

Fast Monitoring of Species-Specific Peptide Biomarkers Using High-Intensity-Focused-Ultrasound-Assisted Trypsic Digestion and Selected MS/MS Ion Monitoring

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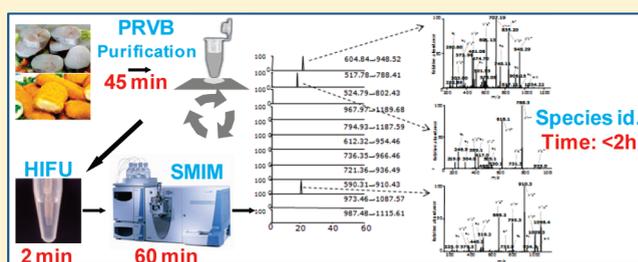
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S Supporting Information

ABSTRACT: A new strategy for the fast monitoring of peptide biomarkers is described. It is based on the use of accelerated in-solution trypsin digestions under an ultrasonic field provided by high-intensity focused ultrasound (HIFU) and the monitoring of several peptides by selected MS/MS ion monitoring in a linear ion trap mass spectrometer. The performance of the method was established for the unequivocal identification of all commercial fish species belonging to the Merlucciidae family. Using a particular combination of only 11 peptides, resulting from the HIFU-assisted tryptic digestion of the thermostable proteins parvalbumins, the workflow allowed the unequivocal identification of these closely related fish species in any seafood product, including processed and precooked products, in less than 2 h. The present strategy constitutes the fastest method for peptide biomarker monitoring. Its application for food quality control provides to the authorities an effective and rapid method of food authentication and traceability to guarantee the quality and safety to the consumers.



Fast monitoring of biomarkers is essential to achieve a rapid response and to make a precise decision in diverse life fields. It is crucial in clinical diagnosis, therapeutic supervision, environmental protection, and food quality control, among others.^{1,2} Currently, the most commonly used approach for the monitoring of peptide/protein biomarkers is based on immunoassays, mainly using ELISA and array techniques.¹ The advantages of these methods are their high specificity and sensitivity. Apart of being time-consuming, a flaw of these techniques is that not always the right antibody is available for each biomarker, making lengthy and expensive work necessary to extend the battery of specific antibodies for new standardized and affordable assays. Therefore, the development of alternative and fast methodologies having high reproducibility, sensitivity, and specificity is necessary.

The emerging targeted mass spectrometry (MS)-based proteomics techniques can constitute an excellent alternative methodology. When these selective and sensitive operating methods are used, the MS analyzer is centered on analyzing only the compound of interest by selected reaction monitoring (SRM) or multiple reaction monitoring (MRM).^{3,4} Monitoring transitions (suitable pairs of precursor and fragment ion m/z) constitute a common assay to identify and quantify biomarkers and, by inference, the change in the corresponding biological condition being studied. This setup provides high analytical reproducibility, a good signal-to-noise (S/N) ratio, and an increased dynamic

range.⁴ The capability of triple-quadrupole (TQ) instruments to selectively isolate a precursor ion and the fragment ion(s) it produces under collision-induced dissociation (CID) is exploited for the experiments using SRM or MRM scan modes.⁵

While SRM and MRM performed on a TQ are the most sensitive scanning modes (low attomolar) with a broad dynamic range (up to 5 orders of magnitude),⁶ their optimization for a definite SRM/MRM assay is time-consuming. More importantly, using these scanning procedures, complete MS/MS spectra are not registered. The MS/MS spectrum of a molecule is of paramount importance to confirm the structure of the compound detected. To solve this problem, new routines, such as MRM-triggered MS/MS using hybrid quadrupole/linear ion trap mass spectrometers, have been explored.⁷ In such assays, when a significant signal for a specific MRM transition is detected, the instrument switches the third quadrupole automatically to the ion trap mode, collecting the full MS/MS spectrum.

Selected MS/MS ion monitoring (SMIM) in an ion trap (IT) mass spectrometer is another scanning mode that allows for a sensitive monitoring of specific molecules, producing complete structural information.⁸ The high scanning speed attainable in

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the IT allows for the production of MS/MS spectra in a fraction of a second, registering the information given by the complete spectra. High-confidence MS/MS spectra are recorded due to the possibility of an averaging of the signal during acquisition. The utility of this operating mode has been demonstrated in several previously published studies,^{8–10} where the possibility of a virtual plotting of all the transitions produced has been discussed.

