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Application of Denaturing High-Performance Liquid Chromatography (DHPLC) for the Identification of Fish: A New Way To Determine the Composition of Processed Food Containing Multiple Species

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ABSTRACT: The identification of fish species in transformed food products is difficult because the existing methods are not adapted to heat-processed products containing more than one species. Using a common to all vertebrates region of the cytochrome *b* gene, we have developed a denaturing high-performance liquid chromatography (DHPLC) fingerprinting method, which allowed us to identify most of the species in commercial crab sticks. Whole fish and fillets were used for the creation of a library of referent DHPLC profiles. Crab sticks generated complex DHPLC profiles in which the number of contained fish species can be estimated by the number of major fluorescence peaks. The identity of some of the species was predicted by comparison of the peaks with the referent profiles, and others were identified after collection of the peak fractions, reamplification, and sequencing. DHPLC appears to be a quick and efficient method to analyze the species composition of complex heat-processed fish products.

KEYWORDS: surimi, DHPLC, fish species identification, *cytochrome b* gene

INTRODUCTION

Mislabeling might be a common practice in the fish industry. Three-quarters of the fish sold in the United States as red snapper (*Lutjanus campechanus*) belong to another species.[1] In another study 16 of 19 samples of surimi-based products were mislabeled.[2] Substitution of fish species can have economic, environmental, health, and product quality consequences. To improve the quality of surimi pastes resulting from lower quality species, more starch and other additives are added to the final product.[3] Environmental consequences result from the fact that mislabeling could adversely affect estimates of fish stock sizes if it influences the reporting of catch data that are used in fisheries management.[1] It could also hide the fishing of endangered species. Mislabeling can lead to involuntary ingestion of fish species that, in highly allergic individuals, can lead to serious or even fatal allergic reactions.[4] Mislabeling may also be involuntary due to fishing of morphologically similar species in the same fishery. It is thus important to have a quick and reliable method that can identify the fish species present in preparations such as surimi.

Surimi is prepared of beheaded boneless fish by mincing, rinsing, dewatering, and concentrating the myofibrillar proteins. The obtained paste is then stored frozen until its use for the preparation of flavored products such as crab-flavored surimi in the form of crab sticks.[5] Surimi is a white paste in which the identification of fish species based on their external morphological features is impossible. Moreover, fish is usually processed to some extent for preservation purposes prior to transportation. Different methods have been developed for the identification of fish species. These include protein-based methods such as isoelectric focusing of proteins,[6] reverse phase high-performance liquid chromatography (HPLC),[7] sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE),[3] or urea isoelectric focusing.[8−10] These techniques depend on the integrity of the fish proteins and are not reliable when products are heat-processed. Enzyme-linked immunosorbent assays (ELISAs) based on the recognition of a heat-stable protein exist, but they can identify only certain genera of fish.[11] Recently, fingerprinting techniques based on the analysis of DNA are being developed. DNA is a robust molecule that is not damaged during extended periods of fish storage or by heat processing and freezing.[2] Direct sequencing is the most efficient method for species identification but can be applied only to pure fish samples containing only one species. Random amplification of polymorphic DNA (RAPDs), single-strand conformation polymorphism (SSCP), and restriction fragment length polymorphism (PCR-RFLP) have been developed.[12,13] The range of application of these methods is, however, limited. Some of them are applicable only for the identification of raw fish, of species in the same family, or of specific species known to be often mislabeled. Amplification of a common to all fish species region of the *cytochrome b* (*cyt b*) gene, followed by sequencing and phylogenetic study was used by another group for the identification of species in surimi-based products.[2] A method allowing the identification of tuna species in commercial cans by minor groove binder probe real-time polymerase chain reaction analysis has also been developed.[14] These methods are efficient; however, they are reliable when one or few species are used and cannot be applied to multispecies products such as crab sticks.

Denaturing HPLC (DHPLC) has been successfully applied for the analysis of different bacterial communities such as urinary tract pathogens,[15] marine microbial populations,[16] intestinal microbiota,[17] and others. This method is based on the amplification of a portion of a conserved bacterial gene, often the 16S rRNA gene, using universal primers. This gene is composed of

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conserved regions that allow the use of universal primers, as well as regions exhibiting interspecies variability which allow the discrimination of amplicons from different species. The amplicons are then separated by HPLC under conditions of partial heat denaturation. Their retention time depends on their thermal stability. Thus, each species has a characteristic DHPLC profile. The analysis of complex samples generates complex profiles that result from the accumulation of the profiles of each species.

