1 **Proteomic analysis of processing by-products from canned and fresh**

2 **tuna: identification of potentially functional food proteins**

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ABSTRACT

Proteomic approaches have been used to identify the main proteins present in processing by-products generated by the canning tuna-industry, as well as in by-products derived from filleting of skeletal red muscle of fresh tuna. Following fractionation by using an ammonium sulphate precipitation method, three proteins (tropomyosin, hemoglobin and the stress-shock protein ubiquitin) were identified in the highly heterogeneous and heat-treated material discarded by the canning-industry. Additionally, this fractionation method was successful to obtain tropomyosin of high purity from the heterogeneous starting material. By-products from skeletal red muscle of fresh tuna were efficiently fractionated to sarcoplasmic and myofibrillar fractions, prior to the identification based mainly on the combined searching of the peptide mass fingerprint (MALDI-TOF) and peptide fragment fingerprinting (MALDI-LIFT TOF/TOF) spectra of fifteen bands separated by 1D SDS-PAGE. Thus, the sarcoplasmic fraction contained myoglobin and several enzymes that are essential for efficient energy production, whereas the myofibrillar fraction had important contractile proteins, such as actin, tropomyosin, myosin or an isoform of the enzyme creatine kinase. Application of proteomic technologies has revealed new knowledge on the composition of important by-products from tuna species, enabling a better evaluation of their potential applications.

Keywords: *Thunnus alalunga*, proteomics, sarcoplasmic, myofibrillar, by-product, food protein.

1. INTRODUCTION

Fish by-products may contain valuable protein and lipid fractions as well as vitamins and minerals highlighting the nutritional and health benefits attributed to fish consumption. Currently, more than 33 million metric tones (23%) of world fish production are destined for non-food uses (FAO, 2008). In addition, the percentages of processing by-products generated from commercial filleting of fish species such as cod, tuna, and trout are even higher, ranging between 60 to 70% (Chen & Jaczynski, 2007). It is anticipated that these high levels of fish by-products are going to be increased during next years as consequence of a raise in fish production in response to expected demand growth for fisheries products (Delgado, Wada, Rosegrant, Meijer & Ahmed, 2003). In this context, there are major ongoing research addressed to improve the management of these by-products in terms of searching new bioactive compounds and developing new technologies that allow a more profitable utilization of this material (Rustad, 2003).

Particularly, given that recent studies have identified a number of bioactive compounds from remaining fish muscle proteins, collagen and gelatine (Kim & Mendis, 2006), the recovery of proteins from fish by-products for their utilization as potential food ingredients is becoming of increasing interest in the food industry (Sanmartin, Arboleya, Villamiel & Moreno, 2009). Therefore, to assess the potential applications for proteins recovered from fish by-products, it is of paramount importance the characterization of the protein fraction of the discarded material. To the best of our knowledge, despite their global economic importance and intensive international trade for canning, protein fractions contained in by-products generated from processing tuna

63   species have not been characterized to date. Likewise, considering that fresh tuna species

64   have high economic value and its protein-rich muscle tissue is a valuable food source, by-

65   products generated from commercial filleting could also be an important and alternative

66   source for functional proteins or peptides.

67   Proteomic techniques have strong potential to accurately characterize the major

68   proteins found in complex food matrices. However, up to now, the use of proteomics in

69   fish biology and aquaculture has been limited (Forné, Abián & Cerdà, 2010).

70   Furthermore, fish protein identification is a challenging task due to the relatively low

71   number of fish protein sequences and expressed sequence tags (ESTs) present in the

72   public databases (Pineiro, Barros-Velazquez, Vazquez, Figueras & Gallardo, 2003;

73   Kjaersgard, Nørrelykke & Jessen, 2006). Therefore, it is essential to obtain protein

74   sequences by either Edman degradation or tandem mass spectrometry which enables

75   identification by matching with homologue proteins from different species as it has been

76   previously reported for hake (Pineiro, Vazquez, Marina, Barros-Velazquez & Gallardo,

77   2001) or cod proteins (Kjaersgard et al., 2006). In this sense, Matrix Assisted Laser

78   Desorption Ionization-Time Of Flight mass spectrometry (MALDI-TOF) provides high

79   sensitivity for peptide mass fingerprinting (PMF). Besides, latest developments in this

80   type of mass spectrometers allow performing tandem mass spectrometry analysis by

81   MALDI-LIFT TOF/TOF for peptide fragment fingerprinting (PFF) (Suckau, Resemann,

82   Schuerenberg, Hufnagel, Franzen, & Holle, 2003).

83   The aim of this study was the fractionation and identification of the main proteins

84   present in processing by-products generated from both commercial canning and filleting

4

85 of tunas species using proteomic approaches by combining peptide mass fingerprinting

86 and peptide fragment fingerprinting (MS/MS) analysis.

87

88 **2. MATERIALS AND METHODS**

89

90 *2.1. By-products homogenization*

91

92 Canned tuna solid by-products were obtained from a local canned fish processing

93 plant. Skin, viscera and red muscle were the main compounds of the raw material, and

94 were collected after an industrial heat treatment at 95ºC for 1h. After deboning and

95 mixing steps, fish wastes were homogenized, freeze–dried and defatted by the Soxhlet

96 method using petroleum ether (Panreac, Barcelona, Spain) at 60ºC during 12 h. The

97 protein, ash, lipid, and moisture contents of the canned by-product were 60.1%, 19.7%,

98 1.0% and 4.4% (w:w), respectively, as determined according to the methods of AOAC

99 (2000).

100 Fresh tuna (*Thunnus alalunga*) red muscle was obtained from filleting wastes at a

101 local fish store situated in the coast (Bermeo, Spain) immediately after the reception of

102 tuna from a close landing centre in order to minimize changes in the quality of the

103 product. Immediately after collection, fish wastes were homogenized using a blender and

104 kept frozen at -20ºC.