The main difficulty when LC–MS is used to monitor biomarkers arises from the complexity of the samples, which may result in false positives. Although the chromatographic gradient time may be properly manipulated to avoid this, lowering the number of components in the sample, when it is possible, may help to simplify the assays. Shorter analysis times are needed when fewer components are present in the sample. Fast and easy protein fractionation or purification steps conducted prior to LC–MS analysis make the analysis simpler and faster.¹¹

Procedures to enhance the protease activity, such as the application of microwaves,¹² high pressure,¹³ or the energy produced by ultrasound,¹⁴ can accelerate the time-consuming trypsin digestion. The application of only 1–2 min of high-intensity focused ultrasound (HIFU) to in-solution tryptic digestions has been reported to achieve an efficiency and reproducibility similar to those obtained by traditional overnight protocols.^{14–16}

A new targeted MS-based strategy for the fast monitoring of peptide biomarkers based on the combination of these methodologies described is proposed and has been applied to the fast authentication of all commercial species from the Merlucciidae family. It is based on (a) the purification of parvalbumins (PRVBs) by heat treatment (time 45 min), (b) their accelerated tryptic digestion using HIFU (time 2 min), and (c) the monitoring of 11 PRVB peptide biomarkers by SMIM in a linear ion trap (LIT) mass spectrometer (time 60 min). Each step was individually adjusted to minimize the time of analysis. With this new and competitive strategy, the unequivocal identification of these closely related fish species in any seafood products, including processed and precooked, can be achieved in less than 2 h.

EXPERIMENTAL SECTION

Reference Species and Commercial Foodstuffs. All the main commercial species from the Merlucciidae family were employed in this study: eleven different hake species, including two different subspecies from *Merluccius australis* and two grenadier subspecies belonging to the *Macruronus novaezelandiae* species (Table 1). Except for European hake, the specimens were frozen on board at $-30\text{ }^{\circ}\text{C}$, with special care in keeping their morphological characteristics in good shape, and shipped by plane to the laboratory for the analyses. The weight of every specimen studied was in the range of 3–6 kg. At least 10 fish belonging to each different species were subjected to taxonomical study according to their anatomical and morphological features by an expert marine biologist and by genetic identification using forensically informative nucleotide sequencing (FINS) at the Food Biochemistry Laboratory of the Marine Research Institute (Vigo, Pontevedra, Spain).^{17–19} Five correctly identified individuals for each of the species were considered as reference species. In addition, for the validation step using commercial real samples, a total of 10 hake foodstuffs were included in the work (Table 1). All samples were analyzed in triplicate.

Table 1. Reference Species and Commercial Foodstuffs from the Merlucciidae Family Considered in This Study^a

species/subspecies	Reference Species common name	origin
<i>M. merluccius</i>	European hake	Spanish coasts
<i>M. capensis</i>	Cape hake	South Africa
<i>M. senegalensis</i>	Senegalense hake	Northwest Africa
<i>M. polli</i>	Benguela hake	Northwest Africa
<i>M. paradoxus</i>	Deep-water Cape hake	South Africa
<i>M. hubbsi</i>	Patagonian hake	South America
<i>M. gayi</i>	Peruvian or Chilean hake	South America
<i>M. australis polylepis</i>	Austral hake	South America
<i>M. australis australis</i>	Austral hake	New Zealand coasts
<i>M. productus</i>	Pacific hake	North America
<i>M. bilinearis</i>	Silver hake	North America
<i>Ma. nov. nov.</i>	Blue grenadier	New Zealand coasts
<i>Ma. nov. magellanicus</i>	Patagonian grenadier	South America

Commercial Products		
code	product presentation	declared species
CP1	raw fillets frozen	<i>M. capensis</i> or <i>M. paradoxus</i>
CP2	raw center cuts frozen	<i>M. australis</i>
CP3	raw loins frozen	<i>M. capensis</i> or <i>M. paradoxus</i>
CP4	raw center cuts frozen	<i>M. capensis</i> or <i>M. paradoxus</i>
CP5	raw center cuts frozen	hake unidentified
CP6	battered precooked sticks frozen	hake unidentified
CP7	battered precooked sticks frozen	hake unidentified
CP8	battered precooked sticks frozen	hake unidentified
CP9	battered precooked sticks frozen	hake unidentified
CP10	battered precooked fillets frozen	hake unidentified

^a Abbreviations: *M.*, *Merluccius* genus; *Ma. nov.*, *Macruronus novaezelandiae*.

Parvalbumin Purification. Sarcoplasmic protein extraction was carried out by homogenizing 5 g of white muscle in 10 mL of 10 mM Tris–HCl buffer, pH 7.2, supplemented with 5 mM phenylmethylsulfonyl fluoride (PMSF), for 30 s in an Ultra-Turrax device (IKA-Werke, Staufen, Germany). In the case of battered precooked foodstuffs the casing was first removed. The sarcoplasmic protein extracts were then centrifuged at 40000g for 20 min at $4\text{ }^{\circ}\text{C}$ (J221-M centrifuge; Beckman, Palo Alto, CA). PRVBs were purified by taking advantage of their thermostability, heating the sarcoplasmic extracts at $70\text{ }^{\circ}\text{C}$ for 5 min.¹⁰ After centrifugation at 40000g for 20 min (J221-M centrifuge), supernatants composed mainly of PRVBs were quantified by the bicinchoninic acid (BCA) method (Sigma Chemical Co., St. Louis, MO).

