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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.

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

Arginine kinase (EC 2.7.3.3) (AK), a monomeric phosphagen-ATP phosphotransferase, widely distributed among invertebrates [20], has been revealed as a potential molecular marker for decapoda species identification, due to the inter-specific variability in its aminoacidic sequence [21,22]. In previous studies, we have characterized the AK proteins from seven closely related shrimp species of commercial relevance by means of tandem mass spectrometry

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Table 1
Penaeid shrimp species considered in the study.

Scientific name[a]	Family	Commercial name	Origin[b]
Pleoticus muelleri	Solenoceridae	Argentine red shrimp	SWA
Pandalus borealis	Pandalidae	Northern shrimp	NA
Penaeus monodon		Giant tiger prawn	IWP and WI
Litopenaeus vannamei		Pacific white shrimp	EP
Fenneropenaeus merguiensis	Penaeidae	Banana prawn	WCP
Fenneropenaeus indicus		Indian white prawn	WI
Farfantepenaeus notialis		Southern pink shrimp	EA
–	–	Penaeus spp.	Commercial origin
–	–	Frozen vannamei shrimp tails	Commercial origin

[a] The taxonomic classification proposed by Pérez-Farfante et al. [9] was adopted.
[b] *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.

(MS/MS) peptide sequencing [23]. Specific peptides than can be used as specific markers for seafood product authentication were found.

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

Once MS technological improvements have allowed fast protein identification, the digestion step has become the main limiting step in the proteomic workflow [25]. Digestion protocols usually take a long time and to reduce this, different strategies have been reported to speed up and simplify it. Heating, microwaves, high pressure, and infrared and ultrasonic energy have been assayed [26]. In this sense, application of less than 60 s of high intensity focused ultrasound (HIFU) has been reported to achieve a digestion efficiency similar to that obtained with overnight protocols [27]. Ultrasonic energy has some advantages over those other strategies, such as low sample requirements, low reagent consumption, and low cost of the equipment, ultrasonic probe [25], which is generally available in research laboratories. Moreover, it can be applied to both *in-gel* and *in-solution* digestion of proteins.

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

2. Material and methods

2.1. Raw material

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

Table 2
Aminoacid sequences of the diagnostic peptides selected.

m/z (z) observed	$(M+H)^+$	Peptide sequence	*P. muelleri*	*P. borealis*	*P. monodon*	*L. vannamei*	*F. merguiensis*	*F. indicus*	*Farf. notialis*
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.

