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Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles

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Abstract:	In the fish food sector, due to a growing globalization of the market, where intentional and unintentional frauds reach alarming levels, the molecular analysis is increasingly used by both Official agencies, to enforce the law on traceability, and private companies, to verify the quality of goods. DNA extraction represents a necessary and critical step for all types of DNA analysis. Among the drawbacks associated with this procedure, there are handling of toxic materials, low DNA yield and low throughput, due to time-consuming manual procedures. In this work, to overcome some of these problems, we developed an alternative method based on a bead milling procedure without proteinase K digestion. The new method was then compared with both a salting out protocol, developed in a previous work, and a commercial kit. Yield, spectrophotometric purity, electrophoretic degradation pattern, and amplificability of the extracted DNA were assessed. In particular, DNA amplificability was evaluated by comparing the band intensity on the gel, after amplification of the 16S rRNA and COI genes with a conventional PCR, and the take-off cycles, after amplification of the 16S rRNA gene with a real-time PCR. The results showed that the bead-based method allowed to obtain acceptable amounts of DNA, with good purity and good characteristics of amplificability. Although the salting out method remains the most effective protocol in terms of pure performances, the bead-milling procedure can be considered a valid alternative, in the light of its lower demand in terms of labor and costs.

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Dear Editor,
we revised the manuscript as suggested and here below you can find our answers, comments and rebuttals.

Reviewer #1: The paper is concerned with the development of a rapid and safe method for the extraction of DNA, with the fundamental characteristic of maximized cost-effectiveness in use in fish. I believe that it is well prepared article and presented and could be accepted for publication after major corrections

Major corrections

In general terms, I believe that the article can be shorter. The main information could be presented without losing its quality in a shorter way.

Also I believe that the authors should check the English grammar.

The manuscript has been edited.

Results and discussion could be shorter. It includes several known facts that can be skipped and replaced by references.

Results and discussion has been shortened as suggested.

Lines 296-314 could be shorter and moved to the methods as it does not contain any results.

The section 3.1 has been removed. The content has been shortened and moved to section 2.1 (line 91-95 and 103-106), which has been renamed as "*2.1 Species choice, tissue collection, sampling and storage*"

Line 346-354 could be deleted as they contained already known information.

Line 346-350 has been completely deleted as suggested, while the information reported in line 351-354 has been shortened and maintained in the manuscript (line 309-311). In our opinion these lines bring essential information to understand the spectrophotometric results. In fact, some information regarding the 260/230 ratio are reported at line 311-315.

Figure 1 could be deleted as the information could be easily described in the text

Figure 1 has been deleted as suggested

Table 1 could be restructured to be shorter and all types to be in one line

Table 1 has been restructured as suggested

Table 2SM is not clear

The table legend has been modified to better explain the meaning of the reported statistical results. As also explained in lines 226 (section 2.7 Statistical analysis), two models were created, which take into consideration the "species" and the "category" factors separately.

Minor corrections

Line 114: It is not clear. Rephrase the sentence

Line 114 has been rewritten

Line140: have should replaced

Line 140 has been checked

Line 273, 274: add reference

As specified in the sentence this evidence came from personal experience. In fact, due to this limitation, we have never used commercial kits for the DNA extraction. For this reason we propose to support the sentence with a personal citation (line 267)

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Development of a simple and cost-effective bead-milling method for DNA extraction from fish muscles

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ABSTRACT

In the fish food sector, due to a growing globalization of the market, where intentional and unintentional frauds reach alarming levels, the molecular analysis is increasingly used by both Official agencies, to enforce the law on traceability, and private companies, to verify the quality of goods. DNA extraction represents a necessary and critical step for all types of DNA analysis. Among the drawbacks associated with this procedure, there are handling of toxic materials, low DNA yield and low throughput, due to time-consuming manual procedures. In this work, to overcome some of these problems, we developed an alternative method based on a bead milling procedure without proteinase K digestion. The new method was then compared with both a salting out protocol, developed in a previous work, and a commercial kit. Yield, spectrophotometric purity, electrophoretic degradation pattern, and amplificability of the extracted DNA were assessed. In particular, DNA amplificability was evaluated by comparing the band intensity on the gel, after amplification of the *16S rRNA* and *COI* genes with a conventional PCR, and the take-off cycles, after amplification of the *16S rRNA* gene with a real-time PCR. The results showed that the bead-based method allowed to obtain acceptable amounts of DNA, with good purity and good characteristics of amplificability. Although the salting out method remains the most effective protocol in terms of pure performances, the bead-milling procedure can be considered a valid alternative, in the light of its lower demand in terms of labor and costs.

Keywords: DNA extraction, bead milling, fish muscle.

INTRODUCTION

The several cases of fraudulent substitution often reported by media have made the unique identification of food a key factor. Consequently, especially in the fishery sector, the DNA based-analytical methods have become increasingly important and are nowadays applied for routine controls, also at the Official level. They represent a valid support not only to improve the compliance and traceability of goods, encouraging the enforcement of the law, but also to raise the protection level of consumers against fish allergies and of endangered animal species (Armani *et al*, 2012a).

In order to meet the demand for reliable and sustainable food traceability systems, any DNA based analytical approach should be both effective and low-cost (Galimberti *et al.*, 2013). Effectiveness is mainly based on the possibility to rely on a successful PCR amplification, which greatly depends on the achievement of a sufficient amount of high quality DNA. In fact, it is fundamental that every extraction method be able to maximize the removal of contaminants that may inhibit PCR. For this reason, DNA extraction is considered the most critical step in the processing of samples for PCR-based analysis. Especially in the fishery sector, which deals with thousands of different species, the choice of the most appropriate technique should be accurately assessed.

Fundamental step of the DNA extraction method is the tissue disruption and the cell lysis. Many chemical and physical disruption methods, followed by enzymatic digestion using proteinase K, have been proposed for tissue lysis. Among those available, the rapid shaking of the samples in the presence of glass or steel beads has been shown to be effective for different kind of matrices of microbial, plant, and animal origin (Dilworth and Frey, 2000; Robe *et al.*, 2003; Allender *et al.*, 2004).

Due to the fact that beads-based tissue-disruption could significantly reduce times and costs, and that, to our knowledge, targeted studies on fish tissues have never been performed, in this work we developed a simple and cost-effective DNA extraction method, based on bead-milling, without

proteolitic enzyme, followed by a salting out procedure. A study was then performed to point out strengths and weaknesses of this method by comparing it with a classical enzymatic digestion coupled with a salting out procedure and with a commercial DNA extraction kit, both from fresh and ethanol preserved tissues, using 38 different species. The yield, the quality and the degradation pattern of the extracted DNA were assessed and their effects on subsequent downstream sample analysis were evaluated by performing conventional and real time PCR.

Considering the high number of fish products marketed worldwide and the increased number of frauds reported, the development of a rapid and safe method for DNA extraction, with minimized costs and good effectiveness, would be required to better support the growing need for molecular analysis.

2. MATERIAL AND METHODS

2.1 Species choice, tissue collection, sampling and storage

Considering that the natural heterogeneity of the tissue composition can affect the efficiency of mechanical disruption, we decided to test the effectiveness of the new DNA extraction procedure by selecting the species on the basis of the fat content, according to the categorization proposed by Ackmann (1989). In fact, fats could contribute to the DNA precipitation and inhibit PCR (Wilson, 1997; Besbes *et al.*, 2011).

Muscle samples were collected from 38 different fish species (Table 1), according to the weight of the specimens. In case of fish with weight greater than 150gr, the tissue was excised from the dorso-lateral muscles of three different specimens and then grossly chopped with scissors. In case of small fish, where a single sampling would not suffices, three mixtures of at least five samples were prepared after skin and bone removal. From each of the three different specimens or mixtures, eighteen samples (one duplicate for each of the extraction method tested, see section 2.3) were produced and extracted.

Considering that collection of tissues belonging to reference specimens, which are often stored in ethanol, since provided by museums or research institutes, represents a prerequisite to develop a

DNA barcoding for the identification of unknown fish samples (Armani *et al*. 2013; Galimberti *et al*., 2013), we also tested our methods on samples maintained in ethanol at 4°C for at least 30 days.