Protein Digestion Using HIFU. PRVB supernatants were subjected to HIFU-assisted trypsin digestion as previously described.¹⁴ A total of 20 μg of heated extract was subjected to in-solution digestion with 1 μg of trypsin without addition of urea, dithiothreitol, or iodoacetamide (Promega, Madison, WI), simultaneously applying HIFU. A high-intensity ultrasonic probe with a 1 mm probe tip (Dr. Hielscher, Teltow, Germany) was set to 50% amplitude and was used to perform the ultrafast digestion for 1 min. Another 1 μg of trypsin was added again to the sample, and the HIFU application was repeated for 1 min.

LC–MS/MS Analysis. Peptide digests were acidified and analyzed by LC–ESI-IT-MS/MS using a Surveyor LC system

coupled to an LTQ LIT mass spectrometer (Thermo Fisher, San Jose, CA). The peptide separation (1 μg) was performed on a 0.18 mm \times 150 mm BioBasic-18 RP column (ThermoHypersil-Keystone) using 0.5% acetic acid in Milli-Q water and in 80% acetonitrile (ACN) as mobile phases A and B, respectively. A 60 min linear gradient from 5% to 40% B, at a flow rate of 1.5–1.7 $\mu\text{L}/\text{min}$, was used. ESI parameters were as follows: spray voltage, 3.5 kV; N_2 flow, 10 arbitrary units; capillary temperature, 200 $^\circ\text{C}$. Peptides were analyzed in positive mode from 400 to 1600 amu (three microscans), followed by four data-dependent MS/MS scans (three microscans), using an isolation width of 3 amu and a normalized collision energy of 35%. Fragmented masses were set in dynamic exclusion for 3 min after the second fragmentation event, and singly charged ions were excluded from MS/MS analysis.

Selected MS/MS Ion Monitoring. SMIM analysis was performed using a Surveyor LC system coupled to an LTQ LIT mass spectrometer (Thermo Fisher, San Jose, CA), as described previously⁸ with minor modifications. The peptide separation (1 μg) was performed on a 0.18 mm \times 150 mm BioBasic-18 RP column (ThermoHypersil-Keystone) using 0.5% acetic acid in water and in 80% ACN as mobile phases A and B, respectively. A 45 min linear gradient from 5% to 40% B, at a flow rate of 1.5–1.7 $\mu\text{L}/\text{min}$, was used. ESI parameters were as described previously. Peptides were detected in the positive ion mode using SMIM.⁸ For this method, the MS instrument was programmed to perform continuous MS/MS scans (five microscans) of doubly charged precursor ions from all candidate peptide biomarkers along the complete chromatographic separation. The normalized collision energy was set to 35%, and a 3 amu mass window was used to fragment selected parent ions.

Mass Spectrometry Data Processing. MS/MS spectra were searched using SEQUEST (Bioworks 3.1 package, Thermo Fisher) against the Teleostei UniProt/TrEMBL database (release 2010_12; 158,545 entries), which also included their respective decoy sequences. The following constraints were used for the searches: semitryptic cleavage with up to two missed cleavage sites and tolerances of 1.8 Da for precursor ions and 0.8 Da for MS/MS fragment ions. The variable modifications allowed were methionine oxidation (Mox), carbamidomethylation of Cys (C*), and acetylation of the N-terminus of the protein (N-Acyl). The database search results were subjected to statistical analysis with the PeptideProphet algorithm (v.4.4).²⁰ The false discovery rate (FDR) was kept below 1%.

The proteins identified in the original and heated sarcoplasmic extracts were submitted to ingenuity pathway analysis (IPA; Ingenuity Systems, Redwood City, CA). Only pathways scoring $-\log(p) \geq 2$, which have >99% confidence of not being generated by chance, were selected.

For the SMIM mode, virtual chromatogram traces were plotted and optimized using QualBrowser software (Thermo Fisher) to show the selected transitions for each parent ion. In addition, MS/MS spectra collected in the SMIM mode were used to validate the peptide identities using SEQUEST as described before.

RESULTS AND DISCUSSION

Strategy for the Fast Monitoring of Species-Specific Peptide Biomarkers. The strategy for the fast monitoring of peptide biomarkers proposed in this work is summarized in Figure 1. This strategy integrates three steps: (a) purification of thermostable proteins (PRVBs) by short heat treatment followed by centrifugation (time 45 min), (b) in-solution trypsin digestion accelerated

using HIFU (time 2 min), and (c) monitoring of 11 species-specific PRVB peptide biomarkers by SMIM using an LIT mass spectrometer (time 60 min). With this strategy, all relevant commercial fish species belonging to the Merlucciidae family can be unequivocally identified in any seafood product, including precooked, in less than 2 h. The detailed results for each step and the validation of this new fast monitoring strategy using unknown hake commercial products are shown in the following sections.

