

A sensitive DNA-based fluorometric method for milk authenticity of dairy products based on spectrally distinct microspheres

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Abstract Milk authenticity of dairy products is of great demand in order to protect consumers from fraudulent products and health risk. Milk-based products produced by goat and sheep milk are located at the top of the list of healthy dairy products, earning the increasing interest of consumers. As a result, these products are extremely vulnerable to adulteration with milk of lower added and nutritional value for profit. In addition, adulterant milks, such as bovine milk, have been accused to cause allergic reactions, especially to sensitive human groups. For these reasons, there is a great need for sensitive and robust analytical methods for milk adulteration detection. This work focused on the development of a DNA-based analytical method based on hybridization assay on spectrally distinct microspheres for adulteration detection of goat and sheep dairy products with bovine milk. The method includes (a) DNA isolation from dairy products, (b) amplification of DNA sequences specific to each animal species and (c) hybridization assay on the surface of distinct microspheres. The identification of the animal origin is accomplished by species-specific oligonucleotide probes coupled to the microspheres and the hybrids formed are detected by a streptavidin–phycoerythrin conjugate. The microspheres are finally analyzed by flow cytometry. The fluorescence emitted by the reporter molecule phycoerythrin is proportional to the concentration of the DNA content in the sample. As low as 0.01% bovine milk in goat milk and 0.05% in sheep milk was able to be detected by the proposed method. The method offers very good detectability, sensitivity and reproducibility.

Keywords DNA · Milk authenticity · Microspheres · Beads · Fluorescence · Flow cytometry

Introduction

The rapid increase of the overconsumption of food stuff, along with the globalization of the market has led the consumers to concern for the food origin, quality and safety. Many products with a high purchasing value are particularly more vulnerable to fraud due to adulteration with similar products of much lower price and nutrition value [1, 2]. In addition, some adulterants may also be serious allergens. This has increased the demand of developing new analytical tools with high detectability and sensitivity for detecting food adulteration. Among others, species identification tools are the most suitable for authenticity test in animal-derived food products.

Dairy products are considered to be one of the seven more attractive foods for adulteration. Especially, milk-based products derived from goat and sheep species are recognized as healthier and with higher nutrition and added value than bovine milk, leading to an increased interest and demand from the consumers. The percentage of adulteration of milk-based products also varies between different countries [1, 3]. Thus, there is a need for detection of the substitution or admixtures of sheep and goat milk with bovine milk aiming to economic benefit, to protect the consumers from fraud. In addition, bovine milk is also responsible for species selective food allergenicity caused mainly in children increasing the demand for milk adulteration detection and proper labeling of commercial products [4].

Many analytical methods have been developed during the last years for milk-based products adulteration detection and animal species identification. These techniques are

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based on the detection of specific proteins or peptides, such as caseins and lactoglobulins, or DNA sequences of different animal species [5].

Milk proteins derived from animal species have been identified by using several analytical techniques such as Electrophoresis, Isoelectric Focusing, Liquid Chromatography, Mass Spectrometry and Immunoassays. Immunochemical techniques that have been used for the adulteration detection of milk products include ELISA using antibodies species-specific to milk proteins. Colorimetric ELISA tests have been applied for the detection of cow milk in goat, ewe, sheep and buffalo milk and cheese with detection limits ranging from 0.001 to 2% [6–9]. An immunochromatographic paper-based strip test was developed for a rapid screening of samples achieving detection of as low as 0.5% of cow milk in sheep cheese samples [10]. Finally, an immunoelectrochemical biosensor using Quantum Dots and a Quartz Crystal Microbalance immunosensor were constructed for goat milk adulteration with detection limits 0.05% and 1 ppm, respectively [11, 12].