2.2 Development of a bead-milling non enzymatic method for total DNA extraction

2.2.1. Protocol optimization. 0.1 mm and 0.5 mm glass beads (SI-BG01, SI-BG05 disruptor beads Scientific Industries, Inc., Bohemia, NY, USA) and 2.5 mm stainless steel beads (precellys 24-Bulk bead, Bertin Technologies Villeurbanne, France) were used to assess their destructive force on fish muscle tissues. The trials were performed using fresh muscle tissue from ten species (Table 1). For the setting up of the bead-milling method, a solution composed of 200 μl of Lysis buffer (500 mM Tris-HCl,100 mM EDTA,100 mM NaCl, 2% w/v SDS, pH 8.0) was added to 200 mg of tissue. The glass beads were used in a 3:2, 1:1, 1:2, 1:6 w/w ratio with respect to the tissue, according to the manufacturer suggestions, whereas the steel beads were added in number of 3, 5, 10 or 20 per 200 mg tissue. The samples were grinded at room temperature (RT) on the Cell disruptor (Disruptor Genie®, Scientific Industries, Inc., Bohemia, NY, USA) at 1200 rpm shaking speed and the extraction was performed according to the method reported in section *2.3.2*. The type and the amount of beads were chosen on the basis of the DNA yield.

Finally, two times of mechanical disruption (30 and 60 min) were tried at both room temperature and 60°C on a thermo-mixer (EuroClone T-shaker, EuroClone S.p.A, Pero, MI, Italy) at 1200 rpm shaking speed. Time and temperature of disruption were chosen by evaluating A260/A280 ratio, A260/A230 ratio, and yield as parameters for selection.

2.2.2. Final Protocols (B30; B60). For each sample, 200 mg of muscle tissue, 10 steal beads and 200 μl of Lysis buffer were put in a 2 ml round bottom tube. The tube was then placed on the T-Shaker preheated at 60°C for 30 and 60 min at 1500 rpm. At the end of the milling step, the samples were centrifuged at 15000 X g for 2 minutes to separate the clear supernatant from the tissues/beads mixture and collected in a clean 1.5 ml tube. The extraction was then completed according to the protocol reported in section 2.3.2, with the exception of those samples that, after the first sodium acetate precipitation, presented a "*cloud no-transparent supernatant phase*". In this case, a step of

reprecipitation with 0.5 volumes of sodium acetate, followed by a double washing with 70% ethanol, were added. The optimized protocol was then evaluated on samples of decreasing weights (200; 100; 50; 25; 10 mg). It was performed on 36 specimens, of which three individuals for each species and three species for each of the four fat categories (Table 1 species in bold) were processed according to methods SO, B30, and B60. The number of beads was proportionally reduced.

2.2.3. *Stainless beads decontamination and sterilization procedure.* After use, the beads were washed carefully under tap water in a Petri plate until cleaned from all tissue debris, then soaked for one hour in a 5% sodium hypochlorite solution. After the removal of the hypochlorite solution, the beads were washed with ultrapure water and ethanol 70%, air dried and then submitted to a cycle in autoclave at 120°C for 30 minutes. The effectiveness of the decontamination procedure was verified by performing a PCR for the amplification of a fragment of the mitochondrial 16S ribosomal RNA (*16srRNA*) (section 2.5.1), in which the autoclaved beads were directly placed into the reaction tubes.

***2.3. Comparison of different extraction procedure*s**

The DNA extraction method developed in this study was compared with other two different methods: a salting out procedure (Armani *et al*. 2011) and a commercial kit, using all the samples (Table 1) before and after the ethanol storage. The ethanol preserved samples were preliminary rehydrated with a 30 minutes washing in 50 mM Tris solution, pH 7.8, before extraction. All the muscle tissues were extracted in duplicate. Considering the low DNA yield obtained with the kit from fresh samples (see section 3.3.1), this procedure was not tested in case of ethanol preserved samples.

2.3.1 Extraction with the commercial kit. 40 mg of tissues were extracted with the EuroGold Tissue DNA Mini Kit (EuroClone S.p.A), following the manufacturer's instructions, with the exception of the final elution, which was obtained with 50μL of deionized sterile water instead of 100-200 μL as suggested by the manufacturer.

2.3.2 Enzymatic digestion and Salting Out extraction protocol (SO). Total DNA extraction was performed starting from 200 mg of tissue, following the protocol of Armani *et al.*, (2011), modified by Armani *et al.*, (2012b). Briefly, after adding 200 µl of lysis buffer and 200 µl of 200 mM Na_2HPO_4, pH 8.0, the tissue was mechanically homogenized with scissors and incubated for one hour digestion with 20 µl of proteinase K, continuously stirring at 1200 X g on a Thermo mixer (EuroClone T-shaker, EuroClone S.p.A, Pero, MI, Italy), at 60°C. The samples were then centrifuged at 15000 X g for 2 min and the upper aqueous phase was collected in a new sterile microcentrifuge tube. The proteins were precipitated adding 0.5 vol. of 4M sodium acetate, pH 8.3. Then the mixture was incubated at RT for 5 minutes and then centrifuged at 15000xg for 5 min. The DNA was precipitated with 0.6 vol. of Isopropanol molecular biology grade (SERVA Electrophoresis GmbH, Heidelberg, Germany), washed once in 70% (v/v) ethanol molecular biology grade (SERVA Electrophoresis GmbH, Heidelberg, Germany) and once in 100% ethanol, air-dried and resuspended in deionized sterile water.

2.4 Qualitative and quantitative determination of total DNA

The overall quality of the extracted DNA with the three different procedures was determined by taking into account purity, yield and integrity of the DNA.

2.4.1. Spectrophotometric assessment and yield. The spectrophotometer NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, U.S.) was used to measure the concentration and the purity of the total DNA on the basis of the UV absorption ratio at 260/280 nm and at 260/230 nm on two subsequent measurements. Subsequently, the yield (µg/mg) was calculated.

2.4.2. Evaluation of DNA integrity by gel electrophoresis. The DNA integrity was checked by gel electrophoresis: 1µg of total DNA of each sample, with the exception of those extracted with the kit for which a lower concentration was used (depending on to the yield of the extraction), was run for 1 h at 240V on a 0.8% agarose gel (GellyPhorLE, Euroclone, UK) prestained with GelRed™ Nucleid Acid Gel Stain (Biotium, Hayward, CA, USA) in 0.5x TBE buffer (pH 8.3). The result was visualized on a UV transilluminator. The DNA sizes were estimated by comparison with two

standard DNA markers, the SharpMass™50-DNA ladder and the SHARPMASS 1 kb-DNA Ladder, (EuroClone S.p.A, Pero , MI, ITALY).

2.5 DNA amplificability

To test the amplificability of the DNA extracted, a conventional PCR and a real-time PCR were performed on 36 specimens (three individuals each species; three species for each of the four categories) (Table 1 species in bold) processed according to all the four methods of extraction.

2.5.1. Conventional PCR.

The DNA samples were amplified by conventional PCR by using two couple of universal primers: 16SaR/16sbr (Palumbi *et al*., 1996) and FISHCOILBC_ts/FISHCOIHBC_ts (Handy *et al*., 2011), for a ~650bp partial sequence of the mitochondrial 16S ribosomal RNA (*16srRNA*) gene and for a ~700bp partial sequence of the mitochondrial cytochrome oxidase I (*COI*) gene, respectively. The amplification reactions were performed in 10 µl containing 1.25 U of Perfect*Taq* DNA Polymerase (5Prime, Gaithersburg, USA), 200 µM each dNTP (dnTPmix, Euroclone S.p.A-Life Sciences Division, Pavia, Italy), 0.25 µM of primer forward and reverse, 1.5 mM of $MgCl_2$ (5Prime, Gaithersburg, USA), 25 ng/µl of BSA (Purified BSA 100X, New England BIOLABS® Inc. Ipswich, MA, USA), and 25 ng of total DNA. After an initial Taq Polymerase activation step (3 minutes at 94°C) both the amplification protocols were set up on a 35 cycles program as follow: 35 cycles of 94° C for 25s, 57.5°C for 15s, 72 °C for 2s (*16s rRNA* gene), 94°C for 30s, 55°C for 30s, 72°C for 30s (*COI* gene). Both the protocols were completed by a final elongation step at 72°C for 5 minutes. The amplification products were resolved on a 2% agarose gel (GellyPhorLE, Euroclone, UK), stained with GelRed™ Nucleid Acid Gel Staining 10000X water solution (Biotium, Hayward, CA, USA) in 0.5X TBE buffer. The final result was visualized on UV transilluminator and DNA fragment size was estimated by comparison with the standard marker SharpMass™50-DNA ladder (Euroclone, S.p.A-Life Sciences Division, Pavia, Italy).

The image of the gels were acquired with a digital camera and then analyzed using the software Image J 1.47t (NIH, Bethesda, USA). The absolute values obtained were normalized as percentage

of the resultant of the sum of every single band within each group, in order to produce a relative estimation of the intensity of each band.

