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An alternative and effective tissue pre-treatment to improve the DNA identification of canned tuna samples

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ABSTRACT

DNA purity represents a major challenge to investigate food authenticity of canned products due to DNA degradation. Herein, we present a low-cost protocol to increase concentration and purity of DNA extracted from canned samples. The experiment mainly consists of: (1) drying the canned tissue in paper filter, (2) washing it with a PBS solution, (3) store in ethanol 96 % at −20°C, and (4) perform DNA extraction. The pre-treated samples showed an increase of both DNA concentration and purity (A260/A280 ratio), indicating that some of the inhibiting molecules were successfully removed. These differences between the two treatments were statistically significant ($p < 0.01$). At the amplification level, the pre-treatment allowed the recovery of complete fragments of the barcode region COX1 with approximately 650 bp. Since obtaining relevant levels of DNA purity and concentration from degraded samples, and the sequencing of large fragments from processed samples represents a difficult task, the presented results demonstrate a positive effect of the proposed protocol. Thus, the combination of this treatment with other methodologies, such as mini-barcoding, and sample types is strongly encouraged.

1. Introduction

Tuna meat represents a widely important seafood product, especially used by canning industry, which involves several steps, such as filleting, freezing, defrosting, cooking, and canning (Pecoraro et al., 2020; FAO, 2024). The morphological mischaracterization makes traditional species identification impossible, favoring the mislabeling occurrence (Wong & Hanner, 2008; Hellberg & Morrissey, 2011; Xing et al., 2020; Zhao et al., 2024), such as substitution of high for lower-values species, or illegal commerce of endangered species (e.g. Pardo & Jiménez, 2020; Eppley & Coote, 2025; Zhang et al., 2025).

In this context, molecular methods represent suitable tools, by using specific primers set to amplify target DNA regions through PCR (Polymerase Chain Reaction), especially the DNA barcode (Cytochrome c Oxidase Subunit I; COXI gene), which is the most traditional DNA-based method for fish identification (Hebert et al., 2003; Rey et al., 2023; Sharrad et al., 2023). However, obtaining high-purity DNA is a pre-requisite for DNA-based methods (Xiong et al., 2019; Tumerkan, 2022) that looks forward to adequate information. Due to the high number of processing steps and the uses of several substances for long-term preservation, the DNA is fragmented into small pieces, many of them uninformative (Rasmussen et al., 2009; Pecoraro et al., 2020), making the obtention of good DNA a major challenge to studies that used using processed samples (Armani et al., 2014).

Different methodologies have been developed for fish products authentication, such as real-time PCR (Servusova, Piskata,2021), PCR-RFLPs (Mata et al., 2020), Mid infrared spectroscopy (Boughattas & Karoui, 2021), and Multiplex PCR (Lee et al., 2022). However, several studies have documented lower success rates (0–39 %) in amplifying large fragments from canned products when compared to others processed products (e.g. Shokralla et al., 2015; Pollack et al., 2018; Sultana et al., 2018; Xing et al. 2020; Roungchun et al., 2022). Thus, the mini-barcode strategies, by amplifying shorter fragments (100–200 bp), have been adopted to identify species from processed samples. Nonetheless, despite presenting a higher success rate than full barcodes (50 % and 39 %, respectively; Pollack et al., 2018), this size-reduction of genetic information can make difficult the differentiation of closely related species, such as tunas (Bucklin et al., 2011).

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Until now, few studies have focused on amending this problem by optimizing the first and most crucial step in DNA authentication methods: DNA recovery (e.g., Faraji et al., 2017; Xiong et al., 2019). Thus, we tested an additional and cost-effective pre-treatment to recover DNA from canned tuna, using a common DNA Extraction Kit from tissue samples, aiming to remove their contaminants, to increase the DNA concentration and purity and improve work routines with processed food samples.

2. Material and methods

2.1. Sample collection, pre-treatment and DNA extraction

The pre-treatment protocol consists of two main steps described below and in Fig. 1. From each canned product, two-three samples were collected. The third step is a conventional tool to preserve tissue samples for molecular procedures.

