Use of Microfluidic Electrophoresis in the Analysis of Milk Proteins- Method Improvement

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RESEARCH ARTICLE

Abstract
Microfluidic electrophoresis (ELFO) has become a widely applicable method that allows the analysis of proteins fractions. ELFO presents advantages that include time reduction low sample consumption, high method efficiency and the integration of results in the context. With sample proper preparation the method becomes accurate and robust and the reagents and consumables used are considerably reduced. This paper describes the adaptation and improvement of a raw milk protein analysis protocol, reported in the standard microfluidic electrophoresis analysis procedures of Agilent Technologies. The described protocol makes adaptations regarding the preparation of biological samples composed of raw milk in the pre-analytical stage with multiple benefits in reporting the final results. Besides the proteins, in the composition of milk, significant percent of fats could be found and the described procedure is practically another step required for the elimination of those interfering substances. This method involves the pre-analytical stage of preparation of raw milk samples, separation and quantification of major milk proteins and total protein content. The improvement proved to have an important beneficial impact on the analysis as the result was very accurate in terms of quantity and quality and also it has drastically reduced the errors in data interpretation of the equipment.

Keywords: microfluidic electrophoresis, milk proteins, quantification, analysis interferences.

INTRODUCTION
In dairy research, the separation and quantification of major milk proteins are fundamental techniques. The methods being extremely accurate and fast, this fact indicates their importance. Microfluidic electrophoresis in the chip, in addition to being a faster method, uses considerably fewer chemicals and materials than traditional techniques. The chip format dramatically reduces the separation time, as well as the consumption of samples and reagents, the system ensuring automatic sizing and quantification in a digital format. Chip gel electrophoresis is performed for the analysis of DNA, RNA, and proteins, on the chip, in the well being loaded the gel, samples, and external standards (ladder). This method is used in proteomics, in the drug development industry, or in obtaining a medical diagnosis. Loaded biomolecules, such as protein micelles, are electrophoretically driven by a voltage gradient - similar to plaque gel electrophoresis. Due to a constant mass-to-charge ratio and the presence of a polymeric matrix (gel), the molecules are separated by size. Detection is based on laser-induced fluorescence (LIF) detection. This information can be used to explain their influence on the biological activity, taste, and functional properties of milk and dairy products and can also be used for product authenticity and history assessment (Mayer, 2012). Thus, for example, heat-induced denaturation and the interactions of whey proteins in milk have been studied in different milk protein systems under a
variety of experimental systems (Pinho, 2012).

Currently, polyacrylamide gel electrophoresis (PAGE), capillary electrophoresis (CE), and high-performance liquid chromatography (HPLC) are used to separate the main protein fractions of milk. These techniques can be coupled with separation equipment, such as ultraviolet spectrometers and mass spectrometers for the quantification of protein fractions (Dziuba et al., 2001, Miralles et al., 2001, Donato and Dalgleish, 2006). The advantages and disadvantages of each of these techniques have been discussed (Wang et al., 2009). In terms of the main advantages, the limits of automation and detection are the most mentioned by the authors. However, the high consumption of toxic reagents, the time required for sample preparation and the high costs of equipment, the physical separation of proteins, and the final integration and quantification of the individual components of proteins are considered to be disadvantages of these techniques.

Recently, the technique of microfluidic electrophoresis on the chip (ELFO) has been developed for the separation and quantification of proteins, with applicability in various fields, such as proteomics, drug development, or medical diagnosis (Poitevin et al., 2009, Li et al., 2010). For proteins, this technique has been reported to be an advanced and automated alternative to the traditional SDS-PAGE method, with the separation and quantification of several samples with significantly reduced analysis time compared to the classical method. Moreover, this technique was recommended due to the precise results it provides and because it uses very small materials and samples, usually less than 0.5 ml total volume per chip (10 samples). The applicability of this technique in highlighting and quantifying milk proteins has been tested and started to be widely used especially since the company producing the equipment Bioanalyzer 2100 - Agilent Technologies has developed an internal standard for this analysis (Nitsche, 2008). Studies on milk and protein have been performed to verify the potential application of this technique in evaluating the distribution of different protein fractions in milk. The authors reported the ability of microchip electrophoresis (ELFO) to rapidly separate and characterize whey proteins (Buffoni et al., 2011). However, it was stated that the results in terms of optimizing the separation of individual proteins are still unsatisfactory when following the methodology recommended by the manufacturer, due to the overlap of signals related to casein fractions. The correct quantification of the percentages of protein fractions depends on the signals obtained. Data obtained with unsatisfactory separation may underestimate or overestimate the amount of protein present, while more efficient separation would provide more accurate results on protein quantification (Anema, 2009).

