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Paper-based DNA biosensor for food authenticity testing

Eleni Th. Bougadi, Despina P. Kalogianni*

Department of Chemistry, University of Patras, Patras 26504, Greece

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ABSTRACT

A paper-based DNA biosensor was developed for food authenticity testing using dairy products as a model. DNA from milk-based samples was isolated, and species-specific DNA sequences were amplified and identified by the biosensor using specific DNA probes. The properties of gold nanoparticles were exploited for visual detection. The biosensor was applied for detection of three species, namely cow, sheep and goat, while as low as 1.6 fmol for cow and goat, and 3.1 fmol for sheep PCR product were detected. Moreover, adulteration down to 0.01% could be detected, based on binary mixtures of cows', ewes' and goats' milk yogurt, containing 0.01 to 5% of cows' yogurt in ewes' and goats' yogurts, respectively. The proposed paper-based DNA biosensor offered 10 times higher detectability than other methods, good specificity and reproducibility, and could be applied easily for the detection of other adulterated food products, such as meat, olive oil and legumes.

1. Introduction

Biosensors have played a key-role in food analysis, as they offer the potential to improve the simplicity, automation, portability and specificity of detection methods, as well as to reduce the time and cost of analysis, and the required volumes of samples and reagents (Rateni, Dario, & Cavallo, 2017). Many and various applications of multitype biosensors have been reported so far. Food authenticity is an attractive challenge to be addressed through biosensor technology, due to the advantages offered and the improvements that have been made in recent years. Testing of raw and processed foods is essential for public health, as well as for the protection of consumers from fraud. However, the use of biosensors is not as widespread as other analytical techniques (Hong et al., 2017). Most biosensors, as well as other methods for food authentication and species identification, target both proteins and nucleic acids. However, DNA is the preferred analyte due to its thermal stability and presence in all cells of the target organism (Danezis, Tsagkaris, Camin, Brusic, & Georgiou, 2016; Lo & Shaw, 2018).

Novel biosensors have been developed for food authentication purposes, targeting various analytes of different origin. For example, Galan-Malo et al., developed a lateral flow immunochromatographic test to detect as low as 0.5% cow milk in milk from other animal species (Galan-Malo, Mendiara, Razquin, & Mata, 2018). Masiri et al., developed a lateral flow immunoassay that could detect as low as 0.01% of raw and 1.0% of cooked horse meat in meat products (Masiri et al., 2017). Magiati et al., were able to detect 0.01% horse and 0.02% pork DNA in binary mixtures, and Xu et al., detected 0.01% mammalian DNA in meat mixtures using a DNA-based lateral flow biosensor, exploiting gold nanoparticles for visual detection (Magiati, Myridaki, Christopoulos, & Kalogianni, 2019; Xu et al., 2017). An electrochemiluminescent biochip on carbon screen-printed electrode was constructed by Azam, Roy, Lim, and Uddin (2018), for meat authentication, based on DNA-luminol interaction and loop-mediated isothermal amplification reaction. The authors were able to detect 0.1 pg/μL of porcine DNA. Another colorimetric biochip, based on a streptavidin-horseradish peroxide conjugate in combination with a chromogenic substrate, was constructed for the detection of 0.1% meat adulteration and 0.1% of cattle, goat and buffalo species in milk admixtures (Beltramo et al., 2017). Moreover, 0.5% of horse meat in beef meat was detected by an amperometric biosensor that used magnetic beads (Ruiz-Valdepeñas Montiel et al., 2017), while multiple DNA targets were detected by DNA arrays for the identification of meat- and fish-based products (Lo & Shaw, 2018).

Electronic tongues and electronic noses have also been used for food authenticity testing. These sensors respond to all volatile compounds present in the samples and the response is converted into a digital record. All digital data are analyzed using statistical models, providing different sensing profiles among different samples (Danezis et al., 2016). Different wine and coffee samples were classified using this technology (Di Natale et al., 1995; Gardner, Shurmer, & Tan, 1992; Strike, Meijerink, & Koudelka-Hep, 1999), while food adulteration was detected in meat and meat-based products (Jia, Liang, Wang, & Wang, 2018), honey (Trifković, Andrić, Ristivojević, Guzelmeric, & Yesilada, 2017) dairy products, olive oil and other oils, fish, saffron, spices, rice,

* Corresponding author.
E-mail address: kalogian@upatras.gr (D.P. Kalogianni).

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tea, juices and beverages (Gliszczyńska-Świgło & Chmielewski, 2017; Peris & Escuder-Gilabert, 2016). Finally, the most recent trends in food authentication are focused on the use of smartphones, as detectors, in combination with several other techniques (Rateni et al., 2017). A novel system was constructed for olive oil authentication, using 3D-printed Lego bricks, LED lights and a smartphone for fluorescence capturing. Intrinsic fluorescent compounds present in oils, such as chlorophylls and polyphenols, were accountable for the emitted fluorescence (Hakonen & Beves, 2018).

