

Optimization of the pepsin digestion method for *anisakis* inspection in the fish industry

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Abstract

During the last 50 years human anisakiasis has been rising while parasites have increased their prevalence at determined fisheries becoming an emergent major public health problem. Although artificial enzymatic digestion procedure by CODEX (STAN 244-2004: Standard for salted Atlantic herring and salted sprat) is the recommended protocol for anisakids inspection, no international agreement has been achieved in veterinary and scientific digestion protocols to regulate this growing source of biological hazard in fish products. The aim of this work was to optimize the current artificial digestion protocol CODEX with the purpose of offering a faster, more useful and safety procedure than the current one for anisakids detection. To achieve these objectives, the existing pepsin chemicals and the conditions of the digestion method were evaluated and assayed in fresh and frozen samples, both in lean and fatty fish species. New conditions were introduced with the objective of being tested, thus improving the current digestion protocol. Results showed that the new digestion procedure considerably reduces the assay time, and it is more handy and efficient (the quantity of the resulting residue was considerably lower after less time) than the largely used from CODEX STAN 244-2004. In conclusion, the new digestion method herein proposed based on liquid pepsin format, is an accurate

reproducible and friendly to use off-site tool, that can be useful in the implementation of screening programs for the prevention of human anisakidosis (and associated gastroallergic disorders) due to the consumption of raw or undercooked contaminated seafood products.

Keywords

Anisakis; CODEX STAN 244-2004; digestion method; fish; liquid pepsin.

1. Introduction

Nematode parasites of *Anisakis* spp. are recurrently found in the abdominal cavity (including gut) and flesh of a large variety of fish and cephalopod species of commercial interest, regularly consumed by humans. The third larval stage is transmitted through the consumption of raw or minimally processed seafood, and may cause pathogenic diseases like gastric or intestinal anisakiasis (Sakanari and McKerrow, 1989; Kikuchi et al., 1990; Esteve et al., 2000; Lopez-Serrano et al., 2003; Nawa et al., 2005; Mineta et al., 2006), and gastro-allergic disorders (Alonso-Gómez et al., 2004; Plessis et al., 2004; Nieuwenhuizen et al., 2006; Audicana and Kennedy, 2008; Hochberg and Hamer, 2010). The significance of this disease affecting both, fish processing and public health, is growing as a consequence of the high incidence and the lasting unawareness of this potential threat among consumers. During the last 50 years, this economical and sanitary problem has been growing as parasites have increased their prevalence, being more relevant in North Atlantic fisheries (Smith and Wootten, 1979; McClelland et al., 1985; Adams et al., 1997; Abollo et al., 2001; Rello et al., 2009). Consequently, several methods have been developed for detection, diagnosis and identification of parasites in fish, from visual inspection (Hartmann and Klaus, 1988), light microscopy (Rijpstra et al., 1988), candling (Wold et al., 2001; Butt et al., 2004), pepsin digestion (Lysne et al., 1995; Lunestad, 2003; Thien et al., 2007; Thu et al., 2007), UV illumination (Adams et al., 1999; Levsen et al., 2005; Marty, 2008), ultrasound (Hafsteinsson et al., 1989; Nilsen et al., 2008), X-Rays (Nilsen et al., 2008), conductivity (Nilsen et al., 2008), electromagnetism (Haagensen et al., 1993; Choudhury and Bublitz, 1994), magnetometry (Jenks et al., 1996), immunodiagnoses (Xu et al., 2010), multilocus electrophoresis (Mattiucci et al., 1997; Abollo et al., 2001), RT-PCR (Fang et al., 2011), real-time FRET (Fluorescence Resonance Energy Transfer) (Monis et al., 2005; Intapan et al., 2008), PCR (Zhu et al., 2002; Abe et al., 2005; Pontes et al., 2005), to Imaging Spectroscopy (Heia et al., 2007). Nevertheless, although all

these methods have been used and are being applied by fishery operators or laboratories as integrated strategies in official and self-control tests, none of them has been accepted as the international reference protocol accomplish with the industrial requirements. That lack of a golden standardization for any of the above given methods, mainly for a fast and easy visual detection, has historically hampered the consensus of parasite detection and diagnosis protocols at the fishing industry, thus reducing consumer confidence towards companies.