105

106 *2.2. Protein extraction and fractionation*

107

108   Canned industry fish by-products were processed following the method described

109   by Maitena, Katayama, Sato & Saeki (2004) with some modifications. Firstly, material

110   was resuspended (1:100 w/v) with 0.16 M KCl (Merck KGaA, Darmstadt, Germany)

111   containing 20 mM Tris-HCl (Sigma-Aldrich, St. Louis, MO) (pH 7.5). After 30 min, the

112   solution was centrifuged at 30,000 x g for 10 min and the supernatant was recovered as

113   *Sample 1*. This step was repeated up to three times, in order to remove sarcoplasmic

114   proteins. The final pellet was then dissolved in 0.5 M KCl (Merck) containing 20 mM

115   Tris-HCl (pH 7.5) for 1 h. After addition of 40% saturation of ammonium sulphate

116   (Panreac), the protein solution was centrifuged at 30,000 x g for 1 h obtaining a

117   precipitate termed *Sample 2*. Afterwards, the ammonium sulphate saturation in the

118   supernatant was raised up to 55%, and again was subjected to centrifugation at 100,000 x

119   g for 1h. Proteins in the precipitated were collected, dissolved in 0.5 M NaCl (Panreac)

120   containing 20 mM Tris-HCI (pH 7.5), and dialyzed against the same buffer to remove

121   ammonium sulfate (*Sample 3*).

122   Regarding the protein extraction from fresh tuna by-products, a method described

123   by Mohan, Ramachandran, Sankar & Anandan (2007) with some modifications was

124   followed. Thus, fresh tuna (*Thunnus alalunga*) red muscle was mixed (1:5 w/v) with

125   sodium phosphate buffer (Merck) 0.1M at pH 7.0 and homogenized using an ultraturrax

126   (Miccra RT-D9[®]). The homogenate was centrifuged at 4 ºC, 12,000 x g for 15 min and

127   the supernatant was collected as *Sarcoplasmic* proteins. Final precipitate was washed

128   thoroughly using the same buffer in order to remove the soluble proteins. Myofibrillar

129   fraction was recovered from the pellet by solubilization (1:5 w/v) with sodium phosphate

130   buffer 0.1M pH 7.0 containing 0.5M NaCl. After homogenization, the solution was

131 centrifuged at 4ºC, 12,000 x *g* for 15 min and the supernatant was collected as a

132 *Myofibrillar* proteins.

133   All the steps in both protein fractionation methods were carried out below 6ºC.

134 Furthermore, the extraction procedures were repeated at least three times in order to

135 evaluate difference between batches. According to the electrophoresis gels, no

136 remarkable differences were observed regarding the number of visualized bands.

137   The protein concentration was determined by the bicinchoninic acid (BCA)

138 method (Smith et al., 1985) using bovine serum albumin (Sigma-Aldrich) as a standard.

139 Thus, total protein content corresponding to the myofibrillar and sarcoplasmic fractions

140 isolated from the homogenized by-products of skeletal red muscle of fresh tuna was 5.5%

141 (w:w).

142

143   *2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)*

144 *procedure*

145

146   For SDS-PAGE analysis, 32.5 μL of the fractionated samples was added to 12.5

147 μL of 4×NuPAGE LDS sample buffer (Invitrogen, CA, USA) and 5 μL of 0.5 M

148 dithiothreitol (DTT, Sigma-Aldrich) and the mixture heated at 70 °C for 10 min. The

149 samples were loaded (20 μL) onto a 4-12% polyacrylamide NuPAGE Novex$^{®}$ Bis-Tris

150 precast gel (Invitrogen), and a continuous MES-SDS running buffer (Invitrogen) was

151 used for proteins extracted from the canned tuna solid by-products and a MOPS-SDS

152 running buffer (Invitrogen) from the proteins derived from the fresh tuna by-products.

153 The gels were run for 35 min (MES-SDS running buffer) and 50 min (MOPS-SDS

154   running buffer) at 120 mA/gel and 200 V and stained using the Colloidal blue staining kit

155   (Invitrogen). Marker proteins used were: myosin (200 kDa), β-galactosidase (116.3 kDa),

156   phosphorylase b (97.4 kDa), BSA (66 kDa), glutamic dehydrogenase (55.4 kDa), lactate

157   dehydrogenase (36.5 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa),

158   lysozyme (14.4 kDa), aprotinin (6 kDa), insulin chain B (3.5 kDa) and insulin chain A

159   (2.5 kDa) (Invitrogen).

160

161   *2.4 In gel-tryptic digestion and peptide extraction*

162

163   Bands were excised manually from 1-D stained electrophoresis gels and subjected

164   to in-gel tryptic digestion according to Shevchenko, Wilm, Vorm & Mann (1996) with

165   minor modifications. The gel pieces were reduced with 30 μL of 10 mM DTT at 56°C for

166   20 min, followed by an alkylation in 30 μL of 50 mM iodoacetamide for 20 min at room

167   temperature in darkness. Then, bands were swollen in a digestion buffer containing 50

168   mM NH_4HCO_3 and 12.5 ng/μL of trypsin (Roche Diagnostics, recombinant, proteomics

169   grade trypsin, Penzberg, Germany) in an ice bath. After 30 min the supernatant was

170   removed and discarded, 20 μL of 50 mM NH_4HCO_3 were added to the gel piece and the

171   digestion allowed to proceed at 37 C overnight. After trypsinization, the supernatant was

172   transferred to an empty eppendorf tube and acidified with 0.1 % TFA.

173

174   *2.5 Sample preparation for MALDI MS analysis*

175

176   Recovered peptides were desalted prior to MALDI analysis by home-made nano-

177   columns consisting of 200 nL of POROS R2 + R3 material (PerSeptive Biosystems,

178   Framingham, MA) as described by Gobom, Nordhoff, Mirgorodskaya, Ekman, &

179   Roepstorff (1999) with some modifications. Columns were equilibrated with 0.1 % TFA

180   and the bound peptides subsequently eluted directly onto the MALDI target with 0.5 µL

181   CHCA solution (20 µg/µL in ACN, 0.1 % TFA, 70:30, vol/vol).

182

183   *2.6 Mass Spectrometric analysis*

184

185   Peptide mass fingerprinting and MS/MS analysis were performed on a Bruker

186   Autoflex III TOF/TOF smartbeam mass spectrometer (Bruker-Daltonics, Bremen,

187   Germany) equipped with an ion LIFT selector and reflector. Positively charged ions were

188   analyzed in reflector mode, using delayed extraction. The ionization is performed by

189   solid-state laser with a 360 nm wavelength in pulses of 200 Hz. Resolution remains

190   always above 7500, throughout the mass window. Routinely, 1400 scans were collected

191   for MALDI-TOF PMF analysis. When feasible, best candidates were chosen in manual

192   fashion for the corresponding LIFT MS/MS PFF analysis (400 MS scans for selection of

193   parental ion, 1600 MS/MS scans for fragments). Spectra were first externally calibrated

194   resulting in a mass accuracy of <50 ppm. Afterwards, internal calibration was performed,

195   reaching an accuracy of <20 ppm. Protein identification was performed by searching

196   NCBInr database (version 09/2009; 10490613 sequences; 3896452119 residues) and

197   using Mascot 2.1 search engine (Matrix Science, Boston, MA). The following parameters

198   were used for database searches: up to two missed cleavages; allowed modifications:

199   carbamidomethylation of cysteine (fixed) and oxidation of methionine (variable). For

200   peptide mass fingerprinting, 0.7 Da of tolerance was allowed for fragment-ion masses

201   obtained by tandem mass spectrometry. In all searches performed, protein scores greater

202   than 63 are considered statistically significant ($p<0.05$).

