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Differentiation of Four Tuna Species by Two-Dimensional Electrophoresis and Mass Spectrometric Analysis

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1. Introduction

Species belonging to the genus *Thunnus* are pelagic predator fishes, commonly known as tuna. The species within this genus are of commercial value, and six of them are considered the most valued in world trade (D.M., MIPAAF, 31 Gennaio 2008). *Thunnus* species originate from a variety of geographic areas, and for this reason the different species can be characterized by the presence of different biological contaminants and sensory characteristics. The species *Thunnus thynnus* has a higher quality and commercial value due to its excellent organoleptic features.

Tuna species are usually consumed as fillets or processed products. The loss of the external anatomical and morphological features makes the authentication of a fish species difficult or impossible and enables fraudulent substitutions (Marko et al., 2004). Species substitution is very common in fish products, due to the profits resulting from the use of less expensive species. For species of tuna, substitutions have both commercial and health implications (Agusa et al., 2005; Besada et al., 2006; Storelli et al., 2010), thus, analytical techniques to differentiate fish species are essential. The development of suitable analytical methods for fish species identification in prepared and transformed fish products is of great interest to enforcement agencies involved with labelling regulations and the authentication of fish in various products to prevent the substitution of fish species (Mackie et al., 2000; Meyer et al., 1995).

Several biochemical techniques enable the study and identification of fillet or minced fish species. Among these methods, isoelectric focusing (IEF) (Etienne et al., 2000; Rehbein et al., 2000; Renon et al., 2001), capillary zone electrophoresis (Acuña et al., 2008), and amplification of selected DNA sequences by the polymerase chain reaction (PCR) have been used for the identification of certain groups of fish species (Espiñeira et al., 2008; Hubalkova et al., 2008; Pepe et al., 2005, 2007; Trotta et al., 2005).

Presently, PCR is the most frequently used technique, as DNA is heat-stable and resistant to heat treatments that may be applied to the tuna during processing. However, obtaining an accurate species identification is very difficult if the species show a high degree of homology as *Thunnus* does (Chow & Kishino, 1995; Lopez & Pardo, 2005; Michelini et al., 2007; Pardo...
& Begoña, 2004; Terio et al., 2010; Viñas & Tudela, 2009). The sequences usually used as species molecular markers are the DNA mitochondrial fragments especially cytochrome b (cyt b) genes and the ribosomal 16S and 12S subunits (Kochzius et al., 2010; Russo et al., 1996; Zehner et al., 1998). Previous studies demonstrated that these molecular markers are not discriminating for Thunnus species, because they have few polymorphisms expressed by point mutations (Bottero et al., 2007).

EU Commission Regulation no. 2065/2001 of 22 October 2001 has established detailed rules for consumer information to be included on labels regarding fish species. Accordingly, it is also necessary to develop new methods to prevent illegal species substitutions in seafood products (EC No 2065/2001). Proteins are playing an increasing role in the international scientific community and proteomics, the large-scale analysis of proteins expressed by a cell or a tissue contributes greatly to the study of gene function (Pandey & Mann, 2000). Recently, proteomics has been applied in the fishing industry with several aims, e.g., to examine the water-soluble muscle proteins from farm and wild fish to show aquaculture effects on seafood quality (Monti et al., 2005) or to elucidate the influence of internal organ colonization by Moraxella sp. in internal organs of Sparus aurata (Addis et al., 2010). Proteomics has also been considered as a tool for species identification in seafood products with interesting results (Carrera et al., 2006, 2007; Chen et al., 2004; López et al., 2002; Piñeiro et al., 1999, 2001).

The aim of this chapter is to examine the potential of proteomics to identify four tuna species through characterisation of specific sarcoplasmic proteins. We investigated T. albacares, T. alalunga, and T. obesus two dimensional gel electrophoresis (2-DE) patterns and also verified the presence of specie-specific proteins for these tuna species. Muscle extracts from four tuna species of the genus Thunnus (T. thynnus, T. alalunga, T. albacares, T. obesus) were evaluated by both mono and 2-DE and mass spectrometric techniques. In preliminary results (Pepe et al., 2010), proteomics was applied for the identification of a species-specific protein in T. thynnus by 2-DE profiles. The analysis of two dimensional gels by ImageMaster™ 2D Platinum software revealed the presence of a protein with a molecular weight of approximately 70 kDa in the T. thynnus' 2-DE pattern, which was absent in the other species. This protein, identified as Trioso fosfato isomerasi (gi46909469) through mass spectrometric techniques might be considered a specific marker. The aim of this chapter was to investigate T. albacares, T. alalunga, and T. obesus 2-DE patterns and verify the presence of species-specific proteins for these tuna species.

