



Note

Authentication of commercially important tuna species landed in Tuticorin coast of Tamil Nadu, India by SE-AFLP method

G. JEYASEKARAN, G. ARUNKUMAR, P. SENTHIL KUMAR, R. JEYA SHAKILA
AND D. SUKUMAR

Fisheries College and Research Institute, Tamil Nadu Fisheries University, Thoothukudi - 628 008, Tamil Nadu, India
e-mail: jeyasekarang@gmail.com

ABSTRACT

Seafood serves as a valuable protein source for human population. Among the seafood, tuna is considered as one of the commercially important species worldwide. Five tuna species namely *Euthynnus affinis*, *Auxis thazard*, *Katsuwonus pelamis*, *Thunnus albacares* and *Thunnus obesus* are commonly landed in the Tuticorin coast of Tamil Nadu. Among the five species, the exportable quality and grade of the meat is as follows: *T. albacares* > *T. obesus* > *K. pelamis* > *E. affinis* > *A. thazard*. In recent years, mislabeling is done by replacing high value tuna meat with low value tuna meat or with other low value fish meat to earn illegally. Usually, the species identification is done based on the morphological features, but this cannot be applied for the processed fish. PCR based identification methods have gained importance in the identification of fish species. PCR-AFLP is one of the molecular based methods, which can differentiate even closely related species. In this study, SE-AFLP method was employed to differentiate the above five tuna species viz., *E. affinis*, *A. thazard*, *K. pelamis*, *T. albacares* and *T. obesus*. Species specific AFLP marker was obtained in the primer combination of EcoR1 for fresh tuna. Band Sharing Index (BSI) analysis was also performed to find the similarities and variation among the five tuna species. AFLP profile of unknown tuna products was compared with the standard AFLP profile and the tuna species authentication was done by analysing BSI score.

Keywords: AFLP, Seafood safety, Species authentication, Traceability, Tuna

Fish is an excellent source of protein having good export potential. It is estimated that about 82.4 million t of fish is globally exported every year (FAO, 2013). In India, nearly 9.84 lakh t of seafood products worth 5.0 billion US \$ have been exported in the year 2013-14 (MPEDA, 2015). As the global trade and market for seafood has grown tremendously, the problem of overexploitation and mislabeling of seafood products keep emerging. Mislabeling mainly occurs in the processed fish products like heat processed fish, fish fillets, fish mince and pickles, since the external morphological features are removed during processing.

There are two main analytical methods for the differentiation of fish species, viz., protein and DNA based methods. Most protein based methods like Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Iso-Electric Focusing (IEF) are found to be effective for fresh fish, but not for processed fishery products, as many proteins get denatured during processing (Teletchea, 2009). Etienne *et al.* (2000) failed to differentiate species of same genus after cooking, particularly *Salmo salar* from *Salmo trutta* and *Oncorhynchus gorboscha* from *Oncorhynchus keta* by SDS-PAGE and Urea IEF techniques.

On the other hand, as DNA is stable in processed condition, molecular method is found to be a reliable identification tool for authentication of seafood species. The usefulness of DNA barcoding in seafood authentication has been described (Nicole *et al.*, 2012). Several molecular based methods like DNA sequencing, species specific Polymerase Chain Reaction (PCR), PCR-Restriction Fragment Length Polymorphism (RFLP), PCR-Amplified Fragment Length Polymorphism (AFLP), multiplex PCR, real time PCR and PCR-Single Strand Conformation Polymorphism (SSCP) have been found successful in differentiating the fish species including the processed products. The potential use of AFLP technology to determine fish and seafood species in processed commercial seafood products and domestic stocks was investigated (Maldini *et al.*, 2006). AFLP technique possesses advantages like requirement of only small amounts of DNA, production of reproducible results, amplification of many restriction fragment subsets by changing the nucleotide extensions on the adaptor sequences, high resolution, applicability to wide variety of DNA samples and no requirement of prior genomic sequence (Vos *et al.*, 1995).

