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Analytical Methods

A quadruplex PCR (qxPCR) assay for adulteration in dairy products

Caterina Agrimonti, Andrea Pirondini, Marta Marmiroli, Nelson Marmiroli *

Department of Life Sciences, University of Parma, v.le Parco Area delle Scienze, 11/A, 43124 Parma, Italy

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ABSTRACT

This study describes the development of a quadruplex quantitative Real Time PCR (qxPCR) based on SYBR®GreenER chemistry, for rapid identification of DNA of cow, goat, sheep and buffalo in dairy products, and for quantification of cow DNA in these products. The platform was applied to: (i) mixes of milks at fixed percentages; (ii) cheeses prepared with the same mixes; (iii) commercial dairy products. The methodology enabled the detection of DNA from cow in mixes of milk and cheeses with a limit of detection (LOD) of 0.1%. When applied to commercial dairy products the qxPCR gave results comparable with each single-plex Real Time PCR.

A good correlation ($R^2 > 0.9$) between peaks' area of derivative of melting curves of amplicons and percentages of cow milk in milk mixes and cheeses, allows for an estimation of cow DNA in a dynamic range varying from 0.1–5% to 1–25%.

1. Introduction

Issued to answer the increasing demand for more transparency in the dairy food chain, the European Regulations require that producers declare the type of milk used in manufacturing (Woolfe & Primrose, 2004). Compliance of dairy products with these Regulations is also mandatory because the persons which are allergic to cow milk (Crittenden & Bennett, 2005) should consume dairy products made only after sheep, goat, or soy milks. The absence of a proper labelling, indicating the possibility of even traces of cow milk in any dairy products, can increase the risk for these persons.

Since 1992 European Union introduced with the Regulation 2081/92 the label of origin: protected designation of origin (PDO), protected geographical indication (PGI) and traditional specialty guaranteed (TSG) to eliminate the unfair competition and misleading of consumers by non-genuine products. This, enforced by EU Regulation 1151/2012 and gradually expanded internationally via bilateral agreements between the EU and non-EU countries, ensures that products genuinely originating in a specific region can be identified in the label.

To ensure the authenticity of dairy products, their geographical origin and the animal species from which the milk has been obtained must be proved. This prompted the development of new analytical methods for milk traceability based on: electrophoresis (Mayer, Bürger, & Kaar, 2012; Molina, Martín-Álvarez, & Ramos, 1999), immunochemistry (Hurley, Ireland, Coleman, & Williams, 2004; López-Calleja et al., 2007), chromatography (De Noni, Tirelli, & Masotti, 1996; Ferreira & Caçote, 2003) and mass spectrometry (Cozzolino, Passalacqua, Salemi, & Garozzo, 2002). These methods, which are very specific, frequently lack in sensitivity and not always are suitable for heat-treated material. A DNA based analysis could be a valid alternative because DNA is extremely persistent during food processing and can retain sequence-specific information retrievable after an amplification reaction (PCR). Molecular methods for identification of animal species contribution to dairy products based upon PCR technology have been developed (Dalvit, De Marchi, & Cassandro, 2007; Mafra, Ferreira, & Oliveira, 2008). The majority of these utilises single-plex end-point or Real-Time PCR for bovine species discrimination in water buffalo mozzarella cheese and for detection of ovine species in other dairy products (Lopparelli, Cardazzo, Balzan, Giaccone, & Novelli, 2007).

Multiplex PCR, a multi-analyte methodology useful to simultaneously detect different DNA targets in a single reaction, was applied to identify the DNA contribute from different animals species in several dairy products (Bottero et al., 2003; Kotowicz, Adamczyk, & Bania, 2007; Mafra, Roxo, Ferreira, & Oliveira, 2007; Tobe & Linacre, 2008). However, co-amplification of different regions of species-specific DNA fragments based on end-point PCR, with subsequent agarose gel electrophoresis did not provide for any quantification of the targets.

