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Abstract: Native isoelectric focusing (IEF) of water-soluble sarcoplasmic proteins was applied to the identification of 14 shrimp species of food interest belonging to the order Decapoda. These species have different commercial values, but due to their phenotypic similarities and carapace removal in their industrial processing, incorrect food labelling and deliberate or inadvertent adulteration can occur. Each of the 14 tested species showed species-specific protein band profiles and intra-specific polymorphism was low, not preventing the correct identification of the species. Therefore, IEF of water-soluble sarcoplasmic proteins allowed the differentiation of the 14 species considered. In addition, sarcoplasmic calcium-binding proteins (SCPs) were identified by tandem mass spectrometry (MS/MS) as the major species-specific proteins in these species, opening the way to further studies focusing on their potential use as specific biomarkers.

Food Chemistry

Vigo, 27 February 2009

Dear Editor of Food Chemistry:

We are sending our manuscript titled “Identification of commercial prawn and shrimp species of food interest by native isoelectric focusing” for your consideration in order to be published in Food Chemistry. Our research group belongs to the Department of Marine Product Chemistry, of the Marine Research Institute (Vigo), and we work on seafood products authentication.

In this study, native isoelectric focusing (IEF) of water-soluble sarcoplasmic proteins was applied to the identification of 14 shrimp species of food interest belonging to the order *Decapoda*. Each of the 14 tested species showed species-specific protein band profiles. Therefore, this simple and fast technique could be usable by fisheries and related industries as a good tool for preventing mislabelling and fraudulent practices, providing a rapid and effective identification and guaranteeing market transparency. In addition, sarcoplasmic calcium-binding proteins (SCPs) were identified by tandem mass spectrometry (MS/MS) as the major species-specific proteins in these species, opening the way to further studies focusing on their potential use as specific biomarkers.

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Sincerely yours,

Ignacio Ortea

Identification of commercial prawn and shrimp species of food interest by native isoelectric focusing

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Running title: Shrimp species identification by IEF.

Abstract

Native isoelectric focusing (IEF) of water-soluble sarcoplasmic proteins was applied to the identification of 14 shrimp species of food interest belonging to the order *Decapoda*. These species have different commercial values, but due to their phenotypic similarities and carapace removal in their industrial processing, incorrect food labelling and deliberate or inadvertent adulteration can occur. Each of the 14 tested species showed species-specific protein band profiles and intra-specific polymorphism was low, not preventing the correct identification of the species. Therefore, IEF of water-soluble sarcoplasmic proteins allowed the differentiation of the 14 species considered. In addition, sarcoplasmic calcium-binding proteins (SCPs) were identified by tandem mass spectrometry (MS/MS) as the major species-specific proteins in these species, opening the way to further studies focusing on their potential use as specific biomarkers.

Keywords: IEF; shrimps; species identification; SCP; MS/MS

1. Introduction

Seafood products include a wide variety of species with a significant importance for food industry. Among them, crustaceans belonging to the order *Decapoda*, comprising prawns, shrimps, lobsters, crayfish, crabs and hermit crabs, are of remarkable commercial interest. This order includes the superfamily *Penaeoidea*, the penaeid shrimps, which are the most important economic resource in the world's crustacean fishery and aquafarming industry (Dall, Hill, Rothlisberg & Staples, 1990; Holthius, 1980; Pérez-Farfante & Kensley, 1997).

Food authentication is a major concern not only for the prevention of commercial fraud, but also for the assessment of safety risks derived from the inadvertent introduction of any food ingredient that might be harmful to human health (Brzezinski, 2005; Civera, 2003; Lockley & Bardsley, 2000). Thus, identification of marine species is an issue of capital relevance for the seafood industry, because of commercial requirements concerning labelling and traceability. In response to consumer concern, global regulations have been implemented to assure complete and correct information, guaranteeing market transparency and avoiding substitution of certain fish species by others of less commercial value (Council Regulation, 2000; Royal Decree, 2004). Anatomical characters are particularly difficult to use for penaeid shrimps species differentiation due to their phenotypic similarities and to the fact that in their processing the external carapace is often removed. This may lead to mislabelled and/or adulterated products, so unintentional fraud may occur. Therefore, the increasing demand of crustaceans in general, and of high quality penaeid shrimps in particular, may be compromised by deliberate or inadvertent adulteration along the food chain by the substitution of higher quality species by others of lower commercial quality. Therefore, it is highly recommendable the development of the necessary analytical tools to make possible distinguishing among closely related species.