Tuna is an important commercial fish having consumer preference all over the world for its delicacy. Very few

species of tuna fetch high price due to their larger size and geographical location. Adulteration of processed tuna with less commercially important species in place of high priced tuna is therefore not permissible. In such a situation, it becomes mandatory to devise a suitable molecular authentication technique to monitor the fraudulence practices. A one step triplex-polymerase chain reaction (PCR) based assay was developed to discriminate between three tuna species *viz.*, yellowfin tuna (*Thunnus albacares*), bigeye tuna (*T. obesus*) and skipjack tuna (*Katsuwonus pelamis*) in fresh, frozen and canned forms using mitochondrial cytochrome *b* gene (Michelini *et al.*, 2007). Identification of four species of tuna *viz.*, bigeye tuna (*T. obesus*), Pacific bluefin tuna (*T. orientalis*), southern bluefin tuna (*T. maccoyii*), and yellowfin tuna (*T. albacares*) by TaqMan probe based Real Time PCR method was reported (Chuang and Chen, 2012). Application of AFLP assay as a tool to distinguish tuna species for seafood authentication is very much limited. Single enzyme AFLP assay has been used to distinguish the level of genetic variation in three *Thunnus* species *viz.*, Atlantic northern bluefin tuna (*T. thynnus*), yellowfin tuna (*T. albacares*) and albacore (*T. alalunga*) (Han and Ely, 2002). Zhang *et al.* (2006) developed AFLP derived SCAR (sequence characterised amplified region) to differentiate Atlantic salmon and rainbow trout. Since the studies on the application of AFLP to authenticate seafood species is very limited, in this study, five major tuna species landed along the coast of Tamil Nadu *viz.*, little tuna (*Euthynnus affinis*), frigate tuna (*Auxis thazard*), skipjack tuna (*K. pelamis*), yellowfin tuna (*T. albacares*) and bigeye tuna (*T. obesus*) were examined by Single Enzyme (SE)-AFLP analysis to obtain species specific banding pattern and molecular markers unique for each species.

For the experiment, five tuna species namely *A. thazard*, *E. affinis*, *K. pelamis*, *T. albacares* and *T. obesus* were collected from fish landing centres along the coast of Tamil Nadu. They were brought to the laboratory in iced condition and morphologically identified using the FAO fish species identification keys (De Bruin *et al.*, 1995).

Genomic DNA from each species of tuna samples was isolated by using DNA extraction kit (HiPurAMultisample DNA extraction kit, HiMedia laboratories Pvt. Ltd., Mumbai, India) and the integrity of DNA was analysed by running onto 1% Agarose gel. DNA concentration was quantified by Biophotometer (Eppendorf AG, Germany). Single enzyme-Amplified fragment length polymorphism (SE-AFLP) analysis was performed as described by Han and Ely (2002). About 100 ng μ l⁻¹ of DNA was digested with 10 U of *Eco*R1 and ligated to adaptor pairs (*Eco*R1 Forward 5'CTCGTAGACTGCGTACC3' and *Eco*R1 reverse 5'AATTGG TACGAGTCTAC3'), in a single reaction tube that contained 1 μ l DNA (100 ng μ l⁻¹), 2 μ l *Eco*R1 buffer

(10X), 10 U *Eco*R1 enzyme, 2 μ l T4 DNA ligase buffer (10X), 5U T4 DNA ligase, 10 pmol of *Eco*R1 forward and reverse adaptor. This reaction mixture was incubated at 37°C for 5 h and the reaction was then stopped by incubating at 60°C for 10 min.

Pre-amplification was done by using *Eco*R1 specific primers having one selective nucleotide beyond the adaptor sequence *Eco*R1 5'GACTGCGTACCAATTC[A]3'. The reaction mixture contained 2 μ l adaptor ligated DNA, 10X PCR buffer [100 mm Tris (pH 9.0), 500 mm KCl, 15 mm MgCl₂ and 0.1% gelatin], 100 mm of each dNTPs, 1.25 U of *Taq* DNA polymerase and 10 pmol of *Eco*R1 primer having one selective nucleotide, *Eco*R1 5' GACTGCGTACCAATTC[A]3'. The optimised cycling conditions were initial denaturation at 94°C for 1 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min and final extension of 5 min at 72°C. Pre-amplified products were diluted 5 times using molecular grade water.