In this context, many authors have improved the method depending on the interest of the analysis (Dupont et al., 2018, Gellrich et al., 2014; Pinho, 2012). Most of these improvements are made in the pre-analytical stage, ie in the sample preparation stage, and aim at ways of extracting and denaturing protein fractions with applicability to certain individual protein species. Therefore, this paper has presented a protocol for the preparation of milk samples, from the pre-analytical stage, which considerably improves the results of the analysis, but does not involve the use of other reagents or modification of the working standard issued by the manufacturer.

MATERIALS AND METHODS

Biological material

5 milk samples belonging to the species Bos taurus were collected from a local farm, in sterile containers and subsequently moved, in 2 ml Eppendorf tubes. These were brought to the laboratory and immediately stored in the freezer at a temperature of -20°C, which ensures the preservation of all component biomolecules to be analyzed.

Sample preparation pre-analytical stage

On the day of analysis, the samples were thawed and homogenized by vortex for 5 minutes. 1 ml of each of the milk samples was divided into two 1.5 ml test tubes. For each sample collected, one of the 1 ml samples was centrifuged for 10 min. at 3500 rpm. Following centrifugation, the lipid components formed an individualized layer on the surface of the supernatant, and the somatic cells formed sediment at the base of the analysis tube. The layer of fat obtained was removed from each sample. Subsequently, 1:10 dilution with deionized water was performed for all samples in the experiment. The protein solution thus obtained followed the analysis protocol specified by the manufacturer. The analysis was performed in parallel for both degreased samples and those containing the raw milk samples.

Analysis of the main proteins in milk

The method of analysis used in this study was microfluidic electrophoresis on the chip using Agilent Bioanalyser 2100 equipment (Agilent Technologies, Waldbrunn, Germany). The Protein 80 Kit was used for the analysis, which contains the analysis chips and all reagents (gel, dye, sample buffer, upper (95 kDa) and lower (1.6 kDa) marker and protein ladder). The milk and protein ladder samples were prepared according to the provided protocol: protein ladder (6 µl) and the sample (4 µl) were treated with denaturing solution (2 µl) in a 0.5 ml tube and were denatured (95°C, 5 min). After cooling, the tubes were briefly centrifuged and 84 µl of deionized water was added to the samples. The face was primed with the provided gel solutions. Subsequently, 6 µl of the prepared protein samples and ladder were loaded on the chip. The face was placed in the bioanalyzer, electrodes were inserted into each well.
by closing the lid and the measurement was started immediately.

The electrophoresis and simultaneous automatic integration procedure took approximately 30 minutes. For protein analysis, the upper and lower markers were used as internal standards. Also, for protein molecular weight standardization the internal ladder was used. The analysis data were collected automatically from Agilent 2100 Expert software.

RESULTS AND DISCUSSIONS

Identification and quantification of individual proteins, variants, and isoforms are necessary because their model is related to milk production traits, composition, nutritional and technological properties (Gustavsson et al., 2014). For example, some variants are related to the chemical composition of milk or functional and technological properties, as illustrated in the manufacture of cheeses (Meza-Nieto et al., 2013). Currently, three techniques: electrophoresis, liquid chromatography, and immunochemistry, are commonly used to identify and quantify individual proteins in milk. From a qualitative point of view, none of these techniques provide conclusive results and are considered to be complementary depending on the target of analysis. Quantitatively, there is a lack of pure protein standards or a certified reference milk sample on the market. This unavailability leads to an erroneous [mass/signal] response factor unless laborious laboratory purification schemes are made, but with the risk of modifying secondary and tertiary structures and influencing the measurement (Dupont et al., 2018).