Specifically, acidified pepsin solution has been largely applied as a confirmatory invasive protocol to detect absence or presence of nematodes in fish products (Lunestad, 2003), and as a tool to quantify parasitic infections and to estimate the number of parasites in the fish musculature (Lysne et al., 1995; Thien et al., 2007; Thu et al., 2007). Some additional variations of the pepsin digestion method from CODEX STAN 244-2004 protocol have been developed by some authors (CX/FFP 08/29/7; Dixon, 2006) with attempts to go further. According to the two definitions of "optimization" provided here ("to achieve maximum efficiency in storage capacity or time or cost" and "to make as effective, perfect, or useful as possible"), the aim of this work was to improve and optimize the current artificial digestion protocol of CODEX by (1) evaluating three different brands of commercial pepsins, (2) implementing new conditions on the basis of the current digestion procedure, and (3) comparing the new practice proposed with the currently used one. As a result, a new analytical methodology is offered based on the modification of the existing artificial digestion of fish flesh provided by CODEX.

2. Materials and methods

2.1. Samples

Fresh and frozen fishes, both of European hake (*Merluccius merluccius*) and Atlantic mackerel (*Trachurus trachurus*), were used as representative samples of lean and fatty fish species, respectively. Three different commercial pepsins were preselected to be evaluated: the recommended reagent in CODEX protocol (pepsin 1), a novel liquid format (pepsin 2) and a commonly used pepsin (pepsin 3). Proteolytic activities indicated by the three manufacturers were 2000 FIP U/g, 660U Ph Eur/ml and 800-2,500 U/mg of protein, respectively. Authors understand that enzymatic activities specified do not need verification because it would not be viable to develop routine protocols, since it should be necessary to perform a check of any pepsin before its use. Therefore, in order to minimize any imprecision related

to the reagents, all of the pepsins used in this study were acquired, stored, prepared and treated properly under the same criteria and under identical conditions (specified by manufacturers).

2.2. Pepsin assays

Briefly, six aliquots of 25g each from both fresh and frozen fish species were digested with the three different pepsins at 37°C during 30 minutes in an ACM-11806 Magnetic Stirrer with thermostated heating Multiplate, using a weight/volume pepsin ratio of 1:20, understanding that ratio as one gram of fish for twenty milliliters of a 0.5% pepsin solution in HCl 0.063M pH 1.5. Undigested muscle residues of each kind of fish and pepsin were weighted and compared, without taking into account the weight due to the parasites in the positive samples.

In order to compare the two pepsins that previously had given higher percentages of digested muscle, appropriate calculations were made to determine the pepsin dose necessary in each case to prepare solutions containing the same proteolytic activity. To this end, density of liquid pepsin (1.215 Kg/m³) and equivalences units were taking into account (Table 1). Enzymatic activity was set at 5000 FIP U/g, because this is the resultant value when applying the CODEX method. One more time, six samples of 25g each of fresh hake and mackerel were digested with the two pepsins during 30 minutes at 37°C, using a weight/volume ratio (1:20). Undigested muscle residues of each kind of fish and pepsin were weighted and compared again, without taking into account the weight due to the parasites in the positive samples.

2.3. Electrophoretic profile

Besides digestions assays, electrophoretic profiles of the two previously selected pepsins were obtained in vertical SDS-PAGE discontinuous gels (10% acrylamide in the separating gel). Electrophoretic separations were carried out at 40 mA/slab, 100V and 150W, using Tris-Tricine buffer (Schägger and von Jagow, 1987) in a Mini Protean® System (BioRad Laboratories, Hercules, USA). Low molecular weight-SDS Marker Kit (GE Healthcare, Buckingham, UK) was employed as reference. The gels were stained with silver, following the protocol described by Heukeshoven and Dernick (1985).