(1) Step 1: Drying – Before the DNA extraction with traditional tissue kits, approximately 50 mg of canned meat were air-dried in filter paper for 10 (ten) minutes to remove part of the solution used to preserve the canned meat. This amount of tissue is based on the indicated by commercial DNA extractions Kits (10–20 mg).
(2) Step 2: Washing – Using a sterilized clamp, the dried samples were transferred to a 1.5 mL microtube and mixed with 500–700 μL (Note that a higher volume could be required depending on the sampled tissue size. But it is important that the entire sample be submerged in the solution) of Phosphate Buffered Saline (1X; pH 7.2; Composition: NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 10 mM, KH_2PO_4 1.8 mM; PBS – Termofisher Scientific®, Gibco™ PBS) using the vortex for 10–15 s. A mechanical mixture could be used in the absence of vortex equipment.
    The mixture was centrifuged at room temperature (25–27°C) for 10 (ten) minutes with a maximum speed of 13,000 rpm (or 17,000 xg). The supernatant discarded using a sterilized pipette tip. This second step was repeated three times, and a final centrifugation was performed in the same conditions described above. The decantation method could be used in this step if the centrifuge equipment is absent.
(3) Step 3: Preserving – After the final centrifugation and discharging of the supernatant, the tissue was transferred to a sterile cryotube or a 1.5 mL microtube containing a sufficient volume of 96 % ethanol to cover the entire sample and stored at −20°C.

We recommend leaving the samples resting for at least 24 h before proceeding with the DNA extraction. Ethanol is responsible for dehydrating the tissue samples, reducing DNA solubility, especially at low temperatures.

The DNA was extracted using the PureLink™ Genomic DNA Mini Kit (Invitrogen™ Life Technologies®) following the manufacturer's instructions. The nano spectrophotometer Nanodrop 2000 was used to quantify the DNA [concentration (ng/μL) and purity (A260/A280 ratio)].

To compare the efficiency of this methodology, the DNA was extracted from canned samples without the pre-treatment, using the same DNA extraction Kit and instructions. Samples labeled from PS1-PS13 represent the pre-treated, and those from NS14-NS26 represent non-pre-treated samples. In addition, we included concentration and purity data from non-processed samples (fin and tissue) collected from skipjack tuna *Katsuwonus pelamis*, as a positive control. These samples were stored in ethanol 96 % at −20°C and labelled as KP1-KP13 (Supplementary Table S1).

2.2. Amplification and sequencing

The full-length COXI barcode region was amplified through PCR using two universal Fish primers pairs described by Ward et al. (2005): FishF1 (5′TCAACCAACCACAAAGACATTGGCAC3′) and FishR1 (5′ TAGACTTCTGGGTGGCCAAAGAATCA 3′), and FishF2 (5′ TCGACTAATCATAAAGATATCGGCAC 3′) and FishR2 (5′ ACTTCAGGGTGACCGAAGAATCAGAA 3′). All reactions were prepared with a final volume of 25 μL containing: 12.5 μL of 2X Taq Pol Master Mix (Cellco®), 0.5 μL of each primer (10 mM), 0.5 μL of magnesium chloride (50 mM), 2 μL of genomic DNA (40 ng/μL), and 9 μL of ultrapure water. The amplification cycle was modified from Ward et al. (2005): 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 52°C for 40 s, 72°C for 1 min, and a final extension at 72°C for 10 min. To improve the quality of the fragments, we tested the addition of 0.2 μL of Taq Polymerase (Cellco®) and