We have adapted this DHPLC method for the analysis of fish species in complex preparations such as crab sticks. We used universal primers to amplify a portion of the *cyt b* gene, which is commonly used in fingerprintring techniques.[2,18] The obtained amplicons were separated by DHPLC. Using peak patterns and/or fraction collection, we were able to estimate the number of fish species in crab sticks and, for most of them, to predict their identity.

MATERIALS AND METHODS

Materials. Thirteen fish species were included in the in silico study: *Trachurus murphyi* (jack mackerel), *Engraulis ringens* (anchoveta), *Exocoetus volitans* (flying fish), *Micromesistius australis* (southern blue whiting), *Micromesistius poutassou* (northern blue whiting), *Theragra chalcogramma* (Alaska pollack), *Saurida elongata* and *Saurida undosquamis* (lizard fish), *Macruronus novaezelandiae* (blue grenadier), *Macruronus magellanicus* (Patagonian grenadier), *Merlucius productus* (Pacific whiting), and *Nemipterus* sp. and *Nemipterus marginatus* (threadfin bream). Twenty-six fish samples were analyzed for their inclusion in the reference library. Seventeen were pastes labeled as containing one fish species and eight were dried whole fish or fillets. The fish pastes were labeled as *Trachurus murphyi*, *Engraulis ringens*, *Exocoetus volitans*, *Micromesistius australis*, *Theragra chalcogramma*, three pastes of *Saurida* sp., two pastes of *Macruronus* sp., *Merlucius productus*, *Nemipterus* sp. and *Nemipterus marginatus*, *Pangasius sutchi* (Pangas), *Hypophthalmichthys molitrix* (silver carp), *Salmo salar* (Atlantic salmon), and *Upeneus* sp. (goat fish/red mullet). Dried fish was *Exocoetus volitans*, *Saurida* sp. (Saury), *Saurida undosquamis*, two *Nemipterus* sp., *Upeneus sulphureus*, *Priacanthus* sp. (bigeye snapper), and *Pennahia* sp. (croaker). All samples were stored at −20 °C.

Ten different commercial crab sticks were also tested. They were purchased at different stores, transported in insulated boxes to the laboratory, and kept at the temperatures indicated on the package until analysis.

In Silico Analysis of Sequences. The DNA sequences of the *cyt b* gene of 13 fish species were retrieved from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and Fishtrace (http://www.fishtrace.org) databases.

The sequences were compared by clustering using CLUSTALX version 2.0.10 software and applying the neighbor-joining method.

DNA Extraction. Total DNA was extracted from 1 g of sample. After an incubation of 45 min at 50 °C in extraction buffer, the sample was centrifuged for 15 min at 3500 rpm. DNA in the aqueous phase was purified on anion-exchange columns (Qiagen Genomic-tip 100/G). After elution, DNA was precipited by adding isopropanol and was centrifuged for 20 min at 12000 rpm. The DNA pellet was then washed with 70% ethanol and dissolved in 100 μL of TE buffer. After spectrophotometric analysis at 260 nm with NanoDrop ND-1000, DNA samples were diluted to a final concentration of 20 ng/μL.

DNA Amplification and Reconditioning PCR. PCR was used to amplify a variable 358 bp fragment of the mitochondrial *cyt b* gene. The primers were adapted from ref 18.

PCR reactions were carried out in a final volume of 25 μL, containing 1× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 0.5 μM of each primer, 100 ng total DNA, 2.5 mM $MgCl_2$, 0.1 mM of each dNTP, and 1 U Taq DNA polymerase (HotGoldStar Eurogentec). PCR cycles were performed on a GeneAmp PCR System 9700 (Applied Biosystems) using the following conditions: 15 min at 95 °C, followed by 30 cycles of 45 s at 94 °C, 45 s at 50 °C, and 1 min at 72 °C. A final extension was performed at 72 °C for 10 min. The size and quality of the PCR products were checked using a 1.5% agarose gel. The amplification products were then subjected to a reconditioning PCR[19] as follows: 5 μL of amplification products was reamplified in a 50 μL mixture of 1× PCR buffer (500 mM KCl, 100 mM Tris-HCl, pH 8.3), 0.5 μM of each primer, 1.5 mM $MgCl_2$, 0.1 mM of each dNTP, 1 U Taq DNA polymerase (HotGoldStar; Eurogentec), and distilled sterile water. After 15 min at 95 °C, five amplification cycles were performed using the conditions described above. The PCR products were purified with the QIAquick PCR Purification Kit (Qiagen).