Chromatographic techniques have also been reported for milk authenticity based on protein analysis mainly of casein. However, the detection limits achieved by chromatographic techniques are not adequate for milk adulteration detection [13]. Spectroscopic techniques that have also been used in milk authenticity tests include Mass Spectrometry (MALDI-TOF-MS, DART-HRMS) based on animal-specific proteins/peptides identification or triacylglycerol profile providing high detection limits ranging between 5 and 50% [14–17]. In addition, Liquid Chromatography, High Performance Liquid Chromatography or Ultra Performance Liquid Chromatography combined to Mass Spectroscopy (LC/HPLC/UPLC-MS) has also been reported with much lower detection limits [18–21]. Capillary Electrophoresis combined with MS (CE-MS), Fourier Transform Near-Infrared (FT-NIR) Spectroscopy, Polyacrylamide Gel Electrophoresis under denaturing conditions in the presence of sodium dodecyl sulphate (SDS-PAGE) and Isoelectric Focusing were further used providing detection limits of 0.25–50% [22–27]. Finally, an effort was made for the construction of a multi-sensor electric tongue based on the detection of several substances present in milk with a high detection of 50% [28].

Moreover, DNA-based techniques have been successfully applied for the discrimination of the animal origin of milk in dairy products. DNA has become the most appropriate analyte comparing to proteins due to high sensitivity and reproducibility of DNA-based techniques [1, 29–31] and since DNA is more stable than proteins during chemical and thermal processing of foods [32]. The detection limits achieved by these methods are comparable to that of protein-based methods. Polymerase Chain reaction (PCR) is an essential step in all DNA-based methods in order to

amplify DNA sequences specific to all animal species studied. PCR has initially been used for the amplification of conserved regions of mitochondrial 12S rRNA genes among different animal species detecting milk adulteration with cow or goat milk [33–36]. The detection was based on agarose gel electrophoresis and staining with ethidium bromide or GelRed with a detection limit of 0.1, 1 and 0.5%, respectively. Similarly, the amplification of the conserved region of mitochondrial cytochrome b gene was also used for the detection of 1% cow milk in goat and sheep species [37]. Moreover, the amplification of mitochondrial *ND1* gene was exploited for identification of 0.1% bovine in yak milk [38]. A new method based on species-specific SNP detection was settled for milk adulteration detection with cow milk. The method includes amplification reaction of the conserved region of κ -casein gene between four different animal species using the same pair of primers and subsequent allele discrimination using minisequencing primer extension reaction and capillary electrophoresis. The authors were able to detect as low as 0.1% proportion of adulteration of buffalo with bovine milk [39]. In addition, the need for the simultaneous detection of more DNA sequences identifying the animal origin led to the development of a quadruplex PCR reaction with subsequent capillary electrophoresis with fluorescently labeled primers for the detection and discrimination of PCR products for the analysis of four animal species (buffalo, cow, sheep and goat) based on mitochondrial specific DNA sequences. However, with this multiplex method only 1% of milk adulteration in binary mixtures was able to be detected [40]. Real-time PCR has subsequently been introduced in milk authenticity analysis offering increased multiplicity and detection limits at the same levels as the aforementioned techniques. The amplification of the conserved regions of mitochondrial cytochrome b and 12S rRNA gene was also used for milk species identification using specific Taqman probes, DNA intercalating fluorescent dyes and High Resolution Melting Analysis providing detection limits of 0.1–2% [32, 41–43]. A quadruplex (cow, goat, sheep and buffalo) real-time PCR based on DNA intercalating fluorescent dye and subsequent T_M (melting-point) analysis has also been developed offering a same detection limit of 0.1% for cow species [44].

In this project, a new sensitive fluorometric DNA-based analytical method on spectrally distinct microspheres was developed for detection of adulteration of goat and sheep yogurt with bovine milk providing lower detection limits than the commonly used techniques for milk adulteration detection. Greek yogurt was chosen for this method development, as yogurt is considered as one of the most popular Greek exported products and is more vulnerable to fraud. In addition, yogurt is a more challenging sample because its complexity is higher than milk. The method focuses on the

detection of the highly conserved region of mitochondrial cytochrome b gene within individual species. The study of this region was chosen due to the fact that most PCR-based milk authenticity methods are usually based on the analysis of mitochondrial DNA sequences, because its sequence is highly conservative within different animal species and gives high copy number in animal cells, increasing the specificity and detectability of the method [45]. We applied the proposed method to dairy products and could detect as low as 0.01% of bovine milk in binary mixtures of bovine/goat yogurts and 0.05% proportion in bovine/sheep yogurt mixtures.