2.4. New assay conditions

Once the best pepsin was selected after the electrophoretic profile was performed, three innovative attributes were introduced and tested during digestions in fresh and frozen samples, with the aim of making more accessible the fish muscle to the enzyme action: (1) the use of the selected pepsin, (2) a new weight/volume ratio for digestion solution (1:10 instead of 1:5 that CODEX protocol recommends) and (3) the homogenization and flattening of the samples before digestion in a blender for food (Smasher® AES Chemunex). For testing the reproducibility and comparing CODEX protocol and the resulting new method after introducing new conditions (hereinafter “LP” protocol), a total of 240 digestions were carried out employing at each time 200 g of fresh and frozen hake and mackerel muscles; 120 digestions following the CODEX protocol and 120 testing the LP protocol. All assays were carried out with a pepsin concentration of 0.5% at an acidified (pH=1.5 with HCl at 0.063M) pepsin enzyme solution, and incubation temperature of 37°C. After finishing every digestion, undigested muscle residues from each fish type and method were weighted, recorded and compared, without taking into account the weight due to the parasites in the positive samples.

2.5. Larvae viability

In order to verify larvae viability during the definitive assays, 40 digestions (5 from each type of fish species, forms of preservation and method) from the 240 digestions that were carried out, were controlled for this aspect. Anisakid-positive samples were arranged by introducing 10 larvae inside anisakid-negative samples of muscle for digestion. All larvae inoculated were extracted from the muscle where they would be introduced, so larvae inoculating fresh fish samples were alive before digestions (not in the case of frozen fish digestions). Separately, 10 live and free (without muscle) anisakid larvae were digested at 37°C in 1000 ml digestion solution following LP protocol in order to check their integrity after 210 minutes of digestion.

3. Results

3.1. Samples and pepsin assays

The significance of digestions after using the three different commercial pepsins at the same concentration (0.5%) and different enzymatic activity between them is shown in Table 2. This table also illustrates digestion conditions during these assays. The two pepsins that provided higher percentages of digested muscle, both for lean and for fatty fish samples, were pepsins 1 and 2.

When both pepsins were compared by equaling their enzymatic activities to 5000 FIP U/g, pepsin 2 showed the fewest fish residue in both types of fish as Table 3 demonstrates. This table also illustrates pepsins proprieties, their enzymatic activity (in FIP units), the required weight used of each one to equal enzymatic activities, and digestion conditions during these assays.

3.2. Electrophoretic profile

SDS-PAGE profile of pepsin 2 extract showed one band with a molecular weight corresponding to pepsin. However pepsin 1 offered a multiple band profile below to that molecular weight (Figure 1), perhaps as autolytic consequence.

3.3. New assay conditions

According to obtained results at initial pepsin assays and due to its proteolytic and handling characteristics, liquid pepsin (n°2) was the selected reagent to test the new conditions (LP protocol) simultaneously to the established and current digestion protocol (CODEX). In order to obtain a maximum weight of 1 g of undigested residue in the faster of the two tested methods, for both procedures fresh samples of *M. merluccius* were digested during 20 minutes, and frozen ones for 15 minutes. The reason why 1 g was the determinant weight in order to establish the digestion time with each pepsin and method is because 1 g was the maximum accorded amount of undigested muscle for getting an easy and rapid finding of parasites. Although *T. trachurus* digestions showed more difficulties during the assays (probably due to muscle characteristics and fat contain), the same criterion of 1 g was followed at the two methods, thus providing more digestion time (45 minutes) to fresh and frozen samples. Results in Table 4 show differences in relation to the amounts of undigested muscle residues from lean and fatty fishes and between procedures. This table also contains digestion protocols conditions, type of fishes and percentages of digested muscle (%).