2. Materials and methods

2.1 Fish samples
In this study, a total of four different tuna species were tested, with three specimens from each species. The whole tuna specimens were identified, according to their anatomical and morphological features, as belonging to T. thynnus, T. alalunga, T. albacares, and T. obesus species at the Department of Animal Science and Food Inspection, University of Naples, "Federico II". T. thynnus and T. alalunga specimens were fished in the Mediterranean Sea and supplied by “Pozzuoli fish market”, T. albacares specimens were fished in the Indian Ocean and supplied by Salerno P.I.F. (Posto di Ispezione Frontaliera), and T. obesus specimens were fished in the South East Atlantic Ocean and were obtained from Philadelphia, Pennsylvania, United States. Fish were frozen on board at – 20 ° C and shipped in insulated boxes to the laboratory. Tuna muscle samples were taken and stored at -80 °C for further analysis.
2.2 Extraction of sarcoplasmic proteins

Raw muscle tissue (3 g) was dipped in 6 mL of 10 mM Tris-HCl buffer at 4 °C, pH 7.2, supplemented with 5 mM PMSF (phenylmethanesulfonylfluoride). Samples were minced with an "Ultra-turrax" at 4 °C, for 30 s at 15,000 g to obtain a homogeneous sample of water-soluble proteins. Minced tissues were centrifuged at 15,000 g at 4 °C for 20 min. The supernatants were then recovered and filtered using Ultrafree CL (0.22 µm) filters, and stored at -20 °C until analysis by electrophoresis (Carrera et al., 2007). The efficacy and the reproducibility of the extraction protocol of sarcoplasmic proteins was evaluated using T. alalunga. The extraction protocol was carried out in triplicate and further checked for quality and quantity by SDS-PAGE.

2.3 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Protein concentration was measured by the Bradford method (Bradford, 1976) using bovine serum albumin as the standard. Proteins (50 µg) were separated on a 12.5% (w/w) polyacrylamide gel at 25 mA/gel constant current. Gels were stained for 50 min with Coomassie Brilliant Blue R-250 and destained with MilliQ grade water.

2.4 Two dimensional electrophoresis (2-DE)

The first dimensional electrophoresis (isoelectric focusing, IEF) was carried out on non-linear wide-range immobilized pH gradients (pH 3-10; 7 cm long IPG strips; GE Healthcare, Uppsala, Sweden) using the Etta IPGphor system (GE Healthcare, Uppsala, Sweden). Analytical-run IPG-strips were rehydrated with 50 µg of total proteins in 125 µl of rehydration buffer and 0.2% (v/v) carrier ampholyte for 12h, at 50 mA, at 20° C. The strips were then focused according to the following electrical conditions at 20°C: 500 V for 30 min, 1000 V for 30 min, 5000 V for 10h, until a total of 15000 V was reached. For preparative gels 100 µg of total proteins were used. After focusing, analytical and preparative IPG strips were equilibrated for 15 min in 6 M urea, 30% (V/V) glycerol, 2% (w/V) SDS, 0.05 M Tris-HCl, pH 6.8, 1% (w/V) DTT, and subsequently for 15 min in the same urea/SDS/Tris buffer solution but substituting the 1% (w/V) DTT with 2.5% (w/V) iodoacetamide. The second dimension was carried out on 12.5% (w/w) polyacrylamide gels (10 cm x 8 cm x 0.75 mm) at 25 mA/gel constant current and 10°C until the dye front reached the bottom of the gel, according to (Hochstrasser et al., 1988) MS-preparative gels were stained for 50 min with Coomassie Brilliant Blue R-250 and destained with MilliQ grade water. The software ImageMasterTM 2D Platinum was used for the analysis of the two dimensional gel images.

2.5 Image analysis

Gels images were acquired with an Epson expression 1680 PRO scanner. Computer-aided 2-D image analysis was carried out using the ImageMasterTM 2D Platinum software. Relative spot volumes (%V) (V=integration of OD over the spot area; %V = V single spot/V total spot) were used for quantitative analysis in order to decrease experimental errors. The normalized intensity of spots on three replicate 2-D gels was averaged and standard deviation was calculated for each condition.

A few initial reference points (landmarks) were affixed for gels alignment, the first step of the image analysis. Landmarks are positions in one gel that correspond to the same position in the other gels. Then, the software automatically detects spots, which represent
the proteins on the gels. The software “matches” the gels, and the corresponding spots are paired. The pair is the association between spots that represent the same protein in different gels. Pairs are automatically determined using ImageMaster powerful gel matching algorithm. The different 2DE images can be compared by synchronized 3-D spots view.

2.6 Protein identification by mass spectrometry
2.6.1 In situ digestion
The analysis was performed on the Comassie blue-stained spots excised from gels. The excised spots were washed first with acetonitrile and then with 0.1M ammonium bicarbonate. Enzymatic digestion was carried out with trypsin (10 ng/µl) in 10mM ammonium bicarbonate pH 8.5 at 4° C for 2 h. The buffer solution was then removed and a new aliquot of the enzyme/buffer solution was added for 16 h at 37° C. A minimum reaction volume, enough for the complete rehydration of the gel was used. Peptides were then extracted washing the gel particles with 1% formic acid and ACN at room temperature.