Materials and methods

Apparatus and reagents

The apparatus used in this study were a thermal cycler obtained from Takara (Shiga, Japan), an ultrasonic cleaner from Branson Ultrasonics (Danbury, CT), the Luminex 100 IS flow cytometer (Luminex, Austin, TX) for the analysis of the microspheres, a Nanophotometer from Implen GmbH (München, Germany), DNA electrophoresis system from Biorad (CA, USA) combined with a digital camera Konica Minolta DYNAX 5D (Konica Minolta Holdings, Tokyo, Japan) and the Gel Analyzer software from Kodak (New York) for gel documentation.

Milk-based products (low-fat yogurt) were purchased fresh from Greek local markets. Nucleospin Food Genomic DNA Isolation Kit from Macherey–Nagel (Düren, Germany) was used for DNA extraction. The reagents used for PCR and gel electrophoresis were deoxynucleoside triphosphates (dNTPs) and DNA markers (Φ X174 DNA-HaeIII Digest) (HT Biotechnology, Cambridge, UK) and Thermostable DNA polymerase (*Taq*) (Invitrogen, Waltham, MA). The reagents used for hybridization assay were fluorescent carboxylated microspheres (Luminex, Austin, TX), 2-(N-morpholino) ethanesulfonic acid (MES) (Sigma, St. Louis, MO), Tetramethylammonium chloride (TMAC) (Fluka Chemie, Buchs, Switzerland), 1-Ethyl-3,3(3-dimethyl aminopropyl)carbodiimide hydrochloride (EDC) (Pierce Chemicals, Rockford, IL), Streptavidin-R-phycoerythrin conjugate (SA–PE) (Molecular Probes, Eugene, OR) and Bovine Serum Albumin (BSA) (Serva, Heidelberg Germany). Oligonucleotides used in this study are listed in Table 1 and were synthesized by MWG-Biotech (Ebersberg, Germany) [4]. Hybridization solution consisted of 50 mM Tris–HCl pH 8.0, 4 mM EDTA pH 8.0, 1 g/L SDS, and 3 M TMAC. TE buffer consisted of 10 mM Tris–HCl and 1 mM EDTA, pH 8.0. Phosphate-buffered saline (PBS) consisted of 0.14 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate and 1.7 mM potassium phosphate, pH 7.4.

Table 1 The sequences of primers and oligonucleotide probes specific for DNA sequences related to cow, sheep and goat animal species

Oligonucleotide name	Sequence (5'→3')
Cow primerF	AGTTAGAGATTGAGAGCCAT- ATACTCTCC
Cow primerR	Biotin-TTGATAAGATCATTGTCTAGTCAT- GTTG
Sheep primerF	Biotin-CCAACATGCCTTTAAACCCTCAA
Sheep primerR	GGAAGTGTAGCCTTCTGACTCG
Goat primerF	CACTTTATCCTCCCATTTCATCATCAC
Goat primerR	Biotin-TCTTTAATGGTGTAGTAAGGGT- GAAATG
Cow probe	NH ₂ -TGGTGACATGCCGCAACTA- GACACG
Sheep probe	NH ₂ -TGCCTTTCCTTCCCCGCCAGTCTC
Goat probe	NH ₂ -CCTCGCCATAGTCCACCT- GCTCTTCC

Conjugation of oligonucleotide probes to the microspheres

Different sets of polystyrene microspheres that contain carboxyl groups on their surface were covalently coupled to 5'-NH₂-oligonucleotide probes specific to the three animal species (bovine, goat and sheep) according to the following protocol. An aliquot containing 1.25×10^5 microspheres was centrifuged for 2 min at 12,900g and washed twice with 25 μ L of 0.1 M MES buffer, pH 4.5. The microspheres were resuspended in 25 μ L of the same buffer and sonicated for 10 min to keep the microspheres in suspension. An amount of 25 pmol of NH₂-oligonucleotide and 1.25 μ L of a freshly prepared solution of 10 g/L EDC, dissolved in water, were added. The mixture was incubated for 30 min at ambient temperature in the dark. The addition of the same amount of EDC was repeated with a fresh solution of EDC, and the mixture was incubated for 30 min as above. The microspheres were pelleted at 12,900 g for 2 min and washed once with 50 μ L of 0.2 mL/L Tween-20 and once with 50 μ L of 1 g/L sodium dodecyl sulfate (SDS). The microspheres were finally resuspended in 125 μ L of TE buffer.

