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Quantitation of Mule Duck in Goose Foie Gras Using TaqMan Real-Time Polymerase Chain Reaction

MIGUEL A. RODRÍGUEZ, TERESA GARCÍA,* ISABEL GONZÁLEZ, LUIS ASENSIO, PABLO E. HERNÁNDEZ, AND ROSARIO MARTÍN

Departamento de Nutrición, Bromatología y Tecnología de los Alimentos, Facultad de Veterinaria, Universidad Complutense, 28040 Madrid, Spain

A real-time quantitative Polymerase Chain Reaction (PCR) method has been developed for the quantitation of mule duck (*Anas platyrhynchos* × *Cairina moschata*) in binary duck/goose foie gras mixtures. The method combines the use of real-time PCR with duck-specific and endogenous control "duck + goose" primers to measure duck content and total foie gras content, respectively. Both PCR systems (duck-specific and duck + goose) were designed on the mitochondrial 12S ribosomal RNA gene (rRNA). The duck-specific system amplifies a 96 bp fragment from duck DNA, whereas the duck + goose system amplifies a 120 bp fragment from duck and goose DNA. The method measures PCR product accumulation through a FAM-labeled fluorogenic probe (TaqMan). The C_t (threshold cycle) values obtained from the duck + goose system are used to normalize the ones obtained from the duck-specific system. Analysis of experimental duck/goose foie gras binary mixtures demonstrated the suitability of the assay for the detection and quantitation of duck in the range of 1−25%. This genetic marker can be very useful to avoid mislabeling or fraudulent species substitution of goose by duck in foie gras.

KEYWORDS: Species identification; 12S rRNA; foie gras; real-time PCR

INTRODUCTION

Within the great variety of foie gras products that can be purchased at the marketplace, first-category foie gras products are the most expensive because they can be prepared from only goose or duck foie gras (*1*). In this category "goose or duck whole foie gras", "goose or duck foie gras", "goose or duck lump of foie gras", and "goose and/or duck foie gras parfait" are included. Whole foie gras is made of only whole lobes, whereas foie gras and lump of foie gras are made of different portions of foie gras lobes agglomerated or rebuilt mechanically, respectively. These three products have to be entirely pure, because mixing duck and goose fat livers is not allowed. However, foie gras parfait contains a minimum of 75% of foie gras and is the only first-category foie gras product in which mixing of duck and goose fat livers is allowed. Goose foie gras, due to its high cost, popularity, and demand, is the most susceptible to substitution using liver or meat from less valuable animal species.

There are no official methods to determine purity or quality of foie gras, and only French legislation (*1*) is used as a reference in all of the European countries that lack specific regulations for this kind of products.

Morphological attributes and sensory differences are commonly used for species identification in foie gras when whole livers or whole lobes are available (*2*, *3*). However, for processed, minced foie gras products, where anatomical features are lost, the accurate identification becomes progressively more difficult, and opportunities for substitution increase, especially when the products are heated and mixed with spices and other ingredients. Protein-based analytical methods for differentiation of goose and mule duck foie gras are scarce and limited to polyacrylamide disc-gel electrophoresis (*4*) and immunological methods (*5*, *6*). The main limitation of electrophoretic methods is that the protein profile of a single species produces a complex banding pattern, and even small amounts of protein from other species will often overlap the species-specific bands, making interpretation of the resulting profile equivocal. Regarding immunoassays, their main drawback is that heat processing may alter the species-specific epitopes (*7*), and there are no references about the use of immunoassays against thermostable proteins to identify duck in foie gras products.

In comparison with proteins, DNA-based methods have proved to be more reliable because of the stability of DNA under the conditions associated with the high temperatures, pressures, and chemical treatments used in the preparation of some food products.

Conventional Polymerase Chain Reaction (PCR) techniques allow the qualitative detection of different animal species in an admixture, but they are not appropriate to achieve the quantitation of species in a product. DNA-based quantitative methods for the detection in meat or meat mixtures are either based on

* Corresponding author (telephone 34-913943747; fax 34-913943743; e-mail tgarcia@vet.ucm.es).

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the quantitative competitive PCR (*8*, *9*), densitometry (*10*), or real-time PCR (*11*−*13*).