2.5.2. Real time PCR.

For the real-time PCR the primers FOR16Spc/REV16Spc designed by Armani *et al*. (2012c) were used for the amplification of a partial ~330bp gene fragment of the *16S rRNA* gene. The DNA was amplified in a RotorGene 6000 thermocycler (Corbett Research, Sidney, Australia) and the reaction was run in 10 µl containing 5 µl of the premixed solution (QuantiTect SYBR Green PCR Kit, Qiagen, Hilden, Germany), 250 nM of primer forward and reverse, and 25 ng tot of DNA template. Cycling conditions were set as follows: initial hold at 95 °C for 10 min; 45 cycles including denaturation 94 °C for 15s, annealing 53 °C for 30s, extension 72 °C for 30s. All samples were run in duplicate with negative and positive controls. The threshold cycle (Ct) value was registered and considered for statistical analysis.

2.6 Estimation of costs and time required by each method

The cost per sample for the three experimental protocols was estimated on the basis of the commercial price of chemicals and disposable items used. The time was estimated on a batch of ten samples from the mechanical disruption of the tissue (with scissor or beads) to the pellet DNA solubilization step.

2.7 Statistical analysis

Normality of data distribution was tested by Shapiro-Wilk W test. Homogeneity of variances was tested by Levene's test.

To test the effect of the factors method (M), species (S) or category (C) (lean, low fat, medium fat, and high fat content) on DNA yield, ratios of absorbance at 260/280 nm (A260/A280), and at 260/230 nm (A260/A230), after extraction, two mixed models with REML estimation of variance were used. In the first model, M, S, and their interaction (M*S) represented the factors with fixed effects, while the random effect was attributed to the individual factor "subject". The second model was built as the previous one, replacing the factor S with the factor C.

Due to the fact that non-homogeneous variances were found, the differences among methods for yield, A260/A280, and A260/A230 were analyzed by Friedman's test for repeated measures followed by Dunn's multiple tests for pairwise comparison of means, for both fresh and ethanol-preserved tissues. The analysis was performed overall and within each category of fish.

To test the effect of the factors M, initial amount of tissue (W), and S on DNA relative yield after extraction, A260/A280, and A260/A230, a mixed model with REML estimation of variance was used: M, W, their interaction (M*W), and S represented the factors with fixed effects, while the random effect was attributed to the individual factor "subject".

The differences among methods of measured values were analyzed by Friedman's test for repeated measures followed by Dunn's multiple tests for pairwise comparison of means.

To test the effect of the factors M and S on the band intensity of the DNA amplified by conventional PCR and on the take-off cycle of the DNA amplified by real-time PCR, a mixed model with REML estimation of variance was used: M, S , and M*S represented the factors with fixed effects, while the random effect was attributed to the individual factor "subject".

A Friedman's test for repeated measures followed by Dunn's multiple tests for pairwise comparison of means was used to compare values related to the band intensity and take-off cycles of PCR performed on the DNA extracted with the four methods.

3. RESULTS AND DISCUSSION

An ideal extraction technique should maximize DNA yield, minimize DNA degradation, and be effective in terms of costs, time, labor, and supplies. Moreover, it should also guarantee the removal of most of the substances that can act as PCR inhibitors (Radstrom *et al.,* 2004; Bessetti 2007). Even though the DNA based methods represent the most used techniques for fish species identification, DNA isolation still represents one of the most time consuming step, which requires active work of operators all along the process.

Among the traditional extraction approaches, the original phenol/chloroform protocol proposed by Sambrook *et al*. (1989) is still widely used. A survey, carried out as a preliminary investigation

of this work and made by analyzing 51 articles published in the last five years, dealing with fish species identification, clearly showed that the phenol/chloroform protocol and the commercial kits are the only extraction methods used (Table 1SM). The phenol/chloroform method, even though applicable to many different species and capable to guarantee high DNA recovery, involves the handling of toxic materials. For this reason, it implies risks for operators and environment. On the other hand, to our experience, the main weakness of the commercial kits is the low DNA yield, if compared with the classical procedures, although DNA purity may be sometimes higher (Author's note). Moreover, commercial kits often present disadvantages such as non-repeatability of the DNA yield and purity declared by the manufacturers (Di Bernardo *et al*., 2007; Akkurt, 2012).

In this light, the development of an user-friendly non-organic-based DNA extraction method could represent the right compromise between the two procedure. Therefore, an alternative salting out method was previously developed for daily utilization in an open-air laboratory environment (Armani *et al.,* 2011) and then effectively used for the isolation of a high-quality total DNA from many species of seafood (Armani *et al*. 2012b; Armani *et al*. 2012c; Armani *et al*. 2013).

In case of non-affinity extraction methods, the initial disruption of the tissue, which relies both on chemicals or physical procedures, is generally the most time-consuming step and can also greatly influence the yield and the quality of the DNA. Although the physical procedures are the most employed, they can be very laborious and require operator training (Burden, 2008). Moreover, all these methods are not easily standardizable and can lead to cross-contamination (Verollet, 2008). In this study, a simple method based on a bead-milling homogenization protocol without proteases was set up, not only to speed up and simplify our previous salting out method, but also to reduce the cost of the extraction, in the light of an increasing request for routinely biomolecular analysis in the fish sector.

3.1 Bead-milling procedure: optimization and final protocol

The cell rupture and the protein digestion are usually achieved by incubation with proteolytic enzymes (proteinase K) for a variable time (Table 1SM). Even though several protocols have been

used for the DNA extraction from fish muscle tissue, the possibility to replace the step of enzymatic proteolysis with only bead mill homogenization has never been assessed (Table 1SM).

One of the most important factor to obtain a good tissue disruption is to properly match the sample size (mass and volume) with a suitable tube size and grinding ball (Burden, 2012). In our protocol, the stain-less steel beads were chosen, due to the fact that they showed better performances, with respect to glass beads, in breaking myo-fibrillar tissue and releasing DNA in solution. In fact, the DNA yield was found to be about 5 times higher (data not shown). Moreover, while the glass beads must be discarded together with the organic precipitate at the end of the disruption step, the steel beads can be re-used after a simple decontamination procedure.

The procedure was optimized at 60°C due to the fact that already at 56°C many cellular proteins and enzymes (including DNAase) are denatured (Lahiri and Schnabel, 1993).

Finally, when samples presented a "cloudy supernatant phase" after the first centrifugation at the end of the lysis step, a further precipitation step with ammonium acetate, followed by a double washing with ethanol 70%, was added to clear the solution. In particular, this occurrence was observed in *Merlangius merlangus*, *Trisopterus minutus capelanus*, *Mullus barbatus*, *Salmo salar*, *Sardina pilchardus* and *Squalus acanthias*.

3.2. Comparison of different extraction procedures.

3.2.1 Yield and spectrophotometric quality. The final yield of an extraction procedure may have some importance, in that it has to provide not only a sufficient amount of DNA, but also a workable concentration proportionally to the number and the type of analyses to be performed. This is particularly important in case of samples provided by museums or collections, which are usually few milligrams.

In general, an A260/A280 ratio between 1.8 and 2.0 indicates uncontaminated DNA (Sambrook and Russell, 2001; Alaey *et al.*, 2005). Lower values indicate contamination by proteins, whereas higher values may be associated to the probable presence of RNA (Varma *et al.*, 2007). The A260/A230 ratio is considered acceptable when ranging in between 1.8 and 2.4. Values lower than

1.8 indicate the residual presence of significant amounts of organic compounds, such as phenol, carbohydrates, or residual guanidine (De Maeseneire *et al*., 2007; Morin *et al.* 2010; http://cancer.ucsf.edu/research/cores/genome/services/genome-analysis-service-analyze).

A260/A230 ratio is less a precise parameter than A260/A280 ratio, which is often reported alone (Besbes, *et al*., 2011; Cawthorn *et al.,* 2011). However, considering that abnormal A260/A230 values may negatively influence downstream analyses, we decided to also consider this parameter to better characterize the DNA obtained with the new method.

The results related to DNA yield, A260/A280, and A260/A230 ratios highlighted a high variability within species (data not shown). This confirms the importance of the role played by operators and the individual factor associated to each specimen. For this reason a mixed model capable to take into consideration such factor of variability (random factor) was chosen to test the influence of the method and category (on the basis of the fat content), or of the method and species on the outcome of the extraction procedures. The statistical analysis showed that method, category and species had a highly significant effect on yield. If the significance associated to method and species remained very high for the other two parameters investigated (A260/A280 and A260/A230), the significance associated to category decreased (Table 2SM). Important to notice that the interaction of the aforesaid factors (method with species or method with category) played an important role as well, indicating that the method influenced yield or absorbance parameters at a different extent in different categories or species.

