

A Molecular Procedure for Detecting Dairy Products Adulteration

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RESEARCH ARTICLE

Abstract

Dairy products are an essential component of human nutrition and therefore are prone to adulteration. Here is presented the application of a DNA-based method, fast and cost-effective to determine the species composition of dairy products by validation of a qualitative PCR detection technique that will be adapted to the domestic market starting from experimental models developed and well defined internationally.


In the process of validating this method, three species of ruminants were considered: beef; sheep, and goat. The reference material was represented by cheeses prepared in strictly controlled percentage mixtures. Identification of species based on milk protein content by using the method of microfluidic electrophoresis has proven to be very accurate. But, the most efficient and accurate methods for detecting species composition of dairy products are based on the detection of specific mitochondrial DNA sequences. The reference materials proved to be optimal for such studies and have been used successfully in the process of validating the method of detecting the composition of commercial cheeses. At the same time, these materials made it possible to set a detection limit of 1% for the fraudulent addition of cow's milk to the composition of dairy products.

Keywords: dairy products, adulteration, multiplex PCR, mitochondrial DNA.

17 INTRODUCTION

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18 Milk and dairy products are an essential component of human nutrition and are most
19 commonly consumed by children and the elderly. Annually, out of the total milk
20 produced in the world, 85% is cow's milk, 11% buffalo's milk, and only 2% sheep's
21 milk and goat's milk. A large part of this milk is processed into dairy products, about
22 60% (Sala et al., 2015). It is considered that 30% of the need for lipids and proteins
23 and 80% of the daily requirement of calcium is provided by dairy products. Fraud by
24 substituting sheep's, goats, or buffalo's milk with cow's milk has become a common
25 practice, and various detection methods such as immunological, electrophoresis,
26 chromatography, and PCR have been developed to detect adulterations with it. Milk
27 was the second most adulterated food reported in the scientific record by 14% (Moore
28 et al., 2012). For quantitative analyzes, a method based on isoelectric focusing and
29 HPLC- for cation exchange of casein has been proposed (Helmut K. Mayer, 2005).

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31 On the other hand, methods for quantifying milk species based on whey protein fraction suffer from a shortcoming, as
 32 this fraction is more sensitive to heating than the casein fraction. Such methods may cause negative falsifications when
 33 sterilized milk or powdered milk has been used in the manufacture of the cheese. Excessive proteolysis during cheese
 34 ripening can also be disadvantageous for quantification. The authors developed capillary electrophoresis to determine the
 35 adulteration of goat's milk products with cow's milk (Cartoni et al., 1999). Analyzes of the mixtures of bovine milk,
 36 sheep's milk, and goat's milk were performed by capillary electrophoresis. The amount of adulteration was quantified by
 37 multivariate regression analysis. (Molina et al., 1999). Lately, DNA molecules have become target compounds for
 38 species identification because they have higher stability compared to proteins. They are also present in most biological
 39 tissues, making them ideal candidates in differentiating and identifying food components, thus becoming a valuable
 40 alternative for protein analysis. Most DNA methods for identifying food species are based on highly specific
 41 amplification of a DNA fragment using the PCR technique. This method has a high potential due to its speed, simplicity,
 42 sensitivity, and specificity (Marfa et al., 2007).

43 This paper presents an internal validation method based on a DNA analysis that is fast, accurate, cost-effective, and
 44 adapts to the requirements of the food industry to determine the authenticity of dairy products, especially specialty
 45 cheeses. This paper aims to meet the requirements of international food safety standards and validate a method for
 46 identifying and quantifying dairy product specifications to meet the demands of the domestic market.

47

48 MATERIALS AND METHODS

49 *Reference material*

50 The cheeses were prepared in our laboratory from fresh cow, sheep, and goat milk purchased from farms in the region
 51 (data not shown). The biological material consists of 10 dairy products, namely 10 kinds of Romanian specialties cheese
 52 purchased at the local market having various compositions and are listed in Table 2.

53 *Primers used in this study*

54 In this work, the primers proposed, by Bottero et al., 2002, were used for the three species targeting the mitochondrial
 55 DNA sequence 12s rRNA and generating fragments of different lengths (Table 1). The first fragments were synthesized
 56 by Eurogentec (Belgium).

