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# Quantitative detection of goats' milk in sheep's milk by real-time PCR

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**Abstract**

The need to support food-labeling legislation has provided a driving force for development of analytical techniques for the analysis of food ingredients. In this study, the development of a method for quantification of goats' milk in sheep's milk mixtures is described. The technique involves the use of a real time PCR technique, based on the amplification of a fragment of the mitochondrial 12S ribosomal RNA gene (rRNA). The method combines the use of goat-specific primers that amplify a 171 bp fragment from goat DNA, and mammalian-specific primers amplifying a 119 bp fragment from mammalian species DNA, which are used as endogenous control. An internal fluorogenic probe (TaqMan) that hybridizes in the "goat-specific" and also in the "mammalian" DNA fragments is used to monitor the amplification of the target gene. A comparison of the cycle number ($C_t$) at which mammalian and goat-specific PCR products are first detected, in combination with the use of reference standards of known caprine content, allows the determination of the percentage of goats' milk in a milk mixture. The assay was used to analyze raw and heat-treated milk binary mixtures (goat/sheep), enabling the quantification of goats' milk in the range 0.6–10%. The reported PCR assay may represent a rapid and straightforward tool applicable to the authentication of milk and other dairy products.


*Keywords:* Species identification; Quantification; 12S rRNA; Goat; Sheep; Milk mixtures; Real-time PCR; Taqman

## 1. Introduction

Food adulteration is a common problem in the dairy industry because it can be easily applied to dairy products. Differences in price and seasonal availability might make it attractive for farmers to adulterate expensive sheep's milk with cheaper goats' milk. In many European countries, laws require producers to state the type of milk used for manufacturing cheese or other dairy products. Apart from the possible economic loss, correct species identification is important for consumers who may have specific food allergies. As a result of this fraudulent practice in the dairy industry, adequate control methods are required to evaluate the authenticity of milk and milk products (De la Fuente & Juárez, 2005; Mafra, Ferreira, Faria, & Oliveira, 2004). Moreover, food analysts are challenged not only by the qualitative detection of goats' milk, but also by the need for its quantitative estimation in milk mixtures.

In the past few years, different protein-based methods (electrophoretic, chromatographic and immunological techniques) and more recently, DNA-based techniques, have been developed and are currently used for species identification in milk and dairy products. Among protein-based methods, capillary electrophoresis (Molina, Martín-Álvarez, & Ramos, 1999), two-dimensional electrophoresis (Chianese et al., 1990), isoelectric focusing of milk caseins (Moio, Sasso, Chianese, & Addeo, 1990) which is the European Community reference method for cows' milk detection (Commission Regulation No. 1081/96), HPLC (Ferreira & Cacote, 2003) and ELISA (Anguita et al., 1997; Hurley, Ireland, Coleman, & Williams, 2004; Moatsou & Anifantakis, 2003) have been widely reported. Although these methods are effective in certain instances, their main drawback is that proteins are denatured during heat and pressure processing, making the detection of the species present in a processed sample more difficult.

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Methods based on protein analysis have been replaced by DNA-based methods, because DNA has the advantage of being a relatively stable molecule, and is more able to withstand heat processing (Behrens, Untan, Brinkmann, Buchloz, & Latus, 1999). Among them, those based on the polymerase chain reaction (PCR) technique have been successfully applied to species identification in dairy products (Bottero et al., 2003; Feligini et al., 2005; López-Calleja et al., 2004, 2005a, 2005b). However, conventional PCR techniques allow the qualitative detection of different animal species in an admixture, but do not result appropriate to achieve the quantification of a target species in a product. DNA-based methods developed for a quantitative detection are either based on quantitative competitive PCR (Wolf & Lüthy, 2001), densitometry (Calvo, Osta, & Zaragoza, 2002) or real-time PCR procedures (Dooley, Paine, Garrett, & Brown, 2004).