Table 1. DHPLC Running Conditions

time (min)	% buffer A	% buffer B	% buffer C
0	51	49	
0.5	46	54	
3.25	40	60	
6.25	30	70	
7.25	20	80	
7.4	0	0	100
7.9	0	0	100
8	51	49	
9	51	49	

DHPLC Analysis. Purified PCR products were analyzed by DHPLC on the WAVE Microbial Analysis System (Transgenomic) using a DNASep HT cartridge (Transgenomic). After equilibration, 5 μL of PCR products was injected in the column, and elution was achieved at a flow rate of 0.9 mL/min. A two-eluant buffer system was used with buffer A (WAVE Optimized Buffer A) composed of 0.1 M TEAA, pH 7, and buffer B (WAVE Optimized Buffer B) composed of 0.1 M TEAA, pH 7, with 25% acetonitrile. Buffer D (WAVE Optimized Buffer D) containing 25% water and 75% acetonitrile was used for the column wash. Gradient rates for the elution are summarized in Table 1. Oven temperature was set to 57.4 °C. Separated PCR products were detected with an HSX-3500 fluorescence detector in the presence of an intercalating agent (WAVE Optimized HS Staining Solution I). The results were analyzed with Navigator software version 1.6.4 (Transgenomic).

Desired fractions were collected using the FCX200 fraction collector. After reamplification, the PCR products were sequenced (MilleGen). The NCBI "Nucleotide" database and the MegaBlast and Blast-n algorithms were used for sequence analysis.

RESULTS AND DISCUSSION

In Silico Analysis of Sequences. Amplification of a variable region of the 16S gene and subsequent DHPLC analysis are efficiently used for the characterization of bacterial communities.[16,17,20,21] We have adapted this method for the characterization of products comprising mixed fish species by amplifying a common portion of the *cyt b* gene. The *cyt b* gene is highly conserved with regions that are variable even in very close species. The sequences of this mitochondrial gene have been determined for many vertebrates, including fish, and have been included in databases.[18]

First, an in silico study was conducted to evaluate the interspecies genetic variability and thus estimate the applicability of this DHPLC method to fish species. The available sequences of

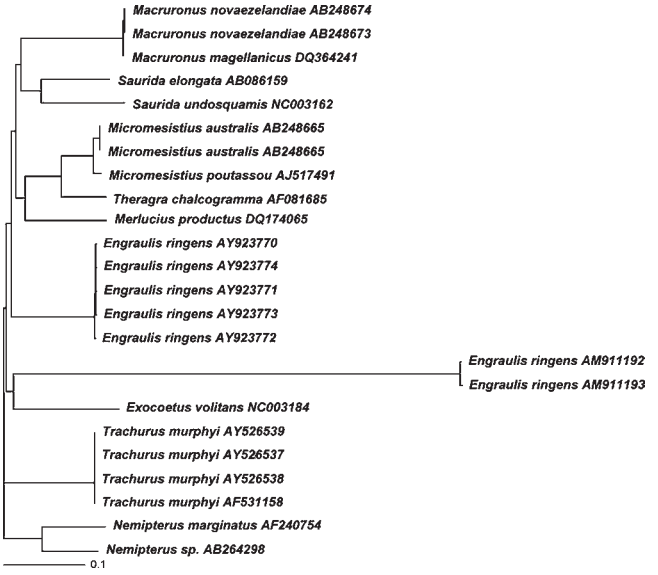

Figure 1. Neighbor-joining tree based on GenBank sequences of fish species commonly used for the preparation of surimi.

the *cyt b* gene of 13 fish species commonly used in surimi were retrieved. These sequences correspond to the fragments that have been previously sequenced; consequently, they can be located at different portions of the gene. We proceeded to their clustering and elaborated a neighbor-joining tree (Figure 1). We thus estimated the degree of genetic variation among the retrieved sequences. The redundant sequences belonging to the same fish species were eliminated. Sequences displaying big genetic differences compared to the others were also eliminated because they correspond to a different portion of the gene and would not be targeted by the chosen universal primers. The sequences that were selected for the subsequent in silico studies are summarized in Table 2.