Selective amplification was done by *Eco*R1 specific primers having three selective bases beyond the adaptor sequence *Eco*R1 5'GACTGCGTACCAATTC[NNN]3' as given in Table 1. The reaction mixture contained 2 μ l pre-amplified DNA, 10X PCR buffer [100 mm Tris (pH 9.0), 500 mm KCl, 15 mm MgCl₂ and 0.1% gelatin], 100 mm of each dNTPs 1.25 U of *Taq* DNA polymerase and 10 pmol of *Eco*R1 specific primer, *Eco*R1 5' GACTGCGTACCAATTC[NNN]3'. The optimised cyclic conditions of the touch-down PCR were: initial denaturation at 94°C for 1 min, followed by 10 cycles of denaturation at 94°C for 1 min, annealing from 65 - 56°C for 1 min with 1°C decrement at each cycle, extension at 72°C for 2 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min, extension at 72°C for 2 min and final extension of 10 min at 72°C. Amplified products were run on 2% agarose gel. The size of AFLP fragments was recorded using Gel Documentation System software version 1.2.0.1 (Alpha Innotech, San Leandro, USA).

Table 1. AFLP adaptors and primers used in the study

Adaptors/Primers	Sequence (5' - 3')
Adaptors	Sequence (5' - 3')
<i>Eco</i> R1 forward	CTCGTAGACTGCGTACC
<i>Eco</i> R1 reverse	AATTGGTACGAGTCTAC
Pre-amplification primer	GACTGCGTACCAATTC
Selective primers	Sequence (5' - 3')
E-AGT	GACTGCGTACCAATTCAGT
E-AGA	GACTGCGTACCAATTCAGA
E-ACG	GACTGCGTACCAATTCACG
E-ACT	GACTGCGTACCAATTCACT
E-ACC	GACTGCGTACCAATTCACC
E-ACA	GACTGCGTACCAATTCACA

Binary data matrix was constructed from the AFLP banding pattern obtained using different *EcoRI* selective primers. The value of '1' represents the presence of band and '0' for the absence of band at the specific loci. Band sharing index (BSI) score was then calculated using the following formula:

$$\text{Band Sharing Index} = \frac{2 N_{ab}}{N_a + N_b}$$

where, N_{ab} - No. of fragments shared between species 'a' and 'b';
 N_a - Total no. of fragments in species 'a', N_b - Total no. of fragments in species 'b'

PCR-AFLP profiles were generated in this study for the first time for the identification of five tuna species available along the Tamil Nadu coast in India. PCR-AFLP profiles have been recognised as a molecular tool to detect fraud in seafood products (Han and Ely, 2002; Maldini *et al.*, 2006). SE-AFLP analysis was adopted in order to reduce the complexity of the AFLP pattern. Double enzyme-AFLP (DE-AFLP) is mainly used with a combination of restriction enzymes, *EcoRI* and *MseI* or *TaqI*, which provide a highly complex pattern that restricts visualisation in agarose gel and requires a capillary gene sequencer for cluster analysis (Xiang *et al.*, 2010). On the other hand, SE-AFLP analysis employs the use of single enzyme, particularly a rare cutter rather than a frequent cutter. Restriction enzyme, *EcoRI* with a cutting site at G'AATTC is the most preferred enzyme for SE-AFLP analysis based on its wide availability and lower cost (Han and Ely, 2002). DNA purity is another important aspect to get good quality AFLP fingerprints. The DNA obtained from different species of tuna was high in molecular size (>3kb). Vos *et al.* (1995) also reported that the DNA must be of high molecular size without any shearing.