2.6.2 MALDI-TOF mass spectrometry
Positive Reflectron MALDI spectra were recorded on a Voyager DE STR instrument (Applied Biosystems, Framingham, MA). The MALDI matrix was prepared by dissolving 10 mg of alpha-cyano-4-hydroxycinnamic acid in 1 mL of acetonitrile / water (90:10 v/v). Typically, 1 µl of matrix was applied to the metallic sample plate, and 1 µl of analyte was then added. Acceleration and reflector voltages were set up as follows: target voltage at 20 kV, first grid at 95% of target voltage, delayed extraction at 600 ns to obtain the best signal-to-noise ratios and the best possible isotopic resolution with multipoint external calibration using a peptide mixture purchased from Applied Biosystems. Each spectrum represents the sum of 1500 laser pulses from randomly chosen spots per sample position. Raw data were analysed using the computer software provided by the manufacturers and are reported as monoisotopic masses. Spectra were manually interpreted, there was no need of any De-isotopic or other post acquisition processing due to the good signal to noise ratio. Peak lists were generated manually and used for proteins identification.

2.6.3 LC-MS/MS analysis
A mixture of peptide solution was analysed by LC-MS/MS analysis using a 4000Q-Trap (Applied Biosystems) coupled to an 1100 nano HPLC system (Agilent Technologies) and Agilent HPLC-Chip/MS. The mixture was loaded on an Agilent reverse-phase pre-column cartridge (Zorbax 300 SB-C18, 5x0.3 mm, 5 µm) at 10 µl/min (A solvent 0.1% formic acid, loading time 5 min). Peptides were separated on a Agilent reverse-phase column (Zorbax 300 SB-C18, 150 mm X 75µm, 3.5 µm), at a flow rate of 0.3 µl/min with a 0% to 65% linear gradient in 60 min (A solvent 0.1% formic acid, 2% ACN in MQ water; B solvent 0.1% formic acid, 2% MQ water in ACN). Nanospray source was used at 2.5 kV with liquid coupling, with a declustering potential of 20 V, using an uncoated silica tip from NewObjectives (O.D. 150 µm, I.D. 20 µm, T.D. 10 µm). Data were acquired in information-dependent acquisition (IDA) mode, in which a full scan mass spectrum was followed by MS/MS of the 5 most abundant ions (2 s each). In particular, spectra acquisition of MS-MS analysis was based on a survey Enhanced MS Scan (EMS) from 400 m/z to 1400 m/z at 4000 amu/sec. This scan
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mode was followed by an Enhanced Resolution experiment (ER) for the five most intense ions and then MS² spectra (EPI) were acquired using the best collision energy calculated on the bases of m/z values and charge state (rolling collision energy) from 100 m/z to 1400 m/z at 4000 amu/sec. Data were acquired and processed using Analyst software (Applied Biosystems).

2.6.4 MASCOT analysis
The mass spectra obtained were then used for protein identification using the MASCOT software that compares peptide masses obtained by MALDI-TOF MS and LC-MS/MS of each spot with the theoretical peptide masses from all the proteins accessible in the databases (Peptide Mass Fingerprinting, PMF). Spectral data were analyzed using Analyst software (version 1.4.1) and MS-MS centroid peak lists were generated using the MASCOT.dll script (version 1.6b9). MS/MS centroid peaks were threshold at 0.1% of the base peak. MS/MS spectra having less than 10 peaks were rejected. MS/MS spectra were searched against NBCI (National Center for Biotechnology Information) database, (2006.10.17 version) using the licensed version of Mascot 2.1 version (Matrix Science), after converting the acquired MS/MS spectra in mascot generic file format. The Mascot search parameters were: taxonomy: Animalia; significance threshold: higher than 50 (according to Mascot scoring system, Pappin et al., 1993), allowed number of missed cleavages 3; enzyme trypsin; variable post-translational modifications, methionine oxidation, pyro-glu N-term Q; peptide tolerance 100ppm and MS/MS tolerance 0.5 Da; peptide charge, from +2 to +3 and top 20 protein entries. Spectra with a MASCOT score <25 having low quality were rejected. The score used to evaluate quality of matches for MS/MS data was higher than 30. However, spectral data were manually validated and contained sufficient information to assign peptide sequence.

Little genomic information is available for Thunnus genus, so protein identification is limited to a scarce number of tuna sequences deposited in the database. Therefore, once a significant protein match was made, protein sequence data were used for BLAST homology searches against other species in the NCBI database.