PRVB Purification and Enzymatic Digestion Accelerated by HIFU. PRVBs, considered as the best protein biomarker for the authentication of Merlucciidae species,^{10,21} were purified from the sarcoplasmic extracts, taking advantage of their thermostability.¹⁰ Figure S-1 in the Supporting Information shows a summary of the protein composition in the extracts before and after the treatment with heat (70 $^\circ\text{C}$ for 5 min). The complete list of proteins and peptides for both samples, identified by LC–MS/MS and Sequest search after a conventional overnight trypsin digestion,²² are presented in the Supporting Information. Protein composition of the original sarcoplasmic extracts revealed more than 125 different proteins involved in 10 relevant functional pathways (Table S-1 in the Supporting Information). After treatment with heat, the majority of identified peptides corresponded to PRVBs (77.87%) (Figure S-1). These results demonstrated that the treatment with heat is a simple, fast, and effective procedure to purify and enrich the samples in only PRVBs.

Purified PRVBs were digested with trypsin using either the conventional overnight procedure or the fast procedure accelerated by HIFU. As reported previously,¹⁴ HIFU-assisted digestion produced results comparable to those obtained by the conventional overnight incubation methods, but in a fraction of time. Moreover, the absence of urea in the digestion buffer prevented undesired peptide side reactions, such as carbamylation of N-termini and Lys residues, which may occur when HIFU is applied in the presence of urea.^{23,24}

The combination of a fast and easy protein purification procedure (time 45 min) with the use of HIFU for the protein digestion (time 2 min) considerably simplified and reduced the time needed for the sample preparation, reflected in the overall time needed for monitoring.

Selection of the Species-Specific Peptide Biomarkers. The next step in the proposed strategy consisted in selecting the smaller number of species-specific peptides, which must be monitored, to effectively identify all the species from the Merlucciidae family. Parvalbumin peptide sequences with a high inter-specific variability, obtained after the extensive de novo sequencing of PRVBs previously published,¹⁰ were used for this purpose. Eleven tryptic peptides were selected on the basis of the information that their combined presence or absence could provide to confidently identify all of the species under study (Table 2). A Basic Local Alignment Search Tool (BLAST) search was performed using the UniProtKB database to validate the uniqueness of the peptide sequences selected. Four of them were present in only one specific species and can be considered as a canonical peptide for each of these Merlucciidae species (S-MER794, S-MER612, S-MER721, S-MER973). The sequences of the rest were shared by PRVBs from several Merlucciidae species or other organisms. However, their use following a specific and systematic combination avoids interferences and allows for a correct discrimination of all the hake species under study. Figure 2 shows the flow diagram for the unambiguous systematic discrimination