203   PMFs and the corresponding tables describing the characterized peptides of all

204   proteins identified in the analyzed by-products are shown in the Supporting Information.

3. RESULTS

3.1 Proteomic identification of by-products from canned tuna

Following the ammonium sulphate precipitation procedure described in Materials and Methods section, different electrophoretic patterns (**Figure 1**, lanes 2 and 3), as well as a strong and highly resolved band of M_r ~36 kDa (**Figure 1**, lane 4) were obtained. Despite the high heterogeneity of the starting material which includes tuna bones, skin, muscle or internal organs, five major electrophoretic bands were visualized after Coomassie staining. These five bands were observed at 1D SDS-PAGE within a molecular weight of 6 and 200 kDa and were cut off for further identification. Three proteins were successfully identified by combination of PMF and PFF analyses (**Table 1** and Supporting Information).

Band no. 3 was identified as ubiquitin, a small protein of 74 amino acids abundantly present in all eukaryotes. It is highly conserved among eukaryotic species and this protein differs only in three residues in yeast and animals (Sharp & Li, 1987). Habu, Ohishi, Mihara & Yanaihara (1994) indicated that the amino acid sequence of tuna ubiquitin is identical to that of mammalian counterparts. The most prominent function of ubiquitin is labeling proteins for proteasomal degradation. Many kinds of stress appear to stimulate ubiquitin expression (Okubo et al., 2002), including heat shock in hamster (Fornace, Alamo, Hollander & Lamoreaux, 1989), chicken (Bond & Schlesinger, 1985) and yeast (Cheng, Watt & Piper, 1994), as well as starvation and respiration stress also reported in yeast (Cheng et al., 1994). Thus, the identification of ubiquitin in by-products

228   from canning tuna is not surprising considering the large number of factors can influence

229   the up-regulation of ubiquitin genes.

230   Analysis of band no. 4, a predominant band present in the solution derived from

231   the second step of the fractionation method (**Figure 1**, lane 3), revealed the unambiguous

232   presence of the β-subunit of hemoglobin (**Table 1**). Furthermore, an additional

233   fractionation step led to the apparent purification of a single and well-resolved band of M_r

234   ~36 kDa (band no. 5, **Figure 1**, lane 4). Proteomic analysis (PMF + PFF) confirmed the

235   presence of only one protein identified as tropomyosin of bluefin tuna from fast skeletal

236   muscle (**Table 1** and Supporting Information). Huang, Ochiai & Watabe (2004) reported

237   that tropomyosin is a mixture of nearly equimolar amounts of two isoforms (designated α

238   and β) in the fast skeletal muscle of bluefin tuna, although only the sequence of the α-

239   type is known so far. These results indicate that the precipitation method reported in this

240   work is useful for obtaining tropomyosin with a high degree of purity from by-products

241   of tuna processed for canning.

242   Regarding bands no. 1 and 2 with M_r ranging between 100 and 200 kDa (**Figure**

243   **1**, lane 2), no positive identification was accomplished by combination of PMF and LIFT

244   analyses.

245

246   *3.2 Proteomic identification of by-products from skeletal red muscle of fresh tuna*

247

248   After fractionation steps, two well-differentiated 1D SDS-PAGE patterns,

249   presumably corresponding to the sarcoplasmic and myofibrillar fractions were obtained

250   (**Figure 2**). Up to 12 bands with M_r ranging between 14 and 116 kDa from the

251 sarcoplasmic fraction and 8 bands with M_r between 14 and 200 kDa from the myofibrillar

252 fraction were excised, tryptic digested and analyzed by MALDI-TOF/TOF (Supporting

253 Information). These bands were selected according to their abundance in both fractions.

254

255   *3.2.1 Myofibrillar fraction*

256

257   The most common myofibrillar proteins in the muscle of aquatic animals are

258 myosin, actin, tropomyosin, and troponins C, I and T representing around 40-60%, 15-

259 30%, 4-6% and 4-6% of the total myofibrillar protein of muscle, respectively, depending

260 upon species (Mackie, 1997). Seven proteins corresponding to the myofibrillar fraction

261 could be identified (**Table 2**). Five bands matched proteins involved in muscle

262 contraction such as myosin (heavy and light chains), actin or tropomyosin. As an

263 example, **Figure 3** shows the sequence coverage map of the combined PMF and five

264 MALDI LIFT-TOF/TOF spectra corresponding to the identification of tropomyosin

265 (**Figure 2**, lane 3, band 5). The peptides released at higher intensities from this band were

266 identified as fragments (by decreasing order of abundance): ^{168}K-R^{178} (*m/z* 1284.8), ^{92}I-

267 R^{101} (*m/z* 1243.7), ^{169}L-R^{178} (*m/z* 1156.7), ^{91}R-R^{101} (*m/z* 1399.8), ^{161}K-R^{167} (*m/z* 894.5),

268 ^{252}T-K^{264} (*m/z* 1552.8) and ^{92}I-R^{105} (*m/z* 1727.9) (**Figure 3A** and Supporting

269 Information). These peptides were obtained as a result of tryptic cleavages occurring at

270 the carboxyl side of basic residues (R and K). Thus, three peptides spanning the region

271 ^{161}K-R^{178} were found as consequence of the tryptic cleavages at bonds ^{160}R-K^{161}, ^{167}R-

272 K^{168} and ^{168}K-L^{169}. Another set of three main peptides was derived from tryptic cleavages

273 at bonds ^{90}R-R^{91}, ^{101}R-A^{102}, ^{91}R-I^{92} and ^{105}R-L^{106}. Finally, peptide ^{252}T-K^{264} was obtained

