrophoretic analysis of lactoferrin preparations and basic milk proteins no less effectively than the apparatus of the «Bio Rad» company.

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ЕЛЕКТРОФОРЕЗ ПРЕПАРАТІВ ЛАКТОФЕРИНУ В АДАПТОВАНОМУ АПАРАТІ ТИПУ СТАДІЄРА ДЛЯ АНАЛІЗУ БІЛКІВ МОЛОКА

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Анотація. Препарати лактоферину, які використовують, як біоактивні добавки при виробництві харчових продуктів містять від 3% до 15% різних білків молока. Важливим питанням є аналіз фракційного складу цих білкових домішок в препаратах лактоферину. Для отримання повної характеристики складу і стану білків домішок доцільно використовувати три системи електрофорезу. Дві з них (нативна і в присутності сечовини) успішно використовуються в адаптованому апараті типу Стадієра. Проте третя важлива система – диск-електрофорезу в присутності додецилсульфату натрію (ДСН) відпрацьована на інших апаратах (зокрема апараті фірми «Bio Rad»). Це ускладнює проведення аналізу і порівнювання результатів. Тому метою нашого дослідження було встановлення можливості використання адаптованого апарату типу Стадієра для електрофоретичного аналізу препаратів лактоферину в присутності ДСН. В роботі було використано знежирене молоко кислотністю 18^0Т, а також три препарати лактоферину, які отримали з ПрАТ «Тернопільський молокозавод». Фракційний склад білків у препаратах лактоферину визначали з використанням трьох систем електрофорезу (електрофорез в присутності сечовини для ідентифікації казеїнів, експрес-електрофорез в нативних умовах для ідентифікації білків сироватки молока та диск-електрофорез в присутності ДСН). Диск-електрофорез в присутності ДСН проводили паралельно в адаптованому апараті типу Стадієра і апараті фірми «Bio Rad». В результаті встановлено, що адаптований апарат типу Стадієра може бути використаний для аналізу молочних білків домішок у цих трьох системах електрофорезу. Порівняльний аналіз трьох препаратів лактоферину на обох апаратах для електрофорезу показав ідентичний якісний склад і близький кількісний склад фракцій домішок та лактоферину, а саме у адаптованому апараті типу Стадієра у препараті Lf_3 вміст лактоферину становив 85±2,5; β-лактоглобуліну – 5±0,7; альбуміну сироватки – 7±0,6; α_{s1}-казеїну – 2±0,4. Для апарату фірми «Bio Rad» вміст фракцій відповідно був наступним: лактоферину – 84±1,9; β-лактоглобуліну – 5±0,4; альбуміну сироватки – 5±0,6; α_{s1}-казеїну – 3±0,5. Це свідчить про можливість порівнювання результатів отриманих на обох апаратах.

Ключові слова: препарати лактоферину, білкові фракції, електрофорез.

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