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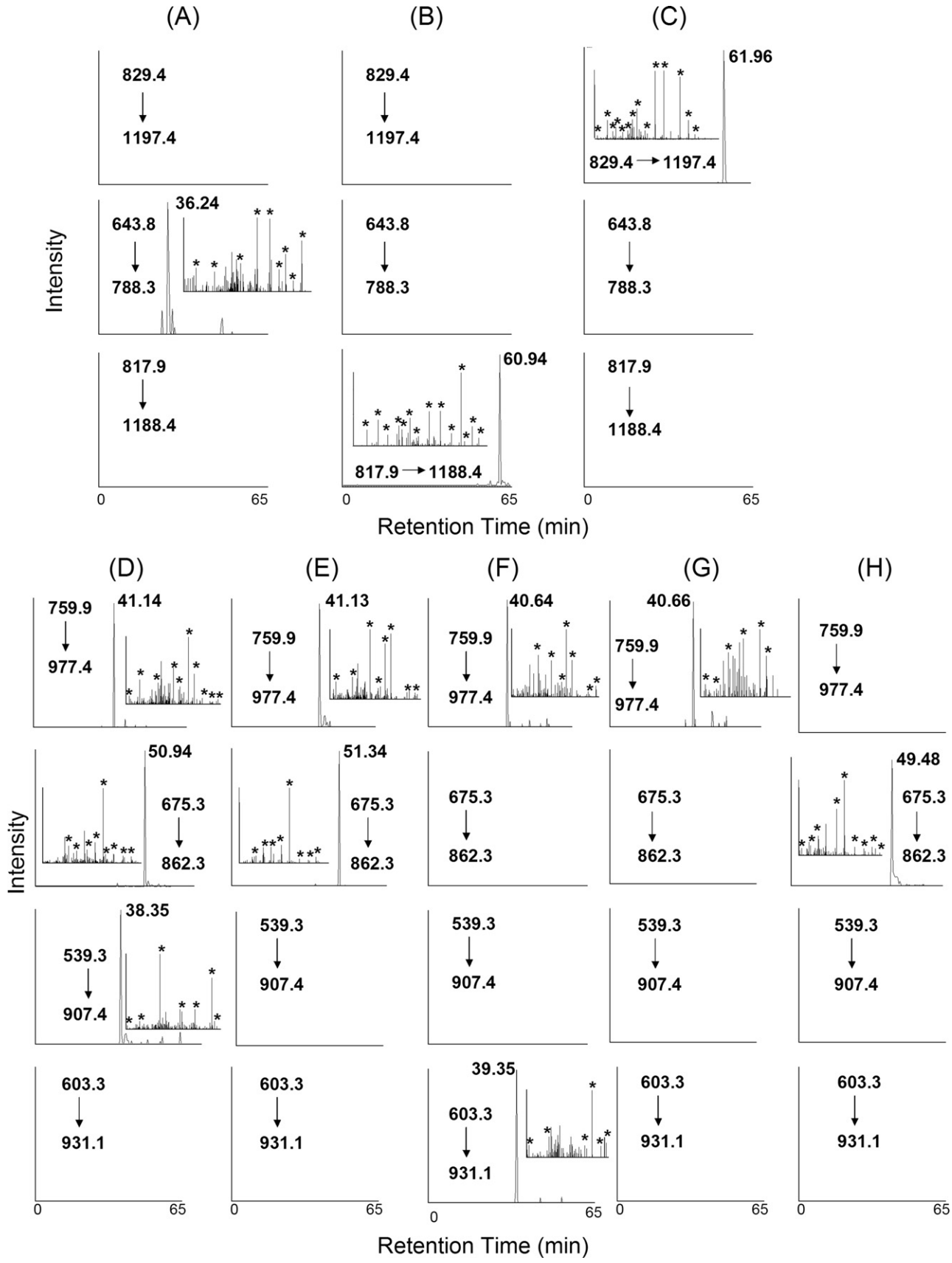

Fig. 1. Discrimination of seven closely related shrimp species by the SMIM of seven AK tryptic peptides. The protein extracts were subjected to HIFU-assisted trypsin digestion followed by HPLC–MS/MS. The IT detector was set to perform a continuous fragmentation of the ions at *m/z* 829.4, 643.8, 817.9, 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.

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 2 × 30 s and the extracts were then centrifuged at 30,000 × *g* 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 μL/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 μscans each) were obtained using an isolation width of 3 μm 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 interface. PicoTips borosilicate glass needles with 1 μm orifice (New Objective, Woburn, MA) were filled with 3–5 μL of sample and used as emitters.