The multiple alignment of the selected sequences with the chosen primers allowed the prediction of the sequences of the fragments that will be amplified. The nucleotide sequence, the CG content, and the length of the fragment determine the thermal stability of a double-stranded DNA. Because universal primers that recognize conserved sequences in all fish species were used, all of the amplicons have the same length. Their sequences are, however, different due to the interspecies variability. Using Navigator software version 1.6.4 (Transgenomic), we predicted their temperatures of partial denaturation (Figure 2). To be separated by DHPLC, the amplicons from different fish species should have different thermal stabilities. Thus, they would present different melting behaviors and consequently different retention times. The differences in the melting temperatures should, however, not be too big to allow the simultaneous analysis of all the amplicons using one elution protocol and one temperature.

As demonstrated in Figure 2, the CG content of the chosen amplicons was between 38.6 and 48.9%. Their temperatures of partial denaturation varied between 56.5 and 59.3 °C. This shows that the amplicons have different thermal stabilities, which should allow their analysis by DHPLC. Moreover, the difference between the lowest and highest temperature is relatively small, 2.8 °C. Therefore, by applying the same temperature and elution gradient, all of the amplicons should be successfully separated

Table 2. Selected DNA Sequences for in Silico Study after Analysis of the Neighbor-Joining Tree

common name	species	GenBank accession no.
jack mackerel	*Trachurus murphyi*	AY526539
anchoveta	*Engraulis ringens*	AY923771
flying fish	*Exocoetus volitans*	NC003184
southern blue whiting	*Micromesistius australis*	AB248665
northern blue whiting	*Micromesistius poutassou*	AJ517491
Alaska pollack	*Theragra chalcogramma*	AF081685
lizard fish	*Saurida elongata*	AB086159
	Saurida undosquamis	NC003162
blue grenadier	*Macruronus novaezelandiae*	AB248674
Patagonian grenadier	*Macruronus magellanicus*	DQ364241
Pacific whiting	*Merlucius productus*	DQ174065
threadfin bream	*Nemipterus* sp.	AB264298
	Nemipterus marginatus	AF240754

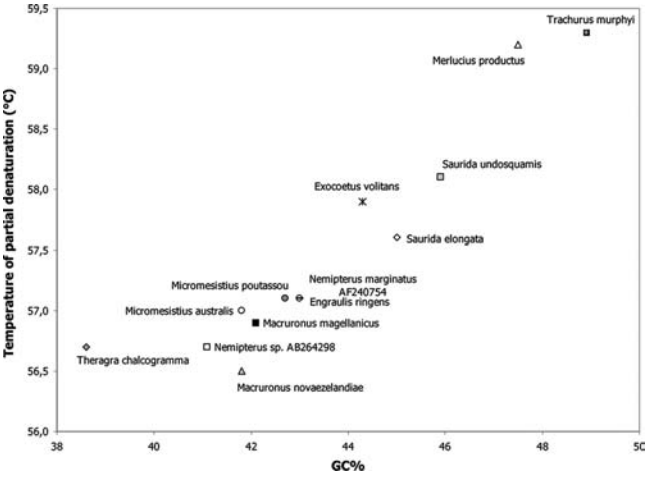

Figure 2. Theoretical temperatures of partial denaturation and CG content of the *cyt b* amplicons.

during the same step of elution. Two possible difficulties should be considered. Several amplicons could have the same temperature of partial denaturation. Their separation should still be possible due to the differences in their nucleotide composition. If, however, they also present the same CG content, their retention times during DHPLC analysis would be the same. Consequently, their profiles would be very similar and would overlap if eluted simultaneously. This was the case for the amplicons from *Nemipterus marginatus*. Two sequences were available for *Nemipterus* sp. in the GenBank database, both within the portion of interest of the *cyt b* gene. One of them led to an amplicon with the same CG content and the same temperature of partial denaturation as the amplicon from *Engraulis ringens* (Figure 2). The separation of these species can rely on the second sequence from *Nemipterus sp*. which presents different characteristics.

Our in silico study showed that the selected amplicons present both sufficient variability in their CG content and a small range of temperatures of partial denaturation. This should render their analysis by DHPLC possible.