For samples stored in ethanol, only yield and A260/A280 are highly influenced by method and species, while the factor “category” is not as important as the two formers. If these results and the results related to fresh samples are taken together, it seems likely that the fat content influenced the process of DNA purification and that ethanol may have leveled down such influence. In fact, it has been previously reported that the extraction of lower DNA yields can be associated with samples with higher fat content (Saunders and Rossi, 2008).

Comparison of performances related to the different methods revealed that, overall, no differences existed between B30 and B60 for all the three parameters (yield, A260/A280, and A260/A230).

As a general outcome, it was found that SO method produced the highest yield and was more efficient in removing contaminants from the DNA solution, with values of A260/A280 and A260/A230 always standing within the optimal range, both in fresh and in ethanol preserved samples. With regard to the methods using milling beads, the yield was, on an average, one-half of the yield obtained with SO method, but about three-fold higher than the yield associated to the kit. Values of A260/A280 were about 2, while A260/A230 were slightly beneath 1.8. Lastly, the kit, only used on fresh samples, provided very low yields, but good A260/A280. On the contrary, unusually very high and very variable levels of A260/A230 were observed.

Even though the commercial kits are used more frequently and are reported to be less technically demanding and safer than classical procedures (Loffler *et al.*, 1997), our previous experience, further confirmed by the results of this work, indicated that, besides the high costs (see section 3.5), one of the main disadvantages is represented by the low yield. In fact, even though the elution buffer was reduced to 50 µl and heated at 70°C before the final centrifugation, the DNA yield remained quite low.

Patterns of performance are similar in the 4 groups, even though differences between methods had different levels of significance. In fact, when comparisons were made within each group, the scenery was variable. The only stable and recurrent evidence was the substantial equality between methods B30 and B60, indicating that the tissue disruption after 30 minutes was practically accomplished and any further elongation of time did not bring any significant improvement. To highlight the fact that, in fresh samples, differences in yield between SO and B30, or B60, or the kit remained highly significant in all the 4 groups for each category, while differences between SO and B30, or B60, in A260/A280 were no more significant in medium and high-fat content groups. Regarding A260/A230, the very high variability observed in samples extracted with the kit and the

lowering of their means in medium and high-fat content groups determined the loss of significance between values of SO and the kit, within this two categories.

As for samples stored in ethanol, differences in yield between SO and both B30 and B60 remained highly significant in all the 4 groups, as well as differences in A260/A280 and in A260/A230.

3.2.2. Decreasing weights. Starting from different amounts of tissue, different DNA relative yields and different values of purity indicators were obtained across the three methods.

Statistical analysis based on the mixed model showed that both initial amount of tissue and method of extraction significantly influenced the relative yield (µg DNA/mg of tissue) of DNA obtained and that such a parameter varied with a significantly different pattern in different methods. Such response was confirmed for both A260/A280 and A260/A230, despite different level of significance associated to the different factors (Table 3SM). The factor species was always found to have a highly significant influence on the three parameters considered.

If marked differences were observed between SO method and bead-milling methods, no significant differences were observed increasing time from 30 to 60 minutes of bead milling. As shown in Figure 1, the pattern of variation are very similar for B30 and B60, once more confirming that 30 minutes of milling is a sufficient time to reach a good level of tissue disruption.

In particular, the initial amount of tissue that gave the highest DNA relative yield for SO method was 50 mg. From lower or higher tissue amounts a significantly lower yield was obtained ($p<0.01$). On the contrary, differences in DNA purity indicators were not significant.

As for bead-milling methods, a net gain in DNA relative yield was observed with the increase of the initial amount of tissue processed. If A260/A280 did not vary appreciably, with only a significantly higher value for 200 mg of tissue with respect to all the other amounts ($p<0.01$), A260/A230 was shown to have a minimum for 100 mg, which significantly differed from the other values ($p<0.05$).

From these results, it is possible to state that the best outcomes in terms of both yield and purity were obtained with the lowest amounts of tissue (25 and 10 mg), with both absorbance ratios around the threshold of the optimal range (Fig. 1). However, the DNA yield being relative, the total amount of DNA obtained would be lower than that obtained starting from higher amount of tissue. This is strictly dependent on the needs and scope of the analysis.

3.2.3. Integrity. The purpose of an extraction method is not only to obtain an acceptable amount of highly pure DNA, but also to limit at most the DNA degradation processes. Agarose gel electrophoresis enables visualization, to some extent, of the degradation level of the extracted DNA. The visual analysis of the DNA degradation pattern did not show any evident difference among the extraction methods tested (data not shown).

3.3. DNA amplificability

In order to test the amplificability of the DNA extracted with different methods, a conventional and a real-time PCR were performed on a subgroup of the fresh tissue samples processed (the same used to test extraction from decreasing weight of tissues). The genes *16srRNA* and *COI* were chosen as target because are among the most used for fish species identification and because require different kind of primers for the amplification (Armani *et al*., 2012a).

3.4.1. Conventional PCR. The DNA was successfully amplified by PCR regardless the extractions procedures, even though some differences were observed among the four methods (Fig 2).

Once again, the mixed model showed that the amplification of the two targeted genes was significantly influenced by both the factors method and species, as well as their interaction, confirming that unknown factors associated to the kind of tissues (likely compositional and depending on the species) may strongly influence the extraction procedure and thus the downstream analysis (Table 4SM). In particular, even though overall means were not very far, significant differences ($p<0.05$) were found only between SO and both B30 and B60, when the *COI* gene was amplified, while significant differences ($p<0.01$) were found between the kit and SO, B30, and B60,

when the gene *16SrRNA* was targeted. The different influence of the extraction methods on the amplificability of different genes may be due to the characteristics of the primer used, whose capability to steadily match the complementary sequence on the DNA is likely influenced, at a different extent, by the presence of residual molecules in the PCR reaction mixtures.

3.4.2. Real time PCR. Starting from the same amount of DNA, the take-off cycle is a function of the PCR efficiency, which in turn is somehow proportional, other than to the characteristics of the primers and the PCR conditions (identical for all the samples), also to the DNA integrity and the presence of potential inhibitors. In this trial, only a fragment of the *16srRNA* gene was amplified, due to the absence of universal primers for the amplification of a short fragment belonging to the *COI* gene.

Statistics showed that both the two factors included in the model (method and species) significantly influenced the take-off cycle ($p<0.001$). The high significance ($p<0.001$) associated to the interaction of the two factors revealed that the factor method had a different effect on different species (Tab. 4).

Overall, direct comparison between methods revealed significant differences between SO and the other three ($p<0.001$), while not significant differences were found between B30, B60, and the kit. On an average, SO was associated to slightly but significantly lower take-off cycles, with respect to the other methods (Fig. 3). The better performance of the downstream amplification in real-time PCR obtained with SO extraction may reflect a higher quality of the extracted DNA and thus a better global performance with regard to the extraction process.

3.4 Cost and time evaluation

The kit was the most expensive procedure with a cost of ~ € 2.25 per sample, which was almost 2.5-fold higher than SO (~ € 0.8) and 3.5-fold higher than the bead-based procedures (Bs) (~ € 0.62). This downside related to the utilization of the kit has already been reported by Ivanova *et al*., (2006). The cost of the Bs was further reduced with respect to SO due to the absence of the enzyme, which is one the most expensive reagents used in the classic extraction procedures. Finally, it must

be underlined that the bead-milling completely replaces the manual work of the operator. This can add some more economical advantage, due to the "working time" saved, which may become conspicuous when a high number of samples have to be processed.

Differences were found comparing the global time required for the entire protocol from the homogenization up to the DNA elution. Overall, the SO required around 3 hrs and 20 min, the B30 2 hrs and 30 min, the B60 3 hrs, while in the case of the kit the total time was extremely variable ranging from 1 hr and 30 min to 3 hrs and 30 min. In fact, as reported by the manufacturer, the lysis step can be extended up to 3 hrs in order to obtain a satisfactory result. Moreover, the total time for SO and the kit can be influenced by the homogenization of the samples by manual scissor shearing, variable from 20 to 40 min, according to the resistance to cutting.

CONCLUSIONS

In this work, a simple, rapid and cost-effective method, based on a mechanical grinding with steel beads followed by a salting out procedure, was developed to isolate DNA from the fish muscle.

From this experimentation emerged that it is not possible to indicate *a priori* the best extraction method for a specific fish species or for a specific gene to be amplified, but general conclusions may be draft on the basis of analysis performed on a number of different species, representative of large categories, such as the very important ones related to the fat content.