DNA extraction

DNA was extracted from samples using the Nucleospin Food Genomic DNA Isolation Kit from Macherey–Nagel according to the manufacturer's instructions including an initial step as follows: an amount of 200 mg of low-fat yogurt from cow, sheep and goat was washed twice with 1 mL of PBS buffer, pH 7.4 by centrifugation for 10 min at 1000 g. The precipitate was dissolved in 200 μ L PBS

buffer [46]. The concentration and the purity of the isolated DNA were determined by UV absorption at 260/280 nm using the Nanophotometer (Implen).

DNA amplification

The isolated DNA was subjected to separate reactions for the amplification of conserved DNA sequences specific to bovine, goat and sheep. The mixture of each reaction had a final volume of 50 μ L and consisted of 50 mM KCl, 20 mM Tris–HCl pH 8.4, 2 mM $MgCl_2$, 200 μ M each of dNTPs, 0.4 μ M each of the primers (specific to bovine, goat or sheep), 1 U of *Taq* DNA polymerase and 5 μ L of the isolated genomic DNA (~5 ng/ μ L). The cycling conditions were: 95 °C for 5 min, followed by 45 cycles at 95 °C for 20 s, 50 °C for 30 s, 72 °C for 30 s and a final extension step at 72 °C for 10 min. The PCR products were biotinylated due to the biotinylated primer (Table 1) used during the amplification reaction. The PCR products were confirmed and quantitated densitometrically by 2% agarose gel electrophoresis and ethidium bromide staining.

Fluorometric hybridization assay on spectrally distinct microspheres

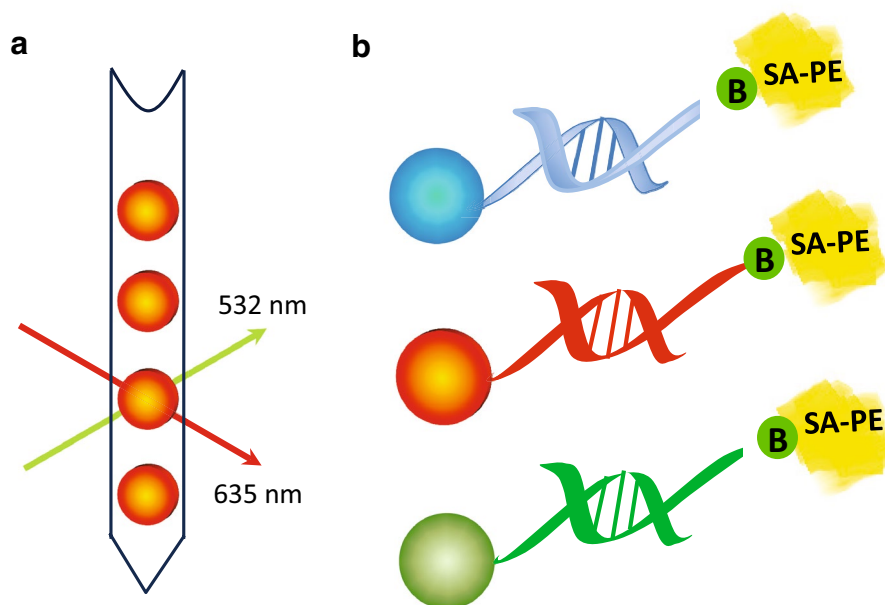
The fluorometric hybridization assay was performed in a total volume of 55 μ L. A 1- μ L volume containing 1000 microspheres of each set of microspheres, coupled to the specific probes for bovine, sheep and goat species that were previously sonicated for 10 min, was added to 49 μ L of the hybridization solution. The mixture was then preheated at 37 °C. Each biotinylated PCR product was denatured for 5 min at 98 °C, placed on ice for 2 min and a 5- μ L aliquot was added to the corresponding microspheres and incubated for 1 h at 37 °C with occasionally stirring. The microspheres were then pelleted by centrifugation at 12,900 g for 2 min and washed once with 50 μ L of the hybridization solution. The microspheres were resuspended in 55 μ L of the hybridization solution and a volume of 12 μ L of SA–PE conjugate (10 mg/L) was added for the detection of the hybrids. The mixture was incubated for 20 min at room temperature, in the dark and the microspheres were then analyzed without extra washing step by the Luminex IS 100 flow cytometer.