The TaqMan quantitative PCR procedure (*14*) is based on the use of a fluorogenic probe that hybridizes within the target sequence bound by usual PCR primers. The probe is labeled with a fluorescent reporter dye on the 5′ end and with a fluorescent quencher dye on the 3′ end. Due to the closeness of the quencher to the reporter, the reporter fluorescence is suppressed. During PCR, the 5′ to 3′ exonuclease activity of *Taq* DNA polymerase degrades the hybridized probe and separates the two dyes. The resulting increase of fluorescence is proportional to the amount of specific PCR products. Moreover, the measurement of fluorescence throughout the reaction by a fluorometer 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. Thus, there is a reduced potential for contamination of the PCR mixture with target DNA because the reaction tubes remain closed throughout the assay.

This paper reports the development of a fluorogenic PCR-based assay to quantify the amount of mule duck (*Anas platyrhynchos* × *Cairina moschata*) in duck−goose binary foie gras mixtures.

MATERIALS AND METHODS

Sample Selection. Whole duck and goose raw fat livers (foie gras), provided by Martiko (Navarra, Spain), were anatomically identified in our laboratory and used as reference samples for the validation of the method. Goose foie gras is bigger than duck foie gras, with a weight of ∼750 g. Both lobes are quite similar in size, and its color is rosy. On the contrary, duck foie gras is triangular in shape, with one of the lobes bigger than the other, and its color is yellow (*3*).

Raw and pasteurized goose and mule duck whole foie gras were provided by Antonio de Miguel (Madrid, Spain). Pasteurized first-category foie gras samples were provided by Imperia foie gras (Gerona, Spain), and commercial sterilized ones were purchased at local delicatessen markets. Seven different commercial brands of foie gras were included in the sampling. Standard commercial pasteurization treatment includes heating at 80 °C for 90 min, whereas sterilization treatments consist of heating at 102−115 °C for 60−75 min.

Samples analyzed included raw whole mule duck and goose foie gras (7 of each species), pasteurized mule duck and goose foie gras (6 of each species), sterilized mule duck and goose foie gras (7 of each species), raw chicken and pork livers (10 of each species), and raw turkey, beef, sheep, and goat muscles (10 of each species). Also, 10 different chicken, turkey, pork, beef, sheep, and goat samples (5 g of each) were pasteurized at 65 °C for 30 min, and 5 g of the same samples were sterilized at 121 °C for 20 min before analysis. Samples from chicken, pork, turkey, beef, sheep, and goat were obtained from local slaughterhouses and markets. All samples were transported to the laboratory under refrigeration, and they were processed immediately or stored frozen at −85 °C until used.

To prepare binary foie gras mixtures (duck in goose), raw foie gras from goose and mule duck were used. Besides, a binary pasteurized foie gras mixture using raw goose foie gras and commercial pasteurized duck foie gras and a binary sterilized foie gras mixture using commercial sterilized duck foie gras and raw goose foie gras were also prepared.

Three different percentages, 1, 10, and 25% (w/w), of duck foie gras were prepared for each duck−goose binary mixture (either raw, pasteurized, or sterilized). Mixtures were made in a final weight of 100 g, using a blender (Sunbeam Oster, Boca Raton, FL).

DNA Extraction. DNA was extracted using the Wizard DNA Cleanup system (Promega, Madison, WI), as described by Rodríguez et al. (*15*). Three separate extractions of each percentage (1, 10, and 25%) were prepared for all of the binary mixtures and were analyzed in this work. DNA concentration was estimated by UV absorption spectrophotometry at a wavelength of 260 nm (*16*).

Construction of a Plasmid DNA for the Standard Curve. In a previous work (*15*), primers *12SFW* (5′-CCACCTAGAGGAGCCT-GTTCT(AG)TAAT-3′) and *12SREV* (5′-TCCGGTACACTTACCT-TGTTACGACTT-3′) were designed for the amplification of a 394 bp fragment in the 12S rRNA gene of mule duck DNA. This duck-specific fragment was amplified, purified, and ligated into the plasmid pGEM-T Easy, using pGEM-T Easy Vector System II (Promega).

Plasmid DNA containing the duck-specific 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 transillumination and analyzed using a Geldoc 1000 UV Fluorescent Gel Documentation System-PC (Bio-Rad Laboratories, Hercules, CA). Plasmid DNA concentration was estimated by absorbance at 260 nm.