Methods based on protein analysis, including sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing electrophoresis (IEF) (Berrini, Tepedino, Borromeo & Secchi, 2006; Chen, Shiau, Noguchi, Wei & Hwang, 2003; Etienne et al., 2001; Mackie et al., 2000; Piñeiro et al., 1999a) and two-dimensional gel electrophoresis (2-DE) (Piñeiro, Barros-Velázquez, Sotelo & Gallardo, 1999b) have been extensively used for fish species identification. Immunoassay (Asensio, González, García & Martín, 2008) and, more recently, mass spectrometry

(MS) methodologies (Carrera, Cañas, Piñeiro, Vázquez & Gallardo, 2006; Mazzeo et al., 2008) have also been used for species identification.

Molecular biological methods as mitochondrial DNA (mtDNA) analysis has been used in polymerase chain reaction (PCR) based studies for fish species identification (Rasmussen & Morrissey, 2008). Among the mtDNA targets, the 16S rRNA gene and the cytochrome oxidase I gene have been reported to serve as good interspecific markers in some crustacean species, although most of these studies were focused not on species identification, but on population structures, phylogeography and phylogenetic relationships (Baldwin, Bass, Bowen & Clark, 1998; Lavery, Chan, Tam & Chu, 2004; Maggioni, Rogers, Maclean & Incao, 2001; Quan, Zhuang, Deng, Dai & Zhang, 2004; Voloch, Freire & Russo, 2005). More recently, two PCR-restriction fragment length polymorphism (RFLP) methods for the detection of crustaceans (Brzezinski, 2005) and penaeid shrimps (Pascoal, Barros-Velázquez, Cepeda, Gallardo & Calo-Mata, 2008) have been proposed.

Although the molecular methods or the proteomic technologies will be the future in the seafood control laboratories, the classic electrophoretic methods such as IEF, which separates proteins based on their differences in isoelectric point (pI), has revealed as a cheaper, faster, easy to apply by the control laboratories and less sophisticated technology than the DNA-based techniques for species identification purposes (Piñeiro et al., 1999a). In this context, little effort has been made to elucidate differences among closely related shrimp species using these protein-based methodologies. Related previous reports described a SDS-PAGE study which was not able to discriminate among close related species (Civera & Parisi, 1991), and two urea IEF studies including only 3 shrimp species (An, Marshall, Otwell & Wei, 1989; Wei, Chen & Marshall, 1990).

In this study, we used native IEF for the unequivocal identification of 14 shrimp species of commercial interest for the food industry worldwide.

2. Materials and methods

2.1. Fish material

14 different prawn and shrimp species of commercial interest were considered (Table 1), 13 species belonging to the superfamily *Penaeoidea* and one species –the northern shrimp *Pandalus borealis*- to the superfamily *Pandaloidea*. Specimens were collected using extractive fishing practices or from aquaculture facilities in different continents worldwide. Shrimps were frozen on board and shipped to our laboratory for the analyses. Six individuals from each species were analyzed. When required, specimens were classified in their respective taxons according to their anatomical external features with the help of a marine biologist from the Marine Sciences Institute (Mediterranean Centre for Marine and Environmental Research, Higher Council for Scientific Research, CMIMA-CSIC, Barcelona, Spain) with expertise in penaeid shrimp taxonomy.

2.2. Extraction of sarcoplasmic proteins

Extraction of the sarcoplasmic proteins was performed by homogenizing 1 g of raw white muscle from each individual in two volumes of milliQ water, using an Ultra-Turrax blender for 3x15 s—with interruptions of 45 s to avoid warming the samples. The extracts were then centrifuged at 30 000g for 15 min at 4°C (J25 centrifuge; Beckman, Palo Alto, USA), and the supernatants were maintained at -80°C until the electrophoretic analysis. Protein concentration in the extracts was determined by the bicinchoninic acid method (Sigma-Aldrich, St. Louis, USA).