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

3. Results and discussion

3.1. Species identification by SMIM in crude protein extracts

The complex peptide pools obtained by in-solution tryptic digestion of unseparated sarcoplasmic proteins were subjected to LC–MS/MS, analyzing only seven precursor ions at *m/z* 829.4, 643.8, 817.9, 759.9, 675.3, 539.3, and 603.3 (Table 2), which correspond to the doubly charged ions from previously described species-specific peptides [23]. Once MS/MS spectra of these precursor ions are recorded, chromatogram traces for the different fragment ions can be obtained. Chromatograms were represented using the ion intensities for fragments at mass to charge ratio (*m/z*) 1197.4 (ion $y''_9{}^+$ from the precursor 829.4), 788.3 (ion $y''_6{}^+$ from the precursor 643.8), 1188.4 (ion $y''_9{}^+$ from the precursor 817.9), 977.4 (ion $y''_8{}^+$ from the precursor 759.9), 862.3 (ion $y''_8{}^+$ from the precursor 675.3), 907.4 (ion $y''_7{}^+$ from the precursor 539.3), and 931.1 (ion b_8 from the precursor 603.3). These combinations of the *m/z* values from a given precursor ion and from a fragment ion produced by it, are known as transitions (precursor *m/z* → fragment *m/z*). In the protein extracts from *P. muelleri*, only the transition 643.8 → 788.3 appeared with a high S/N ratio. Tracing of this transition produced a highly specific peak at a retention time of 36 min (Fig. 1A). The averaged MS/MS spectra obtained around this retention time gave a perfect agreement with the peptide LTNAVNEIEKR (Mr 1286.70). As shown in Fig. 1B, a similar result was obtained for the transition 817.9 → 1188.4 in the *P. borealis* samples; the trace of the corresponding $y''_9{}^+$ fragment (*m/z* 1188.4) also produced a highly specific peak at a retention time of 61 min, and the averaged MS/MS spectra also matched clearly with the corresponding peptide sequence, while no significant signal appeared for the other transitions (Fig. 1B). In contrast, in the extracts from the species belonging to the family Penaeidae, these two peptides could not be detected, as illustrated by the chromatogram traces (Fig. 1C), whereas the specific peak corresponding to the transition 829.4 → 1197.4 eluted at a retention time of 62 min, and the averaged MS/MS also matched clearly with the corresponding peptide sequence. Therefore, these first three transitions could be used to differentiate among *P. muelleri*, *P. borealis*, and family Penaeidae species.

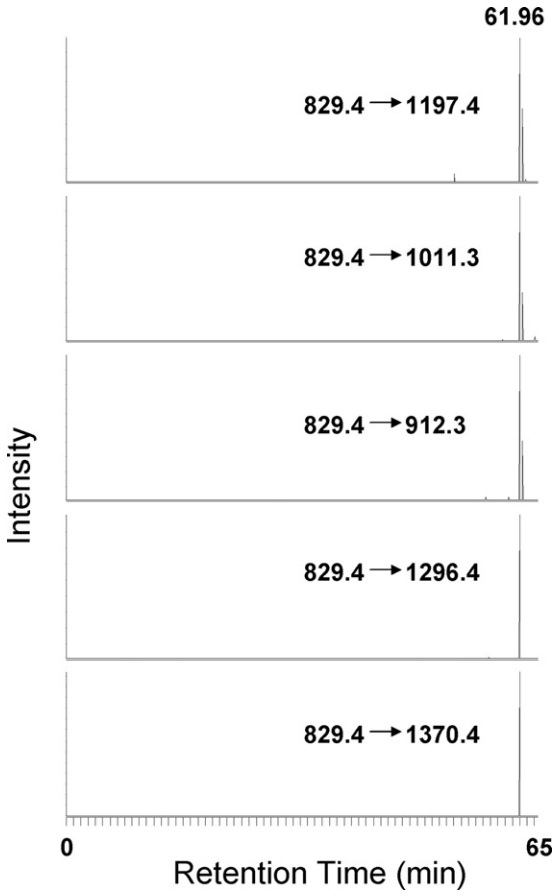

Fig. 2. Chromatogram traces for 5 different ion fragments of the diagnostic peptide TFLVWVNEEDHLR, obtained after the HIFU-assisted trypsin digestion and HPLC-SMIM analysis of a sarcoplasmic extract from *Litopenaeus vannamei*.