274   as a result of tryptic cleavages at bonds ^{251}K-T^{252} and ^{264}K-L^{265} (**Figure 3A**). Five of

275   these peptides, i.e. ^{91}R-R^{101}, ^{92}I-R^{101}, ^{161}K-R^{167}, ^{168}K-R^{178} and ^{169}L-R^{178}, were analyzed by

276   MALDI-TOF/TOF. The information of the peptide mass fingerprinting (PMF), together

277   with that of the peptide fragment fingerprinting (PFF) obtained from the five selected

278   peptides, allowed the unambiguous protein identification. The information derived from

279   the tandem mass spectrometry analysis of the highest scored peptide (i.e., ^{92}I-R^{101}) for

280   this protein is shown in **Figure 3B**. The detected y_n-type ions owing to the free carboxyl-

281   terminus, as well as to the b_n-type ions owing to the free amino terminus, were detected

282   and they are marked in red (**Figure 3B**).

283   In band no. 4 (**Figure 2**, lane 3), two different proteins were identified by

284   combining PMF and PFF, i.e. the enzyme muscle-type creatine kinase CKM1 together

285   with skeletal α-actin. Creatine kinase is known to catalyze, through the consumption of

286   adenosine triphosphate, the conversion of creatine to phosphocreatine, an organic

287   compound capable of storing and providing energy for muscular contraction, whilst

288   skeletal α-actin belongs to the actin family of proteins which are highly conserved

289   proteins involved in various types of cell motility and ubiquitously expressed in all

290   eukaryotic cells. Five MS/MS spectra were acquired from peptides generated from band

291   no. 4. Three of them matched to creatine kinase CKM1: *m/z* 1125.6 (GFTLPPHNSR),

292   *m/z* 1657.9 (TFLVWVNEEDHLR) and *m/z* 1995.0 (GTGGVDTASVGGVFDISNADR),

293   and two matched to α-actin *m/z* 1198.7 (AVFPSIVGRPR) and *m/z* 1790.9

294   (SYELPDGQVITIGNER).

295

296   ***3.2.2 Sarcoplasmic fraction***

297

298   Eight proteins corresponding to 9 bands were identified in the sarcoplasmic

299 fraction (**Figure 2,** lane 2 and **Table 2**). Seven of these bands matched proteins known to

300 be involved in energy metabolism, mainly enzymes of carbohydrate metabolism.

301 Glycolytic enzymes are responsible for quality deterioration of fish after death and before

302 bacterial spoilage (Hui et al., 2006), and they have been proposed as markers for post-

303 mortem changes during storage of fishes such as cod (Gebriel, Uleberg, Larssen,

304 Bjornstad, Sivertsvik & Moller, 2010). Post-mortem biochemical and structural changes

305 in proteins are directly related to sensorial properties and water-holding capacity (Huff-

306 Lonergan & Lonergan, 2005).

307   Glycogen phosphorylase A (band no. 9) participates in the breakdown of

308 glycogen, catalyzing the phosphorolytic cleavage of the α (1-4) glycosidic linkages,

309 releasing glucose-1-phosphate as the reaction product. This enzyme has been reported to

310 be predominant in skeletal muscle of mammalians, among other tissues, in response to its

311 greater demand for specific glycogenolytic control. Furthermore, four enzymes directly

312 involved in glycolysis, and thus serving to breakdown glucose for energy and carbon

313 molecules, were also identified: triose-phosphate isomerase (band no. 19),

314 glyceraldehyde 3-phosphate dehydrogenase (band no. 17), β-enolase (band no. 13) and

315 pyruvate kinase (bands no. 10 and 11). Interestingly, in addition to a long established

316 metabolic function, glyceraldehyde 3-phosphate dehydrogenase enzyme has also been

317 implicated in several non-metabolic processes, such as binding to actin and tropomyosin,

318 and thus, it might have a role in cytoskeleton assembly (Dugaiczyk, Haron, Stone,

319 Dennison, Rothblum & Schwartz, 1983). Thus, interaction of glycolytic enzymes with

320 actin has been suggested to be a mechanism for compartment of the glycolytic

321 pathway (Waingeh, Gustafson, Kozliak, Lowe, Knull & Thomasson, 2006). Additionally,

322 enolase has also been reported as an early marker of human myogenesis (Fougerousse et

323 al., 2001), as well as it has been identified as an early oxidative protein in bonito muscle

324 (Kinoshita, Sato, Naitou, Ohashi, & Kumazawa, 2007). The identified β-isoform is the

325 predominant (~90%) form of enolase in skeletal muscle (Foucault, Vacer, Merkulova,

326 Keller & Abrio-Dupont, 1999). Lastly, the identification of pyruvate kinase in two

327 different bands (no. 10 and 11) of apparently similar electrophoretical mobility might be

328 indicative of the presence of at least two post-translationally modified forms, and/or as

329 consequence of a partial degradation.

330   Isocitrate dehydrogenase, an enzyme that participates in the Krebs cycle

331 catalyzing the oxidative decarboxylation of isocitrate to produce α-ketoglutarate and CO_2

332 while converting NAD^+ to NADH, was detected in the band no. 12. Finally, the enzyme

333 muscle-type creatine kinase, previously matched within the myofibrillar fraction (band

334 no. 4 in **Figure 2**, lane 3), was also identified in the sarcoplamisc fraction as the isomer

335 CKM2 (band no. 14 in **Figure 2**, lane 2). The fact that the tuna red muscle could have at

336 least two isoforms of creatine kinase is consistent with the reported presence of multiple

337 forms of this enzyme in muscle tissue of other fish species, such as carp (Sun, Hui & Wu,

338 1998), antarctic icefish (Winnard, Cashon, Sidell & Vayda, 2003) or zebrafish

339 (Bosworth, Chou, Cole & Rees, 2005).