The real-time quantitative PCR is a powerful technology that is highly accurate, and relatively fast. There have been a number of reports describing the development and application of real-time PCR for the quantitative detection of genetically modified organisms (Yang et al., 2005) and meat and fish species identification (Laube et al., 2003; Lopez & Pardo, 2005; Rodríguez et al., 2004; Sawyer, Wood, Shanahan, Gout, & McDowell, 2003), whereas no reports have been published up to the date, for the quantification of milk in milk mixtures or dairy products.

This technique is commonly based on the use of a TaqMan fluorogenic probe (Holland, Abramson, Watson, & Gelfand, 1991). The probe, labeled with a reporter and a quencher dye, binds to a target DNA between the flanking primers. During PCR amplification, the 5′–3′ exonuclease activity of the *Taq* DNA polymerase cleaves the probe hybridized to the template, releasing the 5′ reporter from the quenching effects of the 3′ quencher. Cleavage of the probe results in an increase of fluorescence, proportional to the amount of template DNA present. The use of fluorescence for detection purposes eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, easing automation of the technique and large-scale sample processing.

The aim of the study described herein was the development of a fluorogenic PCR-based assay to detect and quantify the amount of goats' milk in raw and heat-treated goat/sheep milk mixtures.

## 2. Materials and methods

### 2.1. Sample selection

Authentic samples of pooled raw milk from cow (*Bos taurus*), goat (*Capra hircus*), sheep (*Ovis aries*) and water buffalo (*Bubalus bubalus*) were obtained from a collection tank of a local dairy farm. Also, commercial goats' pasteurized milk were purchased from several retail markets. Samples were transported to the laboratory and were processed immediately or stored frozen at −85 °C until used.

Binary milk mixtures of goats' milk in sheep's milk were prepared for further DNA extraction and PCR analysis. On these mixtures, four different goats' milk percentages containing 0.5%, 1%, 5%, and 10% (vol/vol) were prepared in a final volume of 1 mL. Samples analyzed included raw and heat-treated milks. However, in the case of sheeps' milk, samples were used after being subjected to experimental pasteurization (60 °C, 30 min), due to the unavailability of ovine heat processed milks in the market.

### 2.2. DNA extraction

DNA was extracted using the *Wizard*® DNA Clean-up system (Promega, Madison, WI, USA), as described by López-Calleja et al. (2004). Three independent DNA extractions of each milk percentage (0.5%, 1%, 5%, and 10%) were performed for all binary mixtures, and were analyzed in this work. DNA concentration was estimated by UV absorption spectrophotometry at a wavelength of 260 nm.

### 2.3. Oligonucleotide primers and probes

PCR systems (primers and fluorogenic probe) were designed using Primer Express 2.0 software (Perkin–Elmer/Applied Biosystems Division, Foster City, CA, USA). For that purpose, sequences of the 12S rRNA gene from cow (accession number AJ849533), sheep (AJ849534), goat (AJ849535) and water buffalo (AJ846850) obtained in a previous work (López-Calleja et al., 2005b), were aligned and compared.

Three different primers and a common fluorogenic probe were designed to hybridize in the 12S rRNA gene. A forward primer (*12STAQMANFWM*: 5′-AAAGGACTTGGCGGTGCTT-3′) and a reverse primer (*12STAQMANCH*: 5′-TGACCTAACGTCTTTATGTGTGGTG-3′) were designed for the specific detection of goat (goat-specific PCR system). The same forward primer and another reverse primer (*12STAQMANREVM2*: 5′-GGGTTTGCTGAAGATGGCG-3′) were used as endogenous control for detection of mammalian DNA (mammalian PCR system). A 171 bp fragment of the 12S rRNA gene was selected as a target site for the specific detection of goat. The target site for the mammalian PCR system consisted of a 119 bp fragment of the same gene. Results obtained from the mammalian PCR system were used to normalize those obtained from the goat-specific system as described below.