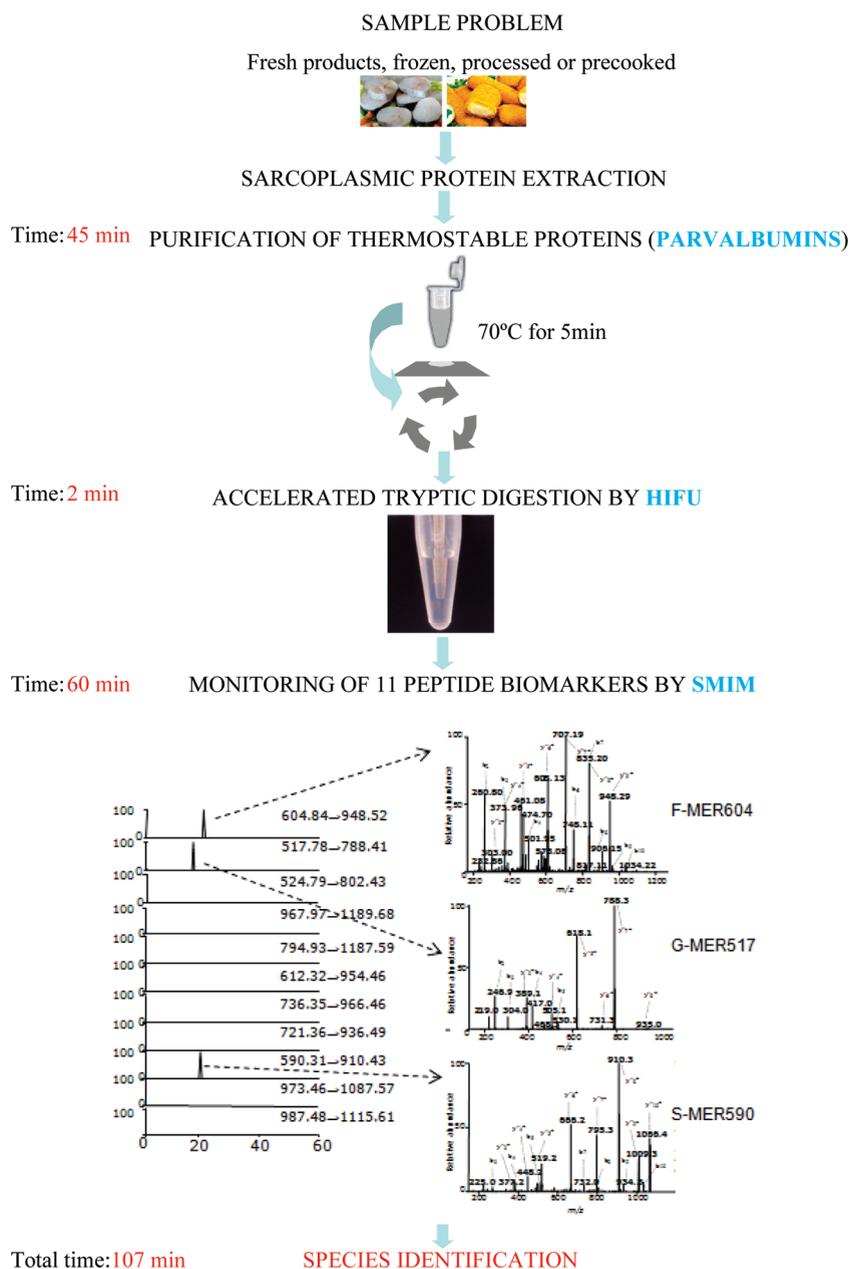


Figure 1. Analytical scheme for the fast monitoring of the species-specific peptide biomarkers proposed in this work.

which can be achieved. According to this scheme, the presence/absence of the peptide F-MER604 determines if any member from the Merlucciidae family is present in the sample. Discrimination between the genera *Merluccius* and *Macruronus* can be achieved by the determination of the presence/absence of peptides G-MER517 and G-MER524, respectively. Within the *Merluccius* genus, the presence/absence of the peptide S-MER967 allows for the classification of hake species into two groups according to their geographic distribution: American hakes (*M. hubbsi*, *M. gayi*, *M. australis polylepis*, *M. australis australis*, *M. productus*, or *M. bilinearis*) or Euro-African hakes (*M. merluccius*, *M. capensis*, *M. senegalensis*, *M. polli*, or *M. paradoxus*). Finally, as can be seen in Figure 2, the combination of the presence/absence of other eight peptides allows for the unambiguous identification of any specific species from the Merlucciidae family.

Fast Identification of Hake Species Using SMIM. For each of the reference hake species, PRVB peptide pools obtained from the accelerated tryptic digestions were subjected to SMIM analysis in an LIT mass spectrometer focusing the MS/MS events on the corresponding precursor ions for the 11 peptides selected. The selected m/z value for each precursor ion corresponded to the predominant charge state, which was +2 for all of them (Table 2). Figure S-2 in the Supporting Information details the MS/MS spectra for each peptide. Once MS/MS spectra were recorded, virtual chromatograms for all the different fragment ions could be obtained. For each of the peptide markers, mass transitions were noted according to the criteria of sensitivity and selectivity. As the peptide mixture used is not too complex, selectivity was not a matter of concern and the transitions chosen in every case were in accordance with the maximum intensity

Table 2. Peptide Biomarkers and Specific Transitions for the Identification of All Species from the Merlucciidae Family^a