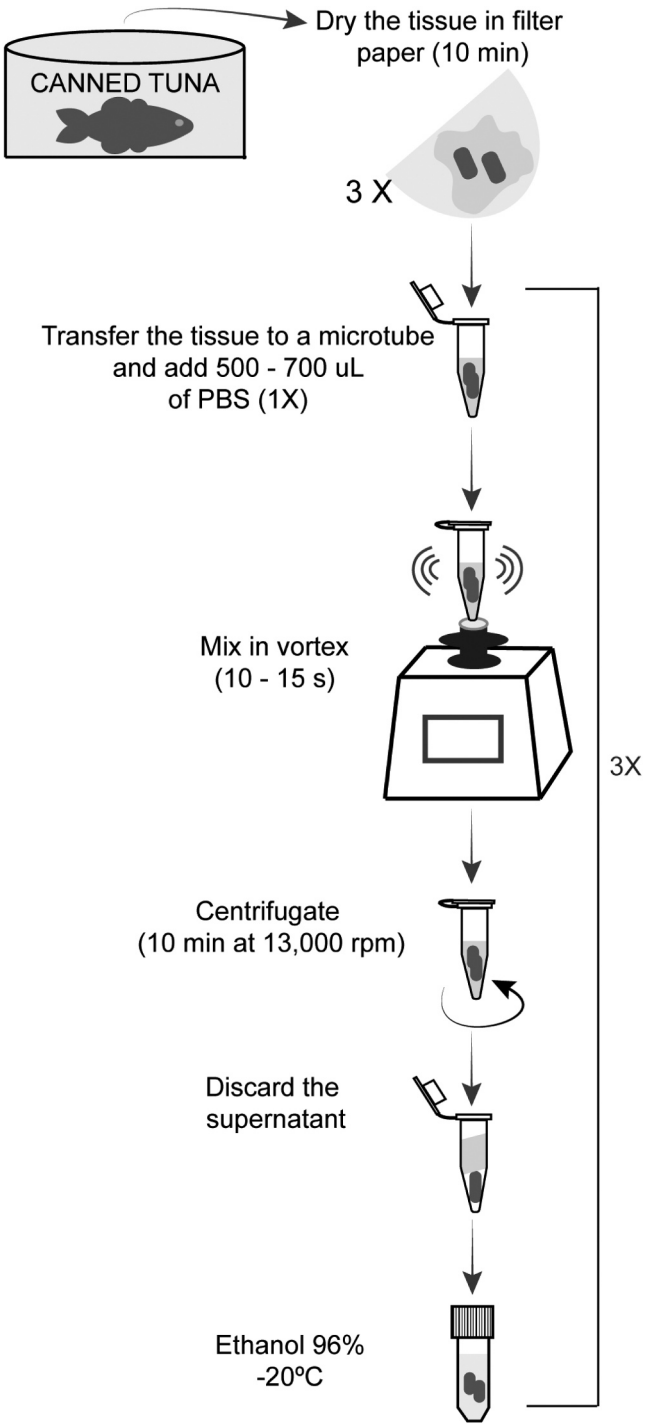

Fig. 1. Pre-treatment scheme to extract DNA from canned tuna.

higher DNA volume (4 μL). The samples were visualized in 1.8 % electrophoresis agarose gel, purified using the Nucleosap (Cellco®) following the manufacture instructions, diluted to 20 ng/μL, and sequenced in the ABI 3500 automatic sequencer (Applied Biosystems).

The electropherogram profiles were visualized using BioEdit v.7.0 software (Hall, 1999) The species identification was confirmed through the degree of similarity obtained by the Nucleotide Basic Local Alignment Search Tool (BLASTn) in National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/).

2.3. Statistical analysis

To test for the data normality and homogeneity of the concentration and purity values, the Shapiro-Wilk (Shapiro & Wilk, 1965) and the Levene tests (Levene, 1960) were performed using the 'tidyverse' package in 'R' (R et al., 2017; https://www.R-project.org/), considering $p < 0.05$.

Both Shapiro-Wilk and Levene tests showed a p-value lower than 0.05 ($p = 0.0017$ and $p = 0.00016$, respectively), rejecting the null hypothesis of data normality and homogeneity for concentration data. Thus, we performed a non-parametric test of Kruskal-Wallis (Kruskal & Wallis, 1952) using the 'tidyverse' package in 'R' (Core Team., 2017; https://www.R-project.org/) to investigate if the differences between pre-treated and non-pre-treated was significant followed by the pairwise Wilcoxon test (Wilcoxon, 1945) under the Bonferroni correction (Rice, 1989).