The TaqMan probe (12SPROBE: 5′-TAGAGGAGCCTGTTCTATAATCGATAAACCCCG-3′) was designed to hybridize in both PCR systems (goat-specific and mammalian) and purchased from Applied Biosystems. The probe was labeled with the fluorescent reporter dye 6-carboxyfluorescein (FAM) on the 5′ end, and with the 6-carboxy-tetramethylrhodamine (TAMRA) fluorescent quencher dye, on the 3′ end.

### 2.4. 5′ Nuclease PCR conditions

The 5′ nuclease PCR was run under generic cycling conditions. The concentrations of primers and probe yielding the highest endpoint fluorescence and the lowest $C_t$ (data not shown), were 300 nM of forward primer (*12STAQMANFWM*), 900 nM of reverse primers (*12STAQMANREVM2* or *12STAQMANCH*), 200 nM of probe (*12SPR- OBE*) and 10 ng of DNA. The TaqMan PCR reactions, using the TaqMan PCR Master Mix reagent (Applied Biosystems), were performed in a total reaction volume of 25 μl in a Microamp® Optical/96-well Reaction Plate (Applied Biosystems) covered with Microamp® Optical caps (8 caps/strip), and were run with the ABI PRISM™ 7700 sequence detection system (PE Applied Biosystems) at the *Centro de Genómica y Proteómica* (Facultad de Biología, UCM).

The following temperature/time program for Taqman™–PCR was applied: 2 min at 50 °C, 10 min at 95 °C, then 40 cycles with 15 s denaturation at 95 °C, and 1 min annealing/elongation at 60 °C. The same program was used to amplify either the goat-specific or the endogenous control (mammalian) PCR fragments. Unless otherwise indicated, all real-time PCR reactions were carried out in duplicate.

### 2.5. Construction of a plasmid DNA for the standard curve

Primers *12STAQMANFWM* and *12STAQMANREVM2* amplified a 119 bp fragment in the 12S rRNA gene of goat DNA. This goat fragment was amplified, purified and ligated into the plasmid pGEM®-T Easy, using pGEM®-T Easy Vector System II (Promega, Madison, Wisconsin, USA).

Plasmid DNA containing the goat fragment was purified using the QIAprep spin Miniprep kit (Qiagen GmbH) following the manufacturer's instructions. The resulting plasmid DNA was electrophoresed in a 0.8% D1 low electroendosmosis agarose gel (Pronadisa, Torrejón, Spain), containing 0.5 μg/mL ethidium bromide in Tris-acetate buffer (0.04 M Tris-acetate and 0.001 M EDTA, pH 8.0) for 45 min at 100 V. The gel was visualized by UV transilumination and analysed using Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA, USA). Plasmid DNA concentration was estimated by absorbance at 260 nm.

Tenfold dilution series of this plasmid, starting from 100 ng DNA, were used as standard curve in real-time PCR, either in the goat-specific or the endogenous control (mammalian) systems.

### 2.6. Data analysis

The amount of goat DNA in an unknown sample was measured by interpolation from a standard curve of threshold cycle ($C_t$) values generated from known starting DNA concentrations (plasmid goat DNA). A comparison of the cycle number at which endogenous and goat-specific PCR products are first detected, in combination with the use of reference standards of known goat content ($C_t$s), was used for assessment of the percentage of goat in a sample. Total DNA quantity in goat/sheep mixtures was determined by reporting the $C_t$ value in the mammalian system ($C_t$m), and goat DNA quantity was fixed by reporting the $C_t$ value in the goat-specific system ($C_t$g). Thus, the $C_t$ corresponding to the percentage of goat DNA of an unknown sample ($C_t$gs), was determined as being the ratio of goat-specific to total DNA threshold cycles with the following equation:

$$C_t\mathrm{gs} = C_t\mathrm{s} * C_t\mathrm{g}/C_t\mathrm{m}$$

where $C_t$s, threshold cycle average value of the standard (plasmid goat DNA) using 10 ng DNA in the endogenous (mammalian) PCR system; $C_t$g, threshold cycle average value of the unknown sample analyzed with the goat-specific PCR system; $C_t$m, threshold cycle average value of the unknown sample analyzed with the endogenous (mammalian) PCR system; and $C_t$gs, threshold cycle value corresponding to the percentage of goat DNA of an unknown sample.

