Elsevier Editorial System(tm) for Food Chemistry

Manuscript Draft

Manuscript Number: FOODCHEM-D-09-00690

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

Article Type: Research Article

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

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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

68 (MS) methodologies (Carrera, Cañas, Piñeiro, Vázquez & Gallardo, 2006; Mazzeo et
69 al., 2008) have also been used for species identification.
70 Molecular biological methods as mitochondrial DNA (mtDNA) analysis has been
71 used in polymerase chain reaction (PCR) based studies for fish species identification
72 (Rasmussen & Morrissey, 2008). Among the mtDNA targets, the 16S rRNA gene and
73 the cytochrome oxidase I gene have been reported to serve as good interspecific
74 markers in some crustacean species, although most of these studies were focused not on
75 species identification, but on population structures, phylogeography and phylogenetic
76 relationships (Baldwin, Bass, Bowen & Clark, 1998; Lavery, Chan, Tam & Chu, 2004;
77 Maggioni, Rogers, Maclean & Incao, 2001; Quan, Zhuang, Deng, Dai & Zhang, 2004;
78 Voloch, Freire & Russo, 2005). More recently, two PCR-restriction fragment length
79 polymorphism (RFLP) methods for the detection of crustaceans (Brzezinski, 2005) and
80 penaeid shrimps (Pascoal, Barros-Velázquez, Cepeda, Gallardo & Calo-Mata, 2008)
81 have been proposed.
82 Although the molecular methods or the proteomic technologies will be the future in
83 the seafood control laboratories, the classic electrophoretic methods such as IEF, which
84 separates proteins based on their differences in isoelectric point (p*I*), has revealed as a
85 cheaper, faster, easy to apply by the control laboratories and less sophisticated
86 technology than the DNA-based techniques for species identification purposes (Piñeiro
87 et al., 1999a). In this context, little effort has been made to elucidate differences among
88 closely related shrimp species using these protein-based methodologies. Related
89 previous reports described a SDS-PAGE study which was not able to discriminate
90 among close related species (Civera & Parisi, 1991), and two urea IEF studies including
91 only 3 shrimp species (An, Marshall, Otwell & Wei, 1989; Wei, Chen & Marshall,
92 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 4ºC (J25 centrifuge; Beckman, Palo Alto, USA), and the supernatants were maintained at -80ºC 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 10ºC 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 H_3PO_4 for anode and 0.1 M β–alanine for cathode (4.0-6.5 pH range); and 1 M H_3PO_4 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 p*I* standard proteins separated in the gel to be analyzed with the p*I* standard pattern chosen as a database standard.

142 *2.5. Protein identification of characteristic bands*

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

153

154 **3. Results and discussion**

155 *3.1. Native IEF*

156 Native IEF profiles of sarcoplasmic proteins, extracted from the muscle of 14 shrimp
157 species, were obtained and the patterns analyzed for species identification. Most of the
158 specific bands described for fish species are in the pH range 4.0-7.0 (Piñeiro et al.,
159 1999b), so a narrow acidic pH range (4.0-6.5) was chosen for the analysis. Six
160 specimens of each of the species were analyzed, in either the same or different gels.
161 Figure 1 represents the fourteen different protein patterns from the shrimp species. Each
162 protein fingerprint is composed of a series of protein bands, which are characteristic for
163 each species, allowing an easy discrimination. IEF patterns exhibited many species-
164 specific bands over a pI range of 4.0-6.5, with most of the bands in the 4.2-5.0 range.
165 Band patterns were scanned, the characteristic bands identified and their p*I* calculated.
166 Table 2 reports a schematic diagram of the patterns showing the average p*I* (±RSD)

values of characteristic bands determined using the LabImage 1D software according to the procedure described in the section 2.4. Firstly, the p*I*s were determined for each gel, and then the average p*I* 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 p*I* 5.22 which was not visible in the other five; two samples of *P. semisulcatus* showed a substitution of a band at p*I* 4.74 by a band at p*I* 5.07; and two samples of *P. monodon* showed a triplet where other four samples had a unique band (p*I* 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, p*I* 4.96; band 2, p*I* 4.76; band 3, p*I* 4.93; band 4, p*I* 4.53; band 5, p*I* 4.49; and band 6, p*I* 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),

