COMPARISON OF REAL-TIME PCR METHODS FOR QUANTIFICATION OF EUROPEAN HAKE (*Merluccius merluccius*) IN PROCESSED FOOD SAMPLES

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ABSTRACT

The quantification of species in commercial products is limited by analytical shortcomings, as most of them provide semiquantitative results. An exception is real-time PCR, which can provide quantitative results using hybridization probes. In the present work, this technique has been applied to the absolute, absolute-relative and relative quantification of the most valued hake species in European markets, *Merluccius merluccius* (European Hake). The best quantification results for this species in binary mixtures with non-target species (*Merluccius capensis*) and using a species-specific real-time PCR MMER_VIC system was achieved using a relative quantification approach (MLL as reference system). Absolute quantification using the MLL nuclear system has been demonstrated as appropriate for the quantification of the *Merluccius* genus in food model samples. This study reveals the impact of different reference systems (MLL and HAKE) in the absolute-relative and relative quantification approaches, showing that the nuclear MLL system performed better than the mitochondrial HAKE system.

Keywords

Species quantification, real-time PCR, European hake, *Merluccius merluccius*, TaqMan system

1. Introduction

The authenticity of seafood products has become newsworthy as various studies and reports have revealed a high degree of mislabelling. One particular aspect of mislabelling is related to processed food, where several ingredients may be present. In this case, ingredients and their quantities should be declared if the ingredient appears in the name of the food, is emphasized on the label, or is usually associated with that name by consumers (EU 1169/2011 and EU1379/2013).

Species identification techniques are well established for most fish, shellfish and molluscs, including a wide range of DNA-based techniques such as species-specific PCR, PCR-RFLPs, SSCPs, PCR-ELISA, FINS, and real-time PCR. (Rasmussen & Morrissey, 2008; Teletchea, 2009). However, there are fewer techniques available for the quantification of species and, with the exception of competitive and real-time PCR, these methods usually give semiquantitative results (Wolf & Lüthy 2001; Marcelino, Guimarães & De-Barros, 2008).

Real-time PCR relies on fluorescence dyes or probes to detect the progress of amplification. Furthermore, the specificity of the reaction increases if a fluorescent hybridization probe complementary to the target sequence is used (Livak, Flood, Marmaro, Giusti & Deetz, 1995; Heid, Stevens, Livak & Williams, 1996; Kutyavin et al. 2000; Lim, Gu Shin, Lee & Hwang, 2011). The multiplexing capability of real-time PCR, using different fluorochromes in the same reaction, allows the detection of DNA from the different species that may be present in a sample (Rasmussen & Morrissey, 2011; Salihah, Hossain, Lubis & Ahmed, 2016). Finally, while real-time PCR provides a molecular tool for the identification and quantification of species, the better-known application of real-time PCR is the detection and quantification of genetically modified organisms (GMO) in food (Engel, Moreano, Ehlert &, Busch, 2006).

TaqMan assays using real-time PCR enable three main types of quantification: absolute, relative and absolute-relative. Absolute quantification refers to the determination of an amount of DNA, either in copy number, concentration or mass (Burns, Valdivia & Harris, 2004; Peirson, Butler & Foster, 2003). In this case, a calibration curve that relates known amounts of template DNA to their corresponding fluorescence signals is required. In the case of relative quantification, a standard curve is not necessary, since quantity is expressed as a percentage of the fluorescence signal of a reference sample (Pfaffl, 2001). The absolute-relative quantification is based on the absolute quantification of a specific DNA amount (i.e., fish species) using a specific system related to the absolute DNA amount quantified using a reference system (i.e., amount of fish DNA).

There are examples in the literature where the quantification of meat species by absolute quantification is employed (López-Andreo, Lugo, Garrido-Pertierra, Prieto & Puyet, 2005; Krcmar & Rencova, 2005; Tasara, Schumacher & Stephan, 2005; Lopparelli,

Cardazzo, Balzan, Giaccone & Novelli, 2007; Rodríguez, García, González, Asensio, Hernández & Martín, 2004 and 2005; Köppel, Ruf, Zimmerli & Breitenmoser, 2008; Köppel, Zimmerli & Breitenmoser, 2009; Fajardo et al. 2008).

In contrast, reports of species quantification in fish products using real-time PCR are still rare, and only a few works have been published, mainly focused on relative quantification in food model samples (Hird et al. 2005; López & Pardo 2005; Nagase et al. 2010; Benedetto, Abete, & Squadrone 2011).

Seafood processed products in which several ingredients may be present include surimi, fish fingers, fish balls or baby foods. Among them, baby foods are among the most highly processed. Processing in baby food includes cooking, grinding and sterilization, and therefore DNA degradation is a challenge not only for species quantification but also for identification. Usually, these foods include a vegetal and a protein source. The position of the name of the protein source in the label determines the minimum amount present, i.e., if the fish name is not first, as in “Vegetables with hake”, at least 8% of hake by weight percent of the total product should be present (Directive 2006/125/EC).