The capillary electrophoresis (EC) technique is usually considered to have advantages of speed, excellent resolution, simplicity, and low operating costs in the analysis of milk proteins compared to plate gel electrophoresis. The parameters applicable to milk proteins were developed in the years 1990–2000 by comparing several types of capillaries as well as buffers. Currently, one of the best results for the separation of milk proteins, a protocol described by Heck et al. in 2008, is obtained with milk samples prepared in a Bis-Tris / 3-morpholinopropanesulfonic buffer (MOPS) of pH 8.6 containing EDTA as calcium chelator, 6M urea and DTT. EC advantages over plate gel electrophoretic methods include greater sensitivity in terms of detection (fluorometry, mass spectrometry), possible separation of uncharged silent variants or oppositely charged molecules (para-k), greater automation, and a smaller amount of evidence (Mayer et al., 2012). However, good reproducibility depends significantly on the quality and preparation of the sample.

In the analysis of the samples, the standard and the indications of the manufacturer were observed. By closing the lid and the measurement was started immediately. The capillary electrophoresis (EC) technique is usually considered to have advantages of speed, excellent resolution, simplicity, and low operating costs in the analysis of milk proteins compared to plate gel electrophoresis. The parameters applicable to milk proteins were developed in the years 1990–2000 by comparing several types of capillaries as well as buffers. Currently, one of the best results for the separation of milk proteins, a protocol described by Heck et al. in 2008, is obtained with milk samples prepared in a Bis-Tris / 3-morpholinopropanesulfonic buffer (MOPS) of pH 8.6 containing EDTA as calcium chelator, 6M urea and DTT. EC advantages over plate gel electrophoretic methods include greater sensitivity in terms of detection (fluorometry, mass spectrometry), possible separation of uncharged silent variants or oppositely charged molecules (para-k), greater automation, and a smaller amount of evidence (Mayer et al., 2012). However, good reproducibility depends significantly on the quality and preparation of the sample.

In our initial analysis, which followed the analysis of the main proteins in cow’s milk, the protocol described by the manufacturer was followed, including in the sample preparation stage. During several tests, it was observed that fresh, unprocessed milk, brought directly from the farm is extremely likely to become a sample with analysis errors. Thus, most samples have shown a residual fluorescent signal, that has appeared as a smear on the gel electrophoresis and has led to serious problems in interpreting the total protein content of the samples (Figure 1).

Moreover, it has been observed that although the major milk proteins, in the form of protein fractions, can be identified on electrophoresis gel as well as on electropherograms, they exceed the margins of error described by the standard in terms of molecular weight, making the interpretation of results rather impossible. As this problem occurred only in the case of milk samples with high-fat content and not in skimmed milk, it was decided to add a pre-analytical stage in the sample preparation stage. The effectiveness of this step is demonstrated by the results obtained in the present study.

In the analysis of the samples, the standard and the indications of the manufacturer were observed, these being described in the material and method section. The pre-analytical step consisted of degreasing of the milk samples, initially performing the dilutions required by the protocol. This appears in the literature as being used in other studies (Gellrich et al., 2014), but it is not specified why there is this deviation from the manufacturer's standard and it is not mentioned that this stage was reached following similar observations. Thus, the five milk samples chosen to highlight the efficiency and importance of adding the degreasing stage were divided into two equal parts, one of them was subjected to the degreasing procedure, and the second was analyzed according to the protocol suggested by the author. A dilution of 1:10 was performed for all samples, which was found to be the ideal dilution in which the main milk proteins can be correctly highlighted. After dilution, the proteins were subjected to the denaturation procedure and analyzed by the capillary microfluidic electrophoresis method.

All 10 samples were migrated on the same chip, under the same conditions. The first five is represented by skim milk, and the next are the parts of milk that have not been subjected to the degreasing process. The samples numbered from one to five represent the five samples of skimmed milk, from six to 10 are the samples with high-fat content. The correspondents, in this case, are sample 1- sample 6, sample 2- sample 7, sample 3- sample 8, test 4 - test 9, test 5 - test 10 (Figure 1).
Figure 1. Virtual ELFO gel: L-Protein ladder, lanes 1-5: skimmed (centrifuged) milk samples; 6-10- raw (uncentrifuged) milk samples.