* Corresponding author. Tel.: +39 0521 905606.
E-mail address: nelson.marmiroli@unipr.it (N. Marmiroli).

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Some PDO cheeses are manufactured from defined amount of each component: e.g. "Murazzano" cheese is made with sheep milk and with, but mot exceeding 40%, of cow milk while in "Casciotta d'Urbino" cow milk should be between 20% and 30%. Assessing the authenticity of commercialised cheeses is a challenge not only for methods of qualitative detection of bovine milk and milk from other species, but also for their quantification. Moreover, quantification of cow milk in dairy products in which it is not permitted needs to assess whether there is a fraud or if it is accidental, i.e. an involuntary contamination.

Multiplex PCR with SYBR Green followed by temperature of melting (T_M) analysis can be a substitute of end-point PCR followed by gel electrophoresis, with a considerable saving of time. T_M analysis has been used mostly in qualitative detection of different DNAs but some recent papers describe also application to quantification of specific targets (Bottari, Agrimonti, Gatti, Neviani, & Marmiroli, 2013; Pafundo, Gullì, & Marmiroli, 2011; Samson, Gullì, & Marmiroli, 2013).

In the present work it is described the development of a unique quadruplex Real Time PCR (qxPCR) platform for the simultaneous detection of milk ingredients (cow, buffalo, sheep, goat) in dairy products and for quantification of cow milk in the same products. This technology was first applied to milk mixtures and to cheeses prepared in laboratory, and then to commercial products.

2. Materials and methods

2.1. Animal samples used

DNA was isolated from raw milk of cow, buffalo, goat and sheep. Reference DNAs were extracted from meat of the same animal species. Primers' specificity was tested on DNA from donkey, horse, chicken, pig and turkey meat.

Mixtures of cow–buffalo, cow–goat and cow–sheep raw milk were prepared in percentages of (%, vol/vol): 0.1–99.9%, 0.5–99.5%, 1–99%, 2.5–97.5%, 5–95%, 10–90%, 25–75% (Table 1S). Mixtures were used to prepare cheeses under controlled conditions with the following procedure: 1 mL of veal rennet (95% chymosin, 5% pepsin) was added to 3 L of raw milk. The milk was warmed to 36 °C for 1 h, stirred for 5 min and warmed again to 36 °C for 30 min. The solution was placed in a strainer and drained for at least 6 h. The obtained cheese was put in a salting brine (1 L of water and 200 g of salt) for 1 h, dried and stored 1 day at room temperature until DNA was extracted.

Commercial buffalo, cow, goat and sheep dairy products were purchased in supermarkets and analysed to evaluate the applicability of the qxPCR to real commercial samples.

2.2. DNA extraction

DNA was extracted from milk, cheese and meat using a CTAB-based method. An amount of 300 mg of sample was incubated overnight at 60 °C under agitation with 4.5 μL of Proteinase K (20 mg mL^{-1}) and 900 μL of CTAB buffer (1.4 M NaCl, 2% (w/v) CTAB (hexadecyltrimethylammonium bromide), 100 mM Tris, 15 mM EDTA, pH 8.0). After centrifugation for 5 min at 20,000×*g*, 900 μL of supernatant was extracted with an equal volume of chloroform. The DNA was precipitated at −20 °C with 90 μL of sodium acetate 3 M pH 5.2 and two volumes of ethanol, for 1 h. After centrifugation at 13,000×*g* for 15 min and washing with ethanol 70%, the DNA was resuspended in 100 μL of sterile deionized water and its concentration was determined with spectrophotometer DU640 (Beckman-Coulter, Fullerton, CA, USA).

2.3. PCR primer design and test

Primers sequence, shown in Table 2S, was obtained using the Primer3 software (Rozen & Skaletsky, 2000) in order to produce amplicons with different lengths and theoretic melting temperatures (T_M). Primers were designed on published sequences in GenBank available at the site of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) and customised by MWG (Ebersberg, Germany). The theoretical T_M of amplicons was calculated with the algorithm OligoCalc (Kibbe, 2007).