Biomarker code	Peptide Sequence	SMIM Transition <i>m/z</i> precursor ion (<i>z</i>) → <i>m/z</i> fragment ion	Retention time (min)	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	Cross-reaction with proteins from other organisms by BLAST
F-MER604	LFLQVFSAGAR	604.84 (2+) → 948.52 (y ⁹ ₉ ⁺)	23.00	■	■	■	■	■	■	■	■	■	■	■	■	-
G-MER517	VFGIIDQDK	517.78 (2+) → 788.41 (y ⁷ ₇ ⁺)	20.00	■	■	■	■	■	■	■	■	■	■	■	■	-
G-MAC524	VFAIIDQDK	524.79 (2+) → 802.43 (y ⁷ ₇ ⁺)	20.00	■	■	■	■	■	■	■	■	■	■	■	■	<i>Xenopus laevis</i> , <i>Xenopus tropicalis</i>
S-MER967	AGDSGDGAIGVDEFAVLVK	967.97 (2+) → 1189.68 (y ⁹ ₁₁ ⁺)	21.50	■	■	■	■	■	■	■	■	■	■	■	■	-
S-MER794	(N-Acyl)AFSGILADADIAAALK	794.93 (2+) → 1187.59 (b ₁₂)	25.00	■	■	■	■	■	■	■	■	■	■	■	■	-
S-MER612	IGVDEFTAMLK	612.32 (2+) → 954.46 (y ⁸ ₈ ⁺)	22.50	■	■	■	■	■	■	■	■	■	■	■	■	-
S-MER736	AEGTFTHGFEFTK	736.35 (2+) → 966.46 (y ⁸ ₈ ⁺)	23.00	■	■	■	■	■	■	■	■	■	■	■	■	-
S-MER721	AEGTFTHGVEFTK	721.36 (2+) → 936.49 (y ⁸ ₈ ⁺)	23.50	■	■	■	■	■	■	■	■	■	■	■	■	-
S-MER590	IGVDEFAAMVK	590.31 (2+) → 910.43 (y ⁸ ₈ ⁺)	22.50	■	■	■	■	■	■	■	■	■	■	■	■	<i>Trachurus japonicus</i> and others ^b
S-MER973	AGDSGDGAIGVDEWAALVK	973.46 (2+) → 1087.57 (y ⁹ ₁₀ ⁺)	23.00	■	■	■	■	■	■	■	■	■	■	■	■	-
S-MER987	AGDSGDGAIGVDEWAVLVK	987.48 (2+) → 1115.61 (y ⁹ ₁₀ ⁺)	22.50	■	■	■	■	■	■	■	■	■	■	■	■	<i>Gadus morhua</i>

^a *m/z* = mass/charge ratio. Key: S1, *M. merluccius*; S2, *M. capensis*; S3, *M. senegalensis*; S4, *M. polli*; S5, *M. paradoxus*; S6, *M. hubbsi*; S7, *M. gayi*; S8, *M. australis polylepis*; S9, *M. australis australis*; S10, *M. productus*; S11, *M. bilinearis*; S12, *Macruronus* spp. A filled square denotes the presence of a peptide biomarker and an empty square the absence of a peptide biomarker. ^b*Sparus aurata*, *Fundulus grandis*, *Fundulus similis*, *Fundulus heteroclitus*, *Hypophthalmichthys nobilis*, *Paralichthys olivaceus*, *Theragra chalcogramma*.