As for milk authentication, real-time PCR is one of the most recent DNA-based methods used for species identification. Real-time PCR has been applied mainly for the detection of 0.1–1% cow species (Cottenet, Blancpain, & Golay, 2011; Dalmasso, Civera, La Neve, & Bottero, 2011; Drummond et al., 2013; Liao, Liu, Ku, Liu, & Huang, 2017; López-Calleja et al., 2007; Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007). Moreover, multiplex real-time PCR has been tested for milk adulteration detection. Two quadruplex real-time PCR reactions have been reported for the simultaneous detection of cow, sheep, goat and buffalo species, or cow, buffalo, ovine and caprine species with a limit of detection (LOD) of 0.2% and 1%, respectively (Domenico, Giuseppe, Rodríguez, & Cammà, 2017; Rentsch et al., 2013). A triplex real-time PCR was developed for the simultaneous detection of 1% bovine and equine milk or 10% bovine and caprine milk, using an endogenous control to avoid false-negative results (Guo et al., 2018, 2019). Real-time PCR with subsequent high-resolution melt (HRM) analysis has been utilized for the detection of cow species with an LOD of 0.1–1% (Ganopoulos, Sakaridis, Argiriou, Madesis, & Tsaftaris, 2013; Sakaridis, Ganopoulos, Argiriou, & Tsaftaris, 2013), for simultaneous detection of goat and sheep with an LOD of 1% (Ganopoulos et al., 2013), as well as for cow, goat, sheep and buffalo species offering a low LOD of 0.1% (Agrimonti, Pirondini, Marmiroli, & Marmiroli, 2015). Hybridization assays on fluorescent beads were also exploited for the detection of adulteration of goat- and sheep-derived dairy products with cow milk with an LOD of 0.01% (Kounelli & Kalogianni, 2017). Finally, next generation sequencing (NGS) has been applied for the identification of four animal species in dairy products, namely cow, goat, cattle and buffalo. The authors were able to detect as low as 10% of adulteration in binary mixtures (Ribani et al., 2018). The above-mentioned methods used for milk adulteration detection are summarized in Table 1. As observed, the proposed DNA biosensor offers greater detectability than the other reported methods for the detection of cow species in milk-based adulterated products.

In this work, a paper-based DNA biosensor was developed for food authentication purposes, using milk-based products as a model. Gold nanoparticles conjugated to streptavidin were used as reporters for visual detection. Finally, binary mixtures of goat- and sheep-based yogurt samples, with different proportions of cow yogurt, were prepared to assess the detectability of milk adulteration by the proposed biosensor.

2. Experimental section

2.1. Materials and equipment

Carboxylated polystyrene beads (5.7×10^6 particles/μL), 2 μm in diameter, were purchased from Polysciences (Warrington, PA, USA), and gold nanoparticles, 40 nm in diameter, from BBI Solutions (Cardiff, UK). The paper-based biosensor consisted of an immunopore FP nitrocellulose membrane (Whatman, Florham Park, NJ, USA) and a sample pad, an absorbent pad and a glass-fibered conjugation pad (Schleicher & Schuel, Dassel, Germany). The Kapa2G Fast *Taq* and Kapa2G Robust PCR kits were purchased from Kapa Biosystems (Wilmington, MA, USA). Terminal transferase was purchased from Takara (Kusatsu, Japan). dNTPs were obtained from Invitrogen (Carlsbad, CA, USA) and biotin dUTP (b-dUTP) was from Applichem (Maryland Heights, MO, USA). The NucleoSpin Food kit was purchased from Macherey-Nagel (Düren, Germany). Agarose and DNA molecular weight markers (*Φ*X174 DNA, *Hae*III digest) for gel electrophoresis were obtained from HT Biotechnology (Cambridge, UK), while ethidium bromide from Research Organics Inc (Cleveland, OH, USA). Bovine serum albumin (BSA) was from Serva (Heidelberg, Germany), while streptavidin (SA) was from Roche (Basel, Switzerland). Sodium hydroxide was from Merck (Burlington, MA, USA) and mono- and dihydrate sodium phosphate were purchased from Lachner (Neratovice, Czech Republic). Tris(hydroxymethyl)aminomethane (Tris) was from Fischer Scientific (New Hampshire, USA), while Tween-20, Triton X-100, Sodium dodecyl sulfate (SDS) and hydrochloric acid were from Sigma-Aldrich (St Louis, MO, USA), glycerol and EDTA were from Carlo Erba (Barcelona, Spain), and 2-(*N*-morpholino)ethanesulfonic acid (MES) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) from AppliChem (Maryland Heights, MO, USA). Oligonucleotides were obtained from Eurofins Scientific (Brussels, Belgium) and are shown in Table S1 (Kounelli & Kalogianni, 2017; Rentsch et al., 2013). Yogurt samples were purchased from a local market. Phosphate-buffered saline (1 × PBS) pH 7.4 contained 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM KH_2PO_4. The 0.1 M phosphate buffer at pH 6.8 was prepared with 46.3 mL Na_2HPO_4 and 53.7 mL NaH_2PO_4. 1 × TE, pH 8.0, consisted of 10 mM Tris, and 1 mM EDTA. The hybridization buffer consisted of 0.22 M NaCl, 0.1 M Tris (pH 8.0) and 0.88 mL/L Triton X-100 and the developing solution of 20 mL/L glycerol, 20 g/L BSA, 2 g/L SDS and 10 mL/L Tween-20 in 1 × PBS pH 7.4.