192 SCP from *Procambarus clarkii* (ABB58783), SCP I from *Pontastacus leptodactylus*
193 (P05946), and SCP αB from *Fenneropenaeus orientalis* (1006232A). In Figure 3,
194 several fragmentation spectra as examples of the SCP peptides found are shown,
195 together with the mass-to-charge ratio (*m/z*) of the precursor ions. Therefore, all the
196 bands studied were identified by MS/MS as isoforms of the SCP, a soluble protein
197 found in invertebrate muscle, and constituent, like parvalbumins, of the EF-hand-type
198 family of the calcium-binding proteins. These proteins, previously described as species-
199 specific proteins (Piñeiro et al., 1999b; Rehbein, 1995), belong to a group of acidic and
200 heat-stable proteins, which accounts for the majority of the water extractable proteins
201 from fish sarcoplasma. Recently, SCPs have also been established as crustacean
202 allergens (Shiomi, Sato, Hamamoto, Mita & Shimakura, 2008), and interspecific
203 variability in their amino acid sequences has been described as the cause of differences
204 in IgE reactivity to SCP in sensitized individuals.
205
206 **4. Conclusions**
207 To the best of our knowledge, the results reported here are representative of the most
208 complete electrophoretic method described to date for the identification of penaeid
209 shrimp species of commercial interest. The specific patterns allowed the unambiguous
210 differentiation of all the 14 tested species. Therefore, this simple and fast technique
211 could be usable by fisheries and related industries as a good tool for preventing
212 mislabelling and fraudulent practices, providing a rapid and effective identification and
213 guaranteeing market transparency. SCPs exhibited extensive polymorphism between
214 species, a very useful property for electrophoretic species identification, as described
215 previously (Rehbein, 1995). Inter-specific variability in the p*I* 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.

Acknowledgements

We thank Ms Lorena Barros for her excellent technical assistance. We also acknowledge members from CETMAR for their helpful collaboration in the collection of specimens for this study. This work was supported by the National Food Program of the INIA (Spanish Ministry for Education and Science) (Project CAL-03-030-C2-2) and by the PGIDIT Research Program in Marine Resources (Project PGIDIT04RMA261004PR) of the Xunta de Galicia (Galician Council for Industry, Commerce and Innovation). I.O. is supported by the FPU program (AP-2004-5826) and B.C. is supported by the RyC program, under the auspices of the Spanish MICINN.

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; p*I*, isoelectric point; RFLP, restriction fragment length polymorphism; RSD, relative standard deviation; SCP, sarcoplasmic calcium-binding protein; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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335 and rock shrimp. *Journal of Food Biochemistry*, *14*(2), 91-102.
336

337

338 **Figure captions**

339

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

342

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

345

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

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361 **Tables**

362

363 Table 1. Shrimp and prawn species considered in this study

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

364 [a]The taxonomic classification proposed by Pérez-Farfante et al. (1997) was adopted.

365 [b]The three initial letters correspond to the FAO codes.

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Table 2. Species-specific band patterns of water-soluble shrimp proteins. Mean p*I* ±RSD (%) were calculated from the patterns of the six shrimps of each species 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
				4.22	

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 [a]P02636, SCP alpha chain; P02635, SCP beta chain; ABB58783, SCP (*Procambarus*

392 *clarkii*); P05946, SCP I; 1006232A, SCP αB.

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Figure(s)
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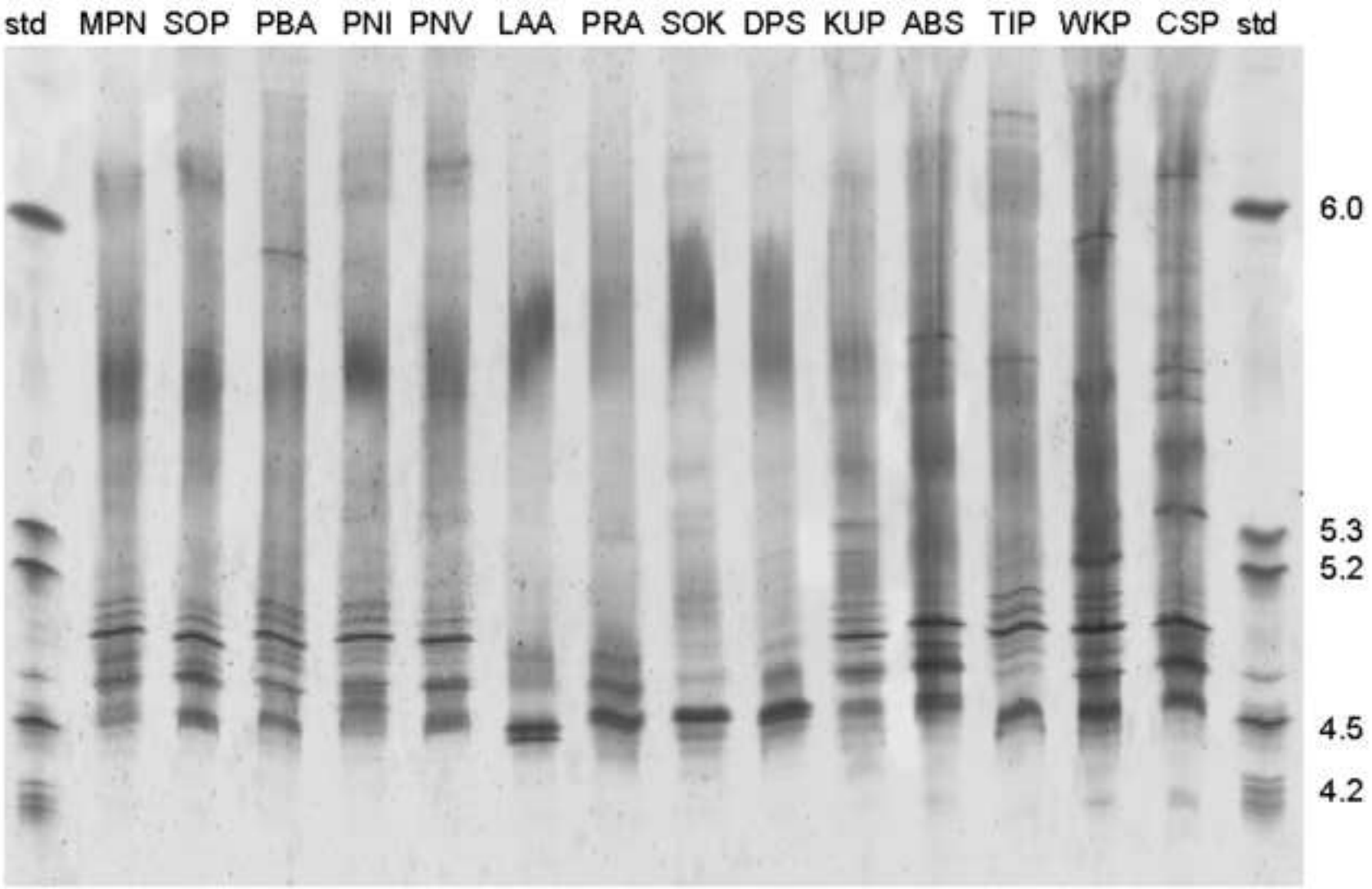