Ten-fold dilution series of this plasmid starting from 100 ng of DNA were used as standard curve in real-time PCR, either in the duck-specific or in the endogenous control (duck + goose) systems.

Oligonucleotide Primers and Probes. The primers and fluorogenic probe used in real-time PCR were designed using Primer Express 2.0 software (Perkin-Elmer/Applied Biosystems Division, Foster City, CA). For that purpose, sequences of the 12S rRNA gene from goose (accession number AJ583550) and mule duck (AJ583548), obtained in a previous work (*15*), were aligned and compared, together with others available in the EMBL database for duck (U59666), chicken (X52392), pork (AJ002189), turkey (U83741), beef (J01394), sheep (NC_001941), and goat (M55541).

Two sets of primers and a common fluorogenic probe (12SPROBE, 5′-ATACCGCCCGTCACCCTCCTCAIAAG-3′) were designed to hybridize in the 12S rRNA gene. One set of primers (*12STAQMANFW*, 5′-AAGCCGGCCCTAGGGC-3′; *12STAQMAND*: 5′-TTACCTCAT-CTTTGGCATTCACG-3′) was designed for the specific detection of mule duck (duck-specific PCR system) in duck−goose binary foie gras mixtures. The other set (*12STAQMANFW*, 5′-AAGCCGGCCCTAGGGC-3′; *12STAQMANREV*, 5′-TCCGGTACACTTACCTTGTTACGACTT-3′) was used as endogenous control for the detection of duck and goose DNA (duck + goose PCR system). As a target site for the specific detection of mule duck, a 96 bp fragment of the 12S rRNA gene was selected. The target site for the duck + goose PCR system consisted of a 120 bp fragment of the same gene. Results obtained from the duck + goose PCR system were used to normalize those obtained from the duck-specific system as described below.

The TaqMan probe was designed to hybridize in both PCR systems (duck-specific and duck + goose) 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-carboxytetramethylrhodamine (TAMRA) fluorescent quencher dye on the 3′ end.

5′ Nuclease PCR Conditions. The 5′ nuclease PCR with a fluorogenic probe was run under generic cycling conditions, and so required the optimization of primer concentration to take into account real differences in primer melting temperature. Different forward and reverse primer concentrations and also different probe concentrations were evaluated to ascertain the effect on C_t and endpoint fluorescent values. The most efficient concentrations, giving a high endpoint fluorescence and a low C_t (data not shown), were 50 nM forward primer (*12STAQMANFW*), 900 nM reverse primers (*12STAQMANREV* or *12STAQMAND*), 150 nM probe (*12SPROBE*), 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 optical adhesive cover and were run with the ABI Prism 7700 sequence detection system (Applied Biosystems) at the Centro de Genómica y Proteómica (Facultad de Farmacia, UCM) with the following program: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C, and 1 min at 60 °C. The same program was used to amplify either the duck-specific or the endogenous control (duck + goose) PCR fragments. Unless otherwise indicated, all real-time PCR reactions were carried out in duplicate.

Data Analysis. During PCR cycling in the ABI Prism 7700, the Sequence Detector software (version 1.7, Applied Biosystems) is able to determine the contributions of each component dye spectra by means of a multicomponenting algorithm. Briefly, a normalized reporter (R_n) value is defined for each reaction tube and ΔR_n, an indication of the magnitude of signal generated by the PCR, is calculated. That value reflects the quantity of fluorescent probes degraded by the 5′ exonuclease activity of the polymerase and fits an exponential function generating a real-time amplification plot for each well. The C_t value is the cycle number at which the amplification plot crosses the threshold. C_t is reported as the fractional cycle number, reflecting a positive result. The threshold is set at 10 times the standard deviation of the mean baseline emission calculated between the 3rd and 15th cycles. In our work, the threshold was set at 0.03 of fluorescence with a baseline range from 3 to 15.