Results

A new and sensitive fluorometric DNA hybridization assay on spectrally encoded microspheres was developed for animal species identification (bovine, sheep and goat) of milk-based products and milk adulteration detection. The proposed analytical method offers greater detectability

than the most commonly used methods for milk adulteration detection with a detection limit as low as 0.01%. This method is an alternative approach for milk authentication in order to protect the consumers from fraud or more significantly from health risks due to allergenicity issues. Allergens can cause serious problems even in traces, thus sensitive detection methods are of great demand. Specifically, in this work, the adulteration of goat and sheep products with bovine milk was studied. The method was based on animal species-specific DNA sequences identification based on hybridization assay on the surface of spectrally distinct microspheres. These polystyrene microspheres have a diameter of 5.6 μ m and contain carboxyl groups at their surface and two fluorescent dyes inertly in different ratios producing up to 100 different sets, giving the opportunity in the future to increase the number of different targets that can be simultaneously analyzed. The method includes: (a) DNA isolation from dairy products (yogurt), (b) amplification of conserved DNA sequences specific to cow, sheep and goat and (c) detection and identification of the amplified DNA sequences by hybridization assay on the surface of different sets of fluorescent microspheres and flow cytometric analysis. The PCR products for the three species were validated based on two methods. (a) Their size was determined by agarose gel electrophoresis and (b) their sequence was confirmed by means of hybridization with specific probes. These two methods provide sufficient validation of the amplified sequences. The amplified DNA sequences are located in the mitochondrial conserved region that codes cytochrome b. The PCR products had size of 83, 88 and 140 bp for cow, sheep and goat, respectively, and were biotinylated at one end via the biotinylated primers used in the PCR. Specific oligonucleotide probes for bovine, goat and sheep, respectively, that carry a NH_2 group at the 5'-end were attached to the surface of three different sets of carboxylated microspheres by using the carbodiimide chemistry with EDC as crosslinker. The biotinylated PCR products specific to the three different animal species were heat-denatured and allowed to hybridize to their complementary probes attached to the surface of specific sets of microspheres. The hybrids were then detected by a streptavidin–phycoerythrin conjugate (SA–PE) and flow cytometry (Fig. 1). The microspheres are analyzed in a row, one by one, by two laser beams. A red laser (635 nm) is used for the classification of the different sets of microspheres by the excitation of the two fluorescent dyes that are contained in each microsphere. A second green laser (532 nm) excites the third fluorophore phycoerythrin that is used as a reporter dye, which fluorescence intensity at 578 nm is proportional to the concentration of the amplified DNA sequence hybridized to the complementary probe. At least 50 microspheres of each set are analyzed in each run. The median value of the measured fluorescence is then used for

Fig. 1 a Analysis of microspheres by the flow cytometer. The microspheres are analyzed in a row passing through two laser beams. A *red* laser (635 nm) is used for the classification of microspheres into groups and a *green* laser (532 nm) induces the fluorescence of the reporter molecule, PE. **b** The hybridization assay on fluorescent microspheres. The biotinylated products are hybridized to the complementary probes attached to the microspheres. The hybrids are detected by SA-PE and analyzed by flow cytometry. B: biotin, SA: streptavidin, PE: phycoerythrin



the construction of calibration graphs. Finally, the method was applied successfully for the authenticity of milk-based products and milk adulteration detection in dairy products.

