Selected Tandem Mass Spectrometry Ion Monitoring for the fast identification of seafood species

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ABSTRACT

Selected tandem mass spectrometry (MS/MS) ion monitoring (SMIM) is the most suitable scanning mode to detect known peptides in complex samples when an ion-trap mass spectrometer is the instrument used for the analysis. In this mode, the MS detector is programmed to perform continuous MS/MS scans on one or more selected precursors, either during a selected time interval, or along the whole chromatographic run. MS/MS spectra are recorded, so virtual multiple reaction monitoring chromatogram traces for the different fragment ions can be plotted. In this work, a shotgun proteomics approach was applied to the detection of previously characterized species-specific peptides from different seafood species. The proposed methodology makes use of high intensity focused ultrasound- assisted trypsin digestion for ultra fast sample preparation, peptide separation and identification by reverse phase capillary LC coupled to an ion-trap working in the SMIM scanning mode. This methodology was applied to the differential classification of seven commercial, closely related, species of Decapoda shrimps proving to be an excellent tool for seafood product authentication, which may be used by fisheries and manufacturers to provide a fast and effective identification of the specimens, guaranteeing the quality and safety of foodstuffs to consumers.

KEYWORDS. ion trap, high intensity focused ultrasound, tandem mass spectrometry, shrimps, SMIM, species identification.

1 Introduction

The assessment of food authentication and origin is a major concern not only for the prevention of commercial fraud [1], but also to avoid the safety risks derived from the inadvertent introduction of any food ingredient that might be harmful for human health [2-4]. The identification of marine species is an issue of primary relevance for the seafood industry, and global commercial requirements concerning labelling and traceability have appeared [5,6]. Seafood products include a wide variety of species with a significant impact in food industry, and among them, crustaceans belonging to the order Decapoda are of remarkable commercial interest. This order includes shrimps, which are one of the most important economic resources in fishery and aquafarming industry [7-9].

External morphological features are particularly difficult to be used in shrimp species differentiation due to their phenotypic similarities and to the fact that they are frequently lost during the manufacturing process. Accordingly, it is highly recommendable the development of the analytical tools necessary to make possible distinguishing between these closely related species, preventing mislabelling and adulteration. Molecular methods for species identification are currently based on DNA or protein analysis, but to date, these methodologies are tedious and time-consuming. Mitochondrial DNA (mtDNA) analysis has been used in polymerase chain reaction (PCR) based studies for fish species identification [10]. Recently, two PCR-restriction fragment length polymorphism (RFLP) based methods for the detection of crustacean [2] and penaeid shrimps [11] DNA have been proposed. Methods based on protein analysis, including sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), isoelectric focusing (IEF) [12-13], two-dimensional gel electrophoresis (2-DE) [14] and immunoassay [15] have been extensively used for fish species identification.

1 Proteomics methods have been proposed as useful tools for the assessment of the
2 authenticity and traceability of marine species in seafood products [16], and some effort
3 has been made to elucidate differences among closely related species using mass
4 spectrometry (MS) [17,18]. However, the need for cheap and rapid screening of a large
5 number of samples has been pushing the development of accurate and sensitive high-
6 tech approaches [19].

7 Arginine kinase (EC 2.7.3.3) (AK), a monomeric phosphagen-ATP
8 phosphotransferase, widely distributed among invertebrates [20], has been revealed as a
9 potential molecular marker for decapoda species identification, due to the inter-specific
10 variability in its aminoacidic sequence [21,22]. In previous studies, we have
11 characterized the AK proteins from seven closely related shrimp species of commercial
12 relevance by means of tandem mass spectrometry (MS/MS) peptide sequencing [23].
13 Specific peptides than can be used as specific markers for seafood product
14 authentication were found.

15 When dealing with complex samples, the selected MS/MS Ion Monitoring (SMIM) is
16 the ion-trap scanning mode most suitable for the detection and quantification of
17 peptides previously sequenced by MS [24]. In the SMIM mode the complete
18 fragmentation spectra of all the selected precursors are recorded along the LC run.
19 Unlike the Selected Reaction Monitoring (SRM) scan, which is based on the selection
20 of only one ion-fragment per precursor-ion selected, the SMIM mode proportionate
21 continuously the whole MS/MS spectra of the selected precursor-ions and
22 chromatogram traces for the different fragment-ions may be plotted.