The amount of duck DNA in an unknown sample is measured by interpolation from a standard curve of C_t values generated from known starting DNA concentrations (plasmid duck DNA). A comparison of the cycle number at which endogenous and duck-specific PCR products are first detected, in combination with the use of reference standards of known duck content (C_ts), is used as the basis of determining the percentage of duck foie gras in a sample. Total DNA quantity in duck/goose mixtures was determined by reporting the C_t value in the duck + goose system (C_td+g), and mule duck DNA quantity was fixed by reporting the C_t value in the duck-specific system (C_td). Thus, the C_t corresponding to the percentage of mule duck DNA of an unknown sample ($C_t$ps) was determined as being the ratio of duck-specific to total DNA threshold cycles with the equation

$$C_t\text{ps} = C_t\text{s} \times C_t\text{d}/C_t\text{d+g}$$

where C_ts is the threshold cycle average value of the standard (plasmid duck DNA) using 10 ng of DNA in the endogenous (duck + goose) PCR system, C_td is the threshold cycle average value of the unknown sample analyzed with the duck-specific PCR system, C_td+g is the threshold cycle average value of the unknown sample analyzed with the endogenous (duck + goose) PCR system, and $C_t$ps is the threshold cycle value corresponding to the percentage of mule duck DNA of an unknown sample.

Besides, the correlation between the variables, threshold cycle (C_t) and concentration ([ ]) is semilogarithmic

$$C_t = b \ln [\,] + a$$

where b is the slope and a is the intercept.

The linearity of the model was verified by analysis of variance (ANOVA): the F value expresses statistically the regression, the coefficient of correlation, r, expresses statistically the correlation between test results obtained (C_t values) and duck concentration in the mixtures, whereas the coefficient of determination, r^2, expresses quantitatively that correlation.

The following parameters were determined for studying the sensitivity of the real-time method (*17*): Cochran's test, which determined whether the variances of the responses obtained for each concentration of duck in the mixtures are homogeneous, for a 0.05 significance level; calibration sensitivity, which corresponds to the slope (as it is a linear model); analytical sensitivity, which is the relationship between the randomness of the test results and the modifications due to the differences in concentration in the mixture and is calculated as a quotient between the calibration sensitivity and mean standard deviation for all concentrations; and 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 and represents the least difference in concentration of duck DNA in the mixture that can be quantified by the analytical method with a significant level. Analytical sensitivity and discriminating capacity are calculated from an average variance of the response $S^2comb.$

$$S^2comb. = \left(\sum(\varphi_i \times S_i^2)\right)\Big/\left(\sum \varphi_i\right)$$

where S_i^2 is the variance of the responses of a certain concentration of the mixture i and φ_i is the degrees of freedom of these responses.

To carry out the validation of the real-time PCR technique developed in this work, three separate DNA extractions of each percentage of duck in the duck−goose foie gras mixtures were assayed in four different days, using two replicates of each sample.

RESULTS AND DISCUSSION

Real-Time PCR System Setup. The primary objective of the PCR system setup was to establish a marker adequate for foie gras quantitation. As target gene for real-time PCR, a fragment of the mitochondrial 12S rRNA gene was selected. The advantage of mitochondrial-based DNA analyses derives from the fact that there are many mitochondria per cell and many mitocondrial DNA molecules within each mitochondrion, making mitochondrial DNA a naturally amplified source of genetic variation (*18*). Besides, the 12S rRNA gene has an acceptable length and an adequate grade of mutation, which can be used to better define species differences, even in closely related species (*19*), and there are various sequences available in the databases such as duck (U59666), chicken (X52392), pork (AJ002189), turkey (U83741), beef (J01394), sheep (NC_001941), and goat (M55541).

To produce a robust method for quantitative foie gras speciation, duck-specific and endogenous control (duck + goose) primers were used in a real-time PCR assay. A major advantage of using an endogenous control (duck + goose PCR system) is that factors affecting amplification such as inhibition, degradation state, and quality of the DNA recovered from a sample can be taken into account by allowing comparison of its amplification response with that of reference standards. This is important given the variable condition of DNA recovered from food samples, which can result in unpredictable amplification.

