

AUTHENTICITY ANALYSIS OF 100% SHEEP'S BRYNDZA FROM SELECTED ESTABLISHMENTS IN THE SLOVAK REPUBLIC

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ARTICLE INFO	ABSTRACT
Received 13. 1. 2024 Revised 16. 5. 2024 Accepted 27. 5. 2024 Published 1. 6. 2024	Cheese was in the past, and still is today, one of the leading commodities. The production of "bryndza" has been preserved over the years, and today it is represented by the Protected Geographical Indication "Slovenská bryndza" (Slovenská bryndza). There are two types of "Slovenská bryndza", mixed or 100% sheep. However, the name 'traditional' belongs only to sheep's bryndza. The high demand for a particular foodstuff also brings risks associated with its quality and fairness. The same is true of the national heritage product, sheep's bryndza. Identifying the originality or authenticity of various animal food products has received considerable attention recently. Our work
Regular article	has focused on the control of adulteration of 100% sheep's bryndza. Purchased samples from different parts of Slovakia were analyzed with a focus on the addition of cow's milk to sheep's bryndza. The reference method for determining the presence of cow's milk in sheep's cheese is based on detecting cow's γ2- and γ3-caseins from cheese after electrophoretic separation on urea-polyacrylamide gels. This method has proven to be reliable and sensitive. We tested sheep's bryndza made from both raw milk and heat-treated milk. The method of checking for adulteration of the cheese proved successful and in one sample we found the presence of cow casein in smaller amounts.
	probably due to accidental contamination. Isoelectric focusing of γ -caseins after plasmolysis is suitable for qualitatively determining sheep and cow proteins in traditional Slovak bryndza.

Keywords: Slovak bryndza; isoelectric focusing; adulteration

INTRODUCTION

Throughout centuries, Slovakia has cherished its rich dairy heritage, and at the heart of this tradition lies the exquisite sheep's lump cheese and the prized sheep's bryndza, celebrated for their exceptional flavors and cultural significance. A Slovakian man named Ján Vagač was the first to manufacture bryndza commercially in 1787. His process yielded a cheese that was fattier than what the shepherds were making, making it easier to spread while improving the shelf life. In the 20th century, further developments in bryndza's production occurred, creating a texture that was even more spreadable. At this time, around 80 producers were making the cheese in Slovakia. Teodor Wallo was among them and enhanced the creaminess by introducing a saline solution method. This gave the Slovakian version of bryndza its own specific quality, distinct from versions in Romania and neighboring countries (Keresteš, 2008). Among Slovakia's most sought-after dairy products are the traditional sheep lump cheese and the traditional sheep's bryndza made from it, which has been awarded the Protected Geographical Indication (PGI) designation. "Slovenská bryndza" (Slovak bryndza) is the traditional cheese of Slovakia (EC, 2007). Typical Slovak dishes like "bryndzové halušky" (a national dish made with dumplings and bryndza cheese), "bryndzové pagáčiky" (scones made with bryndza cheese), "pirohy" (stuffed with bryndza cheese), and many more are created with this cheese. Sheep's bryndza is specific regarding its taste, aroma, and positive properties for the human body. Bryndza cheese includes several predominant lactic acid bacteria (LAB) from the Lactobacillus spp., Enterococcus spp., Lactococcus spp., and Streptococcus spp. (Jurkovič et al., 2006; Berta et al., 2009). It was created by accident and its recipe has been slightly modified to achieve greater health safety and sensory quality or durability (EC, 2008). A lump of matured ewe's cheese, a mixture of this cheese and a lump of cow's cheese, or a combination of a stored lump of matured ewe's cheese and a lump of cow's cheese, is used to make "Slovenská bryndza" a natural cheese. "Slovenská bryndza" must have a minimum of 50% w/w ewe's cheese in its dry matter (MARD SR, 2016). The quality of bryndza cheese depends on many factors, for example milk production period, the quality of ewe milk and the use of starter (Planý et al., 2016). The quality of bryndza cheese including its composition, physico-chemical properties and microbial diversity depends on the quality of the ewe milk (Chebeňová-Turcovská et al., 2011; Pangallo et al., 2014; Sádecká et al., 2014). However, it has retained its essence and effects. The widespread interest in this type of product may mean that these animal products are susceptible to substitution by cheaper milk for economic gain. Milk adulteration is an emerging problem worldwide, affecting consumers, the food industry, and inspection authorities (Zajác et al., 2023). Milk authenticity testing in dairy products is necessary to protect consumers from fraudulent products, mislabelling, and health risks and to prevent unfair competition from the food industry. Thirteen Slovak producers of sheep bryndza from different parts of Slovakia were presented. The experimental research aimed to analyze the authenticity of the sheep's bryndza and check whether it was adulterated by adding milk from another type of dairy animal. The analysis was preceded by defatting the samples in a Soxhlet extractor and obtaining protein powder for the detection of cow proteins. The procedure used in the analysis of bryndza was presented, and finally, the obtained results were presented and compared with other analyzes of sheep bryndza.