PCR amplification and hybridization reactions were performed in a thermal cycler obtained from Takara (Shiga, Japan). Gel

Table 1
An overview of recent DNA-based methods used for milk adulteration.

Method	Animal species	LOD*	References
Real-time PCR	cow	0.1–1%	Liao et al., 2017; Drummond et al., 2013; Cottenet et al., 2011; Dalmasso et al., 2011; López-Calleja et al., 2007; Lopparelli et al., 2007
Multiplex Real-time PCR			
quadruplex	cow, sheep, goat and buffalo	0.2%	Rentsch et al., 2013
quadruplex	cow, buffalo, ovine and caprine	1%	Domenico et al., 2017
triplex	bovine and equine	1%	Guo et al., 2018
triplex	bovine and caprine	10%	Guo et al., 2019
Real-time PCR-HRM			
single	cow	0.1–1%	Ganopoulos et al., 2013; Sakaridis et al., 2013
duplex	goat and sheep	1%	Ganopoulos et al., 2013
Hybridization assays			
fluorescent beads	cow	0.01%	Kounelli & Kalogianni, 2017
biochip	cattle, goat and buffalo	0.1%	Beltramo et al., 2017
Next generation sequencing	Cow, goat, cattle and buffalo	10%	Ribani et al., 2018
Paper-based DNA biosensor	cow	0.01%	This work

* LOD: Limit of Detection.

electrophoretic apparatus was from Bio-Rad (California, USA), while the ultrasonic device was from Branson Ultrasonics (Danbury, CT, USA).

2.2. Samples and DNA isolation

Law-fat yogurt samples derived from three animal species, namely cow, sheep and goat, were purchased from a local market. The animal-origin was reported on the label of each product. Binary mixtures of different amount of cow yogurt in goat's and sheep's yogurt were prepared to assess the detectability of the method. DNA was isolated from all yogurt samples using the NucleoSpin Tissue kit from Macherey-Nagel according to the manufacturer's instructions. The isolated DNA was quantified using the Nanodrop UV/Vis Nanophotometer by Implen (LA, USA). The results are presented in Table S2 in the Supplementary Material. All samples gave similar DNA concentration after the isolation procedure, therefore, the same volume of isolated DNA for each sample was used in the amplification reaction.

2.3. Polymerase chain reaction

The DNA isolated from the three animal species was subjected to amplification reaction using species-specific primers. The Polymerase Chain Reaction (PCR) was performed in 50 μL for each one of the specific DNA sequences for cow, sheep and goat. The PCR mixture contained 1 × Kapa buffer, 1.5 units of Kapa2G Fast or Kapa2G Robust *Taq*, 2 mM $MgCl_2$, 200 μM of each of the dNTPs, 0.4 μM of each of the primers and 5 μL of the isolated DNA of each sample. The amplification reaction was carried out using the following conditions: an initial denaturation step at 95 °C for 5 min, and 45 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The PCR products were then detected and quantified by electrophoresis in a 2.5% agarose gel along with ethidium bromide DNA staining. The concentration of the final PCR products was 54.25 fmol/μL for cow, 59.47 fmol/μL for goat and 39.92 fmol/μL for sheep.