The target site for the specific detection of mule duck in the real-time PCR assay consisted of a 96 bp fragment of the 12S rRNA gene, and the target site for the endogenous control (duck + goose) PCR detection system was a 120 bp fragment of the same gene. The small length of the fragments amplified facilitates PCR amplification and mule duck quantification even in products submitted to severe heat treatments, where the DNA might be highly sheared. The endogenous control was designed to amplify DNA from duck and goose and produced a similar response regardless of the species content for the samples under investigation. Both PCR systems used a common forward primer (*12STAQMANFW*) and a common probe (*12SPROBE*). However, they differ in the reverse primers (*12STAQMAND* in the duck-specific PCR system and *12STAQMANREV* in the duck + goose PCR system) (**Figure 1**). Ten-fold dilution series of a plasmid duck DNA were used to build a calibration curve in both real-time PCR systems (duck-specific and duck + goose) and used as standards to ensure the reproducibility of the data obtained.

Specificity. The duck-specific and the endogenous control (duck + goose) PCR systems were tested for their selectivity and cross-reactivity to different animal species. The duck-specific system amplified a 96 bp fragment from duck DNA (C_t value of 17.2 ± 0.34), whereas no amplification was obtained from chicken, pork, turkey, beef, sheep, goat, and goose DNA (**Table 1**). The duck + goose system amplified a 120 bp fragment from duck and goose DNA (C_t of 18.7±0.22 in mule duck and 18.9 ± 0.19 in goose DNA), whereas no amplification is obtained from chicken, turkey, beef, sheep, goat, and pork DNA. A C_t value of 40.0 is measured if no amplification signal could be detected after 40 cycles (**Table 1**).

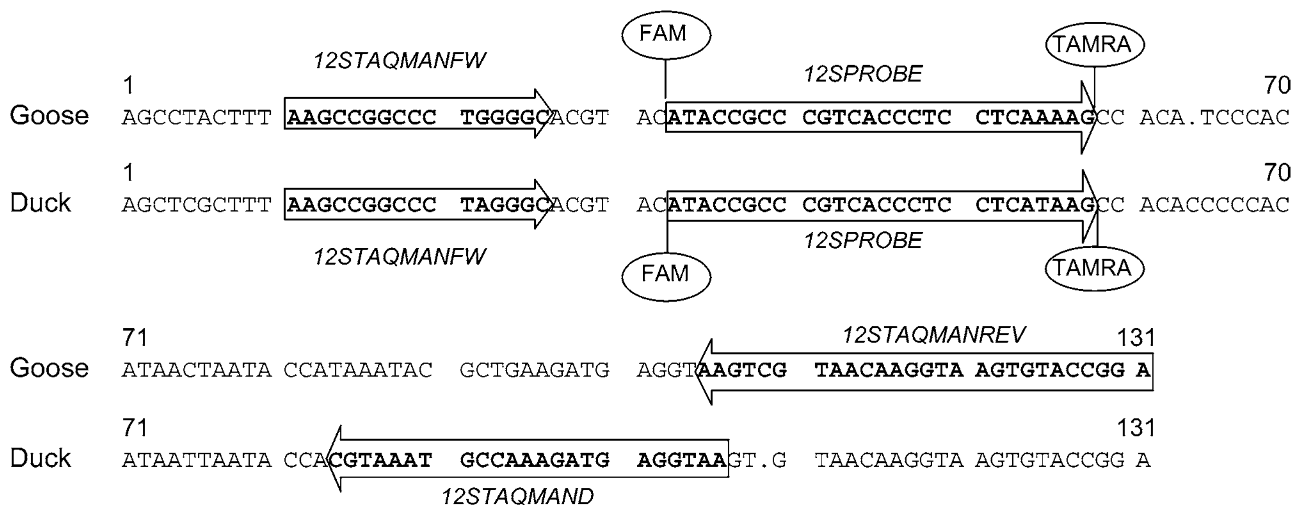

Figure 1. Partial DNA sequence alignment of the 12S PCR products from goose (AJ583550) and mule duck (AJ583548) harboring the designed PCR primers and probe. Primers and probe are boxed in arrows pointing in the 5′ to 3′ direction. The names of the primers and dual-labeled fluorescent probe are indicated. Dots (.) indicate gaps introduced for best-fit alignment.