The first objective of this work is to compare various quantification approaches using real-time PCR: absolute, absolute-relative and relative quantification. We have chosen hake species, in particular, European hake (*Merluccius merluccius*), as a target species since it is usually used in baby foods and is commercially very important in the European Union (Lloris, Matallanas & Oliver, 2005); there are also a variety of similar species that can be used as substitutes.

The first objective was to test the three above-mentioned real-time PCR quantification approaches using different model samples containing hake species, such as DNA extracts and lyophilized muscle.

The second objective was to quantify the *Merluccius* genus using real-time PCR in a food model sample resembling a complex matrix such as the one that may be present in baby food.

2. Materials and Methods

2.1 Hake species and binary mixtures

European hake (*Merluccius merluccius,* abbreviated as Mmer) was used as the target species, whereas Shallow-water Cape hake (*Merluccius capensis*, abbreviated as Mcap) and Deep-water Cape hake (*Merluccius paradoxus,* abbreviate as Mpar) were the non-target species. Specimens (n=5) of the three species were purchased in a local market (Vigo, Spain) and analysed by FINS (forensically informative nucleotide sequencing) to confirm the correct taxonomical assignment.

2.1.1 Binary mixtures of DNA extracts

Approx. 150-250 g of the white muscle of one specimen of each species was minced individually, and 1-2 g was digested in triplicate as described in Sánchez et al. (2009). After digestion, DNA was isolated employing the Wizard DNA Clean-Up System kit (Promega, Spain) following the manufacturer's instructions. The eluted DNA was quantified by the Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen) for dsDNA quantification in a VersaFluor Fluorimeter (Bio-Rad). Up to five specimens per species were used separately to obtain the binary mixtures, and the DNA concentration was adjusted to 25 ng/µL; finally, binary mixtures of DNA extracts were prepared as presented in Table 1.

2.1.2 Binary mixtures of lyophilized muscle

The remaining minced muscle of each specimen of each species (148-248 g) was lyophilized and ground in a domestic grinder for 5 min, followed by 5 min in a ball mill (Fritsch pulverisette) at medium speed. The resulting product was sieved through a 1-mm mesh to obtain homogeneous lyophilized muscle.

Table 1 shows the amounts of lyophilized muscle used to prepare model mixtures; 0.07 g of each mixture was digested following the method of Sánchez et al. (2009) with a slight modification that includes a second addition of lysis buffer, guanidium thiocyanate and proteinase K (≥ 20 Unit/mg). After digestion, DNA was isolated and quantified, and the DNA concentration was adjusted to 25 ng/µL.

2.2 Food model samples for Merluccius spp. quantification

Food model samples were prepared to simulate a commercial baby food product, including cooking and sterilization steps. Two types of food model samples were prepared: standard food samples for the standard calibration curve and test food samples.

2.2.1 Standard food samples

To prepare the standard calibration curve (Figure S2), different amounts of boiled Mcap white muscle (100%, 75%, 50%, 25%, 10%, 5% and 1%) were mixed with different amounts (Table 1) of a complex mixture of the following ingredients: rice (50.2 g),

potatoes (117 g), celery (5.6 g), onion (17.4 g), olive oil (4 g), sunflower oil (3 g), water (250 mL) and salt (0.5 g). Finally, after mixing the hake and matrix, the resulting mixtures were autoclaved at 121 ºC for 20 min.

2.2.2 Test food samples

Three hake species, Mcap, Mmer and Mhub (*Merluccius hubbsi*) were used. The preparation process was the same as described for the standard food samples. Different amounts of white muscle (8, 12 or 20%) of the hake species were mixed with different amounts of the complex mixture (see the composition in 2.2.1). The resulting test food samples are shown in Table S1.

Once the standard and test food samples were prepared, 0.25 g of each were digested in triplicate, and DNA was extracted as described above.

2.3. TaqMan real-time PCR system design and reaction conditions

Three real-time PCR systems were designed and tested for the analysis of quantification approaches. Absolute quantification was tested using a mitochondrial DNA species-specific system (MMER_VIC) and two *Merluccius* genus systems, one mitochondrial (HAKE) and one nuclear (MLL). Absolute-relative quantification was tested with MMER-VIC and HAKE, and relative quantification with MMER-VIC and MLL.

The species-specific real-time PCR MMER_VIC system, designed for the mitochondrial DNA control region in a previous work (Sánchez et al. 2009) was used for the absolute, absolute-relative and relative quantification of Mmer in binary mixture samples.

The primers and probe sequence are as follows: MMERCR4F: CATTYTCYTATATTAACCATTCAGGCAAT, MMERCR5R: TGGGTTGACAGGTTAAATACGAGTAA and MMERCR6TP: VIC-AGAACATTAACATAAAATTAAACT-MGB.