2.4. Preparation of conjugates and tailed-oligonucleotides

2.4.1. Oligonucleotide-functionalized carboxylated polystyrene beads

Oligonucleotides that carried an amino group at one end were conjugated to carboxylated beads for the construction of the test and the control spot of the biosensor. The oligonucleotide NH_2-dT(30), a sequence of 30 bases of thymine, was used for the test spot and an irrelevant oligonucleotide probe that carried an amino group at 5′ end and a biotin moiety at 3′ end was used for the control spot. The conjugation procedure, in detail, was the following: a volume of 12.8 μL of the carboxylated beads was washed with 125 μL of 0.1 M MES buffer, pH 4.5, by centrifugation for 2 min at 15,700 g. The beads were resuspended in 40 μL of the same buffer and sonicated for 2 min. Then, each oligonucleotide (1 μL of 400 pmol/μL) was added separately to the beads along with the coupling reagent EDC (1,25 μL of 0,4 mg/μL in H_2O). The mixture was incubated for 30 min in the dark with frequent stirring. The same addition and incubation time of the coupling reagent was repeated. Then, a volume of 2 μL of Tween-20 (100%) was added, the mixture was centrifuged for 2 min at 15,700 g and washed two times with 100 μL of 2% (v/v) of a Tween-20 solution in 1 × TE, pH 8.0. A volume of 100 μL of 1 × TE at pH 8.0 was used for the final resuspension of the conjugated beads. The conjugates were stored at 4 °C.

Alternatively, for the preparation of the conjugate for the control spot, an oligonucleotide that carried only an amino group at 5′ end was also used. Here, the biotin moieties were introduced at the 3′ end of the probe through a tailing reaction. In more details, after the conjugation reaction, following the same procedure as above, the final resuspension was done in 20 μL of a tailing reaction solution that contained 14 units of terminal deoxynucleotidyl transferase, 100 mM HEPES (pH 7.2), 8 mM $MgCl_2$, 0.1 mM DTT, 0.05 mM of dNTPs mixture and 0.025 mM b-dUTP. The 20-μL reaction was incubated for 1 h at 37 °C and the beads were received by a centrifugation step for 2 min at 15,700 g. Then, the washing and resuspension steps were followed as above.

2.4.2. Streptavidin-functionalized gold nanoparticles (SA-AuNPs)

A volume of 200 μL of gold nanoparticles, 40 nm in diameter, was coupled to 3 μg of streptavidin (SA) from a solution of 1 mg/mL prepared in H_2O. The pH of the gold-nanoparticle solution was firstly adjusted to 6.0 with an appropriate volume of 20 mM pH 6.8 phosphate buffer. The coupling reaction was performed for 2 h at room temperature. To block the area of the uncoupled gold nanoparticles, bovine serum albumin (BSA) was added at a final concentration of 10 mg/L and incubated for 30 min at room temperature. The mixture was then centrifuged at 3300 g for 30 min and the nanoparticles were resuspended in 20 μL of 10 mM pH 6.8 phosphate buffer. A volume of 6 μL of the streptavidin-gold nanoparticles conjugates (SA-AuNPs) was finally used to the paper-based biosensor.

2.4.3. Tailing of the specific probes

The oligonucleotide probe used for cow-species identification carried at its 5′ end a polydeoxyadenosine sequence of 30 bases to enable the detection with the biosensor. For the same reason, a poly(dA) tail was added to the probes specific to goat- and sheep-species through a tailing reaction that contained 28 units of terminal deoxynucleotidyl transferase, 100 mM HEPES (pH 7.2), 8 mM $MgCl_2$, 0.1 mM DTT, 3.5 mM of dATP and 700 pmol of the specific probe in a final volume of 20 μL. The reaction was incubated at 37 °C for 1 h and was finally stopped by the addition of 2 μL of 0.5 M EDTA at pH 8.0.

2.5. Construction of the paper-based DNA biosensor

The paper-based DNA biosensor consisted of an immersion pad, a glass fiber conjugate pad, a nitrocellulose diagnostic membrane and an absorbent pad adjusted properly on a backing pad (Fig. 1). The final size of the sensor was 4 mm × 70 mm. A volume of 1 μL of the conjugated with NH_2-dT(30) beads was placed on the membrane, about 20 mm from the top of the membrane, to form the test spot of the sensor. Similarly, the same volume of the beads conjugated to the biotinylated probe was placed 15 mm from the top of the membrane to form the control spot of the sensor. The spots were left to dry for 10–15 min at room temperature and the biosensor was ready for use.

2.6. Visual detection of PCR products on the paper-based DNA biosensor

After amplification, each PCR product was diluted properly with 0.1 g/L BSA in a total volume of 5 μL and in the presence of its specific probe (1 μL of 0.5 pmol/μL) that carried a poly(dA) tail. The PCR product for cow-specific DNA sequence was placed in the thermal cycler for denaturation at 98 °C for 3 min. The denatured product was then shocked for 2 min in an ice bath and a 2.2-μL volume was added to 7.8 μL of the hybridization buffer pre-heated at 37 °C. The mixture was incubated for 15 min at 37 °C to allow hybridization between the probe and the DNA target.