2.3. IEF

Native IEF was conducted by using Ampholine PAGplate precast polyacrylamide gels in two different pH ranges (pH 4.0-6.5, pH 4.0-5.0; GE Healthcare, Uppsala, Sweden), and was performed at 10°C in a Multiphor II electrophoresis unit (Amersham Biosciences, Sweden) with a thermostatic circulator. Most of the specific bands described for other fish species are in the pH range 4.0-7.0 (Piñeiro et al., 1999b), so narrow acidic pH ranges were chosen. IEF electrode strips were soaked in 0.1 M glutamic acid in 0.5 M H₃PO₄ for anode and 0.1 M β-alanine for cathode (4.0-6.5 pH range); and 1 M H₃PO₄ for anode and 1 M glycine for cathode (4.0-5.0 pH range). 40 µg of total proteins were loaded near the cathode using sample application pieces (GE Healthcare). Two lanes of protein standards in the 3.5-10.7 pH range (IEF Markers 3-10, Invitrogen, Carlsbad, USA) were included in each gel. The running conditions were 1500 V – 50 mA – 30 W, until 4000 V·h was reached, with 30 min prefocusing. 60 minutes after the initiation of electrophoresis, the application pieces were removed to avoid any smearing of proteins on the gel. Following electrophoresis, proteins were fixed for 30 minutes and gels were stained with Coomassie Brilliant Blue (GE Healthcare) equilibrated in 23% ethanol and 8% acetic acid, and dried in air between sheets of cellophane.

2.4. Analysis of patterns

IEF gels were scanned and the acquired images were analyzed using the LabImage 1D software (version 4.1, Kapelan GmbH, Leipzig, Germany). Intra-gel distortions between lines were compensated by aligning the band position of the two lines of standards loaded at different positions on the gel. Different gels were normalized for differences in running conditions by comparing the pattern of pI standard proteins separated in the gel to be analyzed with the pI standard pattern chosen as a database standard.

2.5. Protein identification of characteristic bands

Major characteristic bands were separated in a closer pH range (pH 4.0-5.0) for identification by means of MS analysis. They were excised from gel and subjected to in-gel digestion with trypsin (Roche Diagnostics GmbH, Mannheim, Germany), performed overnight at 37 °C as described elsewhere (Jensen, Wilm, Shevchenko & Mann, 1999). Final digestion solution was dried under vacuum and resuspended in 5 % acetic acid. The produced peptides were subjected to tandem mass spectrometry (MS/MS) analysis, using an ion trap mass spectrometer model LCQ Deca XP Plus (Thermo-Finnigan, San Jose, USA). Peptides were fragmented, obtaining MS/MS spectra; these spectra were then used for database searching against the nr.fasta database (NCBI Resources, NiH, Bethesda, USA) using SEQUEST (Bioworks 3.1 package, Thermo-Finnigan).

3. Results and discussion

3.1. Native IEF

Native IEF profiles of sarcoplasmic proteins, extracted from the muscle of 14 shrimp species, were obtained and the patterns analyzed for species identification. Most of the specific bands described for fish species are in the pH range 4.0-7.0 (Piñeiro et al., 1999b), so a narrow acidic pH range (4.0-6.5) was chosen for the analysis. Six specimens of each of the species were analyzed, in either the same or different gels. Figure 1 represents the fourteen different protein patterns from the shrimp species. Each protein fingerprint is composed of a series of protein bands, which are characteristic for each species, allowing an easy discrimination. IEF patterns exhibited many species-specific bands over a pI range of 4.0-6.5, with most of the bands in the 4.2-5.0 range. Band patterns were scanned, the characteristic bands identified and their pI calculated. Table 2 reports a schematic diagram of the patterns showing the average pI ($\pm$ RSD)

values of characteristic bands determined using the LabImage 1D software according to the procedure described in the section 2.4. Firstly, the pI/s were determined for each gel, and then the average pI values and RSD for each band were calculated. The RSD values varied between 0.0 and 2.3, indicating the good precision for this method. The band patterns were consistent for the six specimens analyzed for each of the species under study. Low intra-specific polymorphism was found, only in 3 of the species: a sample of *F. notialis* showed a significant band at pI 5.22 which was not visible in the other five; two samples of *P. semisulcatus* showed a substitution of a band at pI 4.74 by a band at pI 5.07; and two samples of *P. monodon* showed a triplet where other four samples had a unique band (pI 4.95). However, the presence of these changes did not prevent correct identification of the species. It might be necessary to test larger number of individuals from each species to detect intra-specific differences. However, in the analytical conditions used in this work, the inter-specific band variation is much greater than the intra-specific variation and good enough for correct species identification.