340   Finally, the well-defined and separated band no. 20 was identified by PMF and

341 PFF analyses as myoglobin, a protein abundantly found in the skeletal red muscle, from

342 different tuna species including *Thunnus alalunga* (Albacore), *T. albacares* (Yellowfin),

343   *T. thynnus orientalis* (North Pacific bluefin), *T. thynnus* (Bluefin) and *T. obesus* (Bigeye)

344   (**Figure 4**). However, the highest similarity score "Mowse score" corresponded to

345   myoglobin from *T. alalunga* with a high sequence coverage of 71% that includes the

346   distinctive peptide EHPDTQK derived from the hydrolytic action of trypsin on bonds

347   ^{31}K-E^{32} and ^{38}K-L^{39} (*m/z* 854.3, **Figure 4A**). Likewise, additional peptides released at

348   high intensities were identified as fragments ^{115}A-R^{126} (*m/z* 1129.6), ^{10}C-R^{28} (*m/z* 2108.1),

349   ^{43}F-K^{66} (*m/z* 2267.3), ^{127}N-K^{141} (*m/z* 1663.9) and ^{93}H-K^{101} (*m/z* 1110.6) (Supporting

350   Information). All these abundant peptides were also obtained as a result of tryptic

351   cleavages occurring at the carboxyl side of R and K residues without missed cleavages,

352   with the exception of peptide ^{93}H-K^{101} which contained one missed cleavage (Figure 4A).

353   Furthermore, the C residue at peptide ^{10}C-R^{28} was carbamidomethylated following the

354   treatment with iodoacetamide. Lastly, MS^2 spectra from peptides ^{115}A-R^{126} and ^{10}C-R^{28}

355   (Figure 4B) were dominated by the sequence informative y-ions resulting from cleavages

356   on the carboxy-terminal side of peptide.

357

358   **4. DISCUSSION**

359

360   MALDI has historically been considered a "soft" ionization technique that

361   produces almost exclusively intact protonated pseudomolecular ion species. Later studies

362   have shown that a significant degree of metastable ion decay occurs after ion acceleration

363   and prior to detection. The ion fragments produced from the metastable ion decay of

364   peptides and proteins typically include both neutral molecule losses (such as water,

365   ammonia and portions of the amino acid side chains) and random cleavage at peptide

366   bonds. The observance of these metastable ion decay products in MALDI mass spectra is

367   dependent on the TOF instrumental configuration. As it is indicated in Materials and

368   Methods section, our MALDI TOF spectrometer is equipped with a LIFT pusher/filter

369   that enhance post source decay (PSD) observance, simulating a real TOF/TOF device

370   (Suckau et al., 2003). This is particularly important for protein identification in *Thunnus*

371   *alalunga* (Albacore) whose genome is not sequenced and, consequently, it is essential to

372   obtain protein sequences by tandem mass spectrometry allowing their identification by

373   matching with homologue proteins from different related fish species.

374   Knowledge about quality and composition of fish by-products is essential to

375   obtain a profitable utilization. Results presented in this work show the usefulness of

376   proteomic techniques like peptide mass fingerprinting and peptide fragment

377   fingerprinting performed by MALDI-TOF/TOF to identify proteins extracted from

378   different tuna by-products, although *T. alalunga* is an organism without a sequenced

379   genome. Such information is important for future studies investigating the evaluation of

380   their potential applications.

381   If the recovered protein material is intended to be used for human food products,

382   the knowledge of the nutritional value of these proteins will be essential. It is well-known

383   that tuna species such as *T. alalunga* has proteins of high nutritional value (Castrillón,

384   Navarro & García-Arias, 1996). Regarding essential aminoacids, previous works reported

385   that the composition of proteins from tuna source was of very high quality (Pigott &

386   Tucker, 1990).

387   From a technological point of view, fish myofibrillar proteins have shown

388   excellent functional properties as food ingredients, such as gel-forming ability,

389 emulsifying properties, and water-holding ability, which are related to their solubilization

390 in salt solutions (Saeki & Inoue, 1997). Surimi is a good example of a product created

391 with myofibrillar proteins isolated from previously undervalued fish parts. Likewise, a

392 gel-enhancing effect as well as good water holding and oil binding capacities have been

393 recently reported for sarcoplasmic fish proteins indicating their potential use as a

394 promising food ingredient with good functional properties (Jafarpour & Gorczyca, 2009;

395 Yongsawatdigul & Hemung, 2010).

396   The analyzed by-products could also be suitable sources of bioactive peptides

397 according to the identified proteins. Recently, Je, Qian, Lee, Byun & Kim (2008) and Hsu

398 (2010) reported that protein hydrolysates of tuna dark muscle by-product derived from

399 canned processing possessed strong *in vitro* antioxidative activity. Likewise, peptides

400 possessing ACE (Angiotensin Converting Enzyme) inhibitory properties and, hence, with

401 potential beneficial effects in the treatment of hypertension, derived from muscle proteins

402 of fish species such as bonito or tuna have been previously identified. Thus,

403 glyceraldehyde 3-phosphate dehydrogenase from tuna muscle, protein identified in the

404 band no. 17 of the sarcoplasmic fraction isolated from by-products of red muscle of fresh

405 tuna (**Figure 2**, lane 2, **Table 2**), has an octapeptide (PTHIKWGD) with a potent ACE

406 inhibitory activity (Kohama, Matsumoto, Oka, Teramoto, Okabe & Mimura, 1988). This

407 specific peptide was one of the first identified ACE inhibitory peptides in marine

408 products.

409   Later on, protease digests of bonito muscle had ACE-inhibitory activity in

410 addition to a good taste (Yokoyama, Chiba & Yoshikawa, 1992). These authors

411 demonstrated that the most potent ACE-inhibitory peptides were derived from actin,

412   which has been identified in the band no. 4 of the myofibrillar fraction isolated from by-

413   products of red muscle of fresh tuna (**Figure 2**, lane 3, **Table 2**). In fact, this mixture of

414   peptides derived from bonito actin has been proved to exert not only *in vitro* ACE

415   inhibitory activity but also *in vivo* antihypertensive activity after oral administration in

416   spontaneously hypertensive rats (Fujita, Yokoyama, Yasumoto & Yoshikawa, 1995) and

417   borderline hypertensive humans (Fujita, Yasumoto, Hasegawa & Ohshima, 1997).

418   Consequently, it is commercially available following official approval as a "Food for

419   Specified Health Use" in Japan (Fujita, Yokoyama & Yoshikawa, 2000).

420   Likewise, one peptide (DMIPAQK) derived from muscle-type creatine kinase

421   (Yokoyama et al., 1992), protein identified in this work in both myofibrillar and

422   sarcoplasmic fractions from red muscle, and another heptapeptide (SVAKLEK) derived

423   from tropomyosin (Yamamoto, Ejiri & Mizuno, 2003), which was also identified in by-

424   products from canned tuna in addition to by-products from red muscle, also showed to

425   posses ACE-inhibitory activity.