For DNA sequences specific for sheep and goat, the denaturation of the double-stranded DNA was performed by sodium hydroxide instead of thermal denaturation. A specific amount of the diluted PCR product was mixed with 1 μL of 0.5 pmol/μL of the specific to sheep or goat probe and the volume was fixed to 5 μL with 0.1 g/L BSA. Then, a volume of 5 μL of 0.4 M of NaOH was added and the mixture was incubated for 10 min at room temperature. The solution was neutralized with 5 μL of 0.4 M HCl and 5 μL of 0.4 M Tris pH 7.5 and incubated for 15 min at 37 °C.

Afterwards, a 5-μL aliquot of the pre-hybridized PCR product specific to cow or a 10-μL aliquot of the pre-hybridized sheep- or goat-specific PCR product was placed to the conjugate pad of the sensor just

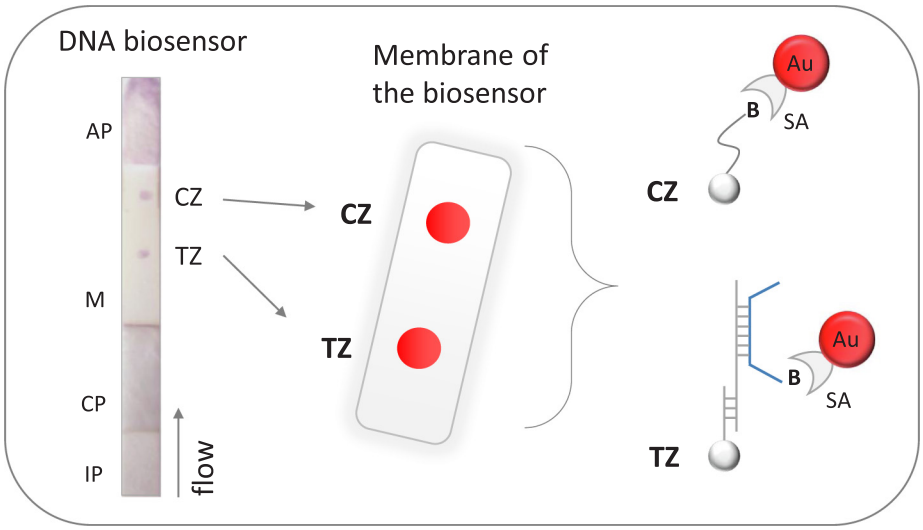

Fig. 1. Left panel: a real image of the paper-based DNA biosensor. The biosensor consists of four parts: an immersion pad that is dipped into the developing solution, a conjugate pad where the SA-gold nanoparticles and the sample are deposited, a diagnostic membrane and an absorbent pad to ensure continuous flow of the reagents. Right panel: the principle of the paper-based biosensor. IP: immersion pad, CZ: conjugate pad, M: membrane, AP: absorption pad. CZ: control zone, TZ: test zone, SA: streptavidin, B: biotin and Au: gold nanoparticles.

above the SA-AuNPs (6 μL) that were pre-deposited on the same pad. The sensor was immediately dipped into 300 μL of the developing solution. After 10–15 min, two red spots were formed on the membrane of the sensor that were visible by naked eye.

3. Results and discussion

In this work, a new paper-based DNA biosensor was developed for food authenticity testing. The sensor was applied for the detection of milk adulteration in dairy products and more specifically for the identification of bovine species in sheep- and goat-derived products (yogurt). Gold nanoparticles were exploited for detection and identification by naked eye of the three animal-specific DNA sequences.

Initially, DNA was isolated from yogurt samples derived from the three analyzed species: cow, sheep and goat. The obtained DNA was then subjected to individual PCRs using species-specific primers, one of which was biotinylated. The biotinylated double-stranded amplified sequences, in proper dilutions, were thermally or NaOH-denatured and hybridized to complementary DNA probes that carried a dA-tail at one end. The hybrids were applied on the conjugate pad of the sensor along with a suitable amount of streptavidin-functionalized gold nanoparticles (SA-AuNPs). The sensor was finally dipped into the developing solution. SA-AuNPs were driven through capillary forces up to the membrane of the sensor, drifting the sample to the same direction. The hybrids were captured onto the test spot of the membrane, through dA-dT hybridization, by the dT(30)-beads conjugates that were pre-deposited at this position, while SA-AuNPs bounded to the hybrids through SA-biotin interaction. The accumulation of AuNPs formed a visible red spot at this area of the strip, confirming the presence of the corresponding species in the sample. The excess of SA-AuNPs was captured to the control spot of the sensor by deposited beads conjugated to a biotinylated probe, forming a second red spot and ensuring that the sensor works perfectly. Both red spots have to be formed to get a positive result. Only the control spot is generated in case of a negative sample. The principle of the paper-based sensor is illustrated in Fig. 1.