Initially, different protocols for sample pretreatment were applied in order to increase the yield of DNA isolation reaction. An extra step at the beginning of the DNA isolation process was finally included: a proper amount of DNA yogurt was washed twice as mentioned in DNA extraction paragraph to remove more effectively possible PCR inhibitors and enrich somatic cells in milk [46]. Subsequently, the DNA amplification reaction was optimized to increase the yield and the specificity of the reaction. The conditions of the reaction studied were the amount of primers, *Taq* DNA polymerase and isolated DNA in the reaction mixture, the temperature of the annealing step, as well as the number of cycles of the reaction. The electropherograms confirmed the optimized conditions. Then, we prepared three separate stock DNA solutions of goat, cow and sheep DNA using yogurt as starting material. Each solution was PCR-tested and found to contain the DNA from the corresponding species only, whereas the DNA from the other two species was undetectable. These experiments proved that our stock DNA solutions were authentic. The stock solutions were used further for the detectability study and the cross-hybridization (specificity) study.

The detectability of the method was then assessed by construction of calibration curves of the three DNA sequences as follows. Aliquots of each PCR product corresponding to bovine, sheep and goat species at concentrations from 0 to 1000 pM were hybridized to the specific oligonucleotide probe on the surface of the microspheres and analyzed by the flow cytometer. The calibration curves are presented in Fig. 2. As low as

4.1 pM of each amplified DNA sequence was detected by this method.

The specificity of the PCR reaction for the amplification of the three DNA sequences from the three different animal species was then determined. In more details, isolated genomic DNA from both sheep and goat was amplified by using the primers specific to “cow” sequence, in order to investigate if a non-specific PCR product is

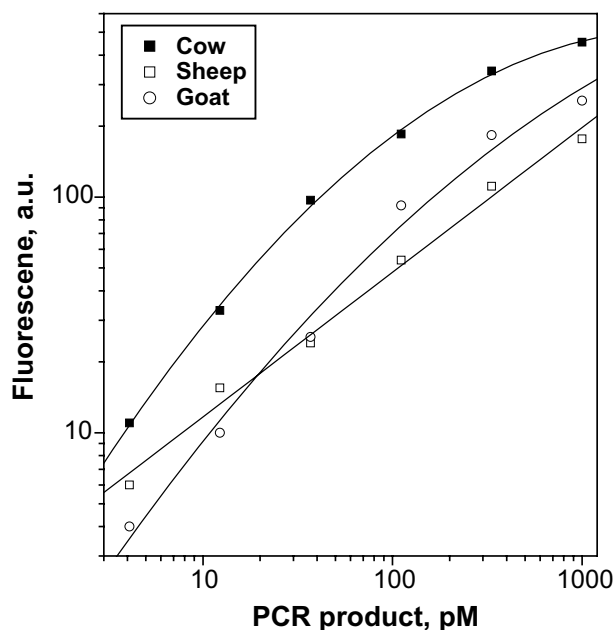


Fig. 2 Calibration graphs of the emitted fluorescence of the reporter molecule versus the concentration of PCR products (0–1000 pM) for cow, sheep and goat species

formed, leading to false-positive results. The electropherograms confirmed the specificity of these primers.

Then, the specificity of the probe specific to bovine species was also studied. Each PCR product (1000 pM) was let to hybridize with the set of microspheres coupled to the probe specific to bovine DNA sequence. The results are shown in Fig. 3. We confirmed that the probe for bovine identification is specific to its corresponding DNA target. The classification of the microspheres into the three different microsphere groups used by the flow cytometer is also presented in Fig. 3.