3.2. Protein identification of major characteristic bands

Major characteristic bands were in-gel digested with trypsin and subjected to MS/MS analysis. To achieve an unequivocal identification of the proteins presented in the specific bands, an optimal separation of the possible marker proteins was needed, so preparative native IEF was performed using an even closer pH range (pH 4.0-5.0). The following bands were selected for mass spectrometry characterization (Figure 2): band 1, pI 4.96; band 2, pI 4.76; band 3, pI 4.93; band 4, pI 4.53; band 5, pI 4.49; and band 6, pI 4.86. Several peptides (between 4 and 10) were produced after tryptic digestion of each of these bands, and all of them matched with some of the five *Decapoda* Sarcoplasmic Calcium-binding Proteins (SCPs) described in databases (Table 3): SCP α chain from *Penaeus sp.* (accession P02636), SCP β chain from *Penaeus sp.* (P02635),

SCP from *Procambarus clarkii* (ABB58783), SCP I from *Pontastacus leptodactylus* (P05946), and SCP α B from *Fenneropenaeus orientalis* (1006232A). In Figure 3, several fragmentation spectra as examples of the SCP peptides found are shown, together with the mass-to-charge ratio (m/z) of the precursor ions. Therefore, all the bands studied were identified by MS/MS as isoforms of the SCP, a soluble protein found in invertebrate muscle, and constituent, like parvalbumins, of the EF-hand-type family of the calcium-binding proteins. These proteins, previously described as species-specific proteins (Piñeiro et al., 1999b; Rehbein, 1995), belong to a group of acidic and heat-stable proteins, which accounts for the majority of the water extractable proteins from fish sarcoplasm. Recently, SCPs have also been established as crustacean allergens (Shiomi, Sato, Hamamoto, Mita & Shimakura, 2008), and interspecific variability in their amino acid sequences has been described as the cause of differences in IgE reactivity to SCP in sensitized individuals.

4. Conclusions

To the best of our knowledge, the results reported here are representative of the most complete electrophoretic method described to date for the identification of penaeid shrimp species of commercial interest. The specific patterns allowed the unambiguous differentiation of all the 14 tested species. Therefore, this simple and fast technique could be usable by fisheries and related industries as a good tool for preventing mislabelling and fraudulent practices, providing a rapid and effective identification and guaranteeing market transparency. SCPs exhibited extensive polymorphism between species, a very useful property for electrophoretic species identification, as described previously (Rehbein, 1995). Inter-specific variability in the pI of SCPs isomers suggest

the potential presence of aminoacid substitutions in their sequences, opening the way to further studies focusing on their potential use as specific biomarkers.

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Abbreviations used

2-DE, two-dimensional gel electrophoresis; IEF, isoelectric focusing; MS, mass spectrometry; MS/MS, tandem mass spectrometry; mtDNA, mitochondrial DNA; m/z , mass-to-charge ratio; PCR, polymerase chain reaction; pI , isoelectric point; RFLP, restriction fragment length polymorphism; RSD, relative standard deviation; SCP, sarcoplasmic calcium-binding protein; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

References

An, H., Marshall, M. R., Otwell, W. S., & Wei, C. I. (1989). Species identification of raw and boiled shrimp by a urea gel isoelectric focusing technique. *Journal of Food Science*, 54(2), 233-236.

241 Asensio, L., González, I., García, T., & Martín, R. (2008). Determination of food
 242 authenticity by enzyme-linked immunosorbent assay (ELISA). *Food Control*, 19(1),
 243 1-8.

244 Baldwin, J. D., Bass, A. L., Bowen, B. W., & Clark, W. H. (1998). Molecular
 245 phylogeny and biogeography of the marine shrimp *Penaeus*. *Molecular*
 246 *Phylogenetics and Evolution*, 10, 399-407.

247 Berrini, A., Tepedino, V., Borromeo, V., & Secchi, C. (2006). Identification of
 248 freshwater fish commercially labelled "perch" by isoelectric focusing and two-
 249 dimensional electrophoresis. *Food chemistry*, 96(1), 163-168.

250 Brzezinski, J. L. (2005). Detection of crustacean DNA and species identification using a
 251 PCR-restriction fragment length polymorphism method. *Journal of Food*
 252 *Protection*, 68, 1866-1873.