Calibration graphs for the three species-specific DNA sequences for cow, sheep and goat were constructed. The PCR products had a size of 83, 88 and 150 bp for cow, sheep and goat DNA sequences, respectively. Each PCR product was diluted properly in 0.1 g/L BSA containing from 1.6 to 50 or 100 fmol of amplified product and analyzed by the paper-based DNA biosensor. As low as 1.6 fmol PCR product for cow and goat and 3.1 fmol sheep-specific amplified DNA sequence were detectable by the sensor (Fig. 2).

To increase the performance of the sensor for milk adulteration detection, the DNA isolated from cow yogurt was subjected to asymmetric PCR. The PCR mixture contained the same ingredients as the amplification reaction described above, but the biotinylated reverse primer was added in 10 × excess (20 pmol) than the forward primer (2 pmol) to obtain a non-symmetric amplified product, in order to enable the subsequent hybridization to its specific probe. After amplification, a volume of 1–5 μL of the asymmetric product was hybridized to 500 fmol of cow-specific probe in a final volume of 5 μL (adjusted with 0.1 g/L BSA). The solution was heat-denatured at 98 °C for 3 min and then placed on ice. After a quick spin down, the 5-μL solution was transferred to the hybridization buffer (5 μL) that was preheated at 37 °C, and the mixture was incubated for 15 min at the same temperature. All the volume (10 μL) was finally applied to the sensor. We observed that the 1-μL assymetric amplified product gave a very good positive signal with the biosensor (Fig. 2).

Afterwards, binary mixtures of milk-based products were prepared to assess the detectability of milk adulteration. Samples of yogurt from the three animal species were accordingly mixed to produce binary admixtures of cow yogurt in sheep and goat yogurt, respectively. The mixtures contained different proportions of cow yogurt ranging from 0.01 to 5%. The DNA of all the mixtures was isolated and subjected to an asymmetric amplification reaction using the cow-specific primers. A 1-μL volume of each amplified product for the cow-sheep mixtures and a 1-μL volume of each one of 10 × diluted PCR asymmetric products for the cow-goat mixtures was then analyzed with the DNA biosensor, as previously described, using the cow-specific probe. As low as 0.01% content of adulteration of sheep and goat yogurt products with cow milk was detected by the proposed paper-based DNA biosensor (Fig. 3).

The specificity of the cow probe was then studied. For this purpose, 50 fmol of sheep- and goat-amplified DNA sequence were analyzed with the sensor using both the cow- and the sheep- or goat-specific probes, respectively. As shown in Fig. 4, the cow probe was strictly specific when the sheep DNA sequence was used and revealed a 3.4% cross-reactivity with the goat DNA sequence.

Finally, the reproducibility of the paper-based DNA sensor was determined. The reproducibility was studied by analyzing, in triplicates, the following samples: i) 50 fmol of double-stranded PCR product, ii) 1 μL of asymmetric amplified product and iii) the binary yogurt mixtures that contained 0.01, 0.1 and 1% content of cow sample. All samples were hybridized to the cow-specific probe and analyzed with the paper-based DNA biosensor, in triplicates (Fig. 5). Upon the formation of the test and the control spots on the membrane of each sensor, the sensors were removed from the developing solution and scanned using the Epson Perfection V500 Photo Scanner (Long Beach, CA, USA). The images of the sensors were further analyzed using the open source image processing program ImageJ. The color density of