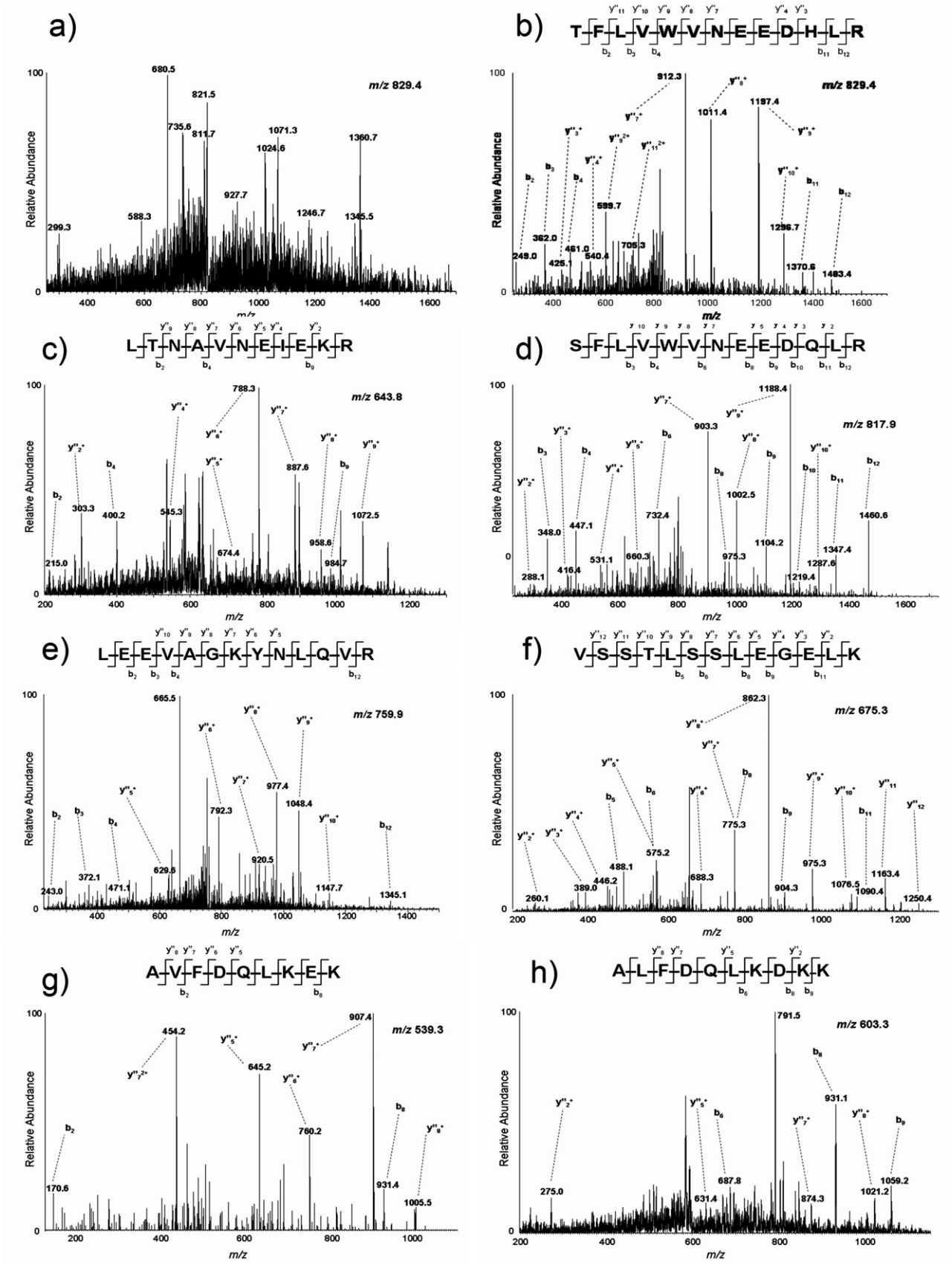

Fig. 3. CID fragmentation spectra of the ions at *m/z* (a) and (b) 829.4, (c) 643.8, (d) 817.9, (e) 759.9, (f) 675.3, (g) 539.3, and (h) 603.3 obtained by off-line direct nanoESI-IT analysis showing the fragment peaks that matched the expected peptide sequences. Sarcoplasmic protein extracts were digested using the fast, HIFU assisted tryptic digestion procedure.

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

It has to be noted that although only one transition per diagnostic peptide has been traced in Fig. 1, the SMIM scanning mode allows the representation of the chromatograms of most of the y and b fragment ions. As an example of this, Fig. 2 shows the chromatogram traces for 5 different transitions, corresponding to the fragments $y''_7{}^+$, $y''_8{}^+$, $y''_9{}^+$, $y''_{10}{}^+$, and b_{11} of the diagnostic peptide TFLVWVNEEDHLR (Fig. S-1a), obtained after the SMIM analysis of a sample from a species belonging to the Penaeidae family.

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

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

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

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

4. Conclusions

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

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 min, guaranteeing food quality, safety and labelling.

Conflict of interest statement

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

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.05.032.

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