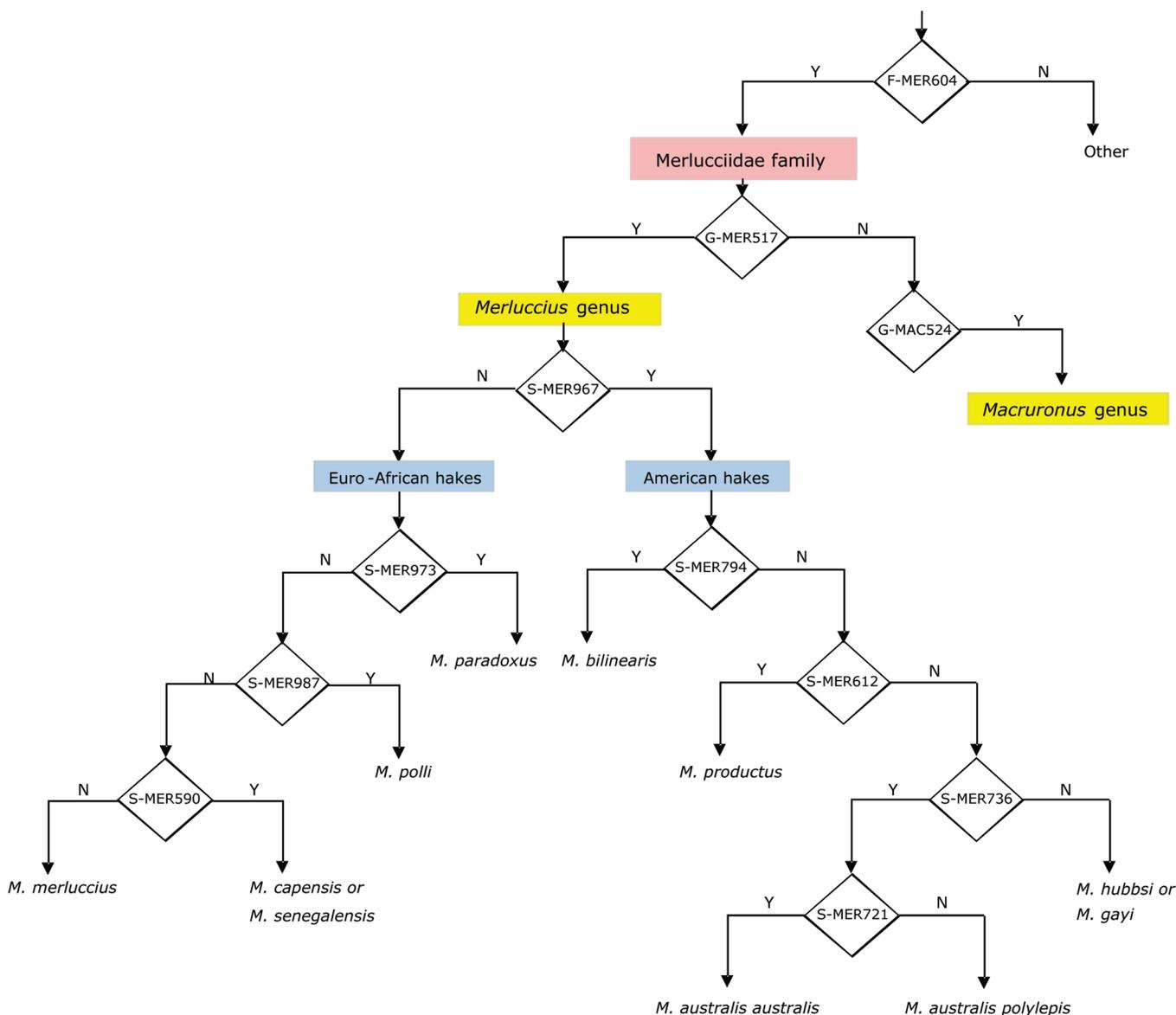


Figure 2. Flow diagram for a systematic discrimination of Merlucciidae species using only 11 specific tryptic peptides from PRVBs. Y denotes the presence and N the absence of a particular peptide.