Even though the comparison performed with other methods showed that the salting out procedure is, on an average, the best in terms of both yield and spectrophotometric quality of the total DNA, the bead-beating protocol allowed to obtain DNA of good quality and in acceptable amount, which suffices for thousands of PCR amplifications, and significantly overcomes that provided by the kit. Moreover, by eliminating the need of a physical disruption performed by the operator, the bead-milling procedure allows to avoid the most undertaking and longest step of all the DNA extraction procedures. This permits to improve the throughput of the procedure by markedly increasing the number of samples processable by a single operator. Lastly, the automatic

tissue-disruption process also makes less important the relationship between goodness of the outcome and ability or expertise of the operator.

In conclusion, the bead-milling method represents a valid alternative to the classical methods of DNA extraction, especially for routine analyses that involve a high number of samples and demand the lowest costs possible.

"Compliance with Ethics Requirements"

The research was performed with founds granted from the University of Pisa.

Armani Andrea declares that he has no conflict of interest.

Tinacci Lara declares that he has no conflict of interest.

Xiong Xiong declares that he has no conflict of interest.

Titarenko Evgeniya declares that he has no conflict of interest.

Guidi Alessandra declares that he has no conflict of interest.

Castigliego Lorenzo declares that he has no conflict of interest.

This article does not contain any studies with human or animal subjects.

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Figure captions

Fig. 1 Performances related to scalar quantities of tissue processed**.**

Fig. 2 Amplification performance of the two genes (A: *COI*; B: *16S*) by conventional PCR, starting from the DNA extracted with the four different methods, from a subgroup of twelve species (lean, n=3; low fat, n=3; medium fat, n=3, high fat, n=3). Quantities are expressed as the relative intensity of the single bands on the gel, corresponding to the percentage related to the sum of the single intensity values calculated on a single gel, where all the samples belonging to a different category were run. Overall values were not reported because of the impossibility of averaging values obtained from different gels. Levels of significance were however calculated by comparing values obtained by parallel comparison of samples run in the same gel.

Fig. 3 Amplification performance in real-time PCR of the DNA extracted with the four different methods, from a subgroup of twelve species (lean, n=3; low fat, n=3; medium fat, n=3, high fat, n=3), expressed as difference (ΔCt) between the take-off cycle (Ct) for each subject and the overall mean.

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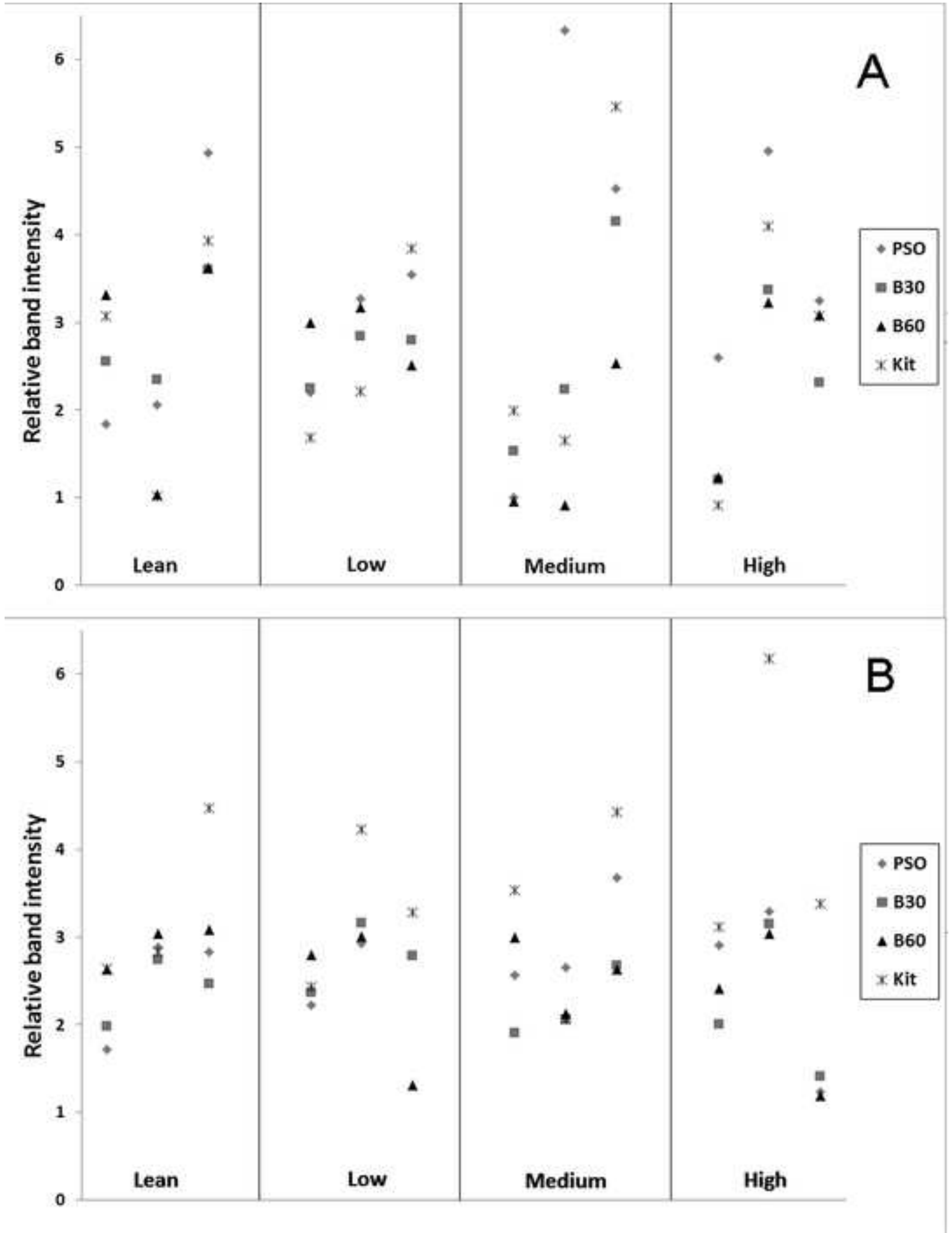

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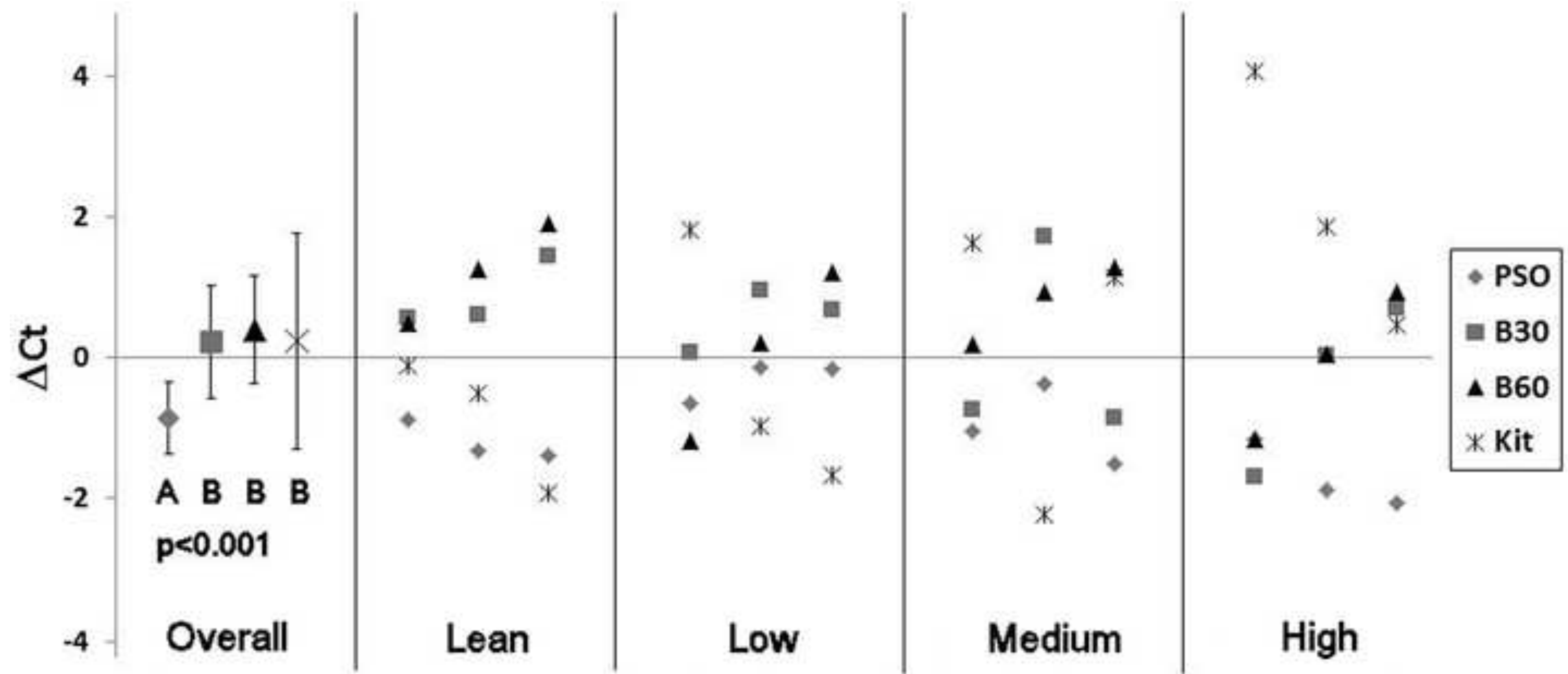