For purity data, both Shapiro-wilk and Levene tests showed a p-value higher than 0.05 ($p = 0.057$ and $p = 0.086$, respectively), accepting the null hypothesis of data normality and homogeneity. Thus, we performed a parametric ANOVA analysis (Kaufmann & Schering, 2007) using the 'tidyverse' package in 'R' (Core Team., 2017; https://www.R-project.org/). To compare the purity and concentration data among muscle/-fin, pre-treated and non-pre-treated samples, a post-hoc test of Dunn (Dunn, 1964) was performed by using the 'FSA' package in 'R' (R et al., 2017; https://www.R-project.org/).

To further validate the robustness of the protocol, a post-hoc power analysis was performed, assessing the adequacy of the sample size in detecting the observed effects. Based on the normality results and statistical tests used, purity data were investigated by though 'pwr' package (Champely et al., 2017) in R (R et al., 2017; https://www.R-project.org/), using the ANOVA observed results. Since Kruskall-Wallis is a non-parametric test, an estimation was constructed by using chi-squared statistic in R. In addition, the confidence intervals of both measurements (concentration and purity) were calculated.

3. Results and discussion

DNA extracted from muscle and fin samples obtained directly from fish specimens of *Katsuwonus pelamis* presented higher concentrations and purity ranges than canned samples (Supplementary Figure S1). In addition, fin/muscle samples also showed smaller deviation in purity ranges than canned samples, indicating the negative effect of the canning process in DNA quality. The differences between the treatments were significant by Kruskall-Wallis test ($p < 0.001$), and the Dunn test indicated that the highest differentiation was observed between muscle/fin and non-pre-treated samples ($p < 0.001$; Z statistic=5.8). Pre-treated and non-pre-treated samples also presented significant values ($p < 0.01$), and these results will be discussed separately.

Most of the DNA extracted from pre-treated samples presented higher concentration values, ranging from 27.2 ng/μL to 94.3 ng/μL (average = 59.28; CI 95 % 35.2–84.1), when compared to non-pre-treated samples which presented a concentration at least around 10 times lower, ranging from 2.1 ng/μL to 29 ng/μL (average = 9.67; CI 95 % 5.1–9.7). In addition, the results of Kruskal-Wallis test showed a significant difference ($p < 0.01$) in the concentration values between the two treatments, indicating a positive effect of the pre-treatment. The 13 samples per treatment presented a large estimated effect (f=0.88), resulting in a power above 99 %, reinforcing the sensitivity to detect the treatment effects.

The A260/A280 ratio represents DNA purity, being a critical parameter in evaluating the DNA recovering success. Pre-treated samples presented a A260/A280 ratio that ranged from 1.42 to 2.05 (average = 1.81; CI 95 %, 1.69–1.93) (Table 1; Fig. 2). To consider the DNA as uncontaminated, the A260/A280 ratio should ideally range from 1.8 to 2.0 (Chang et al., 2016). Outliers' values could indicate contamination by proteins, RNA, or residual presence of salt, phenol or carbohydrates (Armani et al., 2014). Importantly, 8 of the 13 pre-treated samples analyzed presented values of absorbance higher than 1.8, indicating that the substances added during the canning process were successfully removed. In contrast, the A260/A280 ratio showed lower values when DNA was extracted from non-pre-treated samples, ranging from 1.38 to 1.67 (average = 1.52; CI 95 % 1.45–1.59). The differences between the treatments were statistically supported by ANOVA ($p < 0.01$) and they do indicate an interesting innovation offered by the present protocol focused on recovering better DNA quality. To assess the adequacy of the sample size, a post-hoc power analysis was conducted using the observed effect size from ANOVA (f=0.90). This analysis revealed a requirement of a minimum of 6 samples per treatment to achieve 80 % power at a $p < 0.05$. Since the present data with 13 samples per treatment achieved a power of 99.3 %, this indicated a highly sensitive design to detect the improvement of the DNA purity when the treatment was used.