Linearity test, regression line, sensitivity and precision parameters of the goat-specific TaqMan PCR system were evaluated as previously described (Camacho, Torres, Gil-Alegre, Obregón, & Ruiz, 1993). To carry out the validation of the real-time PCR technique developed in this work, three separate DNA extractions from each percentage of goat in the goat/sheep milk mixtures were assayed in different days, using two replicates of each sample.

## 3. Results and discussion

### 3.1. Real-time PCR system set-up

To develop a robust method for quantitative detection of goats' milk in milk mixtures, the use of goat-specific primers and mammalian specific primers (endogenous control) was combined in a real time PCR method. The use of an endogenous control (mammalian PCR system) is important because some factors affecting DNA amplification from the samples can be taken into account. Without the use of the endogenous control, that amplifies any mammalian DNA from the sample, it would be difficult to ascertain whether amplification variations found with the species-specific primers were due to differences in target species content or to other factors such as DNA degradation, inhibition or differences in the amount and quality of the DNA obtained from the sample. By comparison of species-specific versus endogenous control signal obtained from the samples, the inaccuracies caused by the use of standards and unknowns can be reduced. Besides, normalization of the amplification response between unknowns and reference standards is necessary to assure the reproducibility of the assay in different days.

The target site for the specific detection of goat in the real-time PCR assay consisted of a 171 bp fragment of caprine 12S rRNA gene, and the target site for the endogenous control (mammalian) PCR detection system was a 119 bp fragment of the same gene in goat, sheep, cow and water buffalo. The endogenous control was designed to amplify DNA from goat, sheep, cow and water buffalo with the same efficiency. Both PCR systems used a common forward primer (*12STAQMANFWM*) and a common probe (*12SPROBE*). However, they differ in the reverse primers (*12STAQMANCH* in the goat-specific PCR system and*12STAQMANREVM2* in the mammalian PCR system) (Fig. 1). Tenfold dilution series of a plasmid goat DNA were used to build a calibration curve in both PCR systems (goat-specific and mammalian), and used as standards to assure the reproducibility of the data obtained.

### 3.2. Specificity

Both detection systems (goat-specific and endogenous control) were tested for their selectivity and cross-reactions to other milk-producing species. The goat-specific system amplified a 171 bp fragment from goat DNA ($C_t$ value of $20.8 \pm 0.15$), whereas no amplification was obtained from sheep, cow and water buffalo DNA. A $C_t$ value of 40.0 is measured if no amplification signal is detected after 40 cycles. The endogenous system amplified a 119 bp fragment from different mammalian species (goat, sheep, cow, and water buffalo), whereby the observed $C_t$-values differ in most mammalian animals only slightly ($C_t$ of $19.8 \pm 0.14$ in goat, $20.1 \pm 0.16$ in sheep, $21.3 \pm 0.20$ in cow and $20.9 \pm 0.23$ in water buffalo DNA). All tested mammals showed reasonable $C_t$-values between 19.8 and 21.3.

### 3.3. Quantitation limit

Six 10-fold dilution series of plasmid goat DNA starting from 100 ng were prepared as standard curve in real-time PCR, considering 10 ng DNA in the PCR system as 100%. The quantitation limit based on the standard deviation of the response (S) and the slope (b): QL = 10 S/b, for goat-specific PCR was −0.23, which corresponds to 0.58% goat DNA (Fig. 2).