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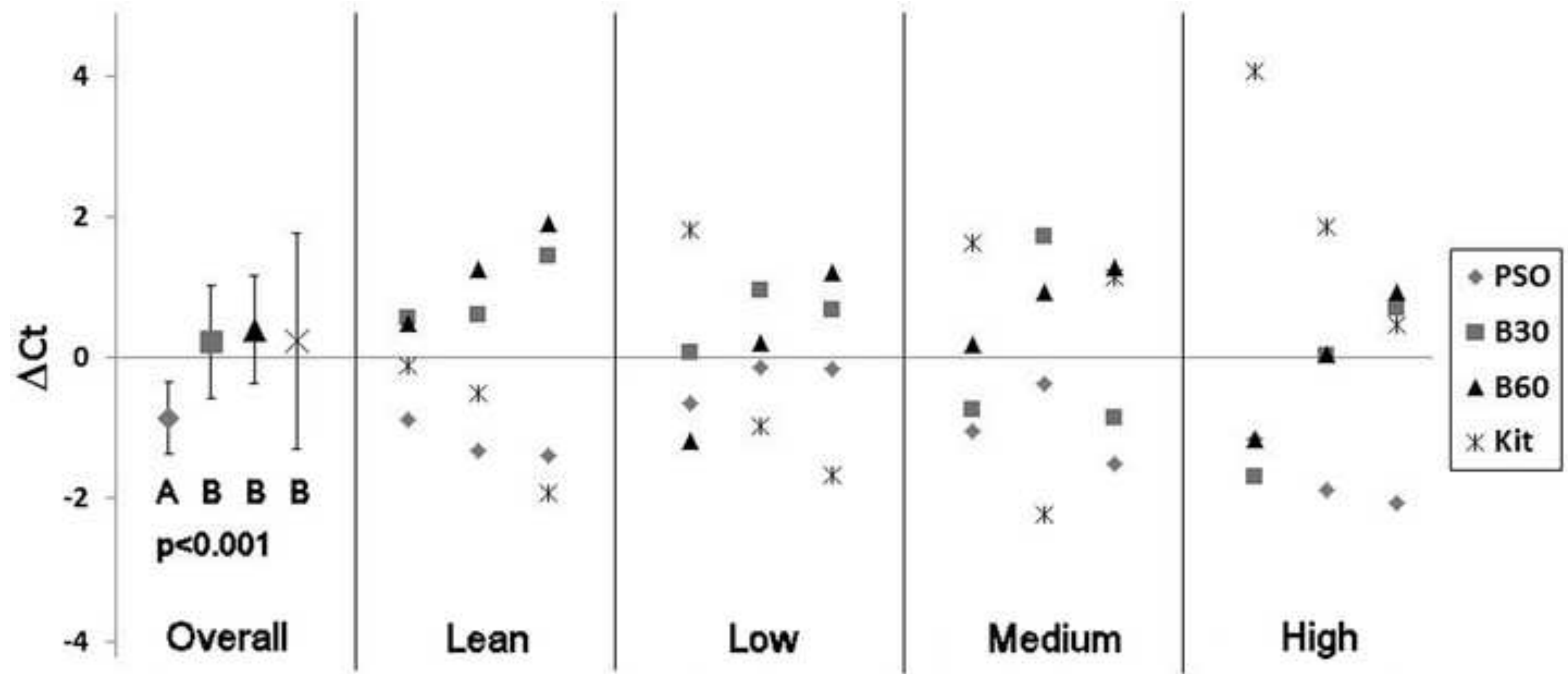

Table
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LEAN (<2%)	**LOW FAT (2-4%)**	**MEDIUM FAT (UP TO 8%)**	**HIGH FAT (> 8%)**
YELLOW GROUPER	BROTULA	TROUT	**SALMON**
Epinephelus awoara	*Brotula multibarbata*	*Onchorhyncus mykiss*	***Salmo salar***
NILE TILAPIA	POOR COD	WHITE STURGEON	**EUROPEAN EEL**
Oreochromis niloticus	*Trisopterus minutus capelanus*	*Acipenser transmontanus*	***Anguilla anguilla***
COD	GRASS GOBY	RED MULLET	GREATER AMBERJACK
Gadus morhua	*Zosterisessor ohpicephalus*	*Mullus barbatus*	*Seriola dumerili*
WHITING	SEABASS	GOLDEN GREY MULLET	**ATLANTIC MACKEREL**
Merlangius merlangus	*Dicentrarchus labrax*	*Liza aurata*	***Scomber scombrus***
HAKE	GILTHEAD SEABREAM	**SARDINE**	
Merluccius capensis	*Sparus aurata*	***Sardina pilchardus***	
GURNARD	ANCHOVY	**SWORDFISH**	
Chelydonictis lucerna	*Engraulis encrasicolus*	***Xiphia gladius***	
STARGAZER	ROUND SARDINELLA	SPINY DOGFISH	
Uranoscopus scaber	*Sardinella aurita*	*Squalus acanthias*	
JOHN DORY	YELLOWFIN TUNA		
Zeus faber	*Thunnus albacares*		
SCORPION FISH	**SMALL-SPOTTED CATSHARK**		
Scorpaena scrofa	***Scyliorhinus canicula***		
BLUESPOTTED SEABREAM			
Pagrus caeruleostictus			
YELLOW GOOSEFISH			
Lophius litulon			
COMMON SOLE			
Solea solea			
GUINEAN SOLE			
Synaptura cadenati			
SPOTTAIL SPINY TURBOT			
Psettodes belcheri			
BLUE SHARK			
Prionace glauca			
SMOOTHHOUND			
Mustelus mustelus			
PORBEAGLE			
Lamna nasus			
RAY			
Raja sp.			

Table 1 Species used in this study grouped on the basis of their fat content according to Ackmann, 1990. The fat content values of the species were retrieved from Prato & Biandolino, (2012); Ozogul & Ozogul (2007) and FAO values (http://www.fao.org/wairdocs/tan/x5916e/x5916e01.htm) In bold the species used for the optimization of the bead extraction method and the real time PCR.

Table
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Table 1SM The table report the DNA extraction method (Commercial kit and Classical procedure) used in article dealing with fish species identification. The procedure market with * did not use enzymatic digestion with proteinase K.

Article	Samples	Method		Reference/ Company
		Commercial kit	Classical procedure	
Identifying Canadian Freshwater Fishes through DNA Barcodes Hubert, *et al*. (2008). Plos One 3(6): e2490.	Fresh	NucleoSpin96 kit		MACHEREY-NAGEL GmbH & Co. KG, D-52313 Düren, Germany
Authentication of Anglerfish Species (Lophius spp) by Means of Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) and Forensically Informative Nucleotide Sequencing (FINS) Methodologies. **Espineira, *et al*. (2008).** *Journal of Agricultural and Food Chemistry* 56 (22): 10594-10599.	Fresh/processed		Phenol-chloroform protocol *	**Rogers, *et al*. (1988)**. Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
DNA barcoding detects market substitution in North American seafood. **Wong & Hanner (2008)**. *Food Research International* 41(8): 828-837	Fresh/frying/ precooked		Glass Fiber protocol	**Ivanova, *et al*. (2006)**. An inexpensive, utomation-friendly protocol for recovering high-quality DNA. Molecular Ecology Notes 6(4): 998-1002.
Detection of Mislabeling in Hake Seafood Employing mtSNPs-Based Methodology with Identification of Eleven Hake Species of the Genus Merluccius. **Machado-Schiaffino, *et al*. (2008).** *Journal of Agricultural and Food Chemistry* 56 (13): 5091-5095.	Fresh/frozen/ precooked		Chelex resin protocol	**Estoup, *et al*. (1996)**. Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes." Molecular Marine Biology and Biotechnology 5(4): 295-298.