These comparative results indicate that both DNA concentration and purity were increased after the pre-treatment application. Although many studies indicate negative effects of the canning process in DNA quality (e.g. Shokralla et al., 2015; Cutarelli et al., 2018; Pollack et al., 2018; Sultana et al., 2018), they lack information on DNA concentration and purity, making direct comparison with the data obtained herein impossible. Despite A260/A280 ratio outside the 1.8–2.0 range may have negative effects on PCR, we stepped forward with both types of recovered DNAs: with and without pre-treatment.

Amplifications using the FishF1/FishR1 primers pair successfully amplify fragments of 650 bp in eight (PS1-PS8) pre-treated samples. Of those, four samples (PS1-PS4) presented well defined bands in agarose gel (Fig. 3). The remaining five samples presented only non-specific/low bands in agarose gel. FishF2/FishR2 primers pair failed to amplify any fragments. Since both primers-set represents are universal primers for COXI amplification in fishes' species, it would be interesting testing different annealing temperatures to optimize their use.

The PCRs reactions using non-pre-treated samples failed in amplifying any fragment using both FishF1/FishR1 and FishF2/FishR2 primers set. In these cases, reactions with higher DNA volume and additional Taq Polymerase were tested, but the results remained negative. The negative control did not show any DNA bands in agarose gel, indicating that no contamination had occurred during the PCR reactions. It is important to say that the occurrence of some failures during the molecular routine is normal. However, comparisons among pre-treated and non-pre-treated samples in terms of COXI-based PCR performances, suggest that positive amplifications are not random and highlight the importance of the pre-treatment protocol proposed in the present study.

Although represented by a small number of samples (N = 8), the positive amplifications represent a striking result, given the difficulty in obtaining large fragments from degraded DNA shown by other studies, especially from processed samples like canned tuna (Shokralla et al., 2015; Sultana et al., 2018; Xing et al., 2020). Alternatively, these studies used the mini-barcode strategy, amplifying shorter fragments of 100–200 bp. However, even shorter fragments present a lower success rate in canned products when compared to other products (Pollack et al., 2018; Xing et al., 2020). Thus, the combination of the pre-treatment described herein, improving the DNA purity, and the amplification of mini-barcode regions is encouraged. However, it is important to

Table 1
Sample information and molecular results of canned tuna samples. In the PCR column, the '√' symbol represents positive amplifications, and the 'X' symbol represents negative amplifications. (C: DNA concentration in ng/μL).

Sample information		DNA extraction			PCR	Purification		Sequencing (fragment length)[a]	BLAST percent identity
Sample ID	Product label description	Pre-treatment	C (ng/μL)	A_{260}/A_{280}		C (ng/μL)	A_{260}/A_{280}		
PS1	Natural solid Tuna	✓	35.2	1.42	✓	518.3	1.20	✓ (683 bp)	*Katsuwonus pelamis* (96.35–98.99 %)
PS2	Natural grated Tuna	✓	52.8	1.93	✓	535.7	1.19	X	-
PS3	Natural grated Tuna	✓	27.1	1.69	✓	567.5	1.22	✓ (682 bp)	*Katsuwonus pelamis* (88–92 %)
PS4	Natural grated Tuna	✓	94.3	1.55	✓	624.7	1.21	✓ (681 bp)	*Katsuwonus pelamis* (97.64–98.77 %)
PS5	Natural grated Tuna	✓	41.2	1.70	✓	533.8	1.18	✓ (673 bp)	*Katsuwonus pelamis* (98.19–99.27 %)
PS6	Natural solid Tuna	✓	75.3	1.82	X	-	-	-	-
PS7	Natural solid Tuna	✓	84.1	1.99	X	-	-	-	-
PS8	Natural solid Tuna	✓	84.6	2.05	X	-	-	-	-
PS9	Natural solid Tuna	✓	82.9	2.04	X	-	-	-	-
PS10	Natural solid Tuna	✓	30.5	1.84	X	-	-	-	-
PS11	Natural solid Yellowfin Tuna	✓	52.1	2.02	✓	332.1	1.14	X	-
PS12	Natural solid Yellowfin Tuna	✓	32.4	1.91	✓	390.8	1.21	X	-
PS13	Natural solid Yellowfin Tuna	✓	78.2	1.6	✓	476.1	1.24	✓ (228 bp)	The fragment obtained did not allow the BLASTn procedure
NS14	Natural solid Tuna	X	5.1	1.6	X	-	-	-	-
NS15	Natural solid Tuna	X	4.5	1.6	X	-	-	-	-
NS16	Natural solid Tuna	X	12.6	1.42	X	-	-	-	-
NS17	Natural solid Tuna	X	8.6	1.56	X	-	-	-	-
NS18	Natural solid Tuna	X	2.1	1.38		-	-	-	-
NS19	Natural solid Tuna	X	5.8	1.42	X	-	-	-	-
NS20	Natural solid Tuna	X	8.2	1.55	X	-	-	-	-
NS21	Natural solid Tuna	X	9.7	1.7	X	-	-	-	-
NS22	Natural solid Tuna	X	8.3	1.65	X	-	-	-	-
NS23	Natural solid Tuna	X	6.4	1.67	X	-	-	-	-
NS24	Natural solid Tuna	X	2.5	1.48	X	-	-	-	-
NS25	Natural solid Tuna	X	22.9	1.37	X	-	-	-	-
NS26	Natural solid Tuna	X	29	1.38	X	-	-	-	-