MATERIAL AND METHODS

Material

We analyzed 13 different samples of sheep's chard. The samples were purchased from shepherd's huts in different towns in Slovakia. The purchased samples were kept in a refrigerator at -12 $^{\circ}$ C until the day of testing.

Methodology

We needed to defeat the bryndza samples before the analysis as it is a high-fat product and we wanted to get the most accurate results. Before isoelectric focusing we separated the fat from the samples on a Soxhlet extractor, the water was evaporated during this process, so we obtained a powder composed of protein and carbohydrate. We performed the extraction three times to separate the fat from the samples as much as possible.

Isoelectric focusing

First, we turned on the heat sink (Serva, Germany) to cool the ceramic plate (Serva, Germany) to 10 °C. The ceramic plate was cleaned with distilled water and wiped dry. We then pipetted 3 ml of the H-shaped cooling mixture. We placed the 0.65 mm GEL-FIXTM polyacrylamide gel fixation film on the prepared plate without air bubbles in a precise position, which was important to record the results in the correct boxes. Prefocusing was started with the Method PrefMil program and was

run at 1000 V, 50 mA, and 10 W for 20 min. We then applied thirteen samples, each of 5 μ l, and two markers (Table 1). We placed electrodes on the gel. The isoelectric focusing program was carried out in three phases at 500 - 2000 V, 20 mA, and 10 - 30 W at different time lengths for a total of 3.5 hours.



Figure 1 Map with locations of sheep's bryndza sample production

Table 1 Location of the sample on the gel

Position on the gel	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Sample number	М	1	2	3	4	5	6	7	8	9	10	11	12	13	М
M															

M - marker

After isoelectric focusing was completed, we carefully removed the gel from the ceramic plate with tweezers and placed it in a tray, which we fixed.

Fixing, coloring, bleaching, and drying

Using fixative solutions, we stabilized the gel. We placed the gel in a plastic container with tweezers, covered it with 20% TCA solution, and covered it. We placed the tray on a shaker (GFL, The Netherlands) where the gel was washed. After 45 minutes, we removed the gel with tweezers and immersed it in 5% TCA solution, in which it was washed for 30 minutes, and covered. We removed the sample and immersed it in the regenerative decolorizing solution for half a minute. This immersion was performed twice. We immersed the gel in a tray of copper sulfate pentahydrate staining solution for 45 minutes and rewashed it at 100 - 200 rpm on a shaker (GFL, The Netherlands). The stained gel was immersed for 20 min in a tub of decolorizing solution. Then we rinsed the gel with distilled water. Washing is important to obtain the correct result. Once the gel was decolored and the desired color was obtained, we placed it on a paper handkerchief and blotted the underside of the foil. The top part with the gel dried in the air.

Photography and analysis of the gel photo

This was followed by photo documentation with Azure biosystems (Dublin) photo equipment designed for photographing gels. The obtained photographs were analyzed using the computer program Gel-Pro Analyzer 6.0. We labeled the protein fractions (bands) captured on the gel in lanes according to their size and density. A report was generated from our recordings in which the migration distances of the proteins on the gel were displayed, and the sizes of the bands (bands) were plotted on graphs according to the height of the peaks. A high concentration of a particular protein in a given band (strip) represents its darker coloration and higher peak height in the graph than other bands.

Canning

Finally, gel preservation was performed by immersion in glycerol solution for 30 min and final drying was done free air drying. We archived the gel obtained.

RESULTS AND DISCUSSION

The aim of the experimental part was to the detection of adulteration of Traditional Slovak Sheep's Bread by the addition of cow's milk. Thirteen samples of sheep's bryndza were purchased for testing, from producers from different parts of Slovakia, shepherd's huts, and commercial establishments. The samples were purchased during the winter months from the end of September to October, i.e. winter bryndza samples. The samples were kept in a freezer at -12 °C, which was then convenient for us when preparing the samples for extraction, as they were easier to grate into smaller pieces. Defatting the samples before isoelectric focusing was the more time-consuming part of the work as we did all the samples in two repetitions making 26 samples. To obtain perfectly fat-free solids, we repeated the extraction three times until the metal container where the fat flowed was empty and dry. Thanks to the triple extraction of the samples, we obtained a representative result recorded on the gel after isoelectric focusing. After isolation and hydrolysis of casein by plasmin, we obtained separated sheep γ 2- and γ 3-caseins in each

sample in the photographs. Other protein fractions (bands) are also captured on the resulting gel without a scattered fat layer. We obtained the following results:



Figure 2 Gel showing protein fractions

Samples analyzed

2. 3.