Primers were firstly tested by PCR on reference DNAs extracted from meat of cow, sheep, goat and buffalo. Presence of amplicons of the expected sizes was checked on 2% agarose gel. Amplicons were purified with the Qiaquick gel extraction kit (Qiagen, Hilden, Germany) and directly sequenced using DTCS Quick Start

Table 1
Threshold cycles (C_T) and efficiency (E) of qxPCR and sxPCR. Results are expressed as averages of three experiments ± SD, p is the probability determined with t-test.

Log_{10} (ngDNA)	C_T			E		
	qxPCR	sxPCR	p	qxPCR	sxPCR	p
Cow						
1.301	18.398 ± 0.0260	18.730 ± 0.306	0.090	1.103 ± 0.243	1.015 ± 0.056	0.276
0.301	21.561 ± 0.062	21.868 ± 0.159	0.114			
−0.699	24.709 ± 0.667	25.138 ± 0.316	0.209			
−1.699	26.479 ± 2.475	28.612 ± 0.569	0.096			
Goat						
1.301	18.984 ± 0.007	18.307 ± 0.030	0.064	1.097 ± 0.166	1.041 ± 0.040	0.306
0.301	21.541 ± 0.173	21.682 ± 0.057	0.090			
−0.699	24.699 ± 0.601	25.105 ± 0.442	0.095			
−1.699	27.855 ± 1.096	27.899 ± 0220	0.473			
Buffalo						
1.301	18.426 ± 0.047	18.329 ± 0.094	0.175	1.126 ± 0.298	1.265 ± 0.404	0.351
0.301	21.567 ± 0.173	21.112 ± 0.110	0.128			
−0.699	24.709 ± 0.442	24.563 ± 0.294	0.181			
−1.699	27.850 ± 1.406	28.048 ± 0.639	0.351			
Sheep						
1.301	17.838 ± 0.144	17.987 ± 0.032	0.129	1.108 ± 0.129	1.213 ± 0.153	0.062
0.301	21.649 ± 1.169	21.904 ± 0.032	0.373			
−0.699	24.181 ± 0.170	24.454 ± 0.558	0.291			
−1.699	27.352 ± 0.396	27.028 ± 0.939	0.266			