253 Carrera, M., Cañas, B., Piñeiro, C., Vázquez, J., & Gallardo, J. M. (2006). Identification
 254 of commercial hake and grenadier species by proteomic analysis of the parvalbumin
 255 fraction. *Proteomics*, 6(19), 5278-5287.

256 Civera, T. (2003). Species identification and safety of fish products. *Veterinary*
 257 *Research Communications*, 27, 481-489.

258 Civera, T., & Parisi, E. (1991). The use of sodium dodecyl sulfate polyacrylamide gel
 259 electrophoresis for the identification of raw and cooked shrimps and crabs. *Italian*
 260 *Journal of Food Science*, 3(2), 9.

261 Council Regulation (2000). Council Regulation (EC) No. 104/2000 of 17 December
 262 1999 on the common organization of the markets in fishery and aquaculture
 263 products. *Official Journal of the European Communities* L 17/22.

264 Chen, T.-Y., Shiau, C.-Y., Noguchi, T., Wei, C.-i., & Hwang, D.-F. (2003).
 265 Identification of puffer fish species by native isoelectric focusing technique. *Food*
 266 *chemistry*, 83(3), 475-479.

267 Dall, W., Hill, B. J., Rothlisberg, P. C., & Staples, D. J. (1990). The biology of
 268 *Penaeidae*. *Advances in Marine Biology*, 27, 1-484.

269 Etienne, M., Jérôme, M., Fleurence, J., Rehbein, H., Kündiger, R., Mendes, R., Costa,
 270 H., & Martínez, I. (2001). Species identification of formed fishery products and high
 271 pressure-treated fish by electrophoresis: a collaborative study. *Food chemistry*,
 272 72(1), 105-112.

273 Holthius, L. B. (1980). FAO species catalogue. Volume 1. Shrimps and prawns of the
 274 world. An annotated catalogue of species of interest to fisheries. *FAO Fisheries*
 275 *Synopsis*, 125(1), 1-271.

276 Jensen, O. N., Wilm, M., Shevchenko, A., & Mann, M. (1999). Sample preparation
 277 methods for mass spectrometric peptide mapping directly from 2-DE gels. In: A. J.
 278 Link, *2-D proteome analysis protocols* (pp. 513-530). Totowa, NJ: Humana Press.

279 Lavery, S., Chan, T. Y., Tam, Y. K., & Chu, K. H. (2004). Phylogenetic relationships
 280 and evolutionary history of the shrimp genus *Penaeus s.l.* derived from
 281 mitochondrial DNA. *Molecular Phylogenetics and Evolution*, 31(1), 39-49.

282 Lockley, A. K., & Bardsley, R. G. (2000). DNA-based methods for food authentication.
 283 *Trends in Food Science & Technology*, 11(2), 67-77.

284 Mackie, I., Craig, A., Etienne, M., Jérôme, M., Fleurence, J., Jessen, F., Smelt, A.,
 285 Kruijt, A., Malmheden Yman, I., Ferm, M., Martinez, I., Pérez-Martín, R., Piñeiro,
 286 C., Rehbein, H., & Kündiger, R. (2000). Species identification of smoked and
 287 gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis,

288 urea isoelectric focusing and native isoelectric focusing: a collaborative study. *Food*
 289 *chemistry*, 71(1), 1-7.

290 Maggioni, R., Rogers, A. D., Maclean, N., & Incao, F. (2001). Molecular phylogeny of
 291 western Atlantic *Farfantepenaeus* and *Litopenaeus* shrimp based on mitochondrial
 292 16S partial sequences. *Molecular Phylogenetics and Evolution*, 18, 66-73.

293 Mazzeo, M. F., Giulio, B. D., Guerriero, G., Ciarcia, G., Malorni, A., Russo, G. L., &
 294 Siciliano, R. A. (2008). Fish authentication by MALDI-TOF mass spectrometry.
 295 *Journal of Agricultural and Food Chemistry*, 56(23), 11071-11076.

296 Pascoal, A., Barros-Velázquez, J., Cepeda, A., Gallardo, J. M., & Calo-Mata, P. (2008).
 297 A polymerase chain reaction-restriction fragment length polymorphism method
 298 based on the analysis of a 16S rRNA/tRNA^{Val} mitochondrial region for species
 299 identification of commercial penaeid shrimps (*Crustacea: Decapoda: Penaeoidea*)
 300 of food interest. *Electrophoresis*, 29(2), 499-509.