426   On the other hand, the genus *Thunnus* comprises many species that differ in

427   commercial value depending on their organoleptic features and, consequently, these

428   species are often subjected to fraudulent substitution (Pepe, Ceruso, Carpentieri,

429   Ventrone, Amoresano & Anastaio, 2010). The proteomic identification of myoglobin in

430   the sarcoplasmic fraction revealed the presence of an *alalunga*-specific peptide

431   (EHPDTQK) that might be used as a specific marker. This fact supports the potential use

432   of proteomic tools for species identification of fish.

433

434   **5. CONCLUSION**

435

436   Procedures described in this work allowed the isolation and characterization of

437   proteins from processing tuna by-products that could be potentially used in a wide

438   number of human food applications. Although further studies are needed to evaluate

439   specific bioactivities, these findings could contribute to expand the use of tuna processing

440   by-products to the development of new commercial applications for the human health

441   promotion.

442

443   **ACKNOWLEDGEMENTS**

444

450

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601

FIGURE CAPTIONS.

603

604 **Figure 1.** SDS-PAGE analyses under reducing conditions showing fractionation of

605 proteins from canned tuna by-products. Lane 1: Marker proteins. Lane 2: sample 1-

606 soluble proteins at 0.16 M KCl 20 mM Tris-HCl (pH 7.5). Lane 3: sample 2 - soluble

607 proteins at 0.5 M KCl, pellet after 40% ammonium sulphate saturation. Lane: 4: sample 3

608 - soluble proteins at 0.5 M KCl, pellet after 55% ammonium sulphate saturation. Labelled

609 bands are described in the text and Table 1.

610

611 **Figure 2.** SDS-PAGE analyses under reducing conditions showing fractionation of

612 proteins from fresh red muscle tuna by-products. Lane 1: Marker proteins. Lane 2:

613 Soluble proteins at 0.1 M sodium phosphate buffer (pH 7) corresponding to the

614 sarcoplasmic fraction. Lane 3: Soluble proteins at 0.1 M sodium phosphate buffer, 0.5 M

615 NaCl (pH 7) corresponding to the myofibrillar fraction. Labelled bands are described in

616 the text and Table 2.

617

618 **Figure 3. A)** MASCOT results obtained for band no. 5 (identified as α-tropomyosin)

619 corresponding to the myofibrillar fraction extracted from fresh tuna by-products. Above

620 is represented the sequence coverage map obtained by PMF. Matched peptides are

621 represented in the table below including: start and end positions of the peptide sequence

622 starting from the amino acid terminal of the whole protein, the observed *m/z*, transformed

623 to its experimental mass (Mr(expt)), the calculated mass (Mr(calc)) from the matched

624 peptide sequence, as well as their mass difference (in ppm), the number of missed

625 cleavage sites for trypsin (Miss), and the peptide sequence. **B)** List of fragmented ions

626 obtained (in red) vs. total predicted ions for MS/MS fragmentation of peptide at

627 2107.0408 Th, which achieve extensive statistical significance (MASCOT score of 137).

628

629 **Figure 4. A)** MASCOT results obtained for band no. 20 (identified as myoglobin)

630 corresponding to the sarcoplasmic fraction extracted from fresh tuna by-products. Above

631 is represented the sequence coverage map obtained by PMF. Matched peptides are

632 represented in the table below including: start and end positions of the peptide sequence

633 starting from the amino acid terminal of the whole protein, the observed *m/z*, transformed

634 to its experimental mass (Mr(expt)), the calculated mass (Mr(calc)) from the matched

635 peptide sequence, as well as their mass difference (in ppm), the number of missed

636 cleavage sites for trypsin (Miss), and the peptide sequence. **B)** List of fragmented ions

637 obtained (in red) vs. total predicted ions for MS/MS fragmentation of peptide at

638 1242.6456 Th.which achieve extensive statistical significance (MASCOT score of 56).

Table 1. Identified proteins of by-products from canned tuna after in-gel trypsin digestion under reducing conditions and combined PMF and PFF analyses.

SDS-PAGE band[a]	Identified protein (NCBI database entry)	Theoretical mass (kDa)	Number of matched peptides / total observed peptides	% Covered sequence	Mowse score	Sequenced peptides by MS/MS (ion score)	Actinopterygii Genera (common name)
3	Ubiquitin (GI:223646272)	8.6	6 / 11	52.7	177	IQDKEGIPPDQQR (68)	*Salmo salar* (Atlantic salmon)
4	β-Hemoglobin (GI:122701)	16.2	3 / 12	16.4	132	CLIVYPWTQR (88)	*Thunnus thynnus* (Bluefin tuna)
5	Tropomyosin (GI:38175083)	32.7	16 / 23	40.0	304	IQLVEEELDRAQER (105)	*Thunnus thynnus* (Bluefin tuna)

[a] Bands corresponding to Figure 1.

Table 2. Identified proteins corresponding to the myofibrillar and sarcoplasmic fractions isolated from by-products of skeletal red muscle of fresh tuna after in-gel trypsin digestion under reducing conditions and combined PMF and PFF analyses.

Protein Fraction	SDS-PAGE band[a]	Identified protein (NCBI database entry)	Theoretical mass (kDa)	Number of matched peptides / total observed peptides	% Covered sequence	Mowse score	Sequenced peptides by MS/MS (ion score)	Actinopterygii Genera (common name)
Myofibrillar	1	Ventricular myosin heavy chain (GI:82175142)	223.3	20 / 50	12	139	ILNPAAIPEGQFIDSR (46) STHPHFVR (25)	*Danio rerio* (Zebra fish)
	2	Myosin, heavy polypeptide 1.1, skeletal muscle (GI:288856329)	222.5	13 / 34	8	78		*Danio rerio* (Zebra fish)
	3	LOC554876 protein (GI:113674097)	24.8	7 / 47	23	76		*Danio rerio* (Zebra fish)
	4	Muscle-type	43.3	12/47	30	236	GTGGVDTASVGGVFDISNADR	*Oreochromis*

		creatine kinase CKM1 (GI:21694041)					(70) GFTLPPHNSR (40) TFLVWVNEEDHLR (29)	*mossambicus* (Tilapia mossambica)
	4	skeletal α-actin (GI:6653228)	42.2	12/47	40	231	SYELPDGQVITIGNER (98) AVFPSIVGRPR (30)	*Sparus aurata* (Sea bream)
	5	Tropomyosin (GI:38175083)	32.8	14 / 51	34	270	IQLVEEELDR (56) KLVIIEGDLER (46) KYEEVAR (20) RIQLVEEELDR (20) LVIIEGDLER (18)	*Thunnus thynnus* (Bluefin tuna)
	7	Fast skeletal myosin Light chain 3 (GI:68132180)	16.2	5 / 18	26	65		*Misgurnus fossilis* (Weatherfish)
Sarcoplasmic	9	Phosphorylase, glycogen (muscle) A (GI:66472494)	97.4	15 / 51	21	126		*Danio rerio* (Zebra fish)