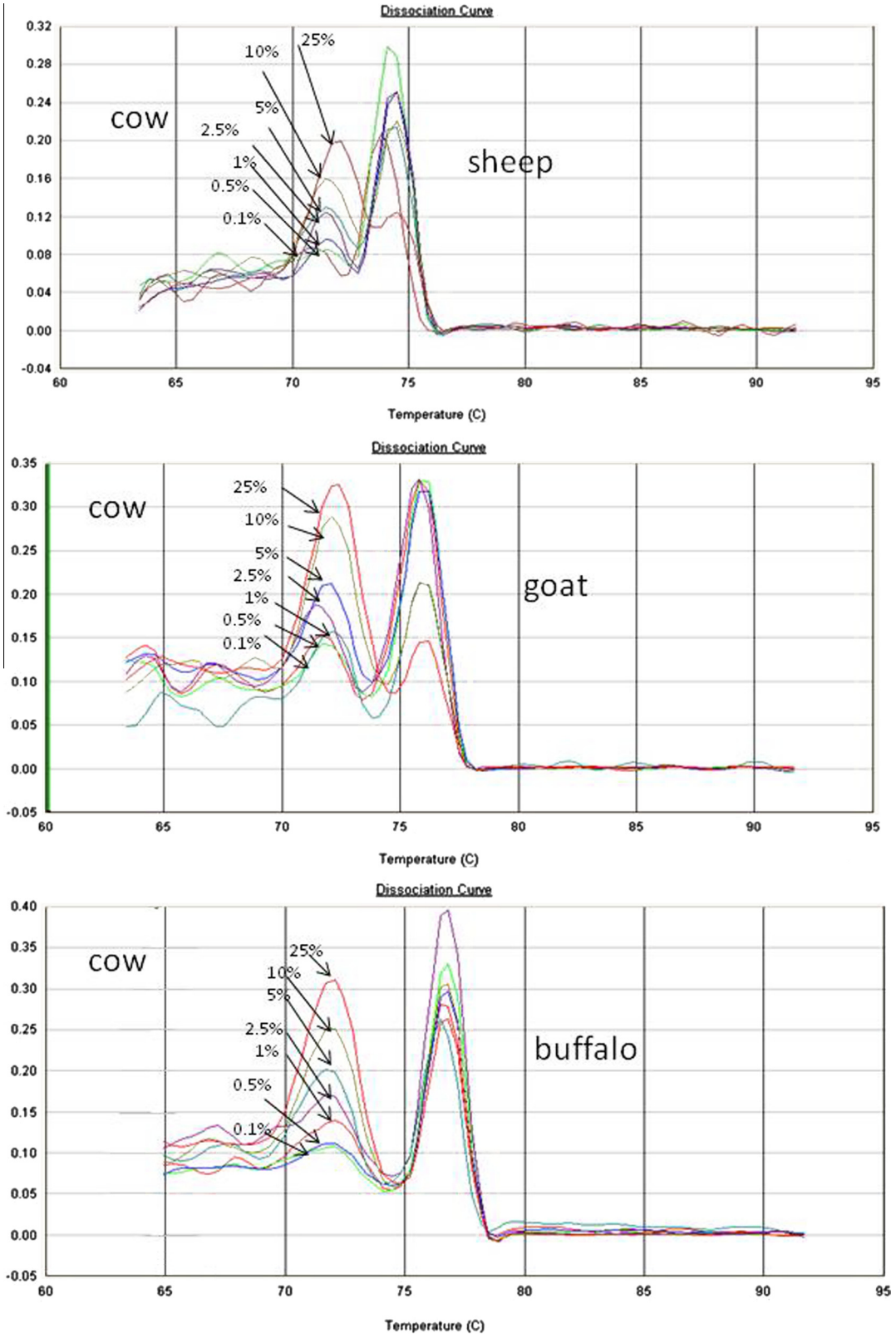

Fig. 1. Dissociation profiles obtained by qxPCR analysis of DNA extracted from binary mixes (cow/sheep, cow/goat, cow/buffalo) of milks at different percentages.

Kit (Beckman-Coulter), according to the manufacturer's instructions. DNA was loaded on CEQ 2000 XL (Beckman-Coulter) and sequence analysis was performed using CEQ 8000 DNA Analysis System Software (Beckman-Coulter). The nucleotide sequences were then analysed with BLASTn algorithm available at the NCBI site.

Primers specificity was tested checking the absence of amplification on DNA extracted from donkey, horse, chicken, pig and turkey meat.

2.4. Development of qxPCR

Species-specific primer concentration was adjusted to obtain comparable C_T values for each species of DNA, comparable with that of single-plex PCR (sxPCR). Tests were firstly conducted using the four pairs of primers on 20 ng of a single species reference DNA and then to a tenfold dilution series: 20, 2, 0.2, 0.02 ng.

A logarithmic plot was generated ($\log_{10}$ of the concentration of DNA versus C_T) by ABI Prism® 7000 SDS software (Applied Biosystems, Foster City, CA) and the standard curve was calculated as $y = ax + b$, where $y = C_T$ (threshold cycle), a = slope of the curve, x = log of DNA amounts, b = intercept of curve on y axis. Efficiency of each reaction (E) was calculated from $E = (10^{-1/a}) - 1$.