Fig. 3 shows the fluorescent profiles of PCR products amplified with the goat-specific and the endogenous control (mammalian) PCR systems from milk samples, plotted against cycle number. Four different percentages of goat (10%, 5%, 1%, and 0.5%) were prepared as goat/sheep milk binary mixtures, and were analyzed using 10 ng DNA. Detection of goat DNA in these mixtures was achieved even in samples containing 0.5% of goat.

Using these percentages as calibration curve in either raw or pasteurized goat/sheep milk binary mixtures, the quantitation limit for goat-specific PCR was −0.22 for raw goat/sheep milk mixtures and −0.2 for pasteurized milk mixtures, which corresponds to 0.59% and 0.6% of goat DNA, respectively (Fig. 4).

### 3.4. Sensitivity, linearity and precision

The sensitivity of the real-time method was determined using the following parameters: (a) *Cochran's test*, which determined whether the variances of the responses obtained for each concentration of goat in the mixtures (raw or pasteurized) were homogeneous; (b) *analytical sensitivity*, which is the relationship between the randomness of the test results and the modifications due to the differ-

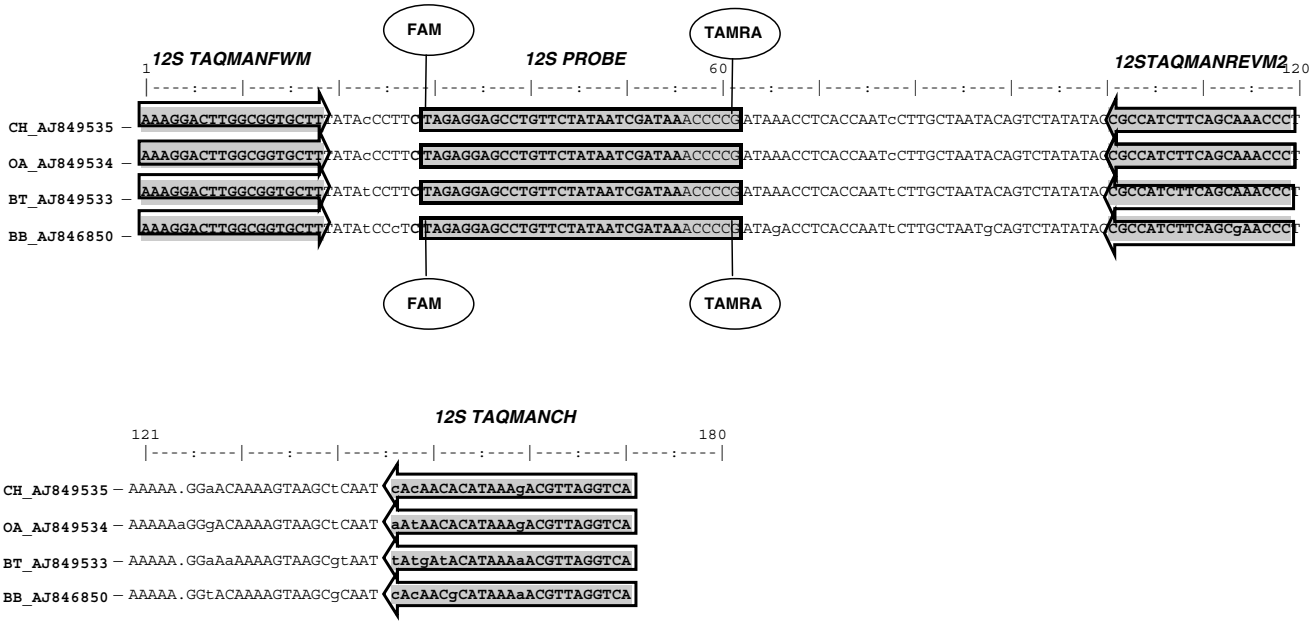
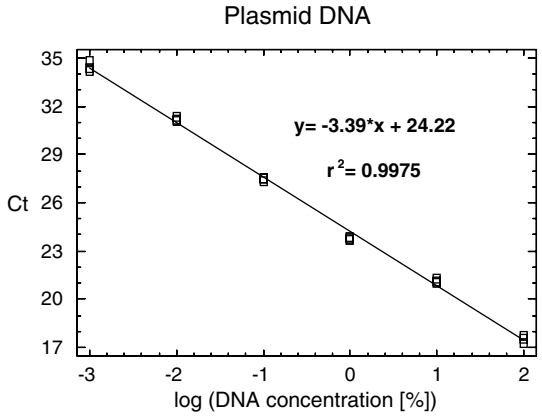