DNA barcoding for the identification of smoked fish products **Smith, *et al*. (2008).** *Journal of Fish Biology* 72(2): 464-471.	Smoked		Phenol–chloroform–ethanol protocol	**Taggart, *et al.* (1992)**. A simplified protocol for routine total DNA isolation from salmonid fishes. Journal of Fish Biology 40(6): 963-965.
Development of a method for the identification of scombroid and common substitute species in seafood products by FINS **Espineira, *et al.* (2009).** *Food Chemistry* 117(4): 698-704.	Fresh/frozen/ processed	NucleoSpin Tissue kit	Chloroform-isoamyl protocol*	MACHEREY-NAGEL GmbH & Co. KG., D-52313 Düren, Germany. **Rogers, et al. (1985).** Extraction of DNA from milligram amounts of fresh, herbarium and mummified plant tissues. *Plant Molecular Biology* 5(2): 69-76.
PCR-based methodology for the authentication of grouper (*Epinephelus marginatus*) in commercial fish fillets. **Asensio, *et al*. (2009).** *Food Control* 20(7): 618-622.	Fresh/processed	Wizard® DNA Clean-Up System		Promega Corporation, 2800 Madison, WI 53711 USA
The Real maccoyii: Identifying Tuna Sushi with DNA Barcodes – Contrasting Characteristic Attributes and Genetic Distances **Lowenstein, *et al*. (2009).** *Plos One* 4(11): e7866	Sushi (fresh)	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
A Validated Methodology for Genetic Identification of Tuna Species (Genus Thunnus) **Vinas, *et al*. (2009).** *Plos One* 4(10):e7606	Fresh		Phenol-chloroform-isoamyl protocol	**Vinas, *et al*. (2004)**. Inter-oceanic genetic differentiation among albacore (Thunnus alalunga) populations. *Marine Biology* 145(2): 225-232.
Identification of shark and ray fins using DNA barcoding **Holmes, *et al*. (2009).** *Fisheries Research* 95(2-3): 280-288.	Fresh/dried		Chelex resin protocol Glass Fiber protocol	**Walsh, *et al*. (1991).** Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. *Biotechniques* 10(4): 506-513. **Ivanova, *et al.* (2006).** An inexpensive, automation-friendly protocol for recovering

				high-quality DNA. *Molecular Ecology Notes* 6(4): 998-1002.
The application of PCR–RFLP and FINS for species identification used in sea cucumbers (Aspidochirotida: Stichopodidae) products from the market **Wen, *et al*. (2010).** *Food Control* 21(4): 403-407.	Fresh/dried	TIANamp Marine Animals DNA Kit		Tiangen Biotech Co. Ltd., Beijing, China
Identification of European Hake Species (Merluccius merluccius) Using Real-Time PCR **Sanchez, *et al*. (2009).** *Journal of Agricultural and Food Chemistry* 57(9): 3397-3403.	Fresh/ canned/ refrigerated/ precooked/battered	Wizard® DNA Clean-Up System		Promega Corporation 2800 Madison, WI 53711 USA
DNA barcoding for conservation and management of Amazonian commercial fish. **Ardura, *et al*. (2010).** *Biological Conservation* 143(6): 1438-1443.	Fresh/processed		Chelex resin protocol	**Estoup, *et al.* (1996).** "Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes." *Molecular Marine Biology and Biotechnology* 5(4): 295-298.
A multiplex-PCR assay for the authentication of mackerels of the genus Scomber in processed fish products. **Catanese, *et al*. (2010).** *Food Chemistry* 122(1): 319-326.	Fresh/canned	FastDNA kit*		MP Biomedicals LLC; QBiogene Division 29525 Fountain Parkway, Solon, OH 44139
Species identification in anchovy pastes from the market by PCR-RFLP technique. **Rea, *et al.* (2009**). *Food Control* 20(5): 515-520.	Fresh/processed samples		Phenol–chloroform protocol	**Sambrook, *et al*. (1987**). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press. Woodbury, NY, USA
Molecular barcoding reveals mislabelling of commercial fish products in Italy **Filonzi, *et al*. (2010).** *Food Research International* 43(5): 1383-1388.	Fresh/processed	Aquapure Genomic DNA kit	Phenol–chloroform protocol	Bio-Rad Laboratories, Inc., 2000 A. Nobel Dr. Hercules,CA 94547 USA **Moore, *et al*. (1999).** Manipulation of DNA." Current protocols in molecular biology, John Wiley & Sons Inc., New York (1999), 211–223
Application of a PCR-RFLP Method to Identify Salmon Species	Canned/smoked/	DNeasy Blood &		QIAGEN GmbH, 40724 Hilden, Germany

in U.S. Commercial Products **Rasmussen, *et al.* (2010).** *Journal of Aquatic Food Product Technology* 19(1): 3-15.	jerky/fresh	Tissue Kit		
Misleading the masses: detection of mislabelled and substituted frozen fish products in South Africa **Von der Heyden, *et al*. (2010).** *Ices Journal of Marine Science* 67(1): 176-185.	Fresh	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Authentication of swordfish (Xiphias gladius) by RT–PCR and FINS methodologies **Herrero, et al. (2011).** *European Food Research and Technology* 233(2): 195-202.	Fresh/frozen/ smoked		Phenol-chloroform protocol *	**Rogers, *et al.* (1988).** Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
FINS methodology to identification of sardines and related species in canned products and detection of mixture by means of SNP analysis systems. **Lago, *et al*. (2011).** *European Food Research and Technology* 232(6): 1077-1086.	Fresh/processed samples		Phenol-chloroform protocol *	**Rogers, *et al.* (1988).** Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
Molecular identification of the black tiger shrimp (Penaeus monodon), the white leg shrimp (Litopenaeus vannamei) and the Indian white shrimp (Fenneropenaeus indicus) by PCR targeted to the 16S rRNA mtDNA **Pascoal, *et al*. (2011**). *Food Chemistry* 125(4): 1457-1461.	Fresh samples	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Duplex real-time PCR for authentication of anglerfish species **Herrero, *et al*. (2011).** *European Food Research and Technology* 233(5): 817-823.	Fresh/precooked		Proteinase K-phenol-chloroform protocol	**Rogers, *et al.* (1988).** Extraction of DNA from plant tissues. Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
Identification of fish species by 5S rRNA gene amplification **Tognoli, *et al*. (2011).** *Food Chemistry* 129(4): 1860-1864.	Fresh		Phenol-chloroform-isoamyl- alcohol protocol	**Aranishi. (2005).** "Rapid PCR-RFLP method for discrimination of imported and domestic mackerel." *Marine Biotechnology* 7(6): 571-575.
DNA barcoding unveils a high rate of mislabeling in a commercial freshwater catfish from Brazil.	Fresh		Phenol-chloroform protocol	**Sambrook, *et al*. (1989).** Molecular Cloning: A Laboratory Manual, Cold Spring Harbor

Carvalho, *et al*. (2011). *Mitochondrial DNA* 22: 97-105.				Laboratory Press. Woodbury, NY, USA
High Level of Mislabeling in Spanish and Greek Hake Markets Suggests the Fraudulent Introduction of African Species **Garcia-Vazquez, *et al*. (2011).** *Journal of Agricultural and Food Chemistry* 59(2): 475-480.	Fresh		Chelex resin protocol	**Moran, *et al*. (2006).** "Identification of highly prized commercial fish using a PCR-based methodology." *Biochemistry and Molecular Biology Education* 34(2): 121-124.
Identification of barramundi (Lates calcarifer) and tilapia (Oreochromis spp.) fillets by DNA- and protein-analytical methods. **Schiefenhoevel, *et al*. (2011).** *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit* 6(2): 203-214.	Fresh		Chloroform-isopropanol protocol	**Rehbein. (2005)**. "Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-strand conformation polymorphism (SSCP) analysis." *European Food Research and Technology* 220(5-6): 625-632.
DNA Barcoding of Catfish: Species Authentication and Phylogenetic Assessment. **Wong, *et al*. (2011).** *Plos One* 6(3):e17812	Fin clip (fresh)	Gentra Puregene Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
DNA barcoding reveals a high incidence of fi sh species misrepresentation and substitution on the South African market **Cawthorn, *et al*. (2012).** *Food Research International* 46(1): 30-40.	Fresh/frozen/processed	SureFood® PREP Allergen Kit		R-Biopharm AG, 64297 Darmstadt, Germany
Chemical characterisation, biogenic amines contents, and identification of fish species in cod and escolar steaks, and salted escolar roe products. **Hwang, *et al*. (2012).** *Food Control* 25(1): 415-420.	Fresh/salted	Chemagic DNA Tissue 10 Kit *		PerkinElmer Chemagen Technologie GmbH 52499 Baesweiler, Germany
A unique specification method for processed unicorn filefish products using a DNA barcode marker. **Yang, *et al*. (2012).** *Food Control* 25(1): 292-302.	Canned/dried/fried	MasterPure™ DNA Purification Kit		Epicentre Biotechnologies, Madison, WI, 53719 USA
Authentication of commercialized crab-meat in Chile using DNA Barcoding. **Haye, *et al*. (2012).** *Food Control* 25(1): 239-244.	Fresh/canned		Phenol-Chloroform method	**Sambrook, *et al.* (1989).** Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press. Woodbury, NY, USA