Real-time PCR amplifications were performed with an ABI PRISM 7000 equipment (Applied Biosystems) using the dsDNA binding dye SYBR®GreenER. PCR was carried out in a final volume of 20 μL containing 1× SYBR®GreenER™ PCR Master Mix (Invitrogen, Carlsbad, California), 20 ng of DNA and different concentrations for each primer pair (Table 2S). All PCR reactions were performed in triplicate. PCRs and T_M analysis were conducted as described by Bottari et al. (2013). The T_M of the amplicons was compared with the T_M of the positive control obtained using reference DNAs (Table 1S).

2.5. Development of a quantitative qxPCR platform

Three experiments were conducted at different times, to quantify cow milk percentages in milk mixes and in cheeses as in Table 1S using qxPCR. The results of three replicates of each experiments were compared.

Derivatives of dissociation curve of amplicons were exported in Excel 2007/XP and each peak' area was calculated with the software Peak Explorer 2.11, available at the site http://peak-explorer.soft112.com. The coefficient of regression (R^2) between peaks' area and percentages of cow milk was calculated with Excel 2007.

3. Results

3.1. Primer specificity

Primer pairs, chosen on the basis of a theoretical T_M, were tested on reference DNAs from cow, buffalo, sheep and goat in single reactions. T_M of the amplicons produced in PCR reactions were consistent with the theoretical values. Nucleotide' sequences of each amplicon were also consistent with those of the target genes reported in Table 2S and available at the NCBI database.

To optimise the qxPCR assay, primer concentration was adjusted to avoid any preferential amplification of targets (Elnifro, Ashshi, Cooper, & Klapper, 2000). Mixes of primers, listed in Table 2S, when tested on 20 ng of DNA of each of the animal species produced amplification profiles comparable with those of the single amplification reaction. Significant differences ($p > 0.05$) between C_T obtained with a qxPCR and with a sxPCR were not found (data not shown).

Table 2
Comparison between results obtained on commercial dairy products analysed by qxPCR and sxPCR. Differences between results obtained with qxPCR and sxPCR are marked with asterisks.

	Declared milk of origin	Profiles obtained by qxPCR	Profiles obtained by sxPCR
Smoked scamorza	Cow	Cow	Cow
Mozzarella	Cow	Cow	Cow
Ricotta	Cow	Cow	Cow
Cooking cream	Cow	Cow	Cow
Whipped cream	Cow	Cow	Cow
Parmesan cheese	Cow	Cow	Cow
Stracchino	Cow	Cow	Cow
Yogurt	Cow	Cow	Cow
Sottilette	Cow	Cow	Cow
Goat's yogurt	Goat	Goat	Goat
Goat's cheese	Goat	Cow/goat	Cow/goat
Goat's butter	Goat	Goat	Goat
Camoscio d'oro	Cow/goat	Cow	Cow/goat*
Sardinia sheep's cheese	Sheep	Sheep	Sheep
Sheep's ricotta	Sheep	Sheep	Sheep
Puglia sheep's cheese	Sheep	Cow/sheep	Cow/sheep
Butterino	Cow/sheep	Cow/sheep	Cow/sheep
Pane Pastore	Cow/sheep	Cow	Cow/sheep*
Sheep's ricotta	Cow/sheep	Cow	Cow/sheep*
Three milk cheese	Cow/ sheep/goat	Cow/sheep	Cow/sheep/goat*
Feta	sheep/goat	Cow/sheep	Cow/sheep/goat*
Buffalo's mozzarella	Buffalo	Cow/buffalo	Cow/buffalo
Buffalo's ricotta	Buffalo	Cow/buffalo	Cow/buffalo
Buffalo's scamorza	Buffalo	Cow/buffalo	Cow/buffalo
Cow's and buffalo's ricotta	Cow/buffalo	Cow/buffalo	Cow/buffalo
Cow's and buffalo's stracchino	Cow/buffalo	Cow/buffalo	Cow/buffalo

Table 3
Regression coefficients between peak' areas in derivative of dissociation curves and percentages of cow milk of samples and range of quantification obtained in three independent experiments with milk mixtures and cheeses. The values are derived from means of peaks' areas obtained with three replicates for each experiment. For cheese, only the results of the most significant experiment are reported.