57

58 **Table 1** Oligonucleotides used as PCR primers

59

Species	Primers 5'-3'	The length of the amplicons (bp)
Bos taurus	5'GTA CTA CTA GCA ACA GCT TA 3'	256
	5'GCT TGA TTC TCT TGG TGT AGA G 3'	
Capra hircus	5'CGC CCT CCA AAT CAA TAA G 3'	326
	5'AGT GTA TCA GCT GCC AGT AGG GTT3'	
Ovis aries	5'ATA TCA ACC ACA CGA GAG GAG AC 3'	172
	5'TAA ACT GGA GAG TGG GAG AT 3'	
Ruminants	5'TAA GAG GGC CGG TAA AAC TC 3'	104
	5'GTG GGG TAT CTA ATC CCA G 3'	

60

61 *Isolation and purification of DNA from the samples under investigation*

62 DNA extraction from cell cultures was performed using the "innuPREP DNA mini" kit (Analytik Jenna, Germany). The
 63 step before DNA isolation was to remove the lipids present in the freshly harvested milk samples. For this purpose, 1 g of
 64 cheese from each sample was resuspended in 5 ml of PBS buffer and evenly distributed in two Grainer centrifuge tubes.
 65 The samples were centrifuged at 14,000 rpm for 20 minutes. After centrifugation, the lipid components formed a single
 66 layer on the surface of the supernatant, and the somatic cells formed sediment at the bottom of the analysis tube.

67 The lipid layer, which contained most of the supernatant, was removed. The cell pellet was resuspended in the remaining
 68 supernatant and then centrifuged again at 14,000 rpm for 20 minutes. At the end of the last centrifugation, the cell pellet
 69 was resuspended in only one milliliter of PBS solution and transferred to 1.5 ml centrifuge tubes. The obtained cell
 70 suspension was subjected to the procedure of DNA isolation and purification.

71 DNA was qualitatively and quantitatively assessed by the spectrophotometric method using the NanoDrop 8000
 72 instrument (ThermoScientific). After quantification, samples were diluted to the same concentration.

73 *PCR analyzes*

74 PCR reactions were performed using Agilent Technologies Surecycler Thermocycler. The reagents that made up the
 75 amplification mixture were as follows: PCR kit, KapaRobust Hot Start 2X (KapaBiosystems, U.S.A.) - 12.5 µl, 20 pmol
 76 of each primer, DNA template - 1 µl, made up to 25µl with distilled water. Amplification conditions were as specified in
 77 the literature (Bottero et al., 2003).

78 *Agarose gel electrophoresis*
79 The resulting PCR products were migrated to agarose gel. The gel was prepared with 2% agarose and TAE Buffer. The
80 staining was performed with ethidium bromide, and the gel was photographed in UV light and analyzed with GelDoc-It
81 Imaging System (UVP England).

82 83 **RESULTS AND DISCUSSIONS**

84 Methods based on the PCR technique are increasingly used in the recognition of milk types with a very low detection
85 limit and are also successfully applied to cheeses and other heat-treated dairy products, where the methods of protein
86 analysis are not can apply (Lopez et al., 2005), (Helmut. K. MAYER, 2005).The use of PCR for quantification in the
87 analysis of food authenticity is still quite rare. The authors described a relatively simple method, not only to detect the
88 addition of cow's milk in sheep's or goat's milk products but also to quantify this addition by the duplex PCR method,
89 using a standardized calibration curve to control the associated problems with DNA extraction and gel preparation (Marfa
90 et al., 2004), (Marfa et al., 2007), (Maudet et al., 2001). For this, the primers described by Bottero et al., (2002) were
91 used, which target a mitochondrial DNA region, 12 S rRNA. They were successfully used in a multiplex PCR reaction
92 aimed at the simultaneous detection of goat's, sheep's, and cow's milk in dairy products on the market. The detection
93 limits described are quite low going up to 0.01% cow's milk in sheep's milk products (Marfa et al., 2008), (De La Fuente
94 et al., 2005). In 2008, Mafra et al. also applied the PCR technique to quantify the adulteration of cow's milk as goat's
95 milk and described that the intensity of the agarose gel strip is proportional to the contamination in the sample.

96 Due to the lack of certified reference material on the market, the first step of the experiment was to validate the PCR
97 multiplex method in our laboratory using the existing reference materials in the laboratory and the mixture of the 3
98 species-specific primers. The pairs of primers used were developed and published by Bottero et al., (2002) stating that
99 they can be multiplexed, thus offering the possibility of identification simultaneous use of the three species in dairy
100 products. The expected dimensions for species-specific amplicons were: bovine 256 base pairs, sheep 172 base pairs,
101 goat 326 base pairs (Figure 1). For the most precise identification of the amplicons specific to each species, the samples
102 certified as 100% bovine, goat, or ovine were used as specific controls.