DHPLC Analysis of Reference Samples: Dried Fish and Fish Paste. Using the data from Figure 2, four fish species (*Theragra chalcogramma*, *Nemipterus* sp., *Saurida undosquamis*, and *Trachurus*

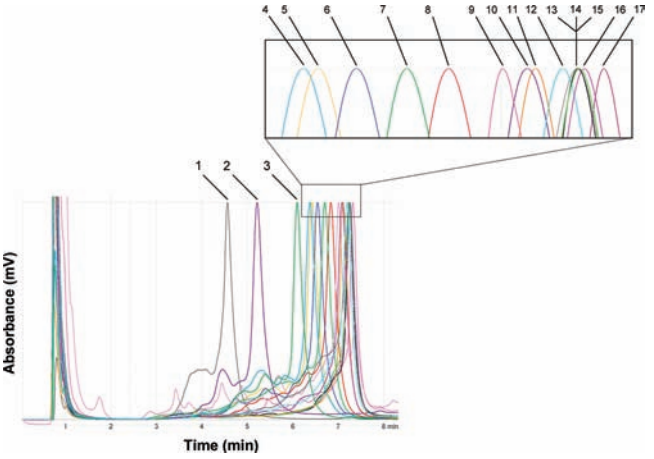

Figure 3. Referent DHPLC profiles obtained with the DNA from fish pastes and dried fish. Peaks: 1, *Pangas sutchi*; 2, *Salmo salar*; 3, *Theragra chalcogramma*; 4, *Micromesistius poutassou*; 5, *Exoceotus volitans*; 6, *Priacanthus* sp.; 7, *Saurida* sp.; 8, *Pennahia* sp.; 9, *Nemipterus* sp.; 10, *Engraulis ringens*; 11, *Hypophthalmichthys molitrix*; 12, *Trachurus murphyi*; 13, *Merlucius productus*; 14, *Saurida undosquamis*; 15, *Upeneus* sp.; 16, *Upeneus sulphureus*; 17, *Nemipterus* sp.

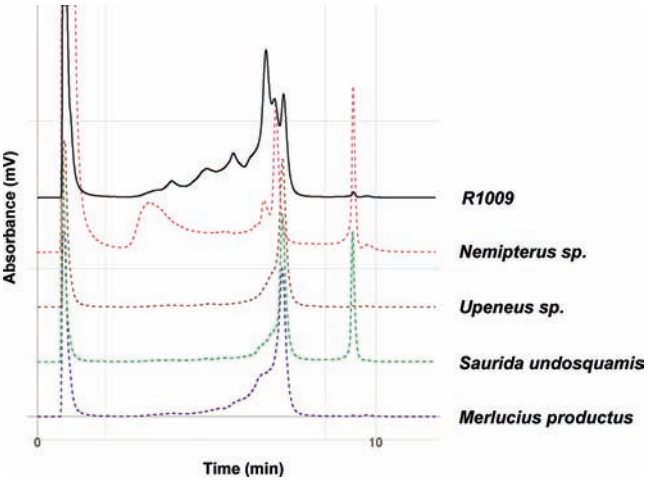

Figure 4. Example of a complex DHPLC profile of a fish paste labeled as *Upeneus* sp. The referent profiles that show the same retention time are presented beneath the sample profile. The last peak corresponds to the washing of the column.

murphyi) were chosen to create a mixture that was used for the establishment of the optimal DHPLC elution conditions. The temperatures of partial denaturation of the amplicons from these species range from 56.7 to 59.3 °C. This range of temperatures is small enough to allow the elaboration of a unique elution protocol but at the same time large enough to encompass the possible temperatures of partial denaturation of all fish species susceptible to analysis. Therefore, the elution conditions should be applicable to all fish species and a unique elution method should be able to separate all of the amplicons present in the analyzed sample.

We then proceeded to the generation of a library of referent DHPLC profiles. The DNA from the fillets, whole fish samples, and fish pastes was extracted. The *cyt b* fragments were then amplified by classic PCR followed by a "reconditioning" PCR. This second PCR, which consists of diluting the amplification product into a fresh reaction mixture followed by amplification for a low number of cycles, increases the specificity of amplification by reducing the formation of heteroduplex molecules. This enhances the resolution of the DHPLC profiles.[19] The obtained amplicons were then separately subjected to DHPLC analysis.

Samples containing amplicons from one sequence generate one major peak. The profiles from such pure samples can be used as referent. The DHPLC profiles resulting from the dried fish and fillets presented a unique major peak with a characteristic retention time, and all of them were included in the reference library. Only eight of the profiles from the fish pastes presented a simple profile containing one major peak, and they were also included in the reference library (Figure 3). The other fish paste profiles were complex, presenting more than one major peak, which was incompatible with their use as a reference.