3. Results

3.1 SDS-PAGE
The protein extraction protocol developed for T. alalunga was used for all examined samples and showed high reproducibility; the extracted proteins were of both good quality and quantity (Figure 1). Protein samples of T. thynnus, T. albacares, T. alalunga, and T. obesus were fractionated by SDS gel electrophoresis as shown in Figure 2. After SDS-PAGE fractionation, some differences could be observed between the different tuna species. SDS-PAGE protein bands, in fact, showed inter-species differences, in particular for proteins with molecular weights lower than 25 kDa.

3.2 Analysis by 2-DE
In order to better elucidate the protein maps of different tuna species, the four samples were subjected to 2D fractionation. Deep analysis of the muscle proteome from the tuna species was undertaken by 2-DE image analysis using the ImageMaster™ 2D Platinum software. The tuna 2-DE images were aligned choosing four landmarks (L1, L2, L3 and L4) in each gel (Figure 3).
Fig. 1. *T. alalunga* SDS-PAGE. Three different protein samples were compared to verify the reproducibility of the extraction protocol.

Fig. 2. *T. alalunga*, *T. albiceps*, *T. obesus*, and *T. thynnus* SDS-PAGE. Proteins with molecular weight lower than 25 kDa are different among species.
Fig. 3. 2DE gel images alignment: landmarks affixing (L1, L2, L3 and L4).

The software correctly detected and aligned spots between the four tuna 2-DE gel images, as reported in Figure 4. The ImageMaster™ 2D Platinum found: 107 total spots on *T. thynnus* 2-DE gel, 93 total spots on the 2-DE gel of *T. alalunga*, 115 total spots on *T. albacares* 2-DE gel and 123 total spots on the 2-DE gel of *T. obesus*.

Fig. 4. Spot detection. Spots from the 2-DE arrayed samples representing proteins are circled in red.
Gel matching of tuna 2-DE images indicated the presence of spots that were both common to the four species, and the presence of spots that were specific for each species (Fig. 5). The software detected 28 specific spots on *T. thynnus* 2-DE gel, 48 specific spots on the 2-DE gel of *T. alalunga*, 65 specific spots on *T. albacares* 2-DE gel and 60 specific spots on the 2-DE gel of *T. obesus*.

Fig. 5. Gel matching: spots circled in green are common to the four tuna species, spots circled in red are not paired and therefore specific for each species.

### 3.3 Identification of non-paired/specie-specific spots

The comparison of the 3-D view of the “not paired” spots in the four 2-DE gel images makes it possible to find the most interesting spots for the characterization of the four tuna species (Fig 6-13). These proteins were considered species-specific markers.

### 3.4 Protein identification

Protein spots were excised from the gel and reduced, alkylated, and in-gel digested with trypsin. The resulting peptide mixtures were analyzed directly by MALDI-TOF MS and/or LC MS/MS. The MS/MS spectra were used to search for a non-redundant match using the in-house MASCOT software, thus taking advantage of the specificity of trypsin and of the taxonomic category of the samples. NCBInr database updates are regularly uploaded to in house version of MASCOT. We filtered identifications restricting to Animalia taxonomy. Molecular weights values that matched within the given mass accuracy of 100 ppm were recorded and the proteins that had the highest number of peptide matches were examined. Protein identification is limited to a scarce number of tuna sequences deposited in the database. Therefore, for proteins identified with low MASCOT score, protein sequence data were used for BLAST homology searches against other species in the NCBI database. The
Fig. 6. An example of a *T. thynnus* spot that might be a specific marker (labeled THY). Equivalent areas on all gels highlighted with a box.

Fig. 7. 3-D view of the *T. thynnus* species-specific spots and 3-D view of the same area from the other species gels. No spot/protein is present in the gel arrayed samples from the other species.
Fig. 8. An example of a *T. alalunga* spot that might be a specific marker (labeled ALA). Equivalent areas on all gels highlighted with a box.

Fig. 9. 3-D view of the *T. alalunga* species-specific spots and 3-D view of the same area from the other species gels. No spot/protein is present in the gel arrayed with samples from the other species.
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Fig. 10. An example of a *T. obesus* spot that might be a specific marker (labeled OBE). Equivalent areas on all gels highlighted with a box.

Fig. 11. 3-D view of the *T. obesus* species-specific spots and 3-D view of the same area from the other species gels. No spot/protein is present in the gel arrayed with samples from the other species.
Fig. 12. An example of *T. albacares* spots that might be a specific marker (labeled ALBA). Equivalent areas on all gels highlighted with a box.