Fig. 1. Partial DNA sequence alignment of the 12S PCR products from goat (AJ849535), sheep (AJ849534), cow (AJ849533), and water buffalo (AJ846850) harbouring the designed PCR primers and probe. Primers and probe are indicated in the Figure, pointing in the 5′–3′ direction. The names of the primers and dual-labelled fluorescent probe are indicated. Dot (·) indicates a gap introduced for best-fit alignment.




| | |
|---|---|
| Total number of samples | 18 |
| "C" Cochran | 0.4602* |
| $S^2$ comb | 0.0054 |
| ANOVA test "F" regression (1) | 6335.14** |
| Analytical sensitivity | -46.1320 |
| Discrimination capacity | -0.0557* |
| Quantitation limit | -0.23 |

* p<0.05
**p<0.01
(1) P value = 0.000

Fig. 2. Linearity test, regression line, sensitivity and precision parameters of the goat-specific TaqMan PCR system using tenfold dilution series of plasmid goat DNA (100 ng–0.01 ng) as standard.

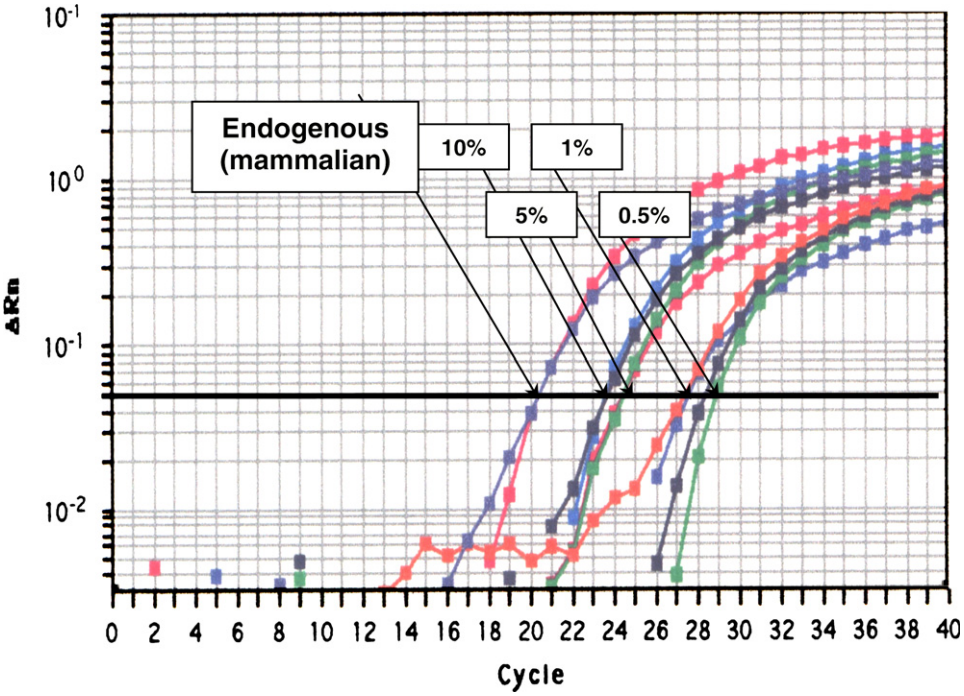



Fig. 3. Fluorescent profiles of PCR products amplified in duplicate with the goat-specific and the endogenous control (mammalian) PCR systems from a goat/sheep milk binary mixture (10%, 5%, 1%, and 0.5% goat), plotted against cycle number.