103



104
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Figure 1 Multiplex PCR reaction validation.

106 a) DNA extracted from 100% bovine material; b) DNA extracted from 100% goat material; c) DNA extracted from 100% sheep material; d) DNA
107 extracted from bovine, caprine, ovine mixtures: 50:25:25; e) DNA extracted from mixtures of bovine, caprine, ovine: 25: 37,5: 37,5; f) DNA extracted
108 from mixtures of bovine, caprine, ovine: 10: 45: 45; g) DNA extracted from mixtures of bovine, caprine, ovine: 5: 47,5: 47,5; h) DNA extracted from
109 bovine, caprine, ovine mixtures: 2,5: 48,75: 48,75 i) DNA extracted from bovine, caprine, ovine mixtures: 1: 49,5: 49,5; j) DNA extracted from
110 mixtures of bovine, caprine, ovine: 0.5: 49.75: 49.75; k) Negative reaction control NTC (negative template control). M- O'Gene Ruler molecular
111 weight marker (ThermoScientific);

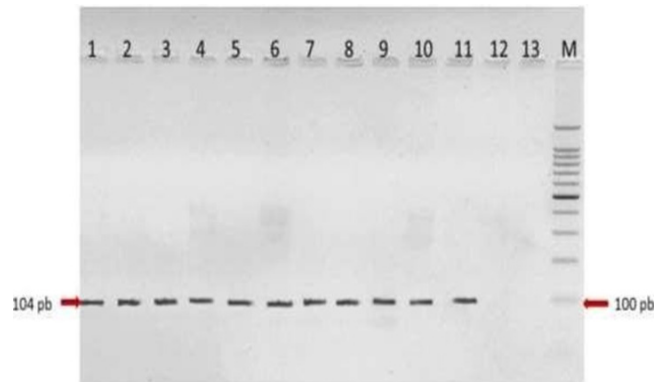
112 From the gel image can be observed the differences between the concentrations of the three species in the final samples,
113 concentration is interpreted according to the intensity of the amplicons. Of course, in such an experiment in which it is
114 desired to identify the adulteration of dairy products, attention must be paid to milk from cattle, because in the vast
115 majority of cases this is the main fraud. The literature does not mention cases in which the products are adulterated with
116 the other two types of milk only if they may accidentally end up in the composition of a certain product. All this is due to
117 purely economic considerations, as cow's milk is much cheaper.

118 Using this method it can be seen that even at a very low percentage concentration of 0.5%, the presence of cow's milk
119 can be identified even in the conditions of preparation of mixtures with a net concentration in favor of the other two
120 species. Following the validation process, a detection limit for bovine milk was set at 1%. However, as a reference
121 sample for the experiments that will be carried out on samples purchased from the market, the sample with the following
122 composition in percentages was chosen: bovine, goat, sheep: 10:45:45. In its choice, it was considered that a 10%
123 concentration of bovine is sufficient to be successfully amplified during the reaction and at the same time, it is low
124 enough to identify the voluntary addition of milk in the composition of cheeses. In order to carry out the experiments
125 from this last stage of our work, dairy specialties included in the cheese category were purchased on the domestic market.

126 The aim was to acquire specialties with a composition as varied as possible to cover all three species considered in this
 127 project, according to Table 2.

128 The products were purchased both from supermarkets and from local producers. These samples were processed in
 129 accordance with the method used for the reference samples. Thus, from the purchased samples were prepared in the
 130 laboratory, samples in the amount of 200 mg, from which total genomic DNA was extracted and purified. Regardless of
 131 the composition of the matrix from which the DNA was extracted, its quantity and quality were sufficient to develop
 132 PCR reactions. After extraction, DNA solutions with a concentration of 200 ng / μ l were prepared.

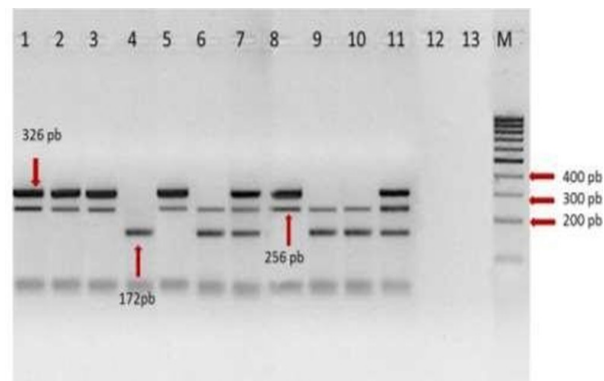
133 The idea of such a pre-analysis is, first of all, the identification in any product, with unknown composition, of the type of
 134 matrix used in its composition. In other words, it is useless to look for ruminant-specific material in a product in the
 135 preparation of which no milk (for example, tofu cheese) was used. In this case, a PCR product was obtained for all
 136 analyzed samples, which demonstrated both the amplifiable quality of the extracted DNA and the presence of ruminant
 137 material in the analyzed samples (Figure 2).