Fig. 1. Ortea *et al.*

Figure(s)

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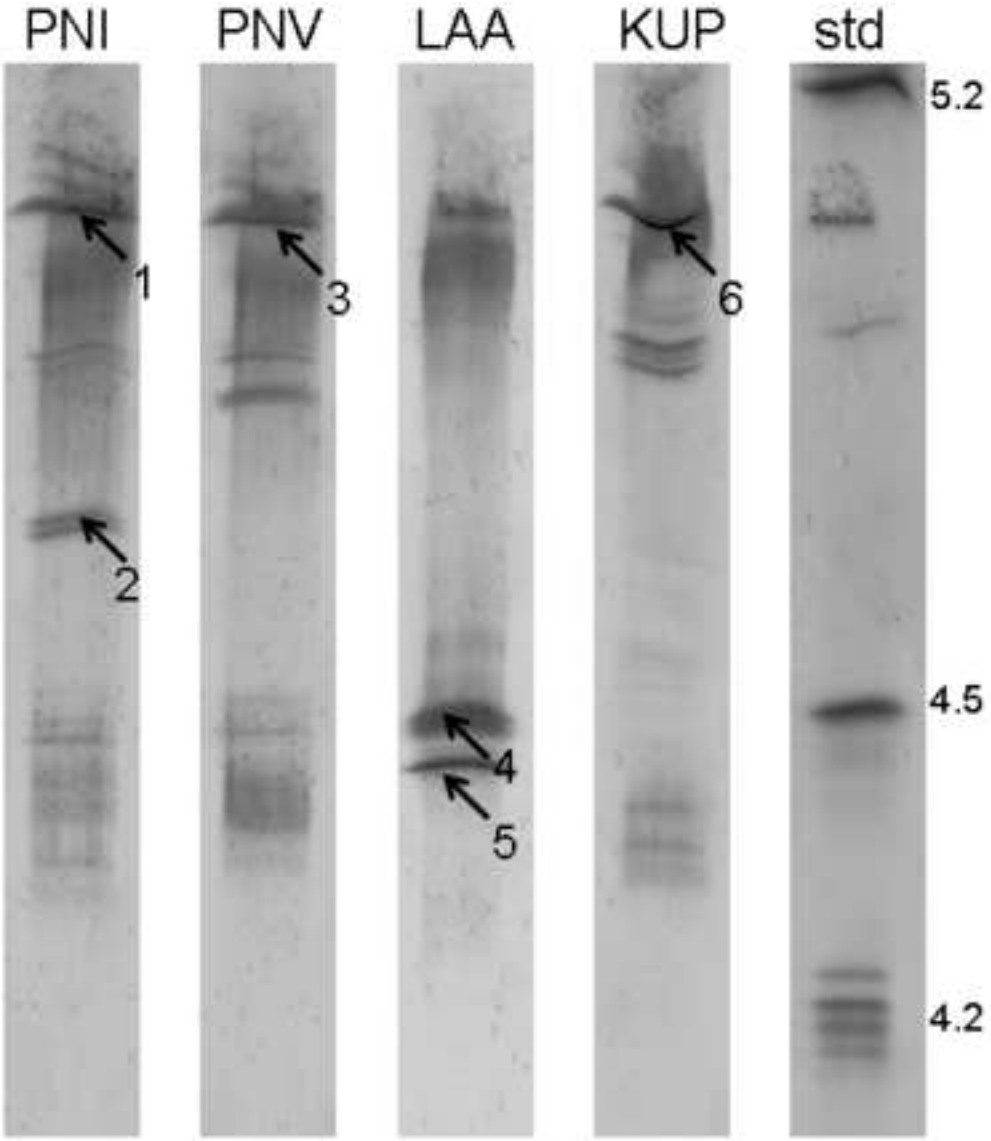