ences in concentration in the mixture, and is calculated as a quotient between the calibration sensitivity (which corresponds to the slope) and mean standard deviation for all concentrations; (c) *discriminatory capacity* which corresponds to the inverse of the analytical sensitivity multiplied by Student's *t* value, with a probability of 0.05 and degrees of freedom corresponding to the number of samples. This parameter represents the least difference in logarithm of concentration of goat DNA in the mixture that can be quantified by the analytical method with a significant level. The discriminatory capacity of the assay was −0.0557 for plasmid DNA, −0.1092 for raw goat/sheep mixture and −0.0894 for the pasteurized goat/sheep mixture.

Normalized $C_t$ values obtained from plasmid DNA and also from goat–sheep mixtures were plotted versus the logarithm of the DNA concentrations to test the linearity (Figs. 2 and 4). The *P*-value in the ANOVA table was <0.01 for all the samples, meaning that there is a statistically significant relationship between the variables ($C_t$ and logarithm of DNA concentrations) at the 99% confidence level in all the milk mixtures analyzed. Linearity was observed for the plasmid goat DNA over six orders of magnitude. The correlation between the two variables, $C_t$ and logarithm of goat concentration, using the plasmid goat DNA as standard, gives a determination coefficient value of 0.9975, which indicates that 99.75% of the variation in the

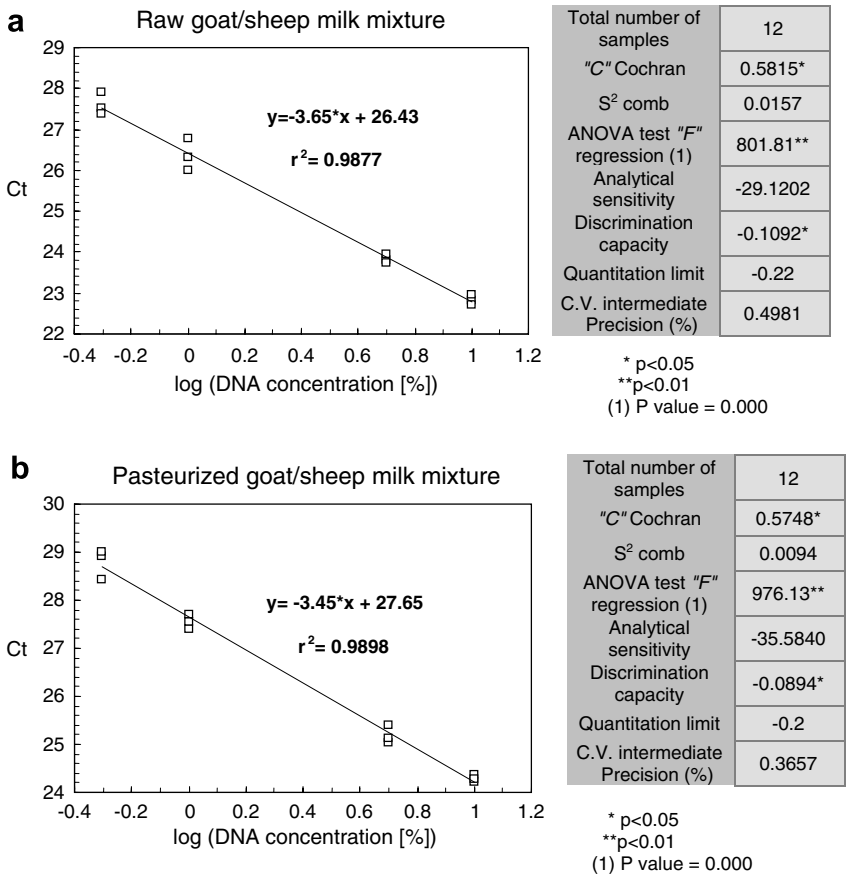



Fig. 4. Linearity test, regression line, sensitivity and precision parameters of the goat-specific TaqMan system using three different DNA extractions of four different goat percentages (10%, 5%, 1%, and 0.5%) of each binary mixture. (a) Raw goat/sheep milk mixture. (b) Pasteurized goat/sheep milk mixture.