Fig. 13. 3-D view of the *T. albacares* species-specific spots and 3-D view of the same area from the other species gels. No spot/protein is present in the gel arrayed with samples from the other species.
BLAST alignment was done for: Triosephosphate isomerase (THY), Pyruvate kinase (ALA) and Fast skeletal muscle troponin T (ALBA). The results of mass spectrometric analysis of the species-specific markers are shown in Table 1.

| Spot ID | Protein                                    | Accession number | Species      | MW (kDa) | pI    | Analysis Method | Score     |
|---------|--------------------------------------------|------------------|--------------|----------|-------|-----------------|-----------|
| THY     | Triosephosphate isomerase [Priapulus caudatus] | gi46909469       | T. thynnus   | 22.9     | 6.51  | LC-MS/MS        | MASCOT 83 |
| ALA     | Pyruvate kinase muscle [Danio rerio]        | gi40786398       | T. alalunga  | 58.6     | 6.54  | LC-MS/MS        | MASCOT 93 |
| ALBA    | Fast skeletal muscle troponin T Subunits [Gadus morhua] | gi20386541       | T. albacares | 27.2     | 9.48  | LC-MS/MS        | MASCOT 92 |
| OBE     | Beta-enolase [Epinephelus coioides]         | gi295792264      | T. obesus    | 47.5     | 6.29  | MALDI           | MASCOT 159|

Table 1. Identification of potential species-marker proteins from 2-DE arrays of the four Thunnus species.

4. Discussion

The specific proteins have important metabolic functions. Pyruvate kinase identified in T. alalunga is an enzyme involved in glycolysis. This protein catalyzes the transfer of a phosphate group from phosphoenolpyruvate (PEP) to ADP, yielding pyruvate and ATP. The specific protein identified in T. Thynnus is triose phosphate isomerase (TPI), a glycolytic enzyme which catalyzes the interconversion of dihydroxyacetone phosphate (DHAP) with D-glyceraldehyde-3-phosphate. TPI plays an important role in glycolysis and is essential for efficient energy production. Beta-enolase was identified in T. obesus and is a muscle-specific enolase (MSE) and is an enzyme of the lyase class that catalyzes the dehydration of 2-phosphoglycerate to form phosphoenolpyruvate. It appears to have an important function in striated muscle development and regeneration. The species-specific T. albacares protein is troponin T, fast skeletal muscle subtype. Troponin T (also symbolized TNTF) is the tropomyosin-binding subunit of troponin, the thin filament regulatory complex which confers calcium-sensitivity to striated muscle actomyosin ATPase activity. Therefore, all the identified species-specific proteins have an important metabolic function. For this reason it is not reasonable to think that these proteins do not exist in the other Thunnus species. But the ImageMaster™ 2D Platinum software did not find these proteins in the same localization of the other species 2D gels, which means that these proteins have a different isoelectric point and molecular weight in the other analysed species. The image analysis was correct for species identification, and it was confirmed by the 3-D view; the different spots are proteins and not artifacts caused by aberrant staining of the gel. So, the presence of the species-specific spot in a different area of the gel could indicate (e.g. pyruvate kinase) a higher rate for glycolysis in T. alalunga. It is important to continue the studies to enhance the knowledge of the identified species-specific spots and to identify other spots that could have species-specific function.
5. Conclusion

Proteomics has been demonstrated to be a useful method to increase scientific knowledge on animals and plants (Pandey & Mann, 2000). The progress in proteomic analytical techniques has enabled more accurate and reliable information for determining species differences (Tyers & Mann, 2003). The realization of a unique fingerprint for a given species is possible through the separation and subsequent identification of specific proteins.

In this study, a proteomic assay for the identification of species-specific markers of commercially important species of the genus *Thunnus* was undertaken. The proteomic fingerprinting of four species of the genus *Thunnus* was obtained using two dimensional electrophoresis followed by protein identification using mass spectrometry. The analysis of the 2-DE images revealed significant differences between the four tuna species investigated. The gel matching (Figure 5) shows that there are several different spots between the species, circled in red. The number of species-specific spots identified by the software is substantial for each *Thunnus* species (28 out of 107 total spots for *T. thynnus*; 48 out of 93 total spots for *T. alalunga*; 65 out of No 115 total spots for *T. albacares* and 60 out of No 123 total spots for *T. obesus*).

The 3-D view of the gels revealed the presence of some red circled spots absent in the same areas from the other species gels. These spots were chosen as species-specific.

The occurrence of species-specific protein spots may be due to differentially expressed proteins only present at low levels or absent in the other species. Thus, 2-DE analysis helped us to identify species-specific proteins, which could be used as specific markers to delineate each species.

The value of a proteomics approach to differentiate tuna species relies on both the ability to obtain the visualization of different protein spots in a 2D map but also the unique identification of the protein candidate by using mass spectral and bioinformatics procedures. Analyses were further enhanced through morphological visualization by 3-D reconstruction of differential spots from the four tuna species. In this way, it was possible to enhance differences and identify highly unique proteins from the *Thunnus* species. This second phase of study further validated the proteomic analysis technique as it confirmed that spots found in different locations and morphology on the 2D gels also corresponded to different proteins.