Mix	R^2 values	Range of quantification (%)
Milk mixtures		
Experiment 1		
Cow–sheep	0.9872	1–25
Cow–goat	0.9401	1–25
Cow–buffalo	0.9892	1–10
Experiment 2		
Cow–sheep	0.9780	2.5–25
Cow–goat	0.9871	2.5–25
Cow–buffalo	0.9584	2.5–25
Experiment 3		
Cow–sheep	0.9943	1–25
Cow–goat	0.9943	0.5–10
Cow–buffalo	0.9973	0.5–10
Cheeses		
Cow–sheep	0.9843	1–10
Cow–goat	0.9233	0.1–5
Cow–buffalo	0.9226	1–10

Efficiency and sensitivity of the qxPCR was assessed in dilutions of reference DNAs (20 ng, 2 ng, 0.2 ng, 0.02 ng) of each species generating a set of standards with high linearity ($R^2 > 0.99$) (Fig. 1S). The t-test showed that C_T and the efficiency of qxPCR were comparable to those in sxPCR ($p > 0.05$) (Table 1).

A negative control made on DNA from donkey, horse, chicken, pig and turkey did not give amplification products using the same primers both in sxPCR and qxPCR.

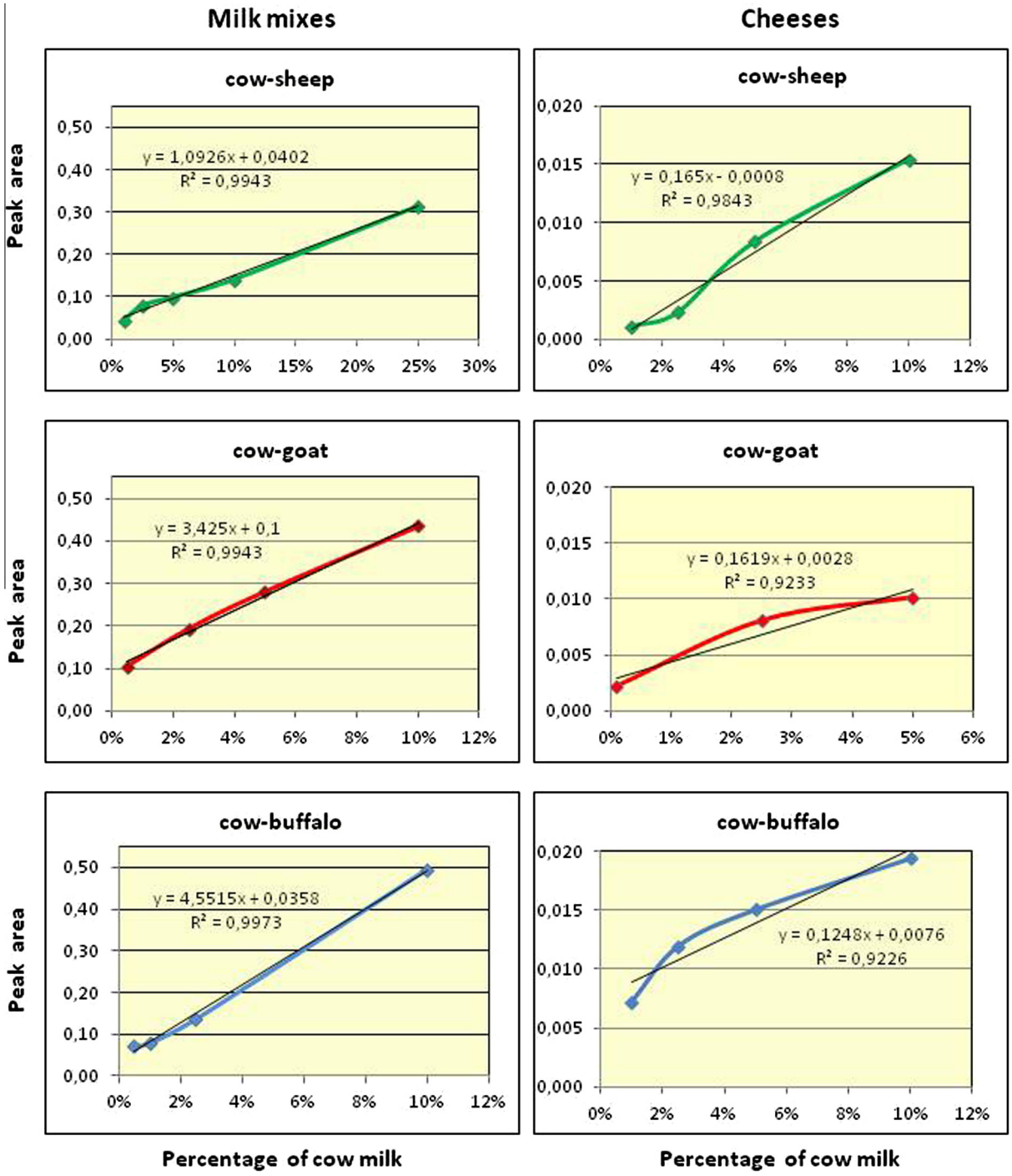

Fig. 2. Regression curves correlating the percentages of cow milk, in milk mixes and in cheeses, with the areas of peaks of derivative of amplicon' dissociation curves.

3.2. Application of qxPCR platform for qualitative analysis of experimental and commercial dairy products

The four primer pairs were mixed and tested on DNA extracted from the milk mixes listed in Table 1S with the qxPCR platform. Amplicons of expected T_M were detected in all mixes (Table 1S). The limit of detection, LOD, (lowest milk percentage yielding visible DNA amplification) of the assay was 0.1% of cow's milk in all mixes (Table 1S). Typical melting profiles obtained by qxPCR in mixes of milks are shown in the Fig. 1.