Likewise, the reference system HAKE was designed in the mitochondrial control region based on a consensus sequence for the genus *Merluccius* and used in absolute-relative quantification in binary mixtures and the absolute quantification in food model samples. Primers and probe sequences are as follows: HAKE_F: CAAGGGTACTAMTYGAAGAYTCACC, HAKE_R: STYTATGGACCTGAAGCTAGGCA and HAKE_P: TGAATTATTCCTGGCATC.

The MLL reference system was used for relative quantification in binary mixture samples of Mmer and for absolute quantification of *Merluccius genus* in food model samples. The system was designed from sequences of the nuclear single copy gene “Mixed Lineage Leukaemia” for the *Merluccius* species and other gadiforms. Primers and probe sequences are as follows: MLL_F: GCCAGCGCCTGTCCC, MLL_R: TTTGCCAGGGTTTCTGTGC and MLL_P: FAM-CACCACAGCCACCT-MGB.

PCR reactions were performed on a total volume of 20 μL in a MicroAmpTM fast optical 96-well reaction plate (Applied Biosystems) and covered with MicroAmpTM optical adhesive film (Applied Biosystems). Each reaction contained 10 μL of TaqMan® Fast Universal PCR Master Mix no UNG Amperase (2X) and 2 μL of DNA. The final probe concentration was 225 nM for all systems, and forward/reverse final concentrations were 900/900 nM for MMER_VIC and HAKE and 50/300 nM for the MLL system. Reactions were run in triplicate on the ABI 7500 Fast instrument (Applied Biosystems) with the standard thermal cycling protocol: 95 ºC for 10 min, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min.

The reparametrized Richards equation was used to accurately model the fluorescence data versus cycle number of PCR amplification (Sánchez, Vázquez, Quinteiro & Sotelo, 2013):

$$F = \frac{F_m}{\left\{1+\exp\left[\left(\frac{\ln a-\left(\frac{a+1}{a}\right)}{\lambda_c - C^*}\right)\left(C^* - C\right)\right]\right\}^a} \quad [1]$$

where F: Fluorescence; F_m: Maximum fluorescence; C: Amplification cycle number; C^*: Position parameter of Richards equation; a: form parameter of Richards equation and related maximum slope of the fluorescence and λ_c: Lag phase or number of cycles necessary to detect fluorescence in the amplification process.

The lag phase parameter (λ_c) obtained from this equation was used to compare the amplification curves. This parameter was proposed by the authors in a previous work (Sánchez, Vázquez, Quinteiro & Sotelo, 2013) as an alternative to the more subjective Ct (Cycle threshold).

2.4. Absolute and absolute-relative quantification

A MMER_VIC standard quantification curve (Figure S1) was used to relate known Mmer DNA amounts (ng) to their amplification signal (λ_c) in absolute quantification approach. The Mmer DNA amount of an unknown sample is determined using the obtained λ_c

value and by calculating the DNA value from the corresponding linear standard equation. Likewise, HAKE and MLL standard quantification curves were used for the absolute quantification of the genus *Merluccius* in food model samples (Figure S1).

The reproducibility of the systems was tested by performing different standard curve assays with each of the systems, MMER-VIC, HAKE (n=36 and n=33, respectively) and MLL (n= 6) (Figure S1). These tests were evaluated using seven orders of magnitude of concentration (from 100 ng down to 0.0001 ng) in the case of MMER_VIC and HAKE, whereas in the case of MLL these concentrations ranged over five orders of magnitude (from 100 ng down to 0.01 ng). Confidence intervals for these curves were calculated with an α=0.05. The limit of detection (LOD) for the HAKE system was 0.001 ng (100% of all replicates were below λ_c =34), whereas LOD for MMER_VIC and MLL it was 0.01 ng (100% of all replicates were below λ_c =32 and λ_c =40, respectively).

In the absolute-relative quantification approach, the percentage of Mmer DNA is calculated with respect to the total DNA from the *Merluccius* genus species using the HAKE system with the following equation:

$$R(\%) = \frac{\text{ng ADN (MMER\_VIC system)}}{\text{ng ADN (reference system)}} \times 100 \qquad [2]$$

where reference systems are HAKE or MLL.

2.5. Relative quantification

Relative quantification (R_q) was used to quantify the Mmer present in the binary DNA extracts and lyophilized samples using the following equation (Pfaffl, 2001):

$$R_q = \frac{E_t^{\ \Delta\lambda_{c,d}\ c-s}}{E_r^{\ \Delta\lambda_{c,r}\ c-s}} \qquad [3]$$

where E_t is the real-time PCR efficiency of the target system (MMER_VIC); E_r is the real-time PCR efficiency of the reference system (MLL); $\Delta\lambda_{c,t}$ (c-s) is the λ_c deviation of the control-sample of the target system and $\Delta\lambda_{c,r}$ (c-s) is the λ_c deviation of the control-sample of the reference system. The S100 sample was chosen as the control sample (Table 1).