23 Once MS technological improvements have allowed fast protein identification, the
24 digestion step has become the main limiting step in the proteomic workflow [25].
25 Digestion protocols usually take a long time and to reduce this, different strategies have

1 been reported to speed up and simplify it. Heating, microwaves, high pressure, and
2 infrared and ultrasonic energy have been assayed [26]. In this sense, application of less
3 than 60 s of high intensity focused ultrasound (HIFU) has been reported to achieve a
4 digestion efficiency similar to that obtained with overnight protocols [27]. Ultrasonic
5 energy has some advantages over those other strategies, such as low sample
6 requirements, low reagent consumption, and low cost of the equipment, ultrasonic probe
7 [25], which is generally available in research laboratories. Moreover, it can be applied
8 to both *in-gel* and *in-solution* digestion of proteins.

9 The objective of this work was the study of the suitability of a shotgun proteomic
10 approach, combining HIFU-assisted ultra fast sample preparation, LC separation and
11 peptide identification by MS using the SMIM scanning mode, as a reliable method for
12 fast and effective shrimp species identification. In addition, the possibility of detecting
13 the target peptides in these samples without LC separation, using static nano-
14 electrospray ionization (ESI)-ion trap (IT) MS, was tested.

15 **2 Material and methods**

16 *2.1 Raw material*

17 Seven different shrimp species of commercial interest were considered (Table 1), five
18 belonging to the family Penaeidae (the penaeid shrimps), *Penaeus monodon*,
19 *Litopenaeus vannamei*, *Fenneropenaeus indicus*, *Fenneropenaeus merguensis*,
20 *Farfantepenaeus notialis*; one to the family Solenoceridae, *Pleoticus muelleri*; and the
21 last, the northern shrimp *Pandalus borealis*, to the family Pandalidae. Specimens were
22 collected, using extractive fishing practices or from aquaculture facilities, in different
23 continents worldwide. Intact shrimps were frozen on board and shipped to our
24 laboratory for the analyses. Special care was taken in keeping their morphological
25 characteristics in good shape. Two groups of samples from commercial sources were

also considered (Table 1): the first batch consisting on whole frozen shrimps imported from a Mozambique fishing-ground and labelled as “*Penaeus* spp.”, and a second batch labelled as “frozen vannamei shrimp tails” and purchased in a retail market in Spain. At least six individuals of each species or commercial origin were analyzed. 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 *Reagents*

All reagents were of analytical grade or better. Acetonitrile (ACN) (Panreac, Barcelona, Spain), acetic acid (Panreac, Barcelona, Spain) and Milli-Q ultrapure water (Millipore, Madrid, Spain) were employed as components of the chromatographic mobile phases. Trysin of sequencing grade was obtained from Roche Diagnostics GmbH (Mannheim, Germany).

2.3 *Extraction of sarcoplasmic proteins*

Sarcoplasmic proteins were extracted by homogenizing 1 g of raw white muscle from each individual in two volumes of milliQ water, using an Ultra-Turrax blender for 2x30 s and the extracts were then centrifuged at 30000g for 10 min at 4°C (J25 centrifuge; Beckman, Palo Alto, CA). Protein concentration in the extracts was determined by the bicinchoninic acid method (Sigma Chemical Co., USA).

2.4 *Peptide sample preparation*

Crude protein extracts were directly subjected to HIFU-assisted trypsin digestion as described previously [27], with some modifications. Extracts with 100 µg of protein each were subjected to in-solution digestion with trypsin at 1:25 protease-to-protein ratio. Final digestion volume was set to 104 µL and trypsin digestion was performed for

60 s under sonication. A Vibra Cell CV 18 (Sonics & Materials, Newton, CT) ultrasonic probe was used with the 2 mm probe tip, and the ultrasonic amplitude was set at 50%. Prior to MS analysis, the tryptic digests were desalted and concentrated using in-tip reverse-phase resins (ZipTip C18, Millipore, Bedford, MA), according to the manufacturer's recommendations.

2.5 MS/MS analysis

Peptide digests were analyzed online by LC-ESI-IT-MS/MS using a LC system model SpectraSystem P4000 (Thermo-Finnigan, San Jose, CA) coupled through an ESI ion source to an IT mass spectrometer model LCQ Deca XP Plus (Thermo-Finnigan). The separation was performed on a 0.18 mm × 150 mm BioBasic-18 RP column (ThermoHypersil-Keystone) using 0.5% acetic acid in water and 0.5% acetic acid in 80% ACN as mobile phases A and B, respectively. A 65 min linear gradient from 5 to 45% B, at a flow rate of 1.4-1.7 μ L /min, was used. SMIM was the scan mode used along the LC separations. The IT was programmed to continuously perform a set of seven MS/MS scans focused on doubly-charged specific precursor ions from previously characterized AK peptides [23]. The fragmentation spectra and sequences of the diagnostic peptides that were monitored are shown in Fig. S-1 in the Supplementary Data and Table 2, respectively, indicating the species in which they must be present according to a previous experimental work [23]. MS/MS scans (5 μ scans each) were obtained using an isolation width of 3 u and normalized collision energy of 35%. Ion chromatograms were plotted using the instrument software to show, for each precursor, a selected product ion.