We have demonstrated that proteomics could be employed to differentiate species when they show contain high degrees of genetic homology (e.g. *Thunnus*). The DNA sequences normally used as species molecular markers are not discriminating for *Thunnus* species (Bottero et al., 2007). Moreover, without the option of a proteomic investigation, it would be necessary to further investigate the genome of each species, to identify genes that may differ between the species. This study shows how the use of proteomics tools is important for species identification.

The future developments of this study should be the identification of other species-specific proteins with metabolic functions characteristic of each species, to then identify species-specific genes. Primers can be subsequently designed for routine molecular biology methods to identify raw and processed fish products by PCR. In fact, PCR is currently routinely used for species identification and maintains this role due to practical attributes such as speed and cost. However, proteomics can provide an immediate and unambiguous identification of protein biomarkers, and in cases where the genomes are similar between species, the analysis of the proteome has a decisive advantage.
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7. References

Acuña, G.; Ortiz-Riaño, E.; Vinagre, J.; García, L.; Kettlun, A.M.; Puente, J.; Collados, L. & Valenzuela, M.A. (2008). Application of capillary electrophoresis for the identification of Atlantic salmon and rainbow trout under raw and heat treatment. J Capill Electrophor Microchip Technol., Vol. 10, No.5-6, pp. 93-9, ISSN 10795383

Addis, M.F.; Cappuccinelli, R.; Tedde, V.; Pagnozzi, D.; Viale, I.; Meloni, M.; Salati, F.; Rogni, T & Uzzau, S. (2010). Influence of Moraxella sp. colonization on the kidney proteome of farmed gilthead sea breams (Sparus aurata, L.). Proteome Sci., Vol. 8, No 50, (12 Oct 2010), pp. 1-8, ISSN 1477-5956

Agusa, T.; Kunito, T.; Yasunaga, G.; Iwata, H.; Subramanian, A.; Ismail, A. & Tanabe, S. (2005). Concentrations of trace elements in marine fish and its risk assessment in Malaysia. Mar Pollut Bull. Vol. 51, No. 8-12, pp. 896-911, ISSN 0025-326X

Besada, V.; Gonzalez, J.J.; Schultz & F. (2006). Mercury, cadmium, lead, arsenic, copper and zinc concentrations in albacore, yellowfin tuna and bigeye tuna from the Atlantic Ocean. Cienc. Mar. Vol. 32, pp. 439-445, ISSN 0185-3880

Bottero, M.T.; Dalmasso, A.; Cappelletti, M.; Secchi, C. & Civera, T. (2007). Differentiation of five tuna species by a multiplex primer-extension assay. J Biotechnol. May 1; Vol. 129, No. 3, (May 2007), pp. 575-80, ISSN 0168-1656

Bradford, M. (1976). A rapid and sensitive method for the quantification of mg quantities of protein. Anal Chem, Vol 72, pp. 248-254, ISSN 0003-2700

Carrera, M.; Cañas, B.; Piñeiro, C.; Vázquez, J. & Gallardo, J. M. (2006). Identification of commercial hake and grenadier species by proteomic analysis of the parvalbumin fraction. Proteomics, Vol. 6, pp. 5278–5287, ISSN 1615-9853

Carrera, M.; Cañas, B.; Piñeiro, C.; Vázquez, J. & Gallardo, J. M. (2007). De Novo Mass Spectrometry Sequencing and Characterization of Species-Specific Peptides from Nucleoside Diphosphate Kinase B for the Classification of Commercial Fish Species Belonging to the Family Merlucciidae. J. Proteome Res. Vol. 6, No. 8, pp. 3070-3080, ISSN 1535-3893

Chen, T.Y.; Shiau, C.Y.; Wei, C.I. & Hwang, D.F. (2004) Preliminary Study on Puffer Fish Proteome Species Identification of Puffer Fish by Two-Dimensional Electrophoresis. J. Agric. Food Chem. Vol. 52, No 8, pp. 2236-2241, ISSN 0021-8561

Chow, S. & Kishino, H. (1995). Phylogenetic relationships between tuna species of the genus Thunnus (Scombridae: Teleostei): inconsistent implications from morphology, nuclear and mitochondrial genomes. J Mol Evol. Vol. 41, No. 6 (December 1995), pp. 741-8, ISSN 0022-2844

Commission Regulation (EC) No 2065/2001 of 22 October 2001 laying down detailed rules for the application of Council Regulation (EC) No 104/2000 as regards informing consumers about fishery and aquaculture products. Off. Jour. of the European Communities. L 278/6.