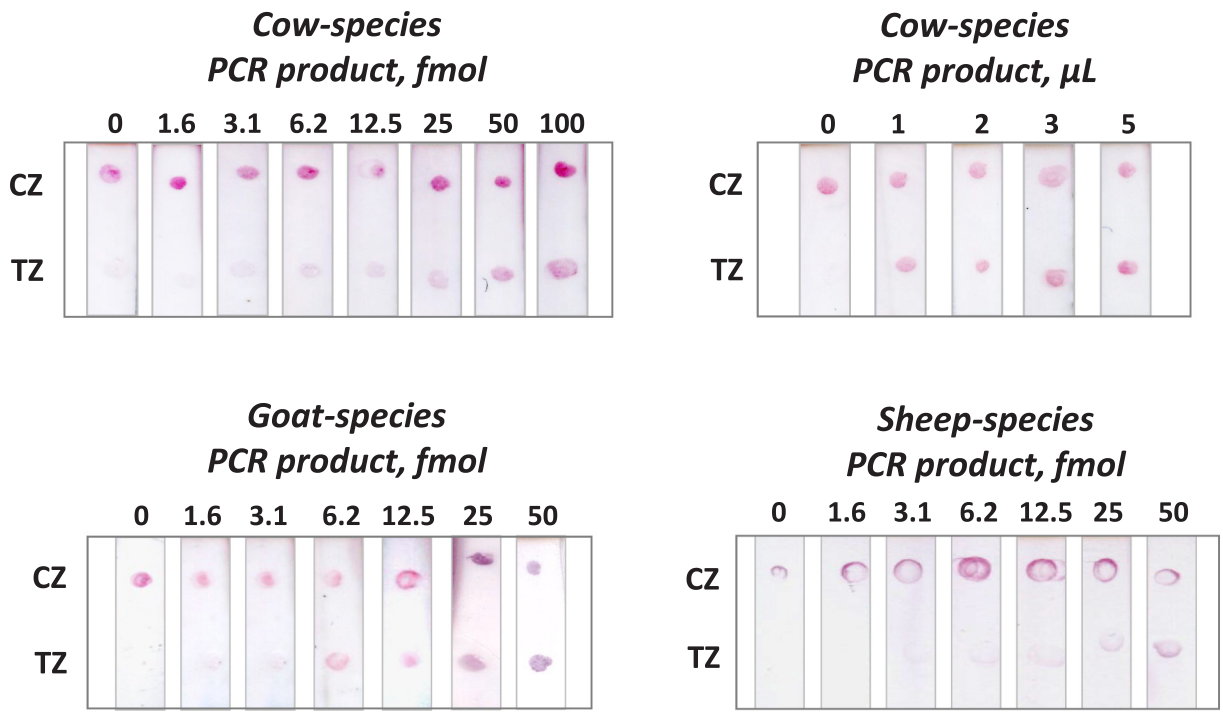

Fig. 2. Calibration graphs of double-stranded (ds) PCR products (0–50 or 100 fmol) for cow, sheep and goat species, as well as for the asymmetric PCR product (1–5 µL) for cow. CZ: control zone, TZ: test zone.

each test spot was measured using this software and the % coefficients of variation (CVs) were calculated for each sample. The %CVs were equal to 4.9% for the 50 fmol of the double-stranded PCR product, 8.9% for the asymmetric PCR product, 0.9% for the 0.01% adulteration content, 2.2% for the 0.1% adulteration content and 3.8% for the 1% adulteration content. The values of the %CVs confirmed the very good reproducibility of the performance of the proposed paper-based DNA biosensor.

4. Conclusions

A paper-based biosensor was developed for food authentication purposes. The biosensor was applied for the identification of three animal species, cow, sheep and goat in cow's, sheep's and goat's yogurt samples based on the visual detection of species-specific DNA sequences after amplification. As low as 1.6 fmol PCR product specific to cow and goat, as well as 3.1 fmol of sheep-specific PCR product were detected, confirming that the proposed biosensor offers high detectability. Also, the biosensor was used for the detection of the adulteration of milk-based products with cow milk. For this purpose, binary mixtures of cow-sheep and cow-goat yogurt samples were prepared and analyzed with the described method. The very low proportion of 0.01% of adulteration was detected by the paper-based biosensor. Compared to other methods used for milk authentication, the proposed DNA biosensor offered about 10 times greater detectability.

In conclusion, the developed paper-based DNA biosensor exhibits very good detectability, sensitivity and reproducibility. It is simple in construction and performance and of low-cost. It is also universal, meaning that it can be applied for the detection of any species-specific DNA sequence and thus other kinds of food adulteration. This is attributed to the fact that the molecular recognition of the species-specific DNA sequences is performed through hybridization of the analyzed DNA-sequence to a complementary oligonucleotide probe prior to the application to the biosensor. The analysis by this paper-based DNA

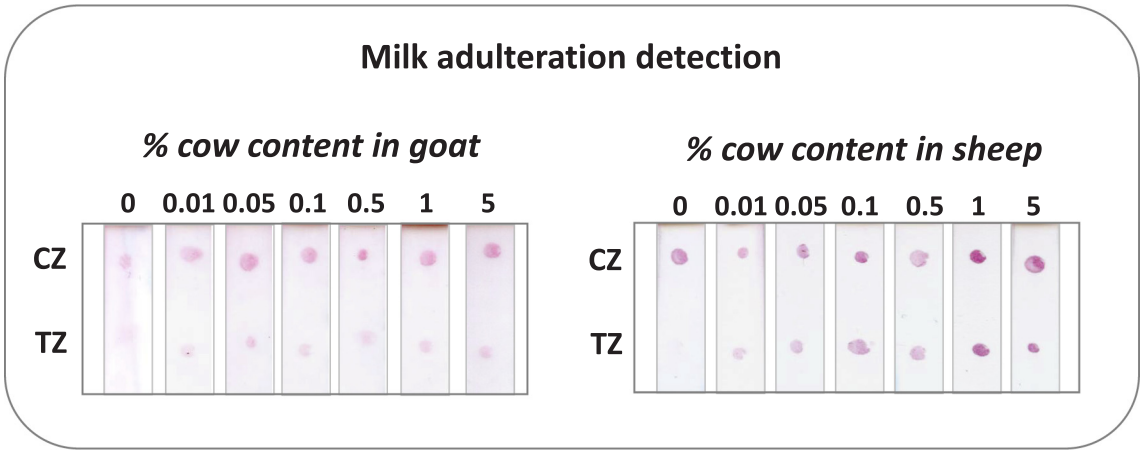

Fig. 3. Calibration graphs for the different adulteration content (0–5%) of cow sample in binary mixtures of cow-goat and cow-sheep yogurt samples. CZ: control zone, TZ: test zone.