All the six selective primers *viz.*, E-AGT, E-AGA, E-ACG, E-ACC, E-ACT and E-ACA provided different banding patterns for raw tuna (Fig.1 and 2). The putative loci detected by E-AGT, E-AGA, E-ACC, E-ACT and E-ACA primers ranged between 200 and 1000 bp, while those detected by E-ACG primer were from <50 bp to 250 bp. The selective primer E-ACG, could not differentiate the five species of raw tuna due to 90% similar banding pattern. The primers E-AGA and E-ACT clearly differentiated the five species of raw tuna than E-AGT and E-ACC primers. The primer E-ACA has shown the least differentiation. AFLP profiles distinguish the species based on band sharing index (BSI) (Maldini *et al.*, 2006). About 50% dissimilarities existed between *K. pelamis* and *A. thazard*; as well as between *T. albacares* and *T. obesus* with E-AGT primer selective amplification. With respect to E-AGA primer, the BSI gave

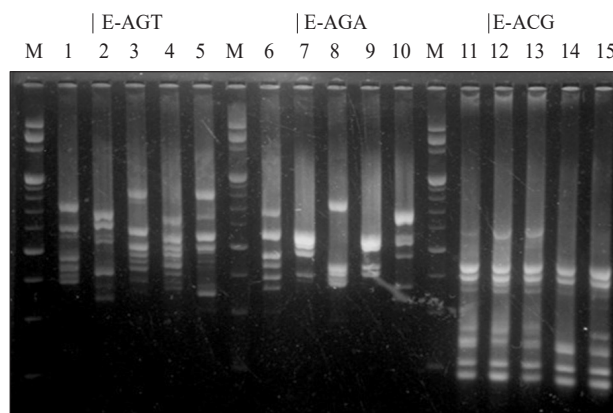


Fig. 1. AFLP profile raw of Tuna species

Lanes 1, 6, 11 - *Euthynnus affinis*, Lanes 2, 7, 12 - *Auxis thazard*, Lanes 3, 8, 13 - *Katsuwonus pelamis*, Lanes 4, 9, 14 - *Thunnus albacares*, Lanes 5, 10, 15 - *Thunnus obesus*, Lane M - 100 bp DNA ladder

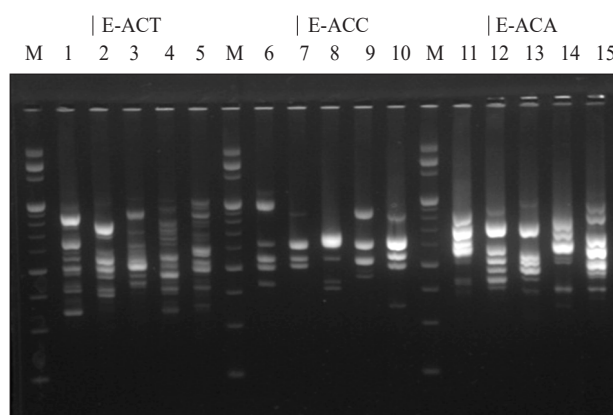


Fig. 2. AFLP profile of raw Tuna species

Lanes 1, 6, 11 - *Euthynnus affinis*, Lanes 2, 7, 12 - *Auxis thazard*, Lanes 3, 8, 13 - *Katsuwonus pelamis*, Lanes 4, 9, 14 - *Thunnus albacares*, Lanes 5, 10, 15 - *Thunnus obesus*, Lane M - 100 bp DNA ladder

a different profile with *E. affinis* having 43% bands different from *T. obesus*. The E-ACG primer was the least effective, as most of the tuna species had only 16-14% dissimilarities. The primer E-ACC expressed good differentiation among tuna species, except that *A. thazard* had 45% disparity with *T. obesus*; and *T. albacares* had 50% bands not sharing with *T. obesus*. The primer E-ACT showed lower differentiation between *E. affinis* and *A. thazard* (33%) and between *A. thazard* and *T. albacares* (33%), but higher disparity between *T. albacares* and *T. obesus* (80%) and hence, proved to be a better selective primer. The E-ACA primer also distinguished the tuna species efficiently with low BSI scores, as *T. albacares* showed only 13% dissimilar banding pattern from that of *T. obesus* (Table 2).