Analysis of a DHPLC Profile by Similarity. In a complex profile each major peak is generated by one amplicon and consequently one species. Thus, the number of species can be estimated by the number of major peaks. Furthermore, it is possible to predict the identity of these species by "similarity analysis". This approach is based on the comparison of the obtained profile with the referent profiles. The similarity in the aspect and retention times between a referent peak and a peak in the sample suggests the presence of the referent species. This approach allows only an estimation of the composition of the analyzed sample. To be identified with certainty, the peak fractions should be collected, reamplified, and sequenced. The obtained sequences can then be compared to sequences in existing databases and the corresponding species identified.

Some of the fish pastes we analyzed generated complex DHPLC profiles composed of up to six major peaks. This suggests that these pastes are composed of more than one fish species even though they are labeled by the manufacturer as containing only one. The comparison of these complex profiles with the referent profiles allowed us to predict some of the species present in each paste (Figure 4).

Two potential difficulties exist when the composition of fish-based products is analyzed with DHPLC. It is possible that several species present identical DHPLC profiles with identical retention times. Consequently, in a complex profile a given peak could match several referent peaks and can thus result from the elution of one or more amplicons. This was the case with the DHPLC profile of the fish paste presented in Figure 4. This fish paste was labeled as *Upeneus* sp. The third major peak matches three referent profiles, from *Upeneus* sp., *Saurida undosquamis*, and *Merlucius productus*, and can therefore indicate the presence of one or more of these species. When there are several matching referent profiles for the same peak, the corresponding fraction can be collected and the amplicons reamplified and sequenced. If only one amplicon is present in the fraction, the corresponding species can be identified by comparison of the obtained sequence with the databases. If more than one amplicon is present, sequencing will not yield satisfying results. The amplicons should then be cloned prior to sequencing and thus identified.

The second difficulty inherent to this method is that the success of the "similarity approach" depends on the volume of the reference library. The rapid identification of a peak is not possible when a matching referent profile is lacking. Again, fraction collection, reamplification, and sequencing can be performed to identify the corresponding fish species.

Table 3. Species Identified by Similarity Analysis of DHPLC Profiles and by Sequencing

sample	peak	species identified by similarity analysis	peak	species identified by sequencing	coverage/similarity (%)	mismatch/gap
R1051	1	*Priacanthus* sp.	3	*Trachurus murphyi*	99/100	0/0
				Trachurus symmetricus	99/99	1/0
R1052	2	*Saurida* sp.	3	*Trachurus murphyi*	100/100	0/0
				Trachurus symmetricus	100/99	1/0
R1054			2	*Lutjanus decussates*	93/88	24/5
			3	*Merluccius hubbsi*	100/85	34/6
				Macruronus magellanicus	100/85	35/6
				Marluccius productus	100/85	35/6
R1062	2	*Theragra chalcogramma*	5	*Trachurus murphyi*	99/100	0/0
	3	*Priacanthus* sp.		*Trachurus symmetricus*	99/99	1/0
	4	*Pennahia* sp.				
R1064	3	*Priacanthus* sp.	3	*Theragra chalcogramma*	90/82	35/7
				Theragra finnmarchica	90/82	35/7
				Gadus morhua	89/80	35/7
			5	*Siganus guttatus*	85/89	17/1
				Premnas biaculeatus	100/86	25/3
R1065	3	*Priacanthus* sp.	6	*Pomacentrus reidi*	95/84	42/1
	4	*Pennahia* sp.		*Anisotremus virginucus*	94/84	43/1
R1066	3	*Priacanthus* sp.	4	*Nemipterus bathybius*	94/89	23/2
				Lutjanus decussates	94/86	30/2
			5	*Macruronus magellanicus*	99/88	23/5
				Merluccius hubbsi	99/88	23/5
				Merluccius productus	99/88	23/5
R1067	2	*Theragra chalcogramma*	1	*Takifugu chinesis*	55/66	45/5
	4	*Priacanthus* sp.	7	*Trachurus murphyi*	100/98	4/3
	5	*Saurida* sp.		*Trachurus symmetricus*	100/98	5/3
R1071	1	*Theragra chalcogramma*	3	*Trachurus murphyi*	100/100	0/0
	2	*Pennahia* sp.				