Additionally, analyses were also performed by off-line nanoESI-IT using an IT mass spectrometer, model LCQ Deca XP Plus (Thermo-Finnigan) equipped with a nanospray

1 interface. PicoTips borosilicate glass needles with 1 μm orifice (New Objective,
2 Woburn, MA) were filled with 3-5 μL of sample and used as emitters.

3 Blank samples were included in the analysis, using the complete reaction protocol
4 with the exception of either, the enzyme (trypsin), the protein extract, or the application
5 of sonication.

6 **3 Results and discussion**

7 *3.1 Species identification by SMIM in crude protein extracts*

8 The complex peptide pools obtained by in-solution tryptic digestion of unseparated
9 sarcoplasmic proteins were subjected to LC-MS/MS, analyzing only seven precursor
10 ions at m/z 829.4, 643.8, 817.9, 759.9, 675.3, 539.3, and 603.3 (Table 2), which
11 correspond to the doubly-charged ions from previously described species-specific
12 peptides [23]. Once MS/MS spectra of these precursor ions are recorded, chromatogram
13 traces for the different fragment ions can be obtained. Chromatograms were represented
14 using the ion intensities for fragments at mass to charge ratio (m/z) 1197.4 (ion y''_9^+
15 from the precursor 829.4), 788.3 (ion y''_6^+ from the precursor 643.8), 1188.4 (ion y''_9^+
16 from the precursor 817.9), 977.4 (ion y''_8^+ from the precursor 759.9), 862.3 (ion y''_8^+
17 from the precursor 675.3), 907.4 (ion y''_7^+ from the precursor 539.3), and 931.1 (ion b_8
18 from the precursor 603.3). These combinations of the m/z values from a given precursor
19 ion and from a fragment ion produced by it, are known as transitions (precursor $m/z \rightarrow$
20 fragment m/z). In the protein extracts from *P. muelleri*, only the transition 643.8 $\rightarrow$
21 788.3 appeared with a high S/N ratio. Tracing of this transition produced a highly
22 specific peak at a retention time of 36 min (Fig. 1A). The averaged MS/MS spectra
23 obtained around this retention time gave a perfect agreement with the peptide
24 LTNAVNEIEKR (Mr 1286.70). As shown in Fig. 1B, a similar result was obtained for
25 the transition 817.9 $\rightarrow$ 1188.4 in the *P. borealis* samples; the trace of the corresponding

1 y''_9^+ fragment (m/z 1188.4) also produced a highly specific peak at a retention time of
2 61 min, and the averaged MS/MS spectra also matched clearly with the corresponding
3 peptide sequence, while no significant signal appeared for the other transitions (Fig.
4 1B). In contrast, in the extracts from the species belonging to the family Penaeidae,
5 these two peptides could not be detected, as illustrated by the chromatogram traces (Fig.
6 1C), whereas the specific peak corresponding to the transition 829.4 $\rightarrow$ 1197.4 eluted at
7 a retention time of 62 min, and the averaged MS/MS also matched clearly with the
8 corresponding peptide sequence. Therefore, these first three transitions could be used to
9 differentiate among *P. muelleri*, *P. borealis*, and family Penaeidae species.

10 To differentiate the species within the family Penaeidae, four other transitions were
11 analyzed. In the extracts from *P. monodon* (Fig. 1D), the transitions 759.9 $\rightarrow$ 977.4,
12 675.3 $\rightarrow$ 862.3 and 539.3 $\rightarrow$ 907.4 were observed, whereas no significant signal was
13 produced for the transition 603.3 $\rightarrow$ 931.1. In the samples belonging to *L. vannamei*
14 (Fig. 1E), the transitions 759.9 $\rightarrow$ 977.4 and 675.3 $\rightarrow$ 862.3 appeared with a high S/N
15 ratio, while neither 539.3 $\rightarrow$ 907.4 nor 603.3 $\rightarrow$ 931.1 were obtained. Within the
16 *Fenneropenaeus* genus, the presence or absence of the transition 603.3 $\rightarrow$ 931.1
17 allowed the differentiation of *F. merguiensis* (Fig. 1F) from *F. indicus* (Fig. 1G),
18 respectively. In addition, the transition 759.9 $\rightarrow$ 977.4 was found in both of them.
19 Finally, extracts from *Farf. notialis* presented the transition 675.3 $\rightarrow$ 862.3, while the
20 other three transitions could not be detected (Fig. 1H). When the averaged MS/MS
21 spectra were analyzed, all of them gave a perfect agreement with the corresponding
22 peptide sequences, as is shown in Fig. 1 insets.