[a] Length before editing and alignment

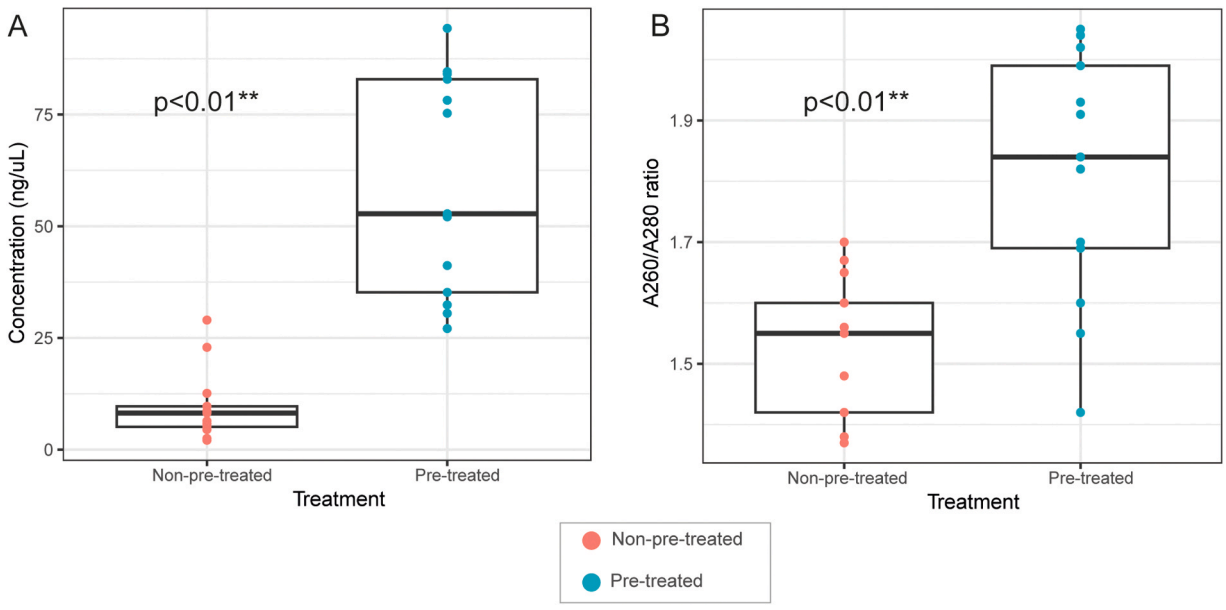

Fig. 2. DNA quantification parameters of (A) DNA concentration (ng/μL) and (B) DNA A260/A280 ratio among pre-treated and non-pre-treated samples. ** Significance at the 0.01 level, as calculated using Kruskal-Wallis and Wilcoxon tests.

highlight that, even when higher purity DNA was used, the DNA fragmentation of the canned samples made it impossible to amplify large regions in some pre-treated samples.