Real-time PCR efficiency was assessed using the following equation:

$$E = 10^{-1/m} \qquad [4]$$

where *m* is the slope of the calibration curve of 10-fold DNA dilutions against the corresponding λ_c value.

2.6. Absolute quantification in a food model

Quantities of the *Merluccius* genus in food model samples were assessed using absolute quantification. In this case, standard quantification curves for HAKE and MLL systems correlating to the natural logarithm of known percentages (Table 1) of hake (in this case the species Mcap) were used in food standard samples against their amplification signal, λ_c (Figure S2). The hake percentage of an unknown sample is obtained by interpolating its λ_c value on the standard curve.

2.7. Accuracy of the quantitative PCR methods

The accuracy of the quantification approach was evaluated as the systematic error (SE) expressed as a percentage of the determined value from the expected value (SE are only absolute values). Means and standard deviation for each quantification approach were calculated (mean ± SD). The means and SE were compared using Student´s t-test, and the corresponding P-value was calculated (Milton, 2001).

3. Results and Discussion

Three main real-time PCR quantification approaches may be used for detecting the amount of different species in a mixture. We have compared these quantification approaches, namely, absolute, absolute-relative and relative quantification, using binary mixtures of DNA extracts and lyophilized muscle and food standard samples. Finally, a validation test was performed with a simulated baby food (test food sample) containing hake and typical ingredients, which were subjected to a thermal process resembling those employed by the industry.

3.1. Absolute quantification

Binary model mixtures of DNA extracts and lyophilized muscle were used for absolute quantification with the MMER_VIC system, using Mmer as the target species and Mpar and Mcap as non-target species.

As can be observed in Figure 1A and 1B, in both types of sample (DNA extracts and lyophilized muscle) and when mixed with both non-target species (Mcap and Mpar), the SE difference (%) between expected and detected DNA varied and ranged from 0% up to 28.0%. The SE differed depending on the non-target species in the case of DNA extract mixtures, while the presence of Mcap produced an overestimation of Mmer, and the presence of Mpar produced the opposite, a subestimation of Mmer. The quantity of the target species does not seem to affect the level of SE.

The average SE by species (mean ± SD) was the same: 12% ± 8.20 when Mcap was the non-target species and 13% ± 6.97 when Mpar was present. Likewise, no significant differences in SEs ($P > 0.001$) were found between binary mixtures of DNA extracts (14% ± 9.37) and lyophilized muscle samples (11% ± 5.97).

These values are similar to those reported by other authors for similar model mixtures of meat species (Koppel et al. 2008, 2009).

3.2. Absolute-relative quantification

3.2.1. Evaluation of Merluccius genus reference systems

One essential component of the absolute-relative quantification approach is the reference system used. The influence of the type of reference system used may determine the final accuracy of the quantification approach. Two reference systems for the determination of the *Merluccius* genus were investigated: one based on mitochondrial DNA (HAKE system) and the other based on nuclear DNA (MLL system). In both cases, an absolute quantification assay using both DNA extracts and lyophilized muscle mixtures of Mmer and Mcap was employed. Table 2 shows the expected (50 ng of DNA) and detected amounts of *Merluccius* genus with both reference systems. Relatively high SEs were observed for every binary mixture analysed with the HAKE system, from 34% up to 118% in the case of DNA extracts and from 10% up to 150% in the case of lyophilized muscle (Table 2). Moreover, in both DNA extract and lyophilized muscle, the SE increases when the percentage of Mcap increases in the mixture, suggesting a higher efficiency and/or affinity of the HAKE system for Mcap. That is, for the same λ_C value, the corresponding DNA concentration is higher for Mcap species than for the other hake species studied. Consequently, if this system is used as a reference system in absolute-relative quantification, the percentage of Mmer will be underestimated if other hake species, for instance Mcap, are present in the mixture.

The other reference system investigated was the nuclear MLL system (99.66% efficiency). As observed in Table 2, the MLL system detected DNA nanograms for the *Merluccius* genus, which remained almost constant and close to the expected 50 ng. SEs were much lower than those obtained with the HAKE system (Table 2). In this case, significant differences ($P>0.05$) were found among average SE values for HAKE and MLL, for both DNA extracts and lyophilized muscle. Furthermore, MLL SEs do not show an increase when any of the species increased, and therefore no differences in affinity or efficiency are expected with these species.

Previous works (Hird et al. 2005; Prado, Boix & Holst, 2012) have shown that nuclear genes can provide more accurate quantification results than mitochondrial genes, as shown here.