Inaccurate labelling detected at landings and markets: The case of European megrims. **Crego-Prieto, *et al.* (2012).** *Fisheries Research* 129: 106-109.	Fresh samples		Chelex resin protocol	**Estoup, *et al.* (1996).** "Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes." *Molecular Marine Biology and Biotechnology* 5(4): 295-298.
Identification of 11 sea cucumber species by species-specific PCR method. **Wen, *et al.* (2013).** *Food Control* 29(1): 287-287.	Fresh/fresh/dried	TIANamp Marine Animals DNA Kit		Tiangen Biotech Co. Ltd., Beijing, China
DNA Barcoding as a Reliable Method for the Authentication of Commercial Seafood Products **Nicole, *et al.* (2012).** *Food Technology and Biotechnology* 50(4): 387-398.	Fresh/frozen/smoked/dried	GenElute Mammalian Genomic DNA Miniprep Kit		Sigma-Aldrich, LLC., St. Louis, Missouri 63103 United States
Forensic DNA analysis reveals use of high trophic level marine fish in commercial aquaculture fish meals **Ardura, *et al.* (2012).** *Fisheries Research* 115: 115-120.	Canned/fresh/ smoked/salty/ dried	QIAamp® DNA Mini Kit		QIAGEN GmbH, 40724 Hilden, Germany
Authentication of the most important species of freshwater eels by means of FINS. **Lago, *et al.* (2012).** *European Food Research and Technology* 234(4): 689-694.	Frozen		Phenol and chloroform protocol *	**Rogers, *et al.* (1988).** Extraction of DNA from plant tissues. In: Plant Molecular Biology Manual. S. Gelvin, R. Schilperoort and D. Verma, Springer Netherlands: 73-83.
Development of real-time PCR assays for the detection of Atlantic cod (Gadus morhua), Atlantic salmon (Salmo salar) and European plaice (Pleuronectes platessa) in complex food samples. **Hird, *et al.* (2012).** *Eu. Food Res.Tech*234(1): 127-136.	Fresh/cooked/ autoclave		Chloroform protocol	**Meyer, *et al.* (1994).** Detection of pork in heated meat products by the polymerase chain reaction. *Journal of AOAC International* 77(3): 617-622.
Application of FINS and multiplex PCR for detecting genuine abalone products. **Chan, *et al.* (2012).** *Food Control* 23(1): 137-142.	Dried/canned		Phenol-chloroform-isoamyl protocol*	**Kang, *et al.* (1998).** A rapid DNA extraction method for RFLP and PCR analysis from a single dry seed. *Plant Molecular Biology Reporter* 16(1): 90-90.
Detection and quantification of tissue of origin in salmon and veal products using methylation sensitive AFLPs. **Rodriguez**	Fresh	NucleoSpin® Tissue Kit		MACHEREY-NAGEL GmbH & Co. KG, D-52313 Düren, Germany

Lopez, *et al*. (2012). *Food Chemistry* 131(4): 1493-1498.				
Identification of tuna species by a real-time polymerase chain reaction technique **Chuang, *et al*. (2012).** *Food Chemistry* 133(3): 1055-1061.	Fresh/canned samples	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Molecular identification of fish species from surimi-based products labeled as Alaskan Pollock. **Keskin, *et al.* (2012).** *Journal of Applied Ichthyology* 28(5): 811-814.	Surimi	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
DNA barcoding commercially important fish species of Turkey **Keskin, *et al*. (2013).** *Molecular Ecology Resources* 13(5): 788-797.	Fresh muscle	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Detection of mislabeled commercial fishery by-products in the Philippines using DNA barcodes and its implications to food traceability and safety **Maralit, *et al*. (2013).** *Food Control* 33(1): 119-125.	Fresh/frozen		Chloroform protocol	**Santos, *et al.* (2010).** A pilot study on the genetic variation of Eastern little tuna (Euthynnus affinis) in Southeast Asia. *Philippine Journal of Science*, 139 (1): 43–50
Ensuring seafood identity: Grouper identification by real-time nucleic acid sequence-based amplification (RT-NASBA) **Ulrich, *et al*. (2013).** *Food Control* 31(2): 337-344.	Fresh/frozen/ ethanol preserved	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Rapid species identification of fresh and processed scallops by multiplex PCR **Marin, *et al*. (2013).** *Food Control* 32(2): 472-476.	Fresh/canned/ boiled		Phenol-chloroform protocol	**Sokolov. (2000).** "An improved method for DNA isolation from mucopolysaccharide-rich molluscan tissues." *Journal of Molluscan Studies* 66: 573-575.
A novel minisequencing test for species identification of salted and dried products derived from species belonging to Gadiformes **Dalmasso, *et al*. (2013).** Food Control 34(2): 296-299.	Fresh/salted/ dried samples	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Commercialization of a critically endangered species (largetooth sawfish, Pristis perotteti) in fish markets of northern Brazil: Authenticity by DNA analysis **Melo Palmeira, *et al*. (2013).**	Fresh/salted samples		Phenol-chlorophorm protocol	**Sambrook, *et al.* (2001**). Molecular Cloning: A Laboratory Manual, *Cold Spring Harbor Laboratory Press. Woodbury, NY, USA*

Food Control 34(1): 249-252.				
DNA barcoding for detecting market substitution in salted cod fillets and battered cod chunks **Di Pinto, *et al*. (2013).** *Food Chemistry* 141(3): 1757-1762.	Salted	DNeasy Blood & Tissue Kit		QIAGEN GmbH, 40724 Hilden, Germany
Development of a rapid genetic technique for the identification of clupeid larvae in the Western English Channel and investigation of mislabeling in processed fish products. **Brechon, *et al.* (2013).** *Ices Journal of Marine Science* 70(2): 399-407.	Fresh/processed	Wizard kit for adult fish	Chelex resin protocol	Promega Corporation, 2800 Madison, WI 53711 USA； **Estoup, *et al.* (1996)**. Rapid one-tube DNA extraction for reliable PCR detection of fish polymorphic markers and transgenes. Molecular *Marine Biology and Biotechnology* 5(4): 295-298.
Differentiation of Sparidae species by DNA sequence analysis, PCR-SSCP and IEF of sarcoplasmic proteins. **Schiefenhoevel, *et al.* (2013).** *Food Chemistry* 138(1): 154-160.	Frozen/processed samples		Chloroform-isopropanol protocol	**Rehbein. (2005).** Identification of the fish species of raw or cold-smoked salmon and salmon caviar by single-strand conformation polymorphism (SSCP) analysis. *European Food Research and Technolog*y 220(5-6): 625-632.

Table 2SM Level of significance, evaluated using two mixed models with REML estimation of variance, of the effects associated to the factors method (M), species (S), and interaction between method and species (M*S) (model 1), or method (M), category (C), and interaction between method and category (M*C) (model 2) on yield, A260/A280, and A260/A230 for fresh and ethanol preserved samples. *** $p<0.001$; ** $p<0.01$; * $p<0.05$; ns=non significant.

	Fresh			**Ethanol**		
	Yield	**A260/A280**	**A260/A230**	**Yield**	**A260/A280**	**A260/A230**
M	***	***	***	***	***	ns
S	***	***	***	***	***	ns
M*S	***	***	***	***	***	ns
M	***	***	***	***	***	ns
C	**	ns	*	ns	ns	ns
M*C	***	*	***	ns	ns	ns

Table 3SM Level of significance, evaluated using a mixed model with REML estimation of variance, of the effects associated to the factors method (M), initial amount of tissue (W), their interaction (M*W), and category (C) on DNA relative yield, A260/A280, and A260/A230. *** $p<0.001$; ** $p<0.01$; * $p<0.05$.

	Yield	**A260/A280**	**A260/A230**
M	***	***	***
W	***	***	*
M*W	***	***	**
S	***	***	***

Table 4SM Level of significance, evaluated using a mixed model with REML estimation of variance, of the effects associated to the factors method (M), species (S), and interaction between method and species (M*S) on the band intensity after conventional PCR of the COI and 16S genes and on the take-off cycle measured by real-time PCR on the gene 16S. *** p<0.001.

	Band intensity		**Take-off**
	COI	*16S*	*16S*
M	***	***	***
S	***	***	***
M*S	***	***	***