Fig. 2. Ortea *et al.*

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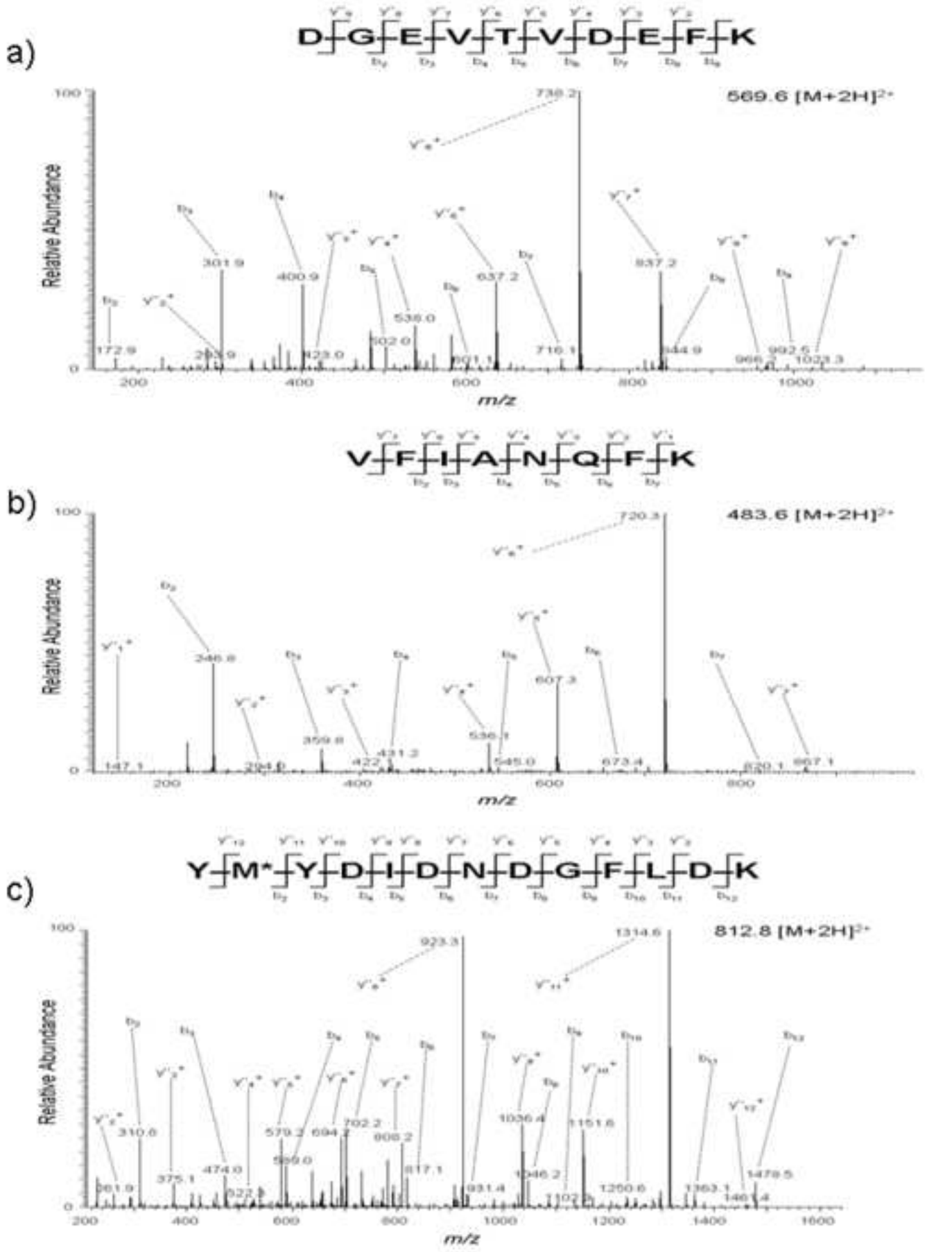

Fig. 3. Ortea *et al.*