3.2.2. Absolute-relative quantification of Mmer using MMER-VIC/HAKE systems

In absolute-relative quantification, the results obtained with the specific absolute quantification (MMER-VIC) are normalized using a reference system, which is used to determine the quantity of all *Merluccius spp.* genus species. In the present design, a mitochondrial control region DNA reference system (HAKE) was tested.

Figure 1C and 1D show the results obtained with this approach. In this case, higher differences between expected and determined values than those obtained with absolute quantification were obtained, although the efficiencies of both systems were similar (MMER-VIC= 92.71% and HAKE=89.91%).

The results show that Mmer was constantly underestimated, with an SE percentage varying from 19% to 57% (excluding S0 samples).

SE (mean ± SD) between non-target species, Mcap and Mpar, were 37% ± 11.26 and 35% ± 11.12, respectively, and as observed with absolute quantification, no significant differences (P> 0.001) were found.

As for the type of sample, the results were broadly similar for both binary mixtures of DNA extracts and lyophilized muscle samples, with an average SE of 38% ± 9.45 and 34% ± 12.25, respectively (no significant differences P>0.001).

3.2.3. Absolute-relative quantification of Mmer using MMER-VIC/MLL systems

Similar to the previous quantification system, MMER-VIC/HAKE, in the case of absolute-relative quantification using MMER-VIC/MLL, the nuclear reference system MLL (99.66% efficiency) was used to normalize the specific detection of Mmer with *Merluccius* genus quantification.

In this case, only binary mixtures of DNA extracts were used. The results have shown that differences between expected and determined values were lower than those obtained with the MMER-VIC/HAKE; SEs for the absolute-relative quantification with MLL ranged from 5% up to 28% (excluding S0 samples). Figure 2 graphically shows the comparison of both absolute-relative systems. When the MLL system was used with binary mixtures of DNA extracts of Mmer+Mcap, the determined percentages were closer to expected values compared to those obtained with MMER-VIC/HAKE. The SE between expected and determined percentages in these samples, excluding S0 samples, ranged between 5% and 27%, while for the HAKE system, the values were between 23% and 46%. These results highlight the unquestionable need for the adequate selection and testing of reference systems in absolute-relative quantification.

3.3. Relative quantification

Mmer relative quantification (R_q) of a target species, as mentioned in section 2.5, relies on the use of a specific system, MMER_VIC, and a reference system. The reference system MLL was selected due to better performance, as shown in section 3.2.1. Equation 3 was used to determine the percentage of Mmer in binary mixtures of both DNA extracts and lyophilized muscle of

Mmer and Mcap. The efficiency of each system was determined; in the case of the specific system, the efficiency was E_{MMER_VIC}= 1.84 and for the reference system E_{MLL}= 1.99.

The determined percentages are shown in Figure 3, together with expected values for both types of binary mixtures. Low SEs, from 3% to 12%, were obtained for binary mixtures of DNA extracts. Most of the lyophilized muscle samples showed SEs ≤18%, except for the S50 sample, with an SE of 30%. This general reduction in SE, for both DNA extracts and lyophilized muscle samples, was derived from similar λ_c values for all samples with the MLL system (data not shown), and the average λ_c value was 21.99 ± 0.09. This further indicates that the MLL system has a similar affinity for the two species present in the mixture. In general, the determined Mmer content does not deviate drastically from the expected content, especially in the case of mixtures of DNA extract samples.

3.4. Quantification of Merluccius spp. in food model samples

Processed baby foods were selected as +.type of processed food to test some of the quantification approaches analysed. The word “hake” on the label of a processed product is used to indicate that the product should contain a *Merluccius* species as an ingredient (Directive 2006/125/EC).

The quantitative capacity of the two TaqMan systems designed to quantify the *Merluccius* genus, one mitochondrial (HAKE) and one nuclear (MLL), was evaluated in food samples resembling commercial baby food using the absolute quantification approach.

The first question addressed was the impact of using a standard curve with one particular species on the genus quantification results. Figure S2 shows the standard curves for HAKE and MLL systems prepared with standard food samples and containing different percentages of Mcap mixed with a food matrix.

These standard curves were used to determine the amount of *Merluccius* genus present in test food samples, which were prepared in the same way as the standard food samples. For this purpose, various samples with Mcap, Mmer or Mhub (8, 12 and 20%) were prepared.

Table 3 shows that the SE values were generally higher for the HAKE system than the nuclear system MLL, which confirms the previous finding that HAKE systems produce higher SEs than MLL. It can also be observed that the SEs increase with decreasing amounts of muscle; the test food sample including Mcap for both systems indicated the possible interference of the matrix. This finding is quite consistent with that described by Hird et al. (2005) in which the matrix effect did not allow the quantification of target species at percentages below 20%. However, the results presented here show that it is possible to quantify similar percentages of target species present in food model samples with lower SEs (see Table 3) than described by these authors.