301 Pérez-Farfante, I., & Kensley, B. (1997). *Penaeoid and sergestoid shrimps and prawns*
 302 *of the world. Keys and diagnoses for the families and genera*. Paris: Éditions du
 303 Muséum National d'Histoire Naturelle.

304 Piñeiro, C., Barros-Velázquez, J., Pérez-Martín, R. I., Martínez, I., Jacobsen, T.,
 305 Rehbein, H., Kündiger, R., Mendes, R., Etienne, M., Jerome, M., Craig, A., Mackie,
 306 I. M., & Jessen, F. (1999a). Development of a sodium dodecyl sulfate-
 307 polyacrylamide gel electrophoresis reference method for the analysis and
 308 identification of fish species in raw and heat-processed samples: A collaborative
 309 study. *Electrophoresis*, 20(7), 1425-1432.

310 Piñeiro, C., Barros-Velázquez, J., Sotelo, C. G., & Gallardo, J. M. (1999b). The use of
 311 two-dimensional electrophoresis in the characterization of the water-soluble protein

fraction of commercial flat fish species. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, 208(5), 342-348.

Quan, J., Zhuang, Z., Deng, J., Dai, J., & Zhang, Y.-p. (2004). Phylogenetic relationships of 12 *Penaeoidea* shrimp species deduced from mitochondrial DNA sequences. *Biochemical Genetics*, 42(9), 331-345.

Rasmussen, R. S., & Morrissey, M. T. (2008). DNA-based methods for the identification of commercial fish and seafood species. *Comprehensive Reviews in Food Science and Food Safety*, 7(3), 280-295.

Rehbein, H. (1995). Food control by applied biochemistry of marine organisms: Comparison of proteins and metabolites from fish and invertebrate muscle. *Helgoland Marine Research*, 49(1), 747-757.

Royal Decree (2004). Royal Decree 121/2004 of 23 January 2004 related to the identification of live, fresh or refrigerated fish and fishery products. Ministry of Agriculture, Fisheries and Food, Spain. BOE number 31, of 5 February 2004.

Shiomi, K., Sato, Y., Hamamoto, S., Mita, H., & Shimakura, K. (2008). Sarcoplasmic calcium-binding protein: identification as a new allergen of the black tiger shrimp *Penaeus monodon*. *International Archives of Allergy and Immunology*, 146(2), 91-98.

Voloch, C. M., Freire, P. R., & Russo, C. A. M. (2005). Molecular phylogeny of penaeid shrimps inferred from two mitochondrial markers. *Genetics and Molecular Research*, 4(4), 668-674.

Wei, C.-I., Chen, H. A. J.-S., & Marshall, M. R. (1990). Use of a modified urea gel isoelectric focusing method for species identification of raw or boiled white, pink, and rock shrimp. *Journal of Food Biochemistry*, 14(2), 91-102.

Figure captions

Fig. 1. Native IEF patterns (pH range 4.0-6.5) of water-soluble sarcoplasmic proteins representative of the 14 shrimp species tested.

Fig. 2. IEF in polyacrylamide gel (pH range 4.0-5.0) of water-soluble sarcoplasmic proteins of four shrimp species, showing bands selected for MS/MS analysis.

Fig. 3. Ion-trap MS/MS spectrum of the SCPs peptides a) DGEVTVDEFK, present in all bands analyzed; b) VFIANQFK, present in bands 1, 2, 3, 4 and 6; and c) YM*YDIDNDGFLDK, present in bands 1, 2, 4, 5 and 6. M*, methionine sulfoxide.