23 It has to be noted that although only one transition per diagnostic peptide has been
24 traced in Fig. 1, the SMIM scanning mode allows the representation of the
25 chromatograms of most of the y and b fragment ions. As an example of this, Fig. 2

1 shows the chromatogram traces for 5 different transitions, corresponding to the
2 fragments y''_7^+ , y''_8^+ , y''_9^+ , y''_{10}^+ , and b_{11} of the diagnostic peptide TFLVWVNEEDHLR
3 (Fig. S-1a), obtained after the SMIM analysis of a sample from a species belonging to
4 the Penaeidae family.

5 Commercial samples were also effectively identified after tracing the seven
6 transitions. In that sense, chromatograms from samples from batch 1 were identical to
7 those plotted in Fig. 1C and 1D, and chromatograms from batch 2 were identical to
8 those represented in Fig. 1C and 1E. The averaged MS/MS spectra around each
9 corresponding retention time also matched the expected peptide sequence. To conclude,
10 samples from batch 1 were identified as *P. monodon*, and those from batch number 2
11 clearly identified as *L. vannamei*. Blank samples were negative for all the transitions
12 (results not shown).

13 3.2 Species identification by off-line nESI of crude extracts

14 The complex unseparated peptide mixtures were also directly analyzed by off-line
15 nanoESI-IT MS. For each sample, ions at m/z 829.4, 643.8, 817.9, 759.9, 675.3, 539.3
16 and 603.3 were subjected to fragmentation and the corresponding MS/MS spectra were
17 recorded, even when they were not visible in full scan. The produced spectra were
18 visually inspected for fragment peaks matching theoretical ion masses from y or b-
19 series of the corresponding peptides. When the MS/MS spectrum did not match the
20 expected peptide sequence, that peptide was considered absent from the sample. For
21 each sample, the presence and absence of each of the seven peptides matched those
22 predicted according to Ortea et al. [23], and those obtained in the SMIM experiment.
23 Fig. 3 shows examples of MS/MS spectra obtained by the blind fragmentation of the
24 seven ions, showing the fragment peaks that matched the expected peptide sequences.
25 Fig. 3a and Fig. 3b are the 829.4 fragmentation spectra representing the absence and the

1 presence of the peptide TFLVWVNEEDHLR, respectively. Several spectra proving the
2 presence of the corresponding peptides are shown in Fig 3c-h.

3 Commonly, mono or multidimensional LC analysis, using orthogonal separation
4 modes, is coupled to tandem mass spectrometry to resolve the complex mixture of
5 peptides obtained when a whole proteome is digested. Separations are needed to prevent
6 the problems in the ionization associated with the coelution of a huge number of
7 peptides [28]. Nevertheless, in the analytical conditions used in this work, the seven
8 diagnostic peptides could be detected by nanoESI-IT-MS/MS without previous LC
9 separation, as is shown in Fig. 3, due to (i) a drastical reduction in the sample
10 complexity obtained by the aqueous cytosolic protein extraction, in a medium
11 containing no salts; and (ii) the selection of AK peptides as targets. This protein is
12 highly abundant in the aqueous extract of shrimp sarcoplasm [17].

13 **4 Conclusions**

14 In this work, a proteomics methodology, combining the speed of HIFU assisted tryptic
15 digestion, the high separation capability of RP-HPLC and the peptide identification
16 ability of MS using the SMIM scanning mode, was used to detect and monitor
17 diagnostic peptides from seven different shrimp species belonging to the order
18 Decapoda. Although a complex peptide pool was used, the combination of retention
19 time and precursor m/z , with the software representation of specific product ions, made
20 possible the differentiation of the seven shrimp species. Although a conventional 3D ion
21 trap was used in this work, this approach, coupling peptide biomarkers with the SMIM
22 configuration, is particularly suitable for linear ion traps, which have a higher scanning
23 speed, thus allowing a higher number of peptides to be monitored and consequently
24 making possible the identification of a larger number of species in a single LC-MS/MS
25 experiment. In addition, this approach can be easily automated, allowing routine high-

throughput analysis of foodstuffs. The complete process, from the arrival of the sample to the identification of the species, takes no longer than 90 minutes.

