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Analytical Methods

Comparison of methods in the recovery and amplificability of DNA from fresh and processed sardine and anchovy muscle tissues

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ABSTRACT

An important condition for a successful PCR amplification is an efficient DNA-extraction procedure out of a complex biological matrix such as canned fish. In this study we compared six extraction methods, including commercial kit, in terms of DNA yield, purity and time requirement. Such methods were applied to distinguish small pelagic fish species (*Sardina pilchardus* and *Engraulis encrasicolus*) among commercial canned products. The quantity and quality of DNA extracted were evaluated using the ratio A_{260}/A_{280}. Data were submitted to principal component analysis (PCA) in order to assess the differences between PCR results of fresh and processed anchovy and sardine muscles. Two main PC characterised the PCR of sardine and anchovy (70% and 69% of all variance): principal component 1 (PC1) (4% and 60%) and principal component 2 PC2 (66.0% and 9%) for sardine and anchovy, respectively. According to the PC1, the PCI/SDS and Chelex extractions (in decreasing order) were positively correlated with results of PCR for both species.

Statistical results confirmed that the quality of DNA, with the highest amplicon length obtained from the fresh and the different canned fish, was best preserved using the SDS/PCI precipitation method.

1. Introduction

DNA-based techniques are considered very efficient for food authentication and the identification of certain fish and fisheries products (Comi, Iacumin, Rantsiou, Cantoni, & Cocolin, 2004; Pepe et al., 2005). Because of it is remarkably stability even at high temperature, the majority of work has focused on exploiting DNA analysis for species identification especially in thermally processed seafood product (Chapela et al., 2007; Pardo & Pérez-Villarreal, 2004).

In such studies, mitochondrial genome (mt DNA) is frequently target because of its relative abundance in respect to nuclear DNA (Mackie et al., 1999; Sebastio, Zanelli, & Neri, 2001) and resistance to degradation following processing (Bartlett & Davidson, 1991). However, the problem of nucleic acid extraction remains critical for downstream application such as polymerase chain reaction (PCR). The application of PCR to amplify the specific fragments of DNA of interest depends on the extraction of DNA from the heterogeneous fish/seafood tissues, which is often the most critical step (Infante et al., 2006). Therefore the isolation of a sufficient amount of high quality DNA is essential for the success of the whole molecular study.

In general, fresh tissue of fish that has not been thermally-treated has the best probability of yielding adequate DNA. Even so the quantity and the quality of the extracted DNA are extremely sample-dependant (Chapela et al., 2007), as a method optimised for the identification of a certain species, is inappropriate to analyse a given sample from a different species with a method that was not designed for that species. Thus DNA isolation from fresh heterogeneous seafood tissues such as sardina and anchovy usually requires some adjustments to habitually used protocols because of the complexity of the matrix which may introduce some degrees of variability into the DNA extraction methods and the efficiency of the DNA amplification (Bauer, Weller, Hammes, & Hertel, 2003).

Several protocols for DNA extraction from pelagic fish samples have been described including the phenol–chloroform-based approaches (Hsieh, Chai, & Hwang, 2005), the CTAB method (Quinta, Gomes, & Teia dos Santos, 2004) with various modifications and the use of commercial kit such as Chelex (Jérôme, Lemaire, Bautista, Fleurence, & Etienne, 2003; Jérôme, Lemaire, Verrez-Bagnis, & Etienne, 2003). In addition, for canned pelagic fish including sardine and tuna, sample pre-treatment is required before DNA extraction to defat the tissues (Olexová, Dovicovicová, & Kuchta, 2004; Peano, Samson, Palmieri, Gulli, & Marmiroli, 2004; Zimmermann, Lüthy, & Pauli, 1998).

The intention of this article is not to revise the commonly used methods but rather to compare and determine which technique consistently yields the highest amount of amplifiable DNA for

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fresh/processed "*Sardina pilchardus* and *Engraulis encrasicolus*" Therefore, the main objective of this study is to evaluate 6 methods of DNA extraction in terms of DNA yield and amplification quality.

2. Materials and methods

2.1. Sample preparation

Different brand of canned sardine (4) and anchovy (1) products in vegetable oil were purchased at local supermarkets in Tunisia. The fresh fish *Sardina pilchardus* and anchovy *Engraulis encrasicolus* were caught by the research vessel "Hannibal" of INSTM during a fishing campaign of small pelagic fish in the North zone of Tunisia, and taxonomically identified with respect to external characters. The salted anchovy were purchased at a local fish market. In the laboratory, fish were stored at −80 °C until analysis.

2.2. Sample pre-treatment

Oil was removed from canned fish by soaking fish muscle overnight in a mixture of chloroform/methanol/water (1:2:0.8). The defatted muscle was recovered by filtration and stored frozen at −80 °C until DNA was extracted.

2.3. DNA extraction

2.3.1. phenol–chloroform–isoamyl alcohol: SDS/PCI method (EX1)

DNA was extracted according to the protocol described by Quinteiro et al. (1998). Sample (0.1 g) was homogenised with 500 μL the extraction buffer A composed of 50 mM Tris–HCl, 100 mM EDTA pH 8.0, 1% SDS, 0.1 mg (3.2 U) of proteinase K (Sigma Aldrich, France). The mixture was incubated 4 h at 55 °C with shaking, then chilled on ice for 30 min. Following a centrifugation at 12,000*g* for 10 min, the supernatant was extracted once with phenol, twice with phenol–chloroform–isoamylic alcohol in a 25:24:1 ratio and once with chloroform, then precipitated with ethanol at −20 °C in the presence of 1/10 volume of sodium acetate 3 M, pH 5. After washing with 70% ethanol and drying at room temperature, the pellet was resuspended in 50 μL TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The DNA extraction for each sample was repeated several time, in this paper the result were reported for $n = 6$ in each case.