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Espíñeira, M.; González-Lavin, N.; Vieites, JM. & Santaclara, FJ. (2008). 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. J Agric Food Chem. Vol. 56, No 22, (26Nov 2008), pp. 10594-9, ISSN 0021-8561

Jérôme, M.; Fleurence, J.; Rehbein, H.; Kündiger, R.; Mendes, R.; Costa, H.; Pérez-Martín, R.; Piñeiro-González, C.; Craig, A.; Mackie, I.; Malmheden Yman, I.; Ferm, M.; Martínez, I.; Jessen, F.; Smelt, A. & Luten, J. Identification of fish species after cooking by SDS-PAGE and urea IEF: a collaborative study. (2000). J Agric Food Chem. Vol. 48, No 7 (Jul 2000), pp. 2653-8, ISSN 0021-8561

Hochstrasser, D.; Augsburger, V.; Pun, T.; Weber, D.; Pellegrini, C. & Muller, AF. (1988). "High-resolution" mini-two-dimensional gel electrophoresis automatically run and stained in less than 6 h with small, ready-to-use slab gels. Clin Chem. Vol. 34, No 1 (Jan 1988), pp. 166-70, ISSN 0009-9147

Hubalkova, Z.; Kralik, P.; Kasalova, J. & Rencova, E. (2008). Identification of gadoid species in fish meat by polymerase chain reaction (PCR) on genomic DNA. J Agric Food Chem., Vol. 56, No 10, (28 May 2008), pp. 3454-9, ISSN 0021-8561

Kochzius, M.; Seidel, C.; Antoniou, A.; Botla, SK.; Campo, D.; Cariani, A.; Vazquez, EG.; Hauschild, J.; Hervet, C.; Hjörleifsdottir, S.; Hreggvidsson, G.; Kappel, K.; Landi, M.; Magoulas, A.; Marteinsson, V.; Nölte, M.; Planes, S.; Tinti, F.; Turan, C.; Venugopal, MN.; Weber, H. & Blohm, D. (2010). Identifying Fishes through DNA Barcodes and Microarrays. PLoS One, Vol. 5, No. 7, (7 Sep 2010), pp. 12620, ISSN 1932-6203

Kojadinovic, J.; Potier, M.; Le Corre, M.; Cosson, R.P. & Bustamante, P. (2007). Bioaccumulation of trace elements in pelagic fish from the Western Indian Ocean. Environ Pollut. Vol. 146, No. 2 (Mar 2007), pp. 548-66, ISSN 0269-7491

López, JL.; Marina, A.; Álvarez, G. & Vázquez, Jesús. (2002). Application of proteomics for fast identification of species-specific peptides from marine species. Proteomics, Vol. 2, No. 12, (Dec 2002), pp. 1658-65, ISSN 1615-9853

Lopez, I. & Pardo, MA. (2005). Application of relative quantification TaqMan real-time polymerase chain reaction technology for the identification and quantification of Thunnus alalunga and Thunnus albacares. J Agric Food Chem., Vol. 53, No 11, (1 Jun 2005), pp. 4554-60, ISSN 0021-8561

Mackie, I. M.; Pryde, S. E.; Gonzales-Sotelo,C.; Medina,I.; Peréz-Martín, R.; Quinteiro,J. ; Rey-Mendez,M. & Rehbein, H. (2000). Possibilità di identificazione delle specie di pesce in scatola. Ind. Conserve, Vol. 75, pp. 59–66, ISSN 00197483

Marko, P. B.; Lee, S. C.; Rice, A. M.; Gramling, J. M.; Fitzhenry, T. M.; Mc Aalister, J. S.; Harper, G. R. & Moran, A. L. (2004). Fisheries: mislabelling of a depleted reef fish. Nature, Vol. 430, No 6997, (Jul 15 2004), pp. 309-10, ISSN 0028-0836

Meyer, R.; Hofeleien, C.; Luphy, J. & Candrian, U. (1995). Polymerase chain reaction-restriction fragment length polymorphism analysis: a simple method for species identification in food. J. AOAC Int., Vol. 78, pp. 1542–1545, ISSN 1060-3271

Michelini, E.; Cevenini, L.; Mezzanotte, L.; Simoni, P.; Baraldini, M.; De Laude, L. & Roda, A. (2007). One-step triplex-polymerase chain reaction assay for the authentication of yellowfin (Thunnus albacares), bigeye (Thunnus obesus), and skipjack

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(Katsuwonus pelamis) tuna DNA from fresh, frozen, and canned tuna samples. J Agric Food Chem., Vol. 55, No 19, (19 Sep 2007), pp.7638-47, ISSN 0021-8561

Monti, G.; De Napoli, L.; Mainolfi, P.; Barone, R.; Guida, M.; Marino, G. & Amoresano, A. (2005). Monitoring Food Quality by Microfluidic Electrophoresis, Gas Chromatography, and Mass Spectrometry Techniques: Effects of Aquaculture on the Sea Bass (Dicentrarchus labrax). Anal. Chem., Vol. 77, pp. 2587-2594, ISSN 0003-2700

Pandey, A. & Mann, M. (2000). Proteomics to study genes and genomes. Nature. Vol. 405, No 6788, pp. 837-846, ISSN 0028-0836

Pappin, DJ.; Hojrup, P. & Bleasby, AJ. (1993). Rapid identification of proteins by peptide-mass fingerprinting. Curr Biol. Vol. 3, No 6, (Jun 1993), pp. 327-32, ISSN 0960-9822