The analysis of the gel shows that the fat residue influences the detector of the equipment, that blue smear being registered as protein content. In normal cow’s milk, mixed, there are 3.3±0.14% protein, 2.7±0.10% casein, 0.45% ±0.05 albumin and 0.1-0.2%±0.03 globulin. The described method can be used only for separation and quantification of major milk proteins: αS1-casein (αS1-CN), β-casein (β-CN), αS2-casein (αS2-CN), k-casein (K-CN), respectively whey proteins, in β-lactoglobulin (β-CN şi α-lactalbumin (α-LA) as well as the quantification of the total protein content. Other protein fractions that are visible on the gel cannot be identified as long as their molecular weight is not detected with accuracy. It is known that in such an analysis that smear might appear as a result of protein degradation as a result of improper sample preparation or to some physiological factors (such as mastitis) but also to technical errors due to improper cleaning of electrode device. Considering all those, those factors must be identified to obtain the most accurate results from the analysis.

The data obtained for the first five samples are in perfect concordance with the results proposed by the Agilent milk protein standard (Nitche, 2008) both in terms of quantity but also in terms of molecular weight with the accepted variation (Table 1). As for the milk samples that were not skimmed it can be noticed that overall, the values were higher than the proposed ones.

<table>
<thead>
<tr>
<th>Sample no</th>
<th>Total Proteins, g/L</th>
<th>κ-casein 45-46.5 kD g/L</th>
<th>α-casein 35.5-37 kD g/L</th>
<th>β-casein 31.5-33 kD g/L</th>
<th>β-lactoglobulin 18-19 kD g/L</th>
<th>α-lactalbumin 12-13 kD g/L</th>
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<tr>
<td>1</td>
<td>32.88</td>
<td>3.5</td>
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<td>3.6</td>
<td>12.7</td>
<td>9.8</td>
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<td>3</td>
<td>31.99</td>
<td>3.4</td>
<td>12.7</td>
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<td>4.6</td>
<td>2.1</td>
</tr>
</tbody>
</table>

Those differences can be noticed also in Figure 2, where the data are presented in a graphical form. The differences are noticeable especially in terms of total protein content where the errors are almost 10 g/L, which represents about 30 % of total content being unacceptable for a quantitative approach. The overestimation of
protein content affects also the proper identification of protein fractions based on estimated molecular weight. It can be observed that the differences in the molecular weight of the principal milk proteins are up to 20 kD when the skimmed milk is compared to the raw milk sample.

![Graph showing differences between total protein content](image)

Figure 2. The differences between the total protein content

As the samples that were skimmed revealed the same pattern as was proposed by the kit producer it was considered that this pre-analytical step is sufficient but necessary in performing this analysis. Also, this procedure proved to be very efficient and by this, a protein chemical purification step is avoided.

**CONCLUSIONS**

The microfluidic electrophoresis (ELFO) is a method suitable for the analysis of major milk protein content providing valuable results in terms of quality and quantity and also being time-efficient. Therefore, the standardization of the method is very valuable; however, in our opinion, some minor but very important adjustments must be performed for the pre-analytical stage with consideration to the chemical composition of the milk sample and the major protein fractions that are to be analyzed.

By performing analyses on raw milk samples our experiment proved that removing the fatty substances is essential for the success of the analysis in terms of both, quality and quantity.

The proposed improvement consists of the addition of a degreasing step that can be easily performed centrifugation in the pre-analytical stage.

**Author Contributions:** O.B. and B.T conceived and designed the analysis; O.G., S.M collected the data; I.H. and C.M contributed data or analysis tools; O.B. and C.T. performed the analysis; O.B., B.T. and I.H. wrote the paper.

**Funding Source:** Authors are required to report all funding sources including grant numbers relevant to the manuscript: “This research was funded by UEFISCDI, grant number PCCDI 7/19.03.2018, code: PN-III-P1-1.2-FPRD-2017.

**Acknowledgments**

Activities under this work were carried out in the *Horia Cernescu Research Unit* in the frame of project “A bio-economical approach of the antimicrobial agents - use and resistance”.

**Conflicts of Interest**

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

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