$C_t$ obtained is due to variation in the goat DNA concentration (Fig. 2).

Linearity of the real-time PCR response was analyzed not only for dilutions of goat plasmid DNA, but also for DNA obtained from the experimental raw and pasteurized milk mixtures. Fig. 4 shows the regression line, sensitivity and precision parameters of goat/sheep milk mixtures. $C_t$ values and goat concentration (%) are related by the equation $C_t = -3.65 \log [\text{goat } (\%)] + 26.43$ ($r^2 = 0.9877$) for raw goat/sheep milk mixtures, and for the pasteurized milk mixtures, the equation was $C_t = -3.45 \log [\text{goat } (\%)] + 27.65$ ($r^2 = 0.9898$). Due to the fact that the concentration of goats' milk in the sample does not influence in the variances of the responses, the precision of this assay has been studied in the complete range of quantification of goats' milk, which goes from 0.6% to 10%. As can be observed, the coefficient of variation obtained from the intermediate precision results for raw (0.4981) and pasteurized (0.3657) goat/sheep milk mixtures, was less than 0.5%. According to the results obtained, the real-time PCR method developed herein allows quantification of goat in the range between 10% and 0.6%, as long as we know how a sample was technologically treated during production. In this way, different calibration curves should be used for raw (Fig. 4a) and pasteurized (Fig. 4b) goat/sheep binary mixtures.

The primary advantage of real-time PCR, in comparison to conventional qualitative PCR (Bottero et al., 2003), is that a large number of samples can be analyzed in a single run. Besides, fluorescence can be measured throughout the PCR, providing real-time analysis of the reaction kinetics and allowing quantification of specific DNA targets. The measurement of fluorescence in the reaction by a fluorometer (7700 system) eliminates the need for post-PCR processing steps, such as gel electrophoresis and ethidium bromide staining of target DNA, which results in a considerable economy in terms of labor and time required. Thus, the risk of carry-over contamination is minimized and so the occurrence of false-positive reactions, due to the fact that the reaction tubes remain closed throughout the assay. Moreover, the threshold cycle is observed when PCR amplification is still in the exponential phase and none of the reaction components is limited. This is the main reason why $C_t$ is a more reliable measure of starting DNA copy number than an endpoint measurement of the amount of accumulated PCR product (Rodríguez et al., 2004). Real-time analysis can facilitate quantification of the amount of goat DNA present in the sample by ascertaining when

(i.e., during which PCR cycle) fluorescence in a given reaction tube exceeds that of a threshold [threshold cycle ($C_t$)]. Comparison between reaction tubes and known standards allows quantification of goat content present in a given sample.

This paper describes the development of a method for quantitative milk identification, based on combining the use of real-time PCR together with universal and species-specific primers. The optimized assay proposed herein is specific, highly sensitive, and applicable to complex food matrices such as milk. Moreover, this method proved to work well even in heat processed milk samples which were expected to contain a large amount of fragmented DNA resulting from the temperature processing (Ferreira & Cacote, 2003).

Real-time quantitative polymerase chain reaction is a powerful technology that is highly accurate, simple and fast and results in elevated sensitivity and specificity, offering a great scope for development of quantitative assays (Heid, Stevens, Livak, & Williams, 1996). Nowadays due to its high cost, it has only a remarkable interest in products with an important economic value. However the enormous utility and possible applications of this technique will make it affordable for most laboratories in the near future. The real-time PCR described in this work could be used to detect minimal amounts of adulteration in milk mixtures and also could be used in inspection programs to enforce labeling regulation of milk and other dairy products.

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