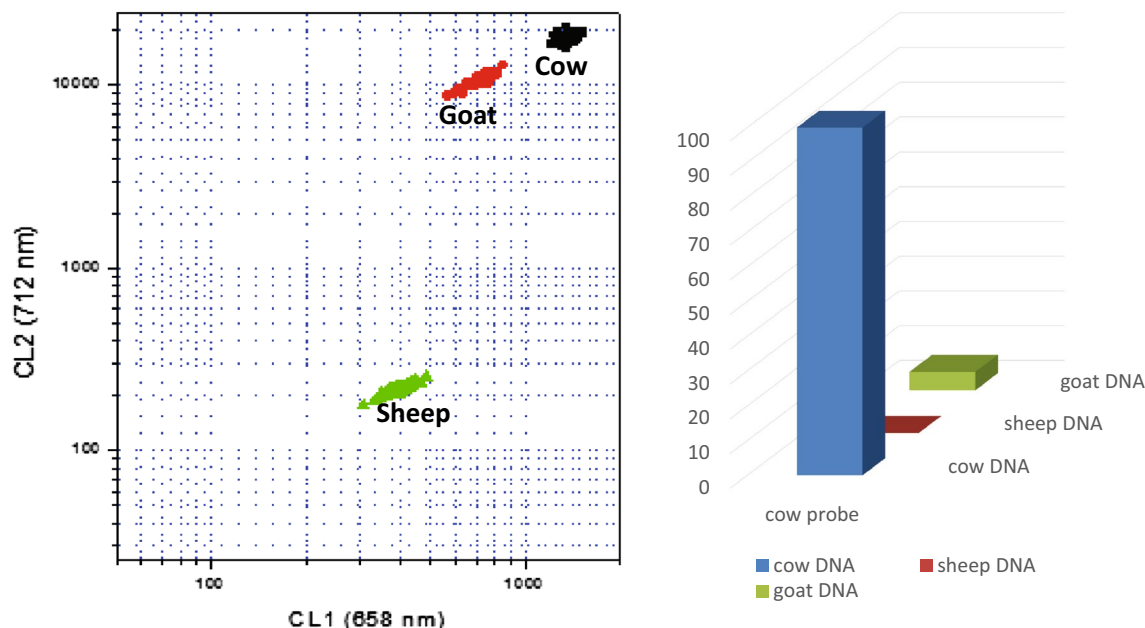


Fig. 3 Left panel the classification of the three groups of microspheres for cow, sheep and goat identification. The ratio of the fluorescence of the two fluorescent dyes at 658 and 712 nm of the micro-

In order to apply this method for authenticity tests of milk-based products and milk adulteration detection, we prepared binary mixtures that contained different proportions (0–5%) of cow yogurt into yogurt from sheep and goat milk, respectively. DNA was isolated from these mixtures and all samples were subjected to PCR using the cow primers to amplify the DNA sequence corresponding to cow species. Then the PCR products were detected by the hybridization assay on the surface of the corresponding microspheres. The results are presented in Fig. 4. As low as 0.01% content of bovine milk in goat yogurt and

spheres is responsible for the separation of analyzed microspheres into different groups. Right panel the specificity study of the probe specific to cow species. CL: channel

Fig. 4 Authenticity study. The fluorescence values are plotted versus the % proportion of cow milk (0–5%) in binary mixtures cow/sheep and cow/goat

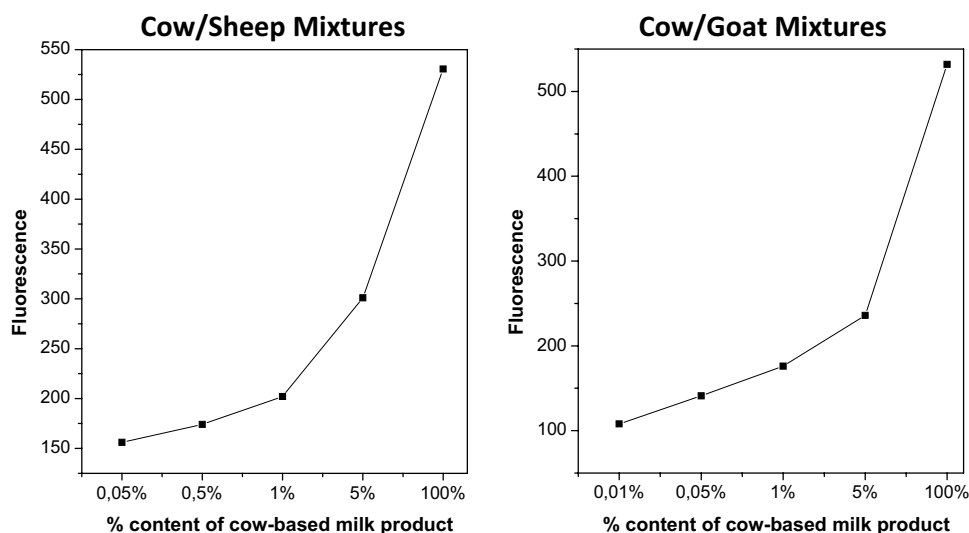


Table 2 Reproducibility within samples

Reproducibility within samples/inter-assay reproducibility		
% content of cow	% CV	
	Cow/sheep mixtures (%)	Cow/goat mixtures (%)
0.05	6.5	3.3
1	2.5	0.5