Table 1. Specificity of Quantitative Real-Time PCR Systems (C_t Values Obtained from 10 ng of DNA)

species	species (scientific name)	duck-specific PCR system	duck + goose PCR system
mule duck	*Anas platyrhynchos* × *Cairina moschata*	17.2 ± 0.34	18.7 ± 0.22
goose	*Anser anser*	37.1 ± 0.41	18.9 ± 0.19
chicken	*Gallus gallus*	40.0	40.0
turkey	*Meleagris gallopavo*	40.0	40.0
pork	*Sus scrofa*	40.0	40.0
beef	*Bos taurus*	40.0	40.0
sheep	*Ovis aries*	40.0	40.0
goat	*Capra hircus*	40.0	40.0

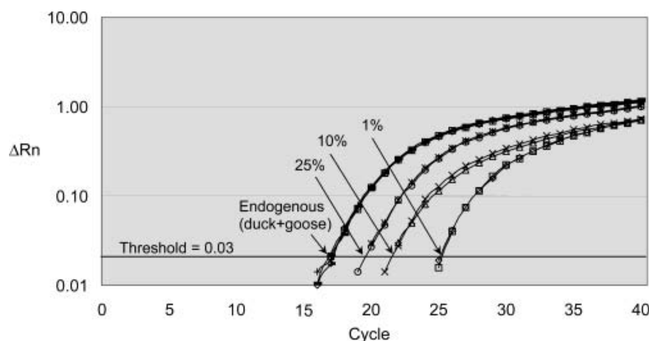

Figure 2. Fluorescent profiles of PCR products amplified in duplicate with both PCR systems [duck-specific and endogenous control (duck + goose)] from a duck/goose foie gras mixture (25, 10, and 1% duck) plotted against cycle number.

Sensitivity (Detection Limit). Six 10-fold dilution series of plasmid duck DNA starting from 100 ng were prepared and used as standard curve in real-time PCR, considering 10 ng of DNA in the PCR system as 100%. The detection limit for duck-specific PCR was 0.001 ng of DNA, which corresponds to 0.01% duck DNA.

Also, 10 ng of DNA obtained from three different percentages of duck foie gras (25, 10, and 1%) in duck−goose binary mixtures was analyzed to study the capability of the assay for quantitation of duck DNA (**Figure 2**). As expected, detection of duck DNA in goose foie gras was achieved even in mixtures containing 1% of duck.

Linearity. To test the linearity, C_t values were plotted versus the logarithm of the DNA concentrations (**Figure 3**), and because the *P* value in the ANOVA table is <0.01, there is a statistically significant relationship between the variables (C_t and logarithm of DNA concentrations) at the 99% confidence level in all of the foie gras mixtures analyzed. Linearity was observed for the plasmid duck DNA over 6 orders of magnitude (**Figure 3A**). The correlation between the two variables, C_t and logarithm of duck concentration, using the plasmid duck DNA as standard, gives a determination coefficient value of 0.9941, which indicates that 99.41% of the variation in the *y*-axis is explained by variation in the *x*-axis (**Figure 3A**).

In this work, different calibration curves were performed for each of the binary mixtures prepared (raw duck/goose foie gras, pasteurized duck/goose foie gras, and sterilized duck/goose foie gras). Panels **B**−**D** of **Figure 3** show the regression line parameters and sensitivity parameters of the mixtures. The method can be used to estimate the duck content in foie gras, as long as we know how a sample was treated technologically during the food production. C_t values and duck concentration (percent) are related by the equation $C_t = -2.71 \log [\text{duck } (\%)] + 22.49$ ($r^2 = 0.8798$) for raw duck/goose foie gras mixtures. For pasteurized mixtures the equation was $C_t = -1.64 \log [\text{duck } (\%)] + 20.10$ ($r^2 = 0.8871$), and for the sterilized foie gras mixtures the equation was $C_t = -2.95 \log [\text{duck } (\%)] + 22.81$ ($r^2 = 0.9319$). The high sensitivity of the real-time PCR method developed herein made it possible to quantify duck content in the range between 25 and 1% in all foie gras mixtures.