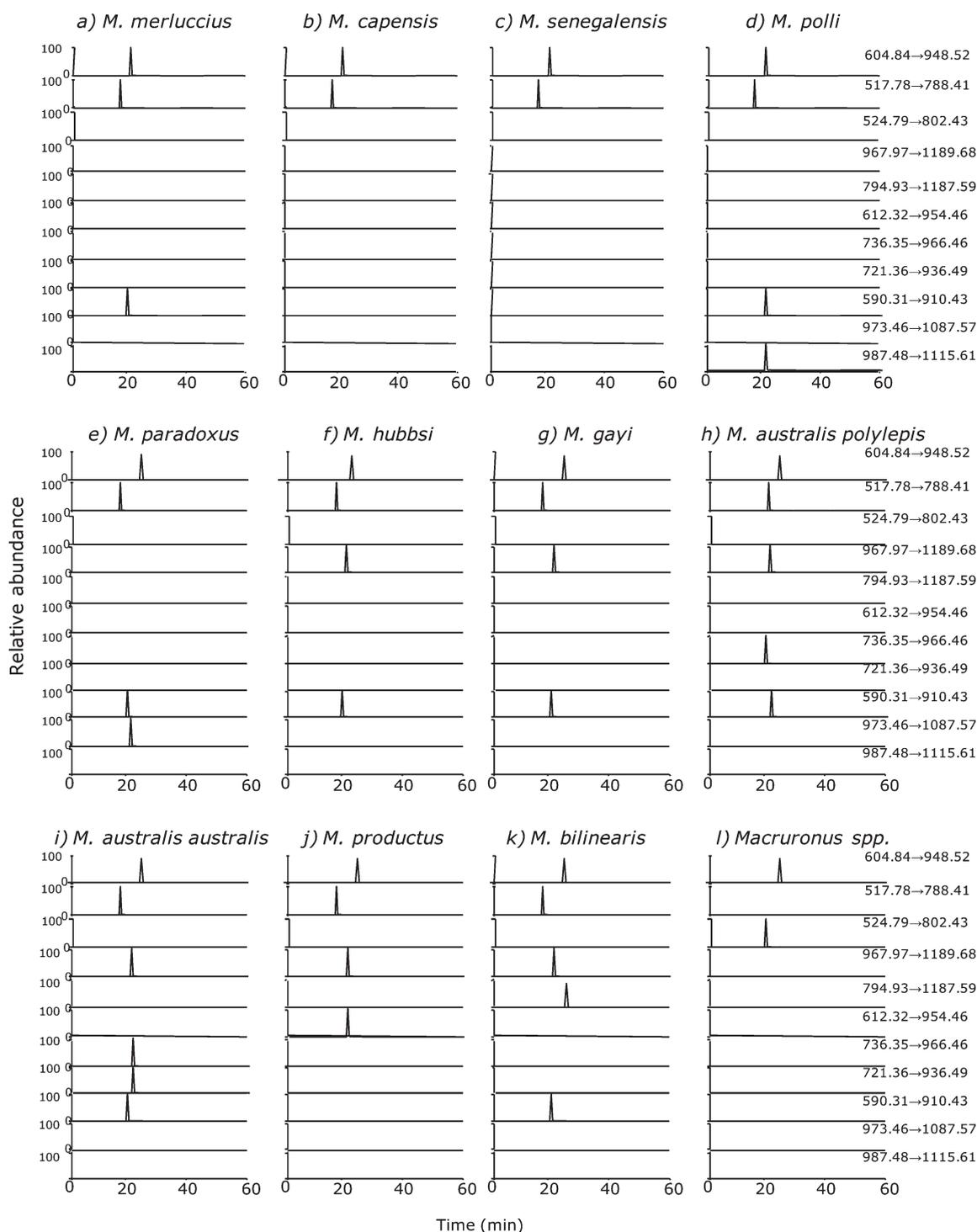


Figure 3. Reference SMIM traces for each Merlucciidae species, plotting the corresponding canonical transition for each PRVB tryptic peptide biomarker.

of the fragments, which mostly corresponded to γ ions. Therefore, the combination of highly sensitive transitions (precursor $m/z \rightarrow$ fragment m/z) (Table 2), together with the use of simple peptide mixtures (coming mainly from PRVBs), made possible the representation of specific transitions with a high signal-to-noise (S/N) ratio. Tracing these transitions for each peptide biomarker, according to the flow diagram described in Figure 2,

made possible unequivocal identification of all the reference hake species (Figure 3).

The results obtained matched precisely those obtained by DNA analysis. However, the time needed for performing both techniques was extremely different. At least 24 h was needed for a genetic identification by FINS, while only less than 2 h was necessary to complete the identification following the proposed

Table 3. Results of the SMIM Assay of Commercial Hake Products^a

product	declared species	species identified by HIFU + SMIM, time <2 h ^b
CP1	<i>M. capensis</i> or <i>M. paradoxus</i>	<i>M. paradoxus</i>
CP2	<i>M. australis</i>	<i>M. australis polylepis</i>
CP3	<i>M. capensis</i> or <i>M. paradoxus</i>	<i>M. paradoxus</i>
CP4	<i>M. capensis</i> or <i>M. paradoxus</i>	<i>M. capensis</i>
CP5	hake unidentified	<i>M. paradoxus</i>
CP6	hake unidentified	<i>M. productus</i>
CP7	hake unidentified	<i>M. paradoxus</i>
CP8	hake unidentified	<i>Macruronus</i> spp.
CP9	hake unidentified	<i>Macruronus</i> spp.
CP10	hake unidentified	<i>M. paradoxus</i>

^a*M.* indicates the *Merluccius* genus. ^bThese results were validated by genetics after 24 h of analysis.

strategy. To our knowledge, this is the fastest method to achieve food species authentication.

Application to Commercial Sample Identification. To validate this new strategy, 10 commercial hake products were subjected to analysis for their authentication. Table 1 summarizes the products that were tested, which had been previously subjected to one or more processing treatments, even precooked. Two main label categories were observed: labels with species declared (four samples) and labels with no declared species, which presented only a general commercial name (i.e., hake) (six samples). Results for the identification of the commercial samples using the designed strategy are shown in Table 3 and in Figure S-3 in the Supporting Information. Samples with complete declared labeling were found to be mostly correct (CP1–CP4). However, some products with no declared species were assigned to species belonging to the *Macruronus* genus (CP8 and CP9). This genus presents a low commercial value and is not as valued by the consumer.²⁵ We have presented here a strategy that allows the fast detection of mislabeling practices in these fish products, and moreover, this work also shows that the use of thermostable proteins allows the application of this fast monitoring method to battered precooked products (CP6–CP10).

CONCLUSIONS

A new strategy for the fast monitoring of species-specific peptide biomarkers for foodstuff authentication has been proposed. The principle it is based on is the use of a fast purification step of the target protein, the acceleration of in-solution protein digestion by HIFU, and the monitoring of several peptides by SMIM in a linear ion trap mass spectrometer. The use of the SMIM mode of scanning allows for a more simple setting of the procedures, giving the possibility of obtaining full MS/MS information necessary for the validation of the structure of the component, and allows the choice of the most adequate transitions for each precursor ion, with attention to the criteria of sensitivity and selectivity. Thus, in combination with a fast purification and digestion step of the target protein, the most sensitive transitions may be considered for all the markers.

With this new strategy, all relevant commercial fish species belonging to the Merlucciidae family present in any seafood product can be unequivocally identified in less than 2 h. The

present strategy constitutes the fastest method for peptide biomarker monitoring by targeted proteomics described up to now, whose application in the food quality control area provide the authorities an effective, competitive, and rapid method of food authentication and traceability that guarantees quality and safety to the consumers.

ASSOCIATED CONTENT

S Supporting Information. Additional information as noted in text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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