Alternatively, an off-line analysis of the unseparated protein digests was tested. This methodology, although is more interactive and less automatizable, also achieved the discrimination of the closely-related species studied in even shorter analysis time.

Both methodologies have demonstrated to be suitable for performing a sensitive, unequivocal, and fastest to date identification of the commercially-relevant shrimp species, allowing the identification of the species that may be present in a certain product in no longer than 90 minutes, guaranteeing food quality, safety and labelling.

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Conflict of interest statement

All authors declare there are no financial/commercial conflicts of interest.

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Figure captions

Fig. 1. Discrimination of seven closely-related shrimp species by the SMIM of seven AK tryptic peptides. The protein extracts were subjected to HIFU-assisted trypsin digestion followed by HPLC-MS/MS. The IT detector was set to perform a continuous fragmentation of the ions at m/z 829.4, 643.8, 817.9, 759.9, 675.3, 539.3, and 603.3. The traces of the selected product ions as a function of retention time are plotted in the chromatograms for the discrimination of (A) *P. muelleri*, (B) *P. borealis*, (C) family Penaeidae species, (D) *P. monodon*, (E) *L. vannamei*, (F) *F. merguiensis*, (G) *F. indicus*, and (H) *Farf. notialis*. Insets show the corresponding averaged MS/MS spectra (relative intensity vs m/z) around the peak apex; asterisks mark fragment peaks matching theoretical ions masses from y or b series.

Fig. 2. Chromatogram traces for 5 different ion fragments of the diagnostic peptide TFLVWVNEEDHLR, obtained after the HIFU-assisted trypsin digestion and HPLC-SMIM analysis of a sarcoplasmic extract from *Litopenaeus vannamei*. **Fig. 3.** CID fragmentation spectra of the ions at m/z (a) and (b) 829.4, (c) 643.8, (d) 817.9, (e) 759.9, (f) 675.3, (g) 539.3, and (h) 603.3 obtained by off-line direct nanoESI-IT analysis showing the fragment peaks that matched the expected peptide sequences. Sarcoplasmic protein extracts were digested using the fast, HIFU assisted tryptic digestion procedure.

Figure 1
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Table 1. Penaeid shrimp species considered in the study

Scientific name ^a	Family	Commercial name	Origin ^b
<i>Pleoticus muelleri</i>	Solenoceridae	Argentine red shrimp	SWA
<i>Pandalus borealis</i>	Pandalidae	Northern shrimp	NA
<i>Penaeus monodon</i>	Penaeidae	Giant tiger prawn	IWP and WI
<i>Litopenaeus vannamei</i>		Pacific white shrimp	EP
<i>Fenneropenaeus merguiensis</i>		Banana prawn	WCP
<i>Fenneropenaeus indicus</i>		Indian white prawn	WI
<i>Farfantopenaeus notialis</i>		Southern pink shrimp	EA
-	-	Penaeus spp.	Commercial origin
-	-	frozen vannamei shrimp tails	Commercial origin

^aThe taxonomic classification proposed by Pérez-Farfante *et al.*⁹ was adopted.
^bOrigin abbreviations: SWA, Southern West Atlantic Ocean.; NA, Northern Atlantic Ocean; IWP, Indo-West Pacific Ocean; WI, Western Indian Ocean; EP, Eastern Pacific Ocean; WCP, Western Central Pacific Ocean; EA, Eastern Atlantic Ocean;

Table 2. Aminoacid sequences of the diagnostic peptides selected.

<i>m/z</i> (z) observed	(M+H) ⁺	Peptide Sequence	<i>P.</i> <i>muelleri</i>	<i>P.</i> <i>borealis</i>	<i>P.</i> <i>monodon</i>	<i>L.</i> <i>vannamei</i>	<i>F.</i> <i>merguiensis</i>	<i>F.</i> <i>indicus</i>	<i>Farf.</i> <i>notialis</i>
539.16 (2+)	1077.58	AVFDQLKEK							
603.26 (2+)	1205.68	ALFDQLKDKK							
675.22 (2+)	1349.71	VSSTLSSLEGELK							
817.76 (2+)	1634.81	SFLVWVNEEDQLR							
829.26 (2+)	1657.82	TFLVWVNEEDHLR							
643.86 (2+)	1286.70	LTNAVNEIEKR							
759.85 (2+)	1518.82	LEEVAGKYNLQVR							

m/z: mass/charge; (■) denotes the presence of a peptide, and (□) the absence.