A standard curve prepared using Mcap was used to quantify either Mmer, Mhub or Mcap. The results were similar for Mcap and Mmer, showing a low SE when 20% of the Merluccius genus was present in the test food samples, especially when MLL was used. These results are consistent with previous reports (Nagase et al. 2010; Prado, Boix & Holst, 2012), which support the use of particular species to determine the quantities of the genus, in this case, Merluccius spp. These authors employed a binary mixture

composed of a target species at various percentages and one non-target species as a standard curve, obtaining quantification results in accordance with the expected levels.

However, the bias between the determined and expected percentage for Argentine hake (Mhub) is higher than for the other two hake species in both the mitochondrial and nuclear systems. This was an unexpected result considering that similar λ_c values were obtained for the reference specimens analysed for these hake species (n=5 for each species) (Mhub, Mmer and Mcap) in the inclusivity assays (data not shown). Even the variance in λ_c values between Mhub and Mcap were closer ($\Delta\lambda_{c\_HAKE}$: 0.54 and $\Delta\lambda_{c\_MLL}$: 0.25) than between Mcap and Mmer ($\Delta\lambda_{c\_HAKE}$: 1.34 and $\Delta\lambda_{c\_MLL}$: 0.74).

A possible explanation is that the specimen employed to prepare the model mixture belongs to a different haplotype than those used for the design of the HAKE and MLL real-time PCR systems. HAKE and MLL sequence alignments (data not shown) did not show intraspecific differences for Mhub in the probe and primer areas.

4. Conclusions

The use of various quantification approaches may significantly impact quantification results. The lowest SE was found for the quantification of Mmer present in binary mixtures of DNA extracts, with Mcap as non-target species quantified with relative quantification using MLL as a reference system (SE between 2.4-12.4%). The other two quantification approaches used performed similarly: absolute quantification with an SE between 0% and 28%, and absolute-relative quantification with an SE between 5% and 27%. With any of the three methods, the greatest SE obtained was always in the sample with the lowest Mmer amount.

Absolute quantification is thus an alternative if a reference system is not available. Moreover, it is clear that for correct absolute-relative quantification, a reference system with the same efficiency for the different hake species is needed.

In addition, this current paper describes the preliminary study of the use of a universal standard calibration curve for the absolute quantification of different hake species in a food model sample. Successful results were obtained for Mcap and Mmer species quantification when the nuclear MLL system was used. However, the determined percentages obtained for Mhub species were deemed unsatisfactory, since too-high bias was observed.

Conflict of interest statement

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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FIGURE CAPTIONS

Figure 1. Absolute quantification of Mmer with the MMER_VIC system in binary mixtures of DNA extracts and lyophilized samples with two non-target species: Mcap (**A**) and Mpar (**B**).

Absolute-relative quantification of Mmer using the MMER_VIC system as a specific system and HAKE as a reference system in binary mixtures of DNA extracts and lyophilized samples with two non-target species: Mcap (**C**) and Mpar (**D**).

Blue bars: expected amount; red bars: determined amount in DNA extracts samples; green bars: determined amount in lyophilized muscle samples.

Figure 2. Absolute-relative quantification of Mmer in binary mixtures of DNA extract samples of Mmer + Mcap using two reference systems: HAKE and MLL. Blue bars: expected percentage of Mmer. Orange bars: determined percentage of Mmer with MLL system. Yellow bars: determined percentage of Mmer with HAKE system.

Figure 3. Relative quantification (**$\%_R$**) of Mmer in binary mixtures of DNA Mmer + Mcap using reference system MLL. Blue bars: expected percentage of Mmer. Red bars: determined percentage of Mmer in DNA extracts samples. Green bars: determined percentage of Mmer in lyophilized samples.

TABLE CAPTIONS

Table 1. Binary mixtures of DNA extracts, lyophilized muscle and standard food sample composition. Amount in μl is shown for mixtures of DNA extracts, while in the case of lyophilized muscle, quantities in grams of each species are presented. The food standards column shows amounts (g) of cooked hake muscle and food matrix used to prepare samples. Mmer: *Merluccius merluc*cius, No Mmer: *Merluccius capensis* or *Merluccius paradoxus*, Hake: *Merluccius capensis* (in this experiment) and Matrix: common food ingredients (e.g., potatoes, onion); quantities of each ingredient are indicated in section 2.2.1. Mmer/Hake quantities S100=100%, S90=90%, S75=75%, …, S0=0%.

Table 2. Performance evaluation of reference systems for absolute-relative quantification of *Merluccius* sp in binary mixtures (DNA extracts and lyophilized muscle samples) of Mmer and Mcap using HAKE and MLL reference systems.

Table 3. Expected and determined percentages of *Merluccius* sp. in test food samples by absolute quantification with HAKE and MLL systems are shown. SE: systematic error between expected and determined percentages, as well as values for each sample, are included.