Table 2. Band sharing index (BSI) of raw tuna species

Band sharing index score					
Species	E-AGT				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
<i>E. affinis</i>	1	0.47	0.53	0.33	0.16
<i>A. thazard</i>	0.47	1	0	0	0.22
<i>K. pelamis</i>	0.53	0	1	0.37	0.18
<i>T. albacares</i>	0.33	0	0.37	1	0.50
<i>T. obesus</i>	0.16	0.22	0.18	0.50	1
Species	E-AGA				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
<i>E. affinis</i>	1	0.20	0.22	0.25	0.55
<i>A. thazard</i>	0.20	1	0	0.33	0.22
<i>K. pelamis</i>	0.22	0	1	0	0.25
<i>T. albacares</i>	0.25	0.33	0	1	0.57
<i>T. obesus</i>	0.55	0.22	0.25	0.57	1
Species	E-ACG				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
<i>E. affinis</i>	1	0.94	0.94	0.86	0.93
<i>A. thazard</i>	0.94	1	1	0.80	0.875
<i>K. pelamis</i>	0.94	1	1	0.80	0.875
<i>T. albacares</i>	0.86	0.80	0.80	1	0.92
<i>T. obesus</i>	0.93	0.875	0.875	0.92	1
Species	E-ACC				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
<i>E. affinis</i>	1	0.38	0.11	0.37	0.32
<i>A. thazard</i>	0.38	1	0	0.33	0.55
<i>K. pelamis</i>	0.11	0	1	0.29	0.11
<i>T. albacares</i>	0.37	0.33	0.29	1	0.50
<i>T. obesus</i>	0.32	0.55	0.11	0.50	1
Species	E-ACT				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
<i>E. affinis</i>	1	0.67	0.44	0.40	0.40
<i>A. thazard</i>	0.67	1	0.50	0.67	0.44
<i>K. pelamis</i>	0.44	0.50	1	0.44	0.22
<i>T. albacares</i>	0.40	0.67	0.44	1	0.20
<i>T. obesus</i>	0.40	0.44	0.22	0.20	1
Species	E-ACA				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
<i>E. affinis</i>	1	0.31	0.17	0.33	0.27
<i>A. thazard</i>	0.31	1	0.46	0.15	0.38
<i>K. pelamis</i>	0.17	0.46	1	0.17	0.27
<i>T. albacares</i>	0.33	0.15	0.17	1	0.13
<i>T. obesus</i>	0.27	0.38	0.27	0.13	1

AFLP appears to provide extremely informative markers for species identification and authenticity testing of unknown fish species (Yu and Guo, 2004). AFLP marker regions were identified in few tuna species from the AFLP

profiles of different raw tuna species (Table 3). AFLP markers were identified with most of the selective primers, except E-ACG. It was found that primers E-ACT and E-ACA alone can differentiate *T. albacares* and *T. obesus* with different

Table 3. AFLP markers of raw tuna species

Primers	AFLP makers (bp)				
	<i>E. affinis</i>	<i>A. thazard</i>	<i>K. pelamis</i>	<i>T. albacares</i>	<i>T. obesus</i>
E-AGT	740	675	575	-	350
E-AGA	450, 370	480	750	-	-
E-ACG	-	-	-	-	-
E-ACC	850, 350	700	-	-	1020
E-ACT	1000	-	400	450	350
E-ACA	-	410	-	730	800

selective markers. The primers E-AGT and E-ACC can also distinguish these two species and each had one AFLP marker specific for *T. obesus* at 350 and 1020 bp respectively. AFLP markers for other small tuna species were also identified using E-AGT and E-AGA primers.

Species specific AFLP marker can be obtained for the products of little known genetic background due to high reproducibility of AFLP, based on the restriction endonuclease digestion and flexibility in the design of primer combinations (Liu and Cordes, 2004). Han and Ely (2002) developed a modified AFLP method using only one restriction enzyme, *EcoRI*, for obtaining species specific patterns for *Morone* and *Thunnus* species, but have not identified any species specific AFLP markers. This was the only report available on the AFLP profiling of tuna species. In the present study, AFLP species specific patterns were additionally obtained for another three species of tuna, in addition to *Thunnus* spp. viz., *T. albacares* and *T. obesus*

All the selective primers examined in this study, except E-ACG, were efficient in distinguishing the raw tuna species. Clear authentication of *T. albacares* and *T. obesus* were achieved using E-ACA and E-ACT primers. Hence, the identified AFLP marker region could be further purified and sequenced to develop selective PCR methodologies for selective identification of processed tuna products after validation.