138 **Figure 2 Testing the amplifiable quality of DNA extracted from commercial products**

139 a) telemea goat / cow; b) Telemea goat; c) Telemea goat; d) Telemea sheep; e) - Goat / cow cheese; f) Sheep curd; g) - Sheep / goat bellows cheese; h)
 140 - Telemea goat; i) - Telemea sheep; j) - Cow / sheep cheese; k) - Positive reaction control, DNA isolated from beef muscle tissue, PTC (positive
 141 template control); l) - Negative reaction control, NTC (negative template control); m) - Environmental contamination control; M- molecular weight
 142 marker O'Gene Ruler (ThermoScientific).
 143
 144

145 This DNA was used as a template in the multiplex PCR reaction. For this reaction, the reference sample with the
 146 following composition in percentages was used as a positive control: bovine, goat, ovine: 10:45:45. (Figure 3)



147 **Figure 3 Multiplex PCR analysis of commercial products.**

148 a) goat / cow telemea; b) - Telemea goat; c) - Telemea goat; d) - Telemea sheep; e) - Goat / cow cheese; f) - Sheep curd; g) - Sheep / goat bellows
 149 cheese; h) - Goat telemea; i) - Telemea sheep; j) - Cow / sheep cheese; k) - Positive reaction control, DNA isolated from beef muscle tissue, PTC
 150 (positive template control); l) - Negative reaction control, NTC (negative template control); m) - Environmental contamination control; M- molecular
 151 weight marker O'Gene Ruler (ThermoScientific).
 152
 153

154 Analyzing Figure 3 it can be said that the method validated in the first part of this study was successfully applied to
 155 commercial products. The results of this study being systematized in Table 2.

156
 157

158 **Table 2** Cheese samples used in this experiment and the result of PCR screening

Number	Biological sample	Provenience/Type	Label	The result of
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			composition	the analysis
1	Cheese	Supermarket / individual packaging	Goat/cow	Goat/cow
2	Cheese	Supermarket / sold by weight	Goat	Goat/cow
3	Cheese	Supermarket / sold by weight	Goat	Goat/cow
4	Cheese	Supermarket / individual packaging	Sheep	Sheep
5	Romanian specialty - Cașcaval	Supermarket / individual packaging	Goat/cow	Goat/cow
6	Romanian specialty (Caș)	Local market / local producer	Sheep	Sheep/cow
7	Bellows cheese(branza de burduf)	Supermarket / individual packaging	Sheep / Goat	Sheep / Goat
8	Cheese	Supermarket / sold by weight	Goat	Goat/cow
9	Cheese	Local market / local producer	Sheep	Sheep/cow
10	Romanian specialty - Cașcaval	Supermarket / individual packaging	Cow / Sheep	Cow / Sheep

159

160 The analysis of the results shows that cheeses made from milk mixtures, one of which is beef, are correctly labeled in
161 terms of composition. Only one sample, sample number 4 - sheep's milk, was free from the addition of cow's milk. As for
162 the other products, it can be said that they were fraudulently added with cow's milk. The results obtained unequivocally
163 demonstrate the applicability of the method based on DNA analysis incorrectly detecting the composition of cheeses, but
164 also the fact that such a method is particularly necessary for proper food control knowing that methods based on protein
165 analysis fail due to their degradation during processing.

166

167 CONCLUSIONS

168 For processed products, the only efficient and accurate methods for detecting species whose milk is a component of dairy
169 products are those based on nucleic acid analysis.

170 The most commonly used methods are those developed for the detection of mitochondrial DNA sequences specific to the
171 species analyzed. The analysis of the cheeses purchased on the domestic market proved the applicability of the validated
172 method. Moreover, the analysis of these products, which led to the detection of fraudulent addition of cow's milk in the
173 composition of two products, underlines the need to use methods based on the analysis of nucleic acids in food control
174 laboratories.