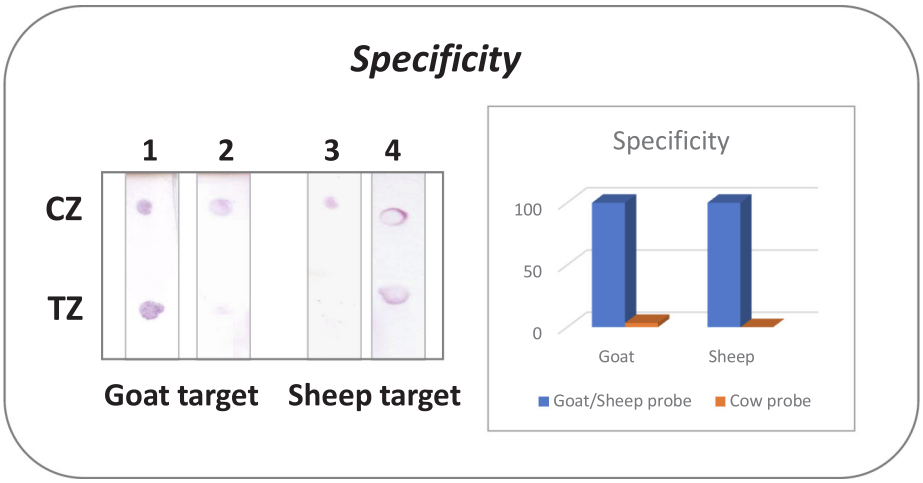

Fig. 4. Specificity study for the cow-specific probe: 50 fmol of each PCR product for goat and sheep species were analyzed by the sensor using both their specific probes along with the cow-specific probe. The biosensors were removed from the developing solution and scanned using a regular Scanner. The obtained images were furtherly analyzed by the software ImageJ. The color intensity of the red test spots formed on the membrane of the sensor was measured, while the normalized data (the value of 100 is attributed to the positive sample for goat or sheep target, respectively) of each test spot are presented as a histogram. 1: 50 fmol of goat PCR product with goat-specific probe, 2: 50 fmol of goat PCR product with cow-specific probe, 3: 50 fmol of sheep PCR product with cow-specific probe and 4: 50 fmol of sheep PCR product with sheep-specific probe, CZ: control zone, TZ: test zone.

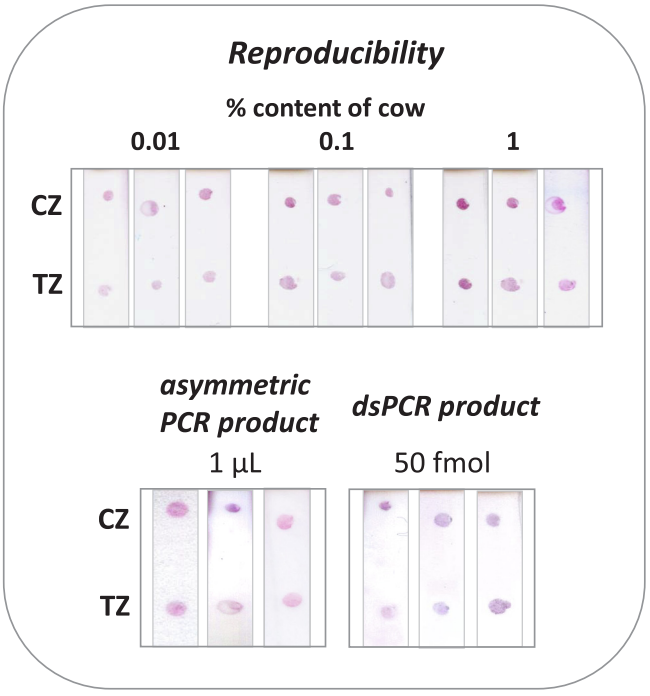

Fig. 5. Reproducibility of the biosensor. The reproducibility was assessed by analyzing (n = 3) the following samples: i) the binary mixtures that contained 0.01, 0.1 and 1% adulteration content of cow species, ii) 1 µL of asymmetric amplified product for cow and iii) 50 fmol of double-stranded PCR product for cow. CZ: control zone, TZ: test zone.

biosensor is rapid and ends within 10–15 min after application of the sample, while the results are eye-detectable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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