DHPLC Analysis of Commercial Crab Sticks. Samples of 10 commercial crab sticks were analyzed using the developed DHPLC method. Following DNA extraction, amplification of the chosen fragment of the *cyt b* gene, and reconditioning PCR, the obtained amplicons were subjected to DHPLC analysis.

Each crab stick sample presented a characteristic DHPLC profile, which reflects its composition. The number of fluorescence peaks allowed us to estimate the number of fish species contained in the analyzed product. The profiles presented between three and seven major peaks, indicating that the samples contained at least between three and seven fish species. Using the reference library and the similarity approach, we partially determined the composition of the analyzed products. The fluorescence peaks identified with this method are summarized in Table 3, second and third columns.

The analysis of sample R1071 is presented in Figure 5 as an example. This sample generated three major peaks; therefore, it is composed of at least three fish species. The retention times of the first and second peak match the retention times of the peaks generated by *Theragra chalcogramma* and *Pennahia* sp. The third peak, however, matches four referent peaks, from *Saurida undosquamis*, *Merlucius productus*, *Trachurus murphyi*, and *Upeneus* sp. The identification of this peak by similarity is therefore impossible.

Among the other tested crab sticks, certain peaks were impossible to identify because of the lack of a matching profile in the reference library. The reference library we created contains only 17 profiles. This is clearly not enough as a large variety of fish can be used to prepare crab sticks. Moreover, the consumption of surimi-based products is expanding, leading to the depletion of the world's resources of the most used fish. Thus, novel species are used for the preparation of surimi. More referent fish species need to be included to expand our library. This will facilitate the identification of a larger number of peaks by similarity analysis.

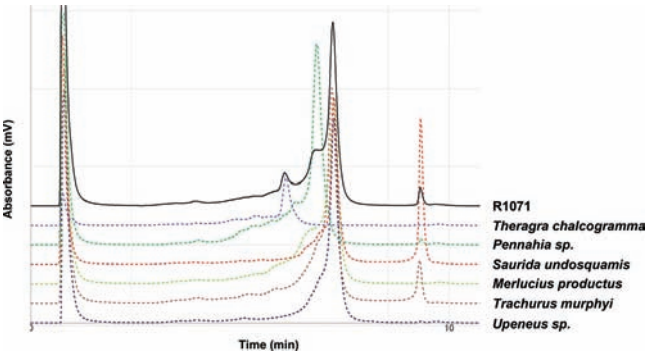

Figure 5. DHPLC analysis of commercial crab sticks. The referent profiles that have the same retention time are shown beneath the sample profile. The last peak corresponds to the washing of the column.

Most of the unidentified major peak fractions from the analyzed crab sticks, as well as some unidentified minor peak fractions, were collected, reamplified, and sequenced. The obtained sequences were compared with the available sequences in the NCBI Nucleotide database. This allowed their identification. The species found in the analyzed crab sticks by similarity analysis and by sequencing are summarized in Table 3.

The sequencing of the third peak from sample R1071 showed that this peak is generated by *Trachurus murphyi*. Moreover, 100% coverage and similarity and 0 gap and mismatch were obtained. For most peak fractions, however, the obtained sequences matched two or three species in the database. This demonstrates one of the limits of this DHPLC-based identification method. In cases when more than one species is identified after sequencing, complementary studies might be useful. The analysis of the coverage/similarity and mismatch/gap values may indicate which species are more likely to be present in the analyzed sample. Databases such as Fishbase contain information about the size, habitat, and special features of each species. This information may allow the elimination of species that are unlikely to be found in surimi because they are very rare, of small size, not edible, or cultivated only in aquariums.

We noted that from all of the analyzed crab sticks the contained fish species were indicated on only two packages. All others were labeled as containing "fish meat". Previous studies have shown that mislabeling, where a high-quality species is substituted by a low-quality species, might be a frequent practice in the fish industry.[2] As the morphological identification of the contained fish species is impossible, it is important to have a reliable and quick method for the identification of species used in fish processed products.

Our results demonstrate that the method we have developed can successfully be applied for the identification of fish species in heavily heat-processed products such as crab sticks. The applicability of this method expands beyond the simple identification of the composition of crab sticks. Apart from the evident economical application, the possibility to identify fish species in food products can be useful in health and environmental issues. As an example, combining this method with the data from Fishbase, the geographical origin of each fish can be estimated. Thus, illegal fishing of endangered species or fishing in prohibited areas could be detected. Moreover, this method could easily be applied to other products composed of a mixture of fish species that undergo heat treatment, such as fish fingers.

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