		10	Pyruvate kinase (GI:197632483)	58.9	9 / 47	20	65		*Salmo salar* (Atlantic salmon)
		11	Pyruvate kinase (GI:197632483)	58.9	11 / 51	19	96		*Salmo salar* (Atlantic salmon)
		12	Isocitrate dehydrogenase 2 (NADP+), mitochondrial (GI:41054651)	50.9	9 / 49	21	72		*Danio rerio* (Zebra fish)
		13	Enolase 3 (β muscle) (GI:68086449)	47.8	9 / 51	27	79		*Danio rerio* (Zebra fish)
		14	Muscle-type creatine kinase CKM2 (GI:21694043)	43.0	15 / 49	32	156		*Oreochromis mossambicus* (Tilapia

							mossambica)
	17	Glyceraldehyde 3-phosphate dehydrogenase (GI:119943230)	36.0	6 / 51	24	63		*Misgurnus anguillicaudatus* (Weather Loach)
	19	Triose phosphate isomerase B (GI:126211567)	26.9	7 / 51	27	70		*Poecilia reticulata* (Guppy)
	20	Myoglobin (GI: 9930588)	15.7	10 / 51	71	301	CWGPVEADYTTIGGLVLTR (137) AGLDAGGQTALR (76)	*Thunnus alalunga* (Albacore tuna)

[a] Bands corresponding to Figure 2.
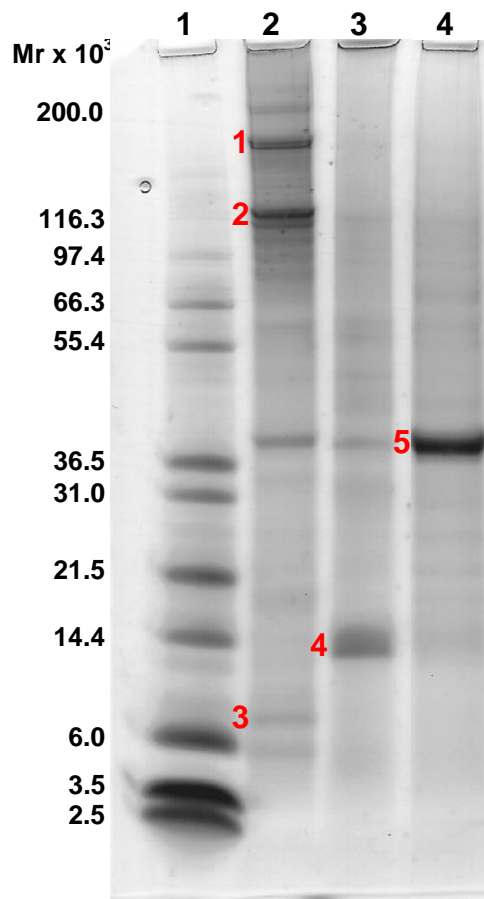
Figure 1. *Sanmartín et al.*
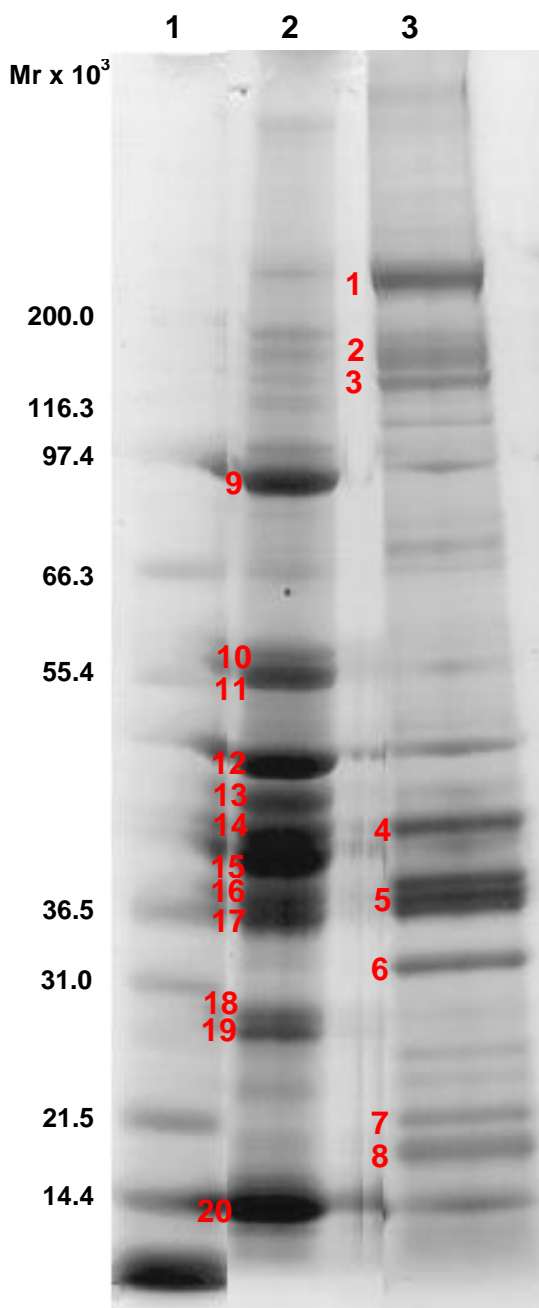
Figure 2. *Sanmartín et al.*