We conclude that real-time PCR enables a desirable and necessary monitoring of foie gras products and allows detection of low levels of contamination and admixture. The sensitive fluorescence detection of the 7700 system allows the threshold cycle to be observed when PCR amplification is still in the exponential phase. This is the main reason C_t is a more reliable measure of starting DNA copy number than an endpoint measurement of the amount of accumulated PCR product. During the exponential phase, none of the reaction components is limiting: as a result, C_t values are very reproducible for reactions with the same starting copy number. This leads to greatly improved precision in the quantitation of DNA (*20*). On the contrary, the amount of PCR product observed at the end of the reaction is very sensitive to slight variations in reaction components. This is because endpoint measurements

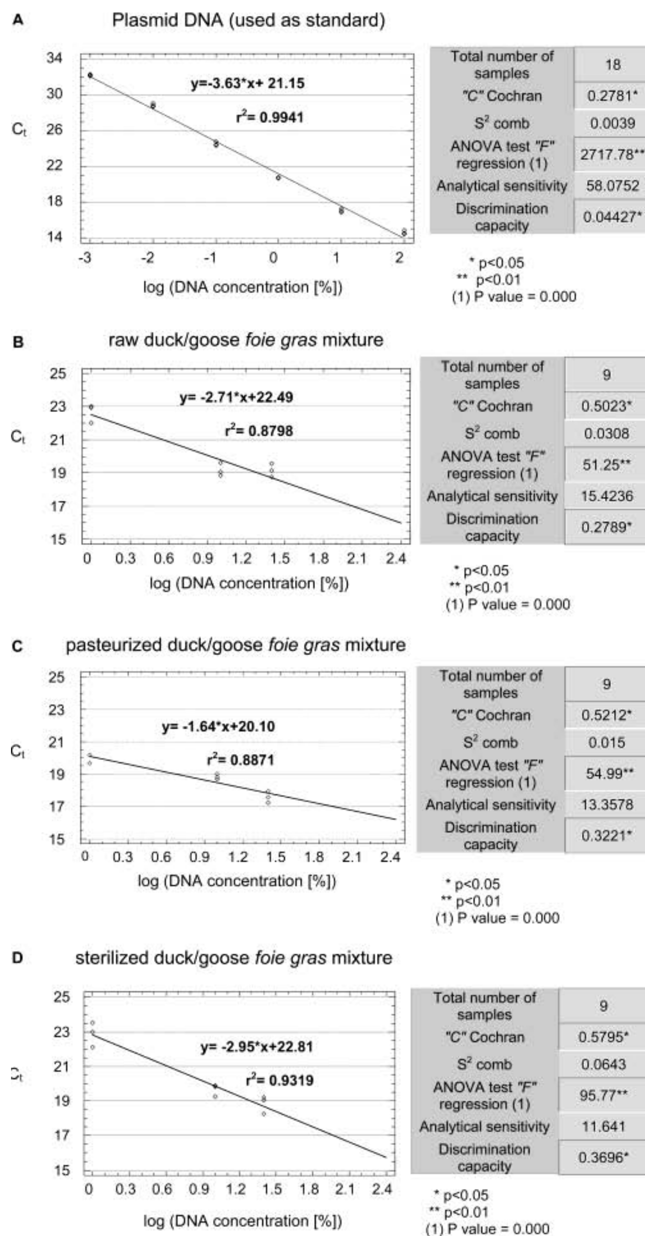

Figure 3. (**A**) Linearity test, regression line parameters, and sensitivity parameters of the duck-specific TaqMan PCR system using 10-fold dilution series of plasmid duck DNA (from 100 to 0.001 ng) as standard. (**B**–**D**) Linearity test, regression line parameters, and sensitivity parameters of the duck-specific Taqman system using three different extractions of three different duck percentages (25, 10, and 1%) of each binary mixture: (**B**) raw duck/goose foie gras mixture; (**C**) pasteurized duck/goose foie gras mixture; (**D**) sterilized duck/goose foie gras mixture.

are generally made when the reaction is beyond the exponential phase and a slight difference in a limiting component can have a drastic effect on the final amount of product.

Real-time quantitative Polymerase Chain Reaction is a powerful technology that is highly accurate, simple, and relatively fast and results in elevated sensitivity and specificity (*21*). Nowadays, due to its high cost, it has only a remarkable interest in products with an important economic value, such as foie gras. However, the enormous utility and possible applications of the technique will make it affordable for most laboratories in the near future. The real-time PCR system described in this work could be used to detect minimal amounts of duck in different mixtures and also could be used in inspection programs to enforce labeling regulation of foie gras and related products.

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