175

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181

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185

186 Conflicts of Interest

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

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189 REFERENCES

190 1. BOLDURA, O.M., POPESCU, S., 2016, PCR: A Powerful Method in Food Safety Field, Polymerase Chain
191 Reaction for Biomedical Applications, Editura InTech.

- 192 2. BOLDURA, O.M., POPESCU, S., BALTĂ, C., MIRCUCU, C., HUȚU, I., AHMADI, M., TULCAN, C., (2017)
193 *Applicability of a duplex end point PCR method in detecting sheep dairy products adulteration by cow milk*
194 *fraudulent addition* Lucr. Șt. Med. Vet., Timișoara, 2017, 50(1): 224-231, DOI: [10.13140/RG.2.2.31851.69922](https://doi.org/10.13140/RG.2.2.31851.69922)
195 3. BOTTERO, M. T., CIVERA, T., NUCERA, D., ROSATI, S., SACCHI, P., TURI, R. M., 2003, A multiplex
196 polymerase chain reaction for the identification of cows', goats' and sheep's milk in dairy products. *International*
197 *Dairy Journal*, 13(4), 277-282.
198 4. BOTTERO, M.T., CIVERA, T., ANASTASIO, A., TURI, R.M., ROSATI, S., 2002, Identification of cow's milk
199 in "buffalo" cheese by duplex polymerase chain reaction. *J Food Prot.*, 65 (2), 362-366.
200 5. CARTONI, G., COCCIOLI, F., JASIONOWSKA, R., MASCI, M., 1999, Determination of cows' milk in goats'
201 milk and cheese by capillary electrophoresis of the whey protein fractions. *J. Chromatogr A*. 846 (1-2), 135-141
202 6. DE LA FUENTE, M. A., JUAREZ, M., 2005, Authenticity assessment of dairy products. *Critical Reviews in*
203 *Food Science and Nutrition*, 45(7-8), 563-585.
204 7. HURLEY, I. P., IRELAND, H. E., COLEMAN, R. C., WILLIAMS, J. H. H., 2004, Application of
205 immunological methods for the detection of species adulteration in dairy products. *International Journal of Food*
206 *Science and Technology*, 39(8), 873-878.
207 8. HUȚU, I., BOLDURA, O.M., POPESCU, S., CHIȘ, C. BALTĂ, C., MIRCUCU, C. (2013). *A multiplex PCR*
208 *approach to the quantification of cow milk in goat products* *Current. Opinion in Biotechnology*, 24, Supplement
209 1, Pages S95, DOI: [10.1016/j.copbio.2013.05.285](https://doi.org/10.1016/j.copbio.2013.05.285).
210 9. MAFRA, I., FERREIRA, I., OLIVIERA, M., 2008, Food authentication by PCR-based methods. *European Food*
211 *Research and Technology*, 227(3), 649-665.
212 10. MAFRA, I., FERREIRA, M.P.L., FARIA, M.A., OLIVIERA, B.P.P., 2004, A novel approach to the
213 quantification of bovine milk in ovine cheeses using a duplex polymerase chain reaction method. *J.*
214 *Agricultural and Food Chemistry*, 52, 4943-4947
215 11. MAFRA, I., ROXO, A., FERREIRA, I., OLIVIERA, M., 2007, A duplex polymerase chain reaction for the
216 quantitative detection of cow's milk in goats' milk cheese. *International Dairy Journal*, 17(9), 1132-1138.
217 12. MAUDET, C., TABERLET, P., 2001, Detection of cows' milk in goats' cheeses inferred from mitochondrial
218 DNA polymorphism. *J. Dairy Research* 68, 229-235
219 13. MAYER, H. K., 2005, Milk species identification in cheese varieties using electrophoretic, chromatographic
220 and PCR techniques. *International Dairy Journal*, 15(6- 9), 595-604.
221 14. MOLINA, E., MARTIN-ALVAREZ, P. J., RAMOS, M., 1999, Analysis of cows', ewes' and goats' milk
222 mixtures by capillary electrophoresis: quantification by multivariate regression analysis. *International Dairy*
223 *Journal*, 9(2), 99-105.
224 15. MOORE, J. C., SPINK, J., LIPP, M., 2012, Development and application of a database of food ingredient fraud
225 and economically motivated adulteration from 1980 to 2010. *Journal of Food Science*, 77(4), R118-R126.
226 16. SALA, C.C, MORAR, A., 2015, *Hygiene and food technology: milk and dairy products, eggs, honey*, Ed.
227 Eurobit, Timișoara.

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