The qxPCR was also tested on DNA from cheeses produced with mixes of milks as mentioned above. The correspondence between PCR profiles and composition of cheeses is reported, with a LOD of 0.1% of cow milk for all experimental cheeses (Table 1S).

Applicability of qxPCR platform was tested on commercial dairy products made with different milk composition (Table 2), comparing the results obtained with sxPCR in which the four primer pairs were utilised separately.

Out of 26 dairy commercial products analysed with qxPCR, 21 (80%) gave profiles similar to those with sxPCR. The 61% of products analysed with qxPCR had a milk origin consistent with that

declared; this percentage raised to 77%, when the analysis was performed with sxPCR.

For other products the results were only partially consistent. In the majority of cases the analysis showed the presence of undeclared cow milk, in others of only one kind of milk (sheep or cow) or of a number of milks lower than declared (Table 2).

3.3. Application of qxPCR for quantitative analyses

Quantification of cow milk by qxPCR in milk mixes and experimental cheeses was obtained by measuring the areas of peaks in derivatives of dissociation curve.

Coefficients of regression (R^2) between area of peaks and percentages of cow milk in mixes, shown in Table 3, were consistent (>0.9) in all experiments, within the dynamic range of percentages of cow milk: 1–25%/1–10% in experiment 1, 2.5–25% in experiment 2 and 0.5–10%/1–25% in experiment 3.

As example, curves from experiment 3 are reported in Fig. 2.

In cheeses, in only one experiment R^2 was >0.9, (Table 3) in the others it was <0.7. In the case of cheese the range of quantification varied from 0.1–5% (cow/goat) to 1–10% (cow/sheep and cow/buffalo).