Figure 3. *Sanmartín et al.*

A)

```
  1 MDAIKKKMQM LKLDKENALD RAEQAESDKK ASEERSKQLE DDLVGLQKKL
 51 KGTEDELDKY SEALKDAQEK LELAEKKATD AEGEVASLNR RIQLVEEELD
101 RAQERLATAL TKLEEAEKAA DESERGMKVI ENRAMKDEEK MELQEIQLKE
151 AKHIAEEADR KYEEVARKLV IIEGDLERTE ERAELSEGKC SELEEELKTV
201 TNNLKSLEAQ AEKYSQKEDK YEEEIKVLTD KLKEAETRAE FAERSVAKLE
251 KTIDDLEDEL YAQKLKYKAI SEELDHALND MTSI
```

     Matched peptides shown in **Bold Red**

Start - End	Observed	Mr(expt)	Mr(calc)	ppm	Miss	Sequence
13 - 21	1073.5678	1072.5606	1072.5513	9	1	K.LDKENALDR.A
36 - 48	1472.7723	1471.7650	1471.7882	-16	1	R.SKQLEDDLVGLQK.K
38 - 48	1257.6832	1256.6760	1256.6612	12	0	K.QLEDDLVGLQK.K
77 - 90	1460.7561	1459.7489	1459.7267	15	1	K.KATDAEGEVASLNR.R
78 - 90	1332.6736	1331.6663	1331.6317	26	0	K.ATDAEGEVASLNR.R
91 - 101	1399.7773	1398.7700	1398.7467	17	1	R.RIQLVEEELDR.A (Ions score 20)
92 - 101	1243.6707	1242.6635	1242.6456	14	0	R.IQLVEEELDR.A (Ions score 56)
92 - 105	1727.9035	1726.8963	1726.8849	7	1	R.IQLVEEELDRAQER.L
153 - 160	940.4485	939.4413	939.4410	0	0	K.HIAEEADR.K
161 - 167	894.4632	893.4560	893.4606	-5	1	R.KYEEVAR.K (Ions score 20)
168 - 178	1284.7732	1283.7659	1283.7449	16	1	R.KLVIIEGDLER.T (Ions score 46)
169 - 178	1156.6689	1155.6616	1155.6499	10	0	K.LVIIEGDLER.T (Ions score 18)
206 - 213	875.4231	874.4159	874.4396	-27	0	K.SLEAQAEK.Y
252 - 264	1552.7724	1551.7652	1551.7304	22	0	K.TIDDLEDELYAQK.L

B)

Monoisotopic mass of neutral peptide Mr(calc): 1242.6456

MS/MS Fragmentation of IQLVEEELDR
Found in gi|38175083, tropomyosin [Thunnus thynnus]

Ions Score: <u>56</u> Individual ions scores > 51 indicate identity or extensive homology (p<0.05).

#	Immon.	b	y	Seq.
1	86.0964	114.0913		I
2	101.0709	**242.1499**	1130.5688	Q
3	86.0964	**355.2340**	*1002.5102*	L
4	72.0808	**454.3024**	*889.4262*	V
5	102.0550	**583.3450**	*790.3577*	E
6	102.0550	712.3876	*661.3151*	E
7	102.0550	841.4302	*532.2726*	E
8	86.0964	954.5142	*403.2300*	L
9	88.0393	1069.5412	*290.1459*	D
10	129.1135		*175.1190*	R

Figure 4. *Sanmartín et al.*

A)

```
  1 MADFDAVLKC WGPVEADYTT IGGLVLTRLF KEHPDTQKLF PKFAGIAQAD
 51 LAGNAAISAH GATVLKKLGE LLKAKGSHAS ILKPMANSHA TKHKIPINNF
101 KLISEVLVKV MQEKAGLDAG GQTALRNVMG IIIADLEANY KELGFTG
```

Matched peptides shown in **Bold Red**

```
Start - End     Observed     Mr(expt)    Mr(calc)       ppm    Miss Sequence
   10 - 28     2108.0934    2107.0861    2107.0408        21       0  K.CWGPVEADYTTIGGLVLTR.L  (Ions score 137)
   32 - 38      854.3234     853.3161     853.3930       -90       0  K.EHPDTQK.L
   43 - 66     2267.2976    2266.2904    2266.2070        37       0  K.FAGIAQADLAGNAAISAHGATVLK.K
   43 - 67     2395.4254    2394.4182    2394.3019        49       1  K.FAGIAQADLAGNAAISAHGATVLKK.L
   76 - 92     1749.9171    1748.9098    1748.8992         6       0  K.GSHASILKPMANSHATK.H
   76 - 92     1765.8901    1764.8829    1764.8941        -6       0  K.GSHASILKPMANSHATK.H  Oxidation (M)
   93 - 101    1110.6326    1109.6253    1109.6345        -8       1  K.HKIPINNFK.L
  115 - 126    1129.5968    1128.5895    1128.5887         1       0  K.AGLDAGGQTALR.N  (Ions score 76)
  127 - 141    1663.8744    1662.8671    1662.8651         1       0  R.NVMGIIIADLEANYK.E
  127 - 141    1679.8795    1678.8723    1678.8600         7       0  R.NVMGIIIADLEANYK.E  Oxidation (M)
```

B)

Monoisotopic mass of neutral peptide Mr(calc): 2107.0408

MS/MS Fragmentation of CWGPVEADYTTIGGLVLTR
Found in gi|118595817, Myoglobin

Ions Score: <u>137</u> **Individual ions scores > 51 indicate identity or extensive homology (p<0.05).**

#	Immon.	b	y	Seq.
1	133.0430	161.0379		**C**
2	159.0917	**347.1172**	1948.0175	**W**

3	30.0338	**404.1387**	*1761.9381*	**G**
4	70.0651	501.1915	*1704.9167*	**P**
5	72.0808	**600.2599**	1607.8639	**V**
6	102.0550	729.3025	1508.7955	**E**
7	44.0495	800.3396	*1379.7529*	**A**
8	88.0393	915.3665	*1308.7158*	**D**
9	136.0757	1078.4299	*1193.6889*	**Y**
10	74.0600	**1179.4775**	*1030.6255*	**T**
11	74.0600	1280.5252	*929.5778*	**T**
12	86.0964	1393.6093	*828.5302*	**I**
13	30.0338	1450.6307	*715.4461*	**G**
14	30.0338	1507.6522	*658.4246*	**G**
15	86.0964	1620.7363	*601.4032*	**L**
16	72.0808	1719.8047	*488.3191*	**V**
17	86.0964	1832.8888	*389.2507*	**L**
18	74.0600	1933.9364	*276.1666*	**T**
19	129.1135		*175.1190*	**R**