2.3.2. Chelex method (EX2)

According to Jérôme, Lemaire, Bautista et al. (2003) and Jérôme, Lemaire, Verrez-Bagnis et al. (2003), 0.1 g of sample was vortexed in 300 μL of a 5% Chelex water solution (Chelex 100 resin Qiagen) with 20 μL of proteinase K (0.1 mg) and 30 μL TE buffer. The mixture was incubated at 56 °C for 4 h to break down all tissue and then heated at 100 °C for 15 min to denature and precipitate resin-bound proteins. Following a centrifugation at 12,000*g* for 10 min, the DNA suspension was stored at 4 °C until use for PCR amplification.

2.3.3. SDS/PCI/NaCl method (EX3)

According to Hsieh et al. (2005), 0.1 g of sample was extracted similarly as the SDS/PCI method, except that NaCl was added to the extraction buffer A to a final concentration of 0.2 M NaCl.

2.3.4. SDS/CI chloroform–isoamyl alcohol method (EX4)

DNA was extracted according to the protocol described by Desalle and Birstein (1996) with slight modification. About 0.1 g of sample was homogenised with the extraction buffer A as for the SDS/PCI method. After overnight incubation at 55 °C, chilling in ices and centrifugation, the supernatant was extracted twice with chloroform–isoamylic alcohol in a 24:1 ratio and once with chloroform, then precipitated with ethanol and resuspended in 50 μL TE buffer.

2.3.5. TRITON/CI chloroform–isoamyl alcohol method (EX5)

This method was inspired from the report of Amita, Vandana, Guleria, and Verma (2002). The muscle tissue (0.1 g) was homogenised in 500 μL of the extraction buffer B (10 mM Tris–HCl, 2 mM EDTA, pH 8.0, containing 50 μL ß-mercaptoethanol 0.2% at final concentration and 50 μL Triton X-100 at 1% final concentration, 0.1 mg (3.2 U) of proteinase K). After incubation for 12 h at 65 °C, the mixtures were centrifuged at 10 min at 12,000*g* at 4 °C. DNA was then extracted with an equal volume of chloroform–isoamylic alcohol. The solution was vortexed vigorously and then centrifuged at 12,000*g* for 10 min. The DNA was precipitated with ethanol in the presence of sodium acetate and resuspended in 50 μL TE buffer.

2.3.6. CTAB/CI method (EX6)

DNA was extracted according to the protocol described by Quinta et al. (2004), with some modification. The muscle tissue (0.1 g) was homogenised in 500 μL CTAB buffer (2% hexadecyl–trimethyl ammonium bromide, 20 mM EDTA, 100 mM Tris–HCl, 1.4 M NaCl, pH 8) added of 50 μL ß-mercaptoethanol 0.2% at final concentration, 0.1 mg (3.2 U) of proteinase K. The mixture were incubated for 12 h at 65 °C, and then centrifuged for 10 min at 12,000*g*. The supernatant was extracted twice with chloroform–isoamylic alcohol and precipitated with ethanol, then resuspended in TE buffer.

2.4. DNA quantification and purity

Extracted DNA was estimated by absorbance at 260 nm. One DO at 260 nm equaling 50 μg/mL DNA. Purity was checked by determining the ratio A_{260}/A_{280} (Sambrock, Fritsch, & Maniatis, 1989).

2.5. PCR amplification

To confirm the spectrophotometric results, and check the suitability of the prepared DNA for molecular identification of the sample origin, the PCR was employed. As processing cause DNA degradation, two pairs of primers were used to amplify the mitochondrial cytochrome *b* gene. The primers cytB1 (5′-CCA TCC AAC ATC TCA GCA TGA TGA AA-3′) and cytB2 (5′-CCC CTC AGA ATG ATA TTT GTC CTCA-3′) described by (Bartlett & Davidson, 1991) amplified a 358 bp fragment; whereas, the primers U24 (5′-CCC ACT TCT CAA AAT TGC TAA CGA CGC-3′) and L252 (5′-ATG CAA ATG AAG AAG AAA GAT GCT CCG TTT-3′) designed for this study, allowed the amplification of a 258 bp fragment. PCR amplification reactions were performed in a final volume of 25 μL. Each reaction mixture contained 100 ng of extracted DNA template, 5 μL of 5× PCR buffer, 50 pmol of each primer, 2 μL of dNTP mix (10 mM of each); 3 mM of $MgCl_2$ and 5 U of Taq DNA polymerase (Go-Taq, Promega, France). The following PCR conditions were used: a denaturation step at 94 °C for 5 min, followed by 35 cycles consisting of 94 °C for 30 s, 47 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. DNA amplification was checked by electrophoresis in 2% agarose gels using TBE buffer (1 mM EDTA, 45 mM Tris borate, pH 8), with ethidium bromide (0.2 μg/mL). PCR products were visualised via ultraviolet trans-illumination before sequencing. The size of the resulting DNA fragments was compared with a commercial 1 kb ladder (Fermentas, France).