Table 3 Reproducibility within runs

% cow content	Reproducibility within run/intra-assay reproducibility			
	Cow/goat mixtures			
	Microspheres count	Mean	SD	RSD (% CV)
0.05	52	156.73	32.70	20.1
	64	154.81	37.32	17.6
	72	151.01	28.88	19.1
1	64	237.48	40.56	17.1
	65	254.74	54.31	21.3
	67	242.34	41.23	17.0

0.05% in sheep yogurt were detected by this method. To ensure the DNA integrity, all samples were subjected in a parallel amplification reaction and hybridization assay for the “sheep” and the “goat” DNA sequence, respectively. The values of the measured fluorescence of all samples were at the highest level of the calibration curves.

Finally, the reproducibility of the method was determined by analyzing in triplicates two different contents of bovine yogurt in cow/sheep and cow/goat yogurt mixtures, respectively. More specifically, the proportions 0.05 and 1% of bovine yogurt in both binary mixtures were selected and their corresponding PCR products were subjected in triplicate to hybridization assay on the fluorescent microspheres attached to the probe specific to bovine. The % CVs of the measured emitted fluorescence were calculated and are presented in Table 2. All CVs were much lower than 10% indicating the good reproducibility of the proposed method. The reproducibility within run was also determined for representative samples (0.05 and 1%) of binary mixtures of cow/goat yogurt. In each run, at least 50 microspheres were analyzed by the flow cytometer. The calculated data are presented in Table 3, while all CVs were lower than 22%.

Conclusions

A new fluorometric DNA-based analytical method for milk adulteration detection was developed. The method offers greater detectability of the most commonly used methods for this purpose, as well as very good sensitivity, robustness

and reproducibility. The method includes isolation of genomic DNA from dairy products from different animal species (bovine, sheep and goat), amplification of the corresponding DNA specific sequence, detection and identification of the origin of the DNA sequence via hybridization assay on spectrally distinct microspheres and flow cytometric analysis. As low as 4.1 pM of amplified DNA sequence corresponding to bovine, goat and sheep was detected by this method. In addition as low as 0.01% content of DNA sequence specific to bovine species was detected in adulterated milk-based products (yogurt) of goat and 0.05% of sheep-based products. Since the detection limit, at present, of cow milk in sheep milk is 1% [47], the proposed analytical method is more than adequate for milk authenticity tests. This method can be further applied for the detection of milk adulteration in many other milk-based products. In conclusion, assays performed in fluorescent microspheres offer two unique advantages: (a) Great potential for multiplexing, since they enable interrogation of up to 100 target sequences simultaneously. There are 100 spectrally distinct fluorescent microspheres available commercially. Furthermore, this multiplexing potential is achieved with a single fluorescent label while all PCR products carry a biotin (by using a biotinylated primer). In comparison, real-time PCR offers detection of up to 6 target sequences and uses different fluorophores and different detection channels for each target, (b) Fluorescent microspheres enable detection of several types of markers simultaneously, including DNA, protein and small molecules, by performing hybridization assays and immunoassays in the same suspension of microspheres. This can be accomplished by mixtures of microsphere sets coupled with either oligonucleotide probes or antibodies. For instance, in future application, we will offer simultaneous multianalyte assays that provide DNA-based species identification along with the detection of protein adulterants or allergens in dairy products and small molecules (e.g., antibiotics). This advantage becomes more important as the complexity of the food sample increases.

Compliance with ethical standards

Conflict of interest Maria L. Kounelli and Despina P. Kalogianni declares that they have no conflict of interest.

Compliance with ethics requirements The article does not contain any studies with human or animal subjects.

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