Figure 1.

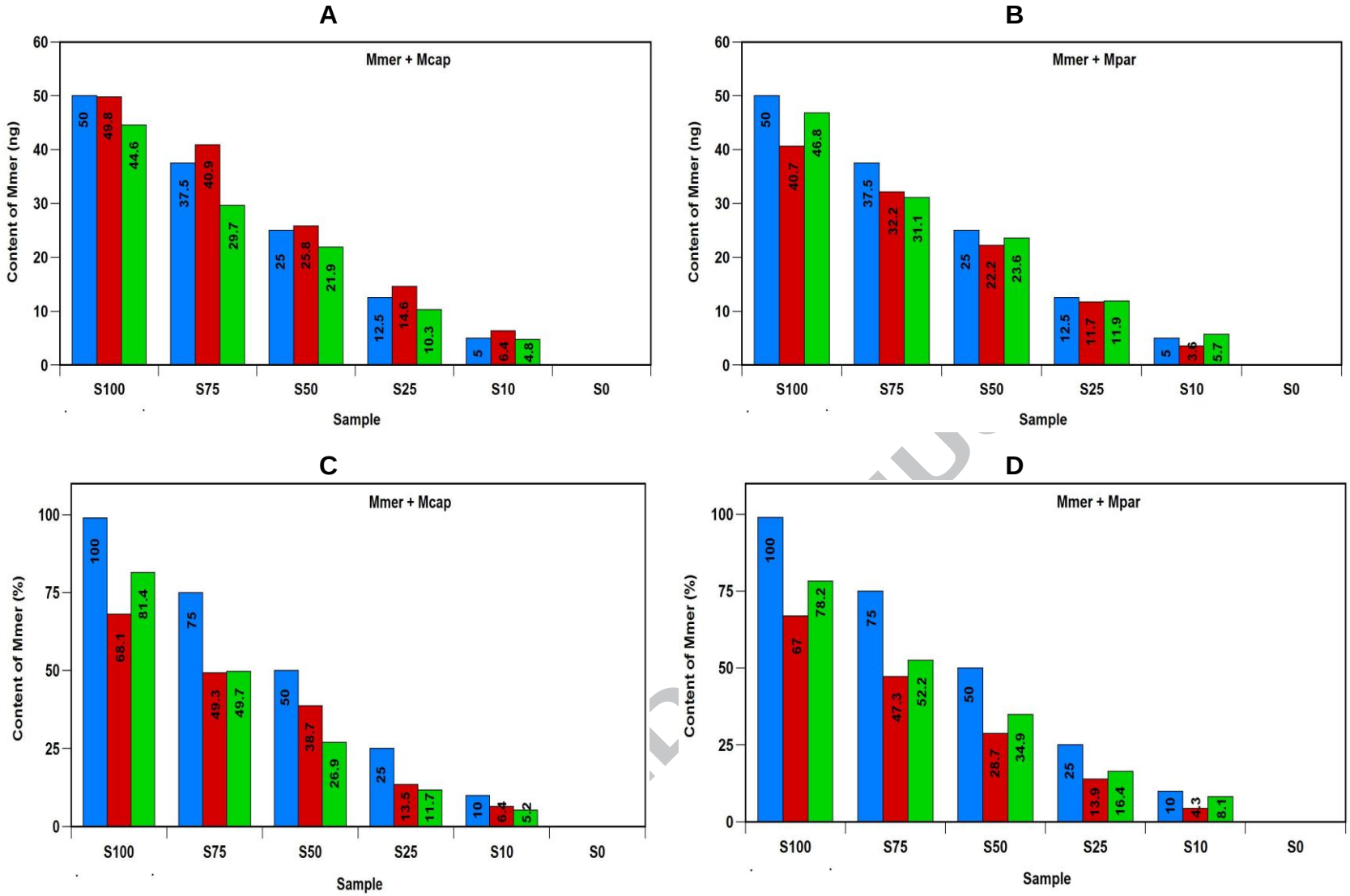


Figure 2.