4.

5.

6.

7.

8.

M - MARKER (numbers 1 and 15 on the gel)

- 1. Salaš Pastierska (number 2 on the gel)
 - Syrmix Zázrivá (number 3 on the gel)
 - Agronova Liptov (number 4 on the gel)

Bryndziareň Turčianske Teplice (number 5 on the gel)

- Syrex Zázrivá (number 6 on the gel)
- Salaš Pružina (number 7 on the gel)
- Bryndziareň Slatina (number 8 on the gel)
- Gemerské ovečky (number 9 on gel)
- 9. Jožko Farmárik (number 10 on gel)
- 10. GreenSheep Zvolenská Slatina (number 11 on the gel)
- 11. Farm Strednica (number 12 on the gel)
- 12. Ecofarm Važec (number 13 on the gel)
- 13. Farm Dlhé nad Cirochou (number 14 on the gel)

The results indicate that no cow's milk was added to samples 2 - 13 (numbers 3 - 14 on the gel) and this means that the samples are fine, have not been adulterated, and are 100% sheep's bryndza.



Figure 3 Gel image showing the thickness, size, and number of fractions present

In the test field of sample number 1. Salaš Pastierska (number 2 on the gel), a small amount of protein not belonging to sheep protein was detected between two bands in the field. There are several reasons or causes for the occurrence of this protein fraction. Due to the weaker intensity of the band, it is difficult to discern the correct reason. It is, however, a band from the cow fraction. The cause of this 'non-sheep' fraction may be an attempt to adulterate the product by adding cow's milk to sheep's milk before making the lump cheese, or improper, inconsistent sanitation of the squepresent in the production area of the plant/shed, or a sales error where the salesperson did not use different tools when serving the customer for each range.



Figure 4 Fractions present on the gel

Isoelectric focusing is a qualitative method that is sensitive and accurate for the detection of cow's milk in mixed samples. Still, it has several limitations: it is not a high-throughput method, it is not quantitative, and the analysis is time-consuming. In addition, the method cannot distinguish between goat and sheep mixtures; the interpretation of the IEF profile can be ambiguous. In addition, IEF does not apply to products made from soy milk because weak interference bands have been observed (**Domenico et al., 2017**).

We agree with the authors' comments on the time-consuming nature of the research, but we think that the number of samples analyzed is also an important factor. With a lower number of samples, we think this method could be more advantageous. However, using more economically expensive chemicals and materials is also a disadvantage of the method. We have found that defeating highfat products is important to obtain more representative results. The sensitivity of the IEF method for distinguishing cow's milk from sheep's cheese is also commented on by Addeo et al. (2019), in their research. The authors inspected an Italian sheep's milk hard cheese with a protected designation of origin in which they found 0.5% bovine milk. They confirm that the method is accurate and sensitive for this type of analysis. We agree with this statement because the gel detected minimal amounts of bovine casein in our sample No 1 (Shepherd's Farm). Adulteration of traditional products occurs virtually all over the world. An important step is to check these practices regularly so that consumers are not deceived and, at the same time, the traditional generational recipes of specific countries are not altered and devalued. A similar problem applies to the traditional national sheep's cheese Travnik (original: Vlašić, Bosnia and Herzegovina). The cheese is made from sheep's milk. However, due to the high demand for this traditional product, some farmers add more than 20% cow's milk to the cheese to increase production. In addition to being sold in markets, this cheese is also exported to other countries. For this reason, Crowley et al. (2018) decided to analyze dozens of samples. The result of the analysis confirmed adulteration in 37% of the samples. Consistent with the research of Zajác et al. (2021), we can confirm the presence of cow y-2-casein at the same position recorded among sheep caseins. In the analysis, they also tested samples of cow and sheep casein alone, as we can see in Figure 5, where the presence of the cow casein fraction can be seen. The hydrolysis of the proteins in the cheeses by the action of plasmin, and the subsequent separation of γ -case in the isoelectric points, is captured in specific pH scale values from 3 to 10 according to the respective animal species. The protein fractions, after reaching their isoelectric point, stop at a given value on the gel. Cow γ -2-caseins and γ -3-caseins are formed by hydrolysis of β -casein by plasmin and have different isoelectric point values compared to sheep y-2-caseins and γ -3-caseins. The isoelectric points of bovine caseins are pI = 7.0 and pI = 6.5 for γ -2-casein and γ -3-casein, respectively. The isoelectric points of sheep caseins are for γ -2-casein pI = 7,2 and γ -3-casein pI = 6,7. Regarding the pI values for sheep γ -caseins, **Domenico et al.** (2017) comment that they can range between pH 6.5 - 7.5. According to our research, the isoelectric points for sheep caseins were values in the range shown in Table 2.