Tables

Table 1. Shrimp and prawn species considered in this study

Scientific name ^a	Commercial name	Origin	Code ^b
<i>Penaeus monodon</i>	Giant tiger prawn	Indo West Pacific	MPN
<i>Farfantepenaeus notialis</i>	Southern pink shrimp	Eastern Atlantic	SOP
<i>Fenneropenaeus merguensis</i>	Banana prawn	Western Central Pacific	PBA
<i>Fenneropenaeus indicus</i>	Indian white prawn	Western Indian	PNI
<i>Litopenaeus vannamei</i>	Pacific white shrimp	Eastern Pacific	PNV
<i>Pleoticus muelleri</i>	Argentine red shrimp	South West Atlantic	LAA
<i>Pandalus borealis</i>	Northern shrimp	Northern Atlantic	PRA
<i>Solenocera agassizii</i>	Kolibri shrimp	Eastern Pacific	SOK
<i>Parapenaeus longirostris</i>	Deepwater rose shrimp	Eastern Atlantic	DPS
<i>Marsupenaeus japonicus</i>	Kuruma prawn	Indo West Pacific	KUP
<i>Farfantepenaeus aztecus</i>	Brown shrimp	Western Atlantic	ABS
<i>Penaeus semisulcatus</i>	Green tiger prawn	Indo West Pacific	TIP
<i>Melicertus latisulcatus</i>	Western king prawn	Indo West Pacific	WKP
<i>Farfantepenaeus brevisrostris</i>	Crystal shrimp	Eastern Pacific	CSP

^aThe taxonomic classification proposed by Pérez-Farfante et al. (1997) was adopted.

^bThe three initial letters correspond to the FAO codes.

369 Table 2. Species-specific band patterns of water-soluble shrimp proteins. Mean pI
370 $\pm$ RSD (%) were calculated from the patterns of the six shrimps of each species
371 analyzed. The RSD values varied between 0.0 and 2.3.

MPN	SOP	PBA	PNI	PNV	LAA	PRA	SOK	DPS	KUP	ABS	TIP	WKP	CSP
											6.28		
											6.22		
6.13				6.10									6.11
6.05				6.04									
												5.88	
		5.83	5.79										
	5.65									5.68	5.65		
	5.60			5.60						5.63			
				5.56						5.57			5.57
	5.52			5.52									5.50
				5.34									
5.21		5.17										5.19	5.23
											5.14		
5.10			5.10								5.09		
		5.07									5.07	5.08	
5.04			5.04	5.03									
	5.00	5.00							5.00		4.99	5.02	
4.95	4.95	4.93	4.96	4.93						4.97	4.94	4.94	
			4.91						4.91				4.93
4.86	4.87	4.88	4.86	4.87		4.89		4.86	4.86	4.88	4.89	4.86	4.88
		4.84		4.82		4.84					4.82		4.85
	4.80								4.81	4.80			4.79
4.75	4.74	4.74	4.76	4.74		4.74			4.74		4.74	4.77	4.75
	4.72		4.73	4.72					4.72				
									4.70				
			4.64					4.64		4.66			
4.61						4.62						4.61	4.62
	4.60		4.59			4.60	4.60	4.60	4.59	4.60	4.59		
		4.57		4.57		4.58	4.57	4.58	4.56				
4.54										4.55			
			4.52	4.51	4.53		4.50	4.51			4.51	4.53	4.53

	4.48	4.49	4.49	4.48	
				4.42	4.44
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389 Table 3. Sequences found that matched with *Decapoda* Sarcoplasmic Calcium-binding
390 Protein peptides.

sequence	100% matched with protein ^a	Found in band no.
NLWNEIAELADFNK	P02636, P02635, ABB58783, P05946, 1006232A	1, 2, 6
DGEVTVDEFK	P02636, P02635, 1006232A	1, 2, 3, 4, 5, 6
VFIANQFK	P02636, P05946, 1006232A	1, 2, 3, 4, 6
NDFECLAVR	P02636, P02635, 1006232A	1, 2
VGLDEYR	P02636, 1006232A	1, 2, 3, 6
EIDDAYNK	P02636, 1006232A	1, 2
AGGLTLER	P02636, 1006232A	2, 3
GEFSAADYANNQK	P02636, 1006232A	3, 6
YMYDIDNDGFLDK	P02635	1, 2, 4, 6
YM*YDIDNDGFLDK	P02635	1, 2, 4, 5, 6
SAFADVK	P02635	1, 2, 6
AGGINLAR	P02635	5
VTLIEGR	P02635	5
YM*YDIDNNGFLDK	ABB58783, P05946	3, 4, 6

391 ^aP02636, SCP alpha chain; P02635, SCP beta chain; ABB58783, SCP (*Procambarus*
392 *clarkii*); P05946, SCP I; 1006232A, SCP αB.

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Fig. 1. Ortea *et al.*

Fig. 2. Ortea *et al.*

Fig. 3. Ortea *et al.*