Except for the PS2, PS11 and PS12 samples, the sequencing of the pre-treated samples provided good quality sequences. The electropherograms presented only a few non-identified nucleotides in the beginning of the sequence, which is normally expected in Sanger sequencing. The base peaks were well defined, and the fragment lengths ranged from 673 bp to 682 bp (Table 1), showing that the full COXI barcode region was obtained. This result allowed the identification of the skipjack tuna (*Katsuwonus pelamis*), in the canned products analyzed. An ambiguous sequence of 228 bp was obtained from the PS13 sample

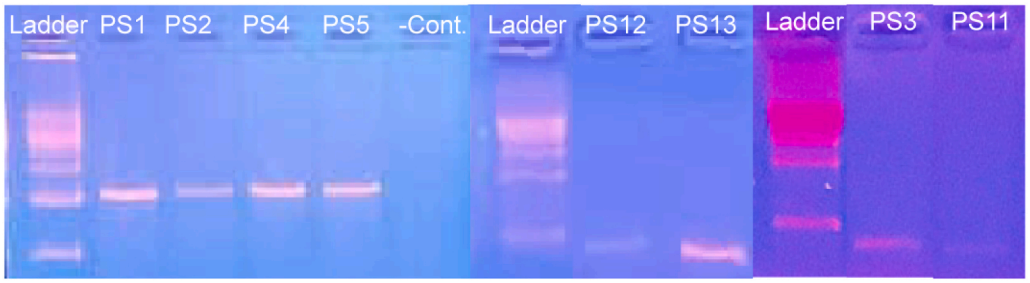

Fig. 3. Agarose gel showing positive amplifications of the COXI gene from pre-treated samples. (-Cont: negative control).

and, for that, the BLASTn tool failed in identifying the similarities in the database.

Nagalakshmi et al. (2016) investigated the species authenticity of different sample types (fresh, frozen, canned, ready to cook, and ready to eat) and they found that the canned tuna presented the lowest amplification length (200–300 bp) when compared to the other samples (550–650 bp), as observed by Pollack et al. (2018). Due to the low DNA purity, which can make the amplification throughout PCR and sequencing steps in terms of identifying species from processed meat samples difficult, the improvement of DNA concentration and purity, plus the sequencing of the full barcode COXI region, even in few samples, highlights the importance of the protocol proposed and tested herein.

4. Conclusions

Overall, the protocol offered herein showed significant improvements of both DNA concentration and purity from canned tuna samples when compared to those non-pre-treated. These improved results enabled complete COXI amplification in eight pre-treated samples. Of these, we obtained complete COXI sequences for four of the total thirteen samples. This represents a remarkable result since several studies have revealed the low success of the DNA-based species identification methods carried out on products from different processes, especially canning. However, it is important to mention that the present study is limited by a small sample size. In the context of food fraud investigation, food safety and overall food certification, this study must serve as a baseline experiment for new improved techniques concerned the development of better strategies to identify species using degraded DNA. In addition, the combination of the methodological improvements offered here with other methods, such as mini barcoding, is strongly encouraged, including other sample types in the tests.

CRediT authorship contribution statement

Mônica L. Adam: Writing – review & editing. **Paulo Travassos:** Writing – review & editing, Project administration, Funding acquisition. **Queiroz-Brito Maria Clara G.:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis. **Rodrigo A. Torres:** Writing – review & editing, Supervision, Conceptualization.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2025.108439.

Data availability

Data will be made available on request.

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Glossary

DNA: Deoxyribonucleic acid
PCR: Polymerase chain reaction
PCR-RFLP: Restriction fragment length polymorphism polymerase chain reaction
COXI: Cytochrome c Oxidase Subunit I
PBS: Phosphate Buffered Saline
BLASTn: Nucleotide Basic Local Alignment Search Tool
RNA: Ribonucleic acid