Table 2 Identified protein fractions and their isoelectric points

Protein fraction	Isoelectric point
γ-2-casein/ γ-2-casein	7.48 - 7.56
γ-3-casein/ γ-3-casein	6.53 - 6.74

Zajác et al. (2021) state that the IEF laboratory method is suitable for determining the proportion of sheep and cow lump cheeses in Slovak bryndza if the producer has followed the production process given by legislation without incorrect technological steps.



Figure 5 Gel slide with caseins of different origin

The adulteration problem has also been detected and controlled in Romania, where cow's milk is also added to buffalo, goat, and sheep milk. To avoid unfair competition between producers, methods have been devised to identify the authenticity of dairy products. Most of them are based on milk protein analysis. However, the European methodology for identifying the authenticity of IEF cheeses by analysis of gamma casein fractions has the disadvantage in this case that goat's and sheep's milk cannot be distinguished. Therefore, the study by Balteanu et al. (2011) aimed to test the possibility of using all alleles described so far to distinguish between the milk of these species. Interspecies milk mixtures were prepared from reference samples containing all described genetic variants of the four farmer species, combinatorially with each other. The IEF behavior of each major milk protein and their species-specific genetic variants were used to distinguish each milk type, including differentiation between goat and sheep milk, and cow and Romanian buffalo milk. The preliminary results of their work indicate the applicability of this proposed method for the authentication of dairy products. They also indicate the possibility of using β -casein CBT as a marker to identify the authenticity/origin of Romanian buffalo cheeses. According to the analysis by Illoul et al. (2020) of sheep milk from the Ouled-Djellal and Rembi sheep species, reared in North Africa, we can agree and confirm the presence of α -caseins in our samples, despite the different breed from the Slovak breed. The analysis results were obtained the same way using isoelectric focusing.



Figure 6 Negative gel image with different types of caseins



Figure 7 Negative gel image confirming α-s- caseins

Figure 8 is a photograph of the gel after isoelectric separation of γ -casein from cheese samples along with one sample of 100% cow and one sample of 100% sheep protein. The results of the research by **Špoljaric et al. (2013)** confirm the adulteration of sheep's cheese by the addition of cow's milk.



Figure 8 Negative gel image with caseins of different animal species

Comparing our result (Fig. 9) and the result (Fig. 10) arrived at by the same isoelectric focusing method by **Sienkiewicz et al. (2006**), the protein found can be considered to be consensual, and hence would imply that we have captured bovine (cow) γ -2-casein in the analysis.



Figure 10 Close-up of bovine casein in sheep's cheese

CONCLUSION

We have been checking the adulteration of 100% sheep's bryndza in Slovakia. Samples purchased from different parts of Slovakia were analyzed with a focus on adding cow's milk to sheep's bryndza. The reference method for determining the presence of cow's milk in sheep's cheese is based on detecting cow's γ 2- and γ 3caseins from cheese after electrophoretic separation on urea-polyacrylamide gels. This method has proven to be reliable and sensitive. We tested sheep's bryndza made from both raw milk and heat-treated milk. The method for checking adulteration of cheese proved successful and we found the presence of cow casein in one sample in a minor amount, probably due to unintentional contamination. Isoelectric focusing of γ -caseins after plasmolysis is suitable for qualitatively determining sheep and cow proteins in traditional Slovak bryndza. The method was more time-consuming with our number of samples. We defatted the samples by triple extraction on a Soxhlet extractor, thus obtaining representative samples recorded after isoelectric focusing on the gel without fat spots around the protein fractions. The method can be shortened by omitting the staining procedure after isoelectric focusing and photographing or scanning the unstained fixed gel. From the results of our analysis, we can confirm the honesty of Slovak producers and their focus on preserving tradition and producing quality traditional products. Although we evaluate the obtained results of the work positively, our positive result should not represent an omission of controls in the following years. It is important to check the adulteration of traditional Slovak bryndza on a targeted basis in regular and random monitoring so that any attempts at adulteration can be traced and reported publicly if illegal practices are detected. Announced checks on random samples by the official inspection bodies would provide a degree of respect and order in the area of adulteration of sheep's bryndza. They would also create some certainty for honest producers and their competitiveness, as the conditions for producers would be uniform and determined by legislation. Slovak inspection authorities focus on checking sheep's bryndza and sheep's lump cheese for microbiological safety, an important indicator of quality and safety for consumers. However, checks on the composition and authenticity of this product with a protected recipe should not be neglected and this aspect of product quality should also be monitored. The adulteration of food with a protected geographical indication has a major impact on all stakeholders, i.e. producers, farmers, consumers, businesses, government, and food inspection authorities. Similarly, the establishment of reliable analytical methods is essential to ensure the integrity of technological production and to build consumer confidence. A decline in consumer confidence could lead to economic losses and loss of traditions.

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