Acknowledgements

Authors gratefully acknowledge the Science and Engineering Research Board, Ministry of Food Processing Industries (SERB-MFPI), Govt. of India, New Delhi, India for the financial assistance extended to this project. The authors thank the Vice-Chancellor, Tamil Nadu Fisheries University, Nagapattinam for his encouragement in carrying out this research work.

References

Chuang, P. S., Chen, M. I. and Shiao, J. C. 2012. Identification of tuna species by a real-time polymerase chain reaction technique. *Food Chem.*, 133: 1055-1061.

De Bruin, G. H. P., Russell, B. C. and Bogusch, A. 1995. *FAO species identification field guide for fishery purposes. The marine fishery resources of Sri Lanka*, Food and Agriculture Organization of the United Nations, Rome, Italy, p. 316-320.

Etienne, M., Jerome, M. and Fleurence, J. 2000. Identification of fish species after cooking by SDS-PAGE and urea IEF: a collaborative study. *J. Agri. Food Chem.*, 48: 2653-2658.

FAO 2013. *Global capture fisheries production statistics for the year 2011*. Fisheries and Aquaculture Department. Food and Agriculture Organization of the United Nations. www.fao.org/fishery/capture/en. (Accessed 20 July 2015)

Han, K. and Ely. B. 2002. Use of AFLP analyses to assess genetic variation in *Morone* and *Thunnus* species. *Mar. Biotechnol.*, 4: 141-145.

Liu, Z. J. and Cordes, J. F. 2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, 238: 31-37.

Maldini, M., Marzano, F. N., Fortes, G. G., Papa, R. and Gandolfi, G. 2006. Fish and seafood traceability based on AFLP markers: Elaboration of a species database. *Aquaculture*, 261: 487-494.

Michelini, E., Cevenini, L., Mezzanotte, L., Simoni, P., Baraldini, M., Laude, L. D. and Roda, A. 2007. One-step triplex-polymerase chain reaction assay for the authentication of yellowfin (*Thunnus albacares*), bigeye (*Thunnus obesus*) and skipjack (*Katsuwonus pelamis*) tuna DNA from fresh, frozen and canned tuna samples. *J. Agri. Food Chem.*, 55: 7638-7647.

MPEDA 2015. *Marine Products Export Development Authority*. <http://www.mpeda.com/> (Accessed 30 April 2015).

Nicole, S., Negrisolo, E., Eacher, G., Mantovani, R., Patarnello, T., Erickson, D. L., Kress, W. J. and Barcaccia, G. 2012. DNA barcoding as a reliable method for the authentication of commercial seafood products. *Food Technol. Biotechnol.*, 50(4): 387-398.

Teletchea, F. 2009. Molecular identification methods of fish species: reassessment and possible applications. *Rev. Fish Biol. Fish.*, 19: 265-293.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Van de Lee, T. and Hornes, M. 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Res.*, 23: 4407-4414.

- Xiang, S. R., Cook, M., Saucier, S., Gillispie, P., Socha, R., Scroggins, R. and Beaudette, L. A. 2010. Development of Amplified Fragment Length Polymorphism - derived functional strain-specific markers to assess the persistence of 10 bacterial strains in soil microcosms. *Appl. Environ. Microbiol.*, 76: 7126-7135.
- Yu, Z. and Guo, X. 2004. Genetic analysis of selected strains of eastern oyster (*Crassostrea virginica*) using AFLP and microsatellite markers. *Mar. Biotechnol.*, 6: 575-586.
- Zhang, J., Wang, H. and Cai, Z. 2006. The application of DGGE and AFLP- derived SCAR for discrimination between Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). *Food Control*, 18(6): 672-676.