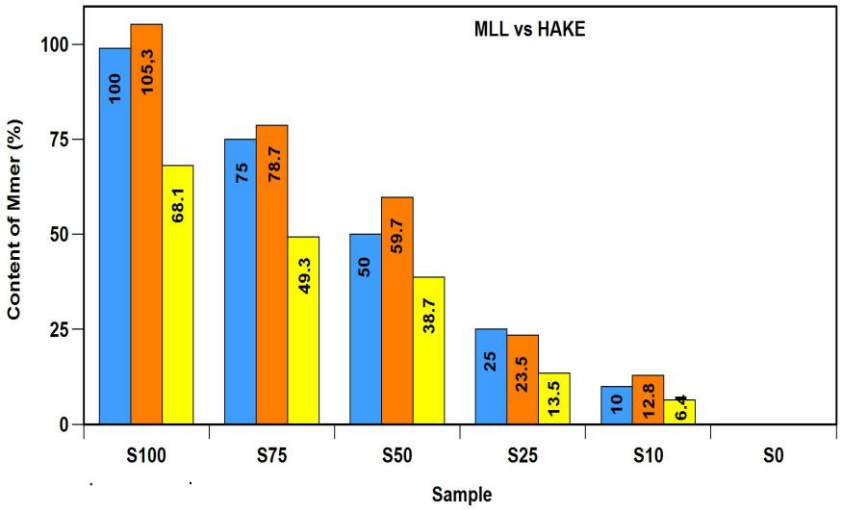
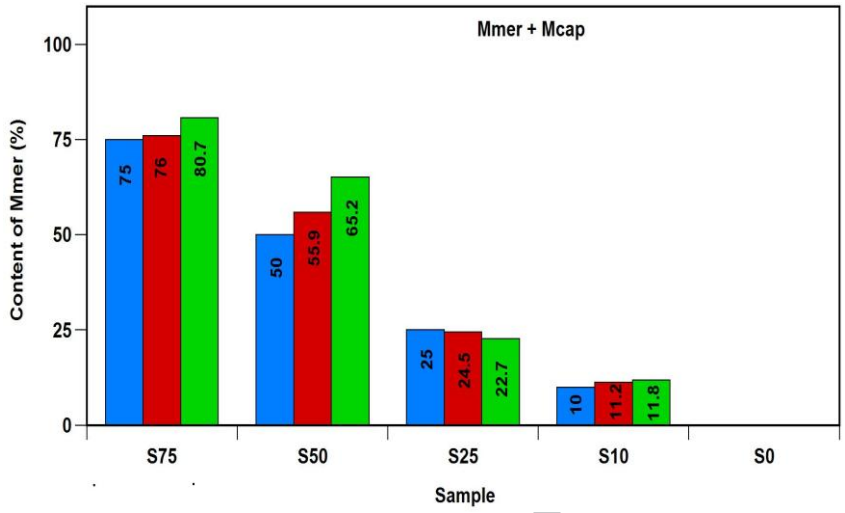


Figure 3.



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	Extracts (μL)		**Lyophilised (g)**		**Food Standards (g)**	
Sample	**Mmer**	**No Mmer**	**Mmer**	**No Mmer**	**Hake**	**Matrix**
S100	20	0	1	0	10	0
S90	18	2	0.90	0.10	-	-
S75	15	5	0.75	0.25	7.5	2.5
S50	10	10	0.5	0.5	5	5
S25	5	15	0.25	0.75	2.5	7.5
S10	2	18	0.1	0.9	1	9
S5	-	-	-	-	0.5	9.5
S1	-	-	-	-	0.1	9.9
S0	0	20	0	1	-	-

Table 2. Performance evaluation of reference systems for absolute-relative quantification of *Merluccius sp* in binary mixtures (DNA extracts and lyophilized muscle samples) of Mmer and Mcap using HAKE and MLL reference systems.

		Detected ng				SE%			
		Extracts		Lyophilised		Extracts		Lyophilized	
Sample	**Expected ng**	**HAKE**	**MLL**	**HAKE**	**MLL**	**HAKE**	**MLL**	**HAKE**	**MLL**
S100	50	73	56	55	63	46	12	10	26
S75	50	83	57	60	61	66	14	20	22
S50	50	67	55	81	62	34	10	62	24
S25	50	108	61	88	47	116	22	76	-6
S10	50	100	52	92	56	100	4	84	12
S0	50	109	58	125	48	118	16	150	-4
Mean	***50***	***88***	***57***	***80***	***56***	***80***	***13***	***67***	***16***

Table 3. Expected and determined percentages of *Merluccius sp*. in test food samples by absolute quantification with HAKE and MLL systems are shown. SE: systematic error between expected and determined percentages, as well as λ_c values for each sample, are included.

		HAKE			MLL		
Sample	**Expected %**	**λ_c**	**Detected**	**SE**	**λ_c**	**Detected %**	**SE**
Mcap	8	22.23	4.59	43	27.02	6.24	22
Mcap	12	21.05	7.56	37	25.87	10.40	13
Mcap	20	18.86	19.06	5	24.40	20.08	0
Mmer	12	20.29	10.43	13	25.99	9.87	18
Mhub	8	23.60	2.58	68	28.09	3.89	51
Mhub	12	22.60	3.92	67	27.04	6.18	49
Mhub	20	20.73	8.67	57	25.76	10.92	45

HIGHLIGHTS

- First work comparing DNA quantification approaches in fish model samples.
- Quantification approaches included absolute, absolute-relative and relative approaches.
- Real-time PCR relative quantification method provided the most accurate results.
- Relative quantification could be considered for standardised quantification of hake.