Pardo, MA. & Pérez-Villareal, B. (2004). Identification of commercial canned tuna species by restriction site analysis of mitochondrial DNA products obtained by nested primer PCR. Food Chemistry. Vol. 86, pp. 143–150, ISSN 0308-8146

Pepe, T.; Trotta, M.; di Marco, I.; Cennamo, P.; Anastasio, A. & Cortesi, ML. (2005). Mitochondrial cytochrome b DNA sequence variations: An approach to fish species identification in processed fish products. J. Food Prot. Vol 68, pp. 421-425, ISSN 0362-028X

Pepe, T.; Trotta, M.; Di Marco, I.; Anastasio, A.; Bautista, J.M. & Cortesi, M.L. (2007). Fish species identification in surimi-based products. J Agric Food Chem. Vol. 55, No. 9 (May 2007), pp. 3681-5, ISSN 0021-8561

Pepe, T.; Ceruso, M.; Carpentieri, A.; Ventrone, I.; Amoresano, A. & Anastasio A. (2010). Proteomic analysis for the identification of Thunnus genus three species. Vet Res Commun. Vol. 34, Suppl. 1, pp. 153-S155, ISSN 0165-7380

Piñeiro, C.; Barros-Velázquez, J.; Stelo, CG. & Gallardo, JM. (1999). The use of two-dimensional electrophoresis in the characterization of the water-soluble protein fraction of commercial flat fish species. Eur. Food Res. Technol., Vol. 208, Numbers 5-6, pp. 342-348, ISSN 1438-2377

Piñeiro, C.; Vázquez, Jesús.; Marina, AI.; Barros-Velázquez, J. & Gallardo, JM. (2001). Characterization and partial sequencing of species-specific sarcoplasmic polypeptides from commercial hake species by mass spectrometry following two-dimensional electrophoresis. Electrophoresis, Vol. 22, No. 8, (May 2001), pp. 1545-52, ISSN 0173-0835

Rehbein, H.; Kündiger, R.; Pineiro, C. & Perez-Martin, RI. (2000). Fish muscle parvalbumins as marker proteins for native and urea isoelectric focusing. Electrophoresis, Vol. 21, No 8, (May 2000), pp.1458-63, ISSN 0173-0835

Renon, P.; Colombo, M.; Colombo F.; Malandra, R. & Biondi, P. A. (2001). Computer-assisted evaluation of isoelectric focusing patterns in electrophoretic gels: Identification of smoothhounds (Mustelus mustelus, Mustelus asterias) and comparison with lower value shark species. Electrophoresis. Vol. 22, pp. 1534-1538, ISSN 0173-0835

Russo, C.; Takezaki, N. & Nei, M. (1996). Efficiencies of different genes and tree-building methods in recovering a known vertebrate phylogeny. Mol. Biol. EVol., Vol. 13, No. 3, (Mar 1996), pp. 525-536, ISSN 0737-4038
Storelli, M.M.; Barone, G.; Cuttone, G.; Giungato, D. & Garofalo, R. (2010) Occurrence of toxic metals (Hg, Cd and Pb) in fresh and canned tuna: public health implications. Food Chem Toxicol. Vol. 48, No. 11, (Nov 2010), pp. 3167-70, ISSN 0278-6915

Terio, V.; Di Pinto, P.; Decaro, N.; Parisi, A.; Desario, C.; Martella, V.; Buonavoglia, C. & Tantillo, M.G. (2010) Identification of tuna species in commercial cans by minor groove binder probe real-time polymerase chain reaction analysis of mitochondrial DNA sequences. Mol Cell Probes. Vol. 24, No.6, pp. 352-6, ISSN 0890-8508

Trotta, M.; Schönhuth, S.; Pepe, T.; Cortesi, M.L.; Puyet, A. & Bautista, J.M. (2005). Multiplex PCR method for use in real-time PCR for identification of fish fillets from grouper (Epinephelus and Mycteroperca species) and common substitute species; J Agric Food Chem., Vol. 53, No 6 (23 Mar 2005), pp. 2039-45, ISSN 0021-8561

Tyers, M. & Mann, M. (2003). From genomics to proteomics. Nature, Vol. 422, No 6928, (13 Mar 2003), pp. 193-7, ISSN 0028-0836

Viñas, J. & Tudela, S. (2009). A validated methodology for genetic identification of tuna species (genus Thunnus). PLoS One. Vol. 4, No. 10, (Oct 2009), pp. 7606, ISSN 1932-6203

Zehner, R; Zimmermann, S. & Mebs, D. (1998). RFLP and sequence analysis of the cytochrome b gene of selected animals and man: Methodology and forensic application. Int. J. Leg. Med. Vol. 111, pp. 323-327, ISSN 0937-9827
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