4. Discussion

Polymerase Chain Reaction (PCR), sxPCR and qxPCR, are suitable methods to detect animal species origin in milk and in dairy products. At the purpose, single-plex (Bania, Ugorski, Polanowski, & Adamczyk, 2001; Di Pinto, Conversano, Forte, Novello, & Tantillo, 2004), duplex (Mafra et al., 2008), and triplex end-point PCR have been used (Bottero et al., 2003).

Real Time PCR platform combined with SYBR Green (SG) chemistry, less specific than TaqMan probes but also less expensive (Giglio, Monis, & Saint, 2003), offers the possibility, by melting curve analysis, of checking the amplicon specificity, as with gel electrophoresis. The use of qxPCR with SG chemistry can save in time and in cost avoiding gel electrophoresis but testing simultaneously different DNA targets.

The primers described in this work were designed on the sequence of cytochrome b and 12S RNA (Dalmasso, Civera, La Neve, & Bottero, 2011) and used in qxPCR to amplify DNA from several animal species with considerable efficiency.

The qxPCR platform described in this paper had a LOD for cow milk as low as 0.1%. This LOD, assessed also for cheese, was similar to the LODs of sxPCR with SG, as reported by Lopparelli et al. (2007) and Mayer et al. (2012), but lower than the 0.5% reported by Bottero et al. (2003) for a multiplex end point PCR, and much lower than the 2% reported by Dalmasso et al. (2011) as LOD for a PCR with a single primer pairs for the allelic discrimination between cow and buffalo.

When qxPCR was applied to milk authenticity in dairy products purchased at supermarkets it detected the presence of undeclared cow milk, in particular in buffalo cheeses; these results, validated by sxPCR, confirmed that addition of cow milk in cheese made with milk from water buffalo, is a frequent fraud, as reported by Dalvit et al., 2007 and by the Central Inspectorate for Quality Protection and Repression of Frauds in alimentary products (ICQRF) of the Italian Ministry of Agriculture and Forestry (MiPAAF).

In few cases, qxPCR, failed to detect goat and sheep milk in cheeses, that were detected in a sxPCR reactions, but results obtained either with qxPCR or sxPCR were consistent in 80% of the samples analysed. Therefore qxPCR may be a valuable tool for screening a large number of samples, when results are not consistent with the label of products, they can be validated by sxPCR to solve any doubtful cases.

In conformity to disciplinary of dairy production, it is required also a quantification of milk composition. Quantitative PCR based on hydrolysis probes has been reported in literature to detect (Dalmasso et al., 2011) and quantify (Lopparelli et al., 2007) cow milk in buffalo mozzarella, in single or duplex configuration. The costs of this approach and its limitation to two or few targets make it of a restricted applicability.

In this paper we have reported the possibility of quantifying cow milk in dairy products with qxPCR based on SG, by establishing a correlation between the peaks area in the dissociation curves of each amplicon and the amount of DNA in the sample.

For cheeses, the general performance was lower than in milk mixes. Efficiency of sxPCR with SG in milk mixes is comparable with sxPCR with TaqMan probes, but for cheeses TaqMan probes gave better results (Lopparelli et al., 2007). Effectively, the amount (quantity) and quality of DNA obtained are lower in cheese than in milk, due to the higher processing: homogenisation, filtration, centrifugation, heating, acidification and coagulation.

Correlation, in milk mixes, between the area of each amplicon' peak and the percentage of the corresponding milk, suggested that this type of quantification is feasible and reliable. Indeed, this procedure requires a standard curve made with reference mixes of milk at fixed percentages of each specific milk. The percentage of milk in an unknown sample after amplification of DNA and peaks' area determination is obtained placing this value on the standard curve.

For cheeses the quantification is further complicated by the higher complexity of the matrix. The availability of appropriate standards for dairy products, some available in European Union (Mayer et al., 2012), may help to estimate whether a milk compositional labelling of PDO cheese were feasible or not.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2015.04.017.

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