New conditions introduced and assayed (liquid pepsin, weight/volume ratio of 1:10 and the flattened of the samples before digestion), gave higher percentages of digested muscle (a lower quantity of resulting residue) after less time, both for lean and fatty fish species, than the CODEX protocol.

3.4. Larvae viability

Concerning larvae viability tests, after both CODEX and LP digestion protocols for both type of fishes and for both forms of preservation, all larvae introduced were recovered in perfect conditions; live larvae were recovered still alive and showing a good mobility, resembling to mobility showed before digestions (Figure 2). Moreover, 10 live and free larvae were submitted to 210 minutes of digestion following the LP protocol, and after this time the same quantity of larvae was recovered without mobility but completely intact.

4. Discussion

Nowadays, due to the low confidence of other traditional parasite detection methods like the widely used visual inspection of abdominal cavity (Llarena-Reino et al., 2012), the norm by CODEX (CODEX STAN 244-2004) is considered the current recommended procedure for anisakids detection and counting in certain fish species and commercial displays. However, due to the lack of an officially legislated reference standard, not for CODEX protocol neither for any of the traditionally used formulas, there is no consensus in *modus operandi* to accomplish with artificial digestions for anisakids detection. An example of a similar approach in terms of performance and objectives, which has been sharply and effectively legislated, is diagnosis method for trichinellosis. Traditionally different detection protocols and variations had been used for meat inspections and for studies concerning *Trichinella* (Forbes and Gajadhar, 1999; Leclair et al., 2003; Gajadhar et al., 1996 and 2009). Since January 2006 a Commission Regulation of the European Community of 5 December 2005 (EC No. 2075/2005) has laid down specific rules on official controls for *Trichinella* in pig meat. This detailed law forces to carry out the magnetic stirrer protocol for pooled-sample digestion in fresh pig meat. Afterward, some authors concluded that pepsin powder potentially caused severe allergic reactions to sensitive people (Marqués et al., 2006) and workers (Maddox-Hyttel et al., 2007) who handled the chemical, thus constituting a health risk. Simultaneously, the Commission Regulation of the European Community of 24th October 2007 (EC No. 1245/2007) modified Annex I of the regulation (EC) No 2075/2005, allowing the use of liquid pepsin to detect *Trichinella* in meat. Similarly, during the present study the artificial digestion protocol from CODEX has been revised in depth, detecting some limitations and disadvantages in the powder pepsin and in the conditions, when new changes have been introduced and assayed. During the first assay carried out with the three pepsins (artificial digestions at concentrations of 0.5%; Table 2), pepsin 3 offered the lowest proteolytic activity. Due to this, authors decided remove it from the study. After selected and assayed pepsins 1 and 2 for the second test (preparing digestion solutions with the same proteolytic activity; Table 3), pepsin 2 gave better results; higher percentage of digested muscle (or lower weight of undigested muscle) than 1. Therefore, liquid pepsin (pepsin 2)

provided more effectiveness. It also offered an easier handling at work procedures than pepsin 1 when using both enzymes. Moreover and as mentioned above, liquid enzyme avoids possible allergic reactions that pepsin in powder form may cause. Additionally, the study of the purity by means of the SDS-PAGE silver staining profile was determinant to qualify pepsin 2 as the cleanest, purest, fastest and the most versatile and efficient of both. This was the reason why this liquid enzyme was selected as the most interesting pepsin to be assayed applying the new conditions, in a comparative test between both pepsins and both procedures (CODEX and LP). Therefore, since there is a non standardized safer optional method for *Trichinella* detection, it seems reasonable to consider a similar non standardized safer alternative method for anisakids detection as well, due to the important, increasing and urgent requirement of its use.

When LP procedure was performed with pepsin 2 by introducing the new improved conditions suggested in this study (different pepsin, weight/volume ratio of 1:10 and the flattened of the samples before digestion), it was observed that the new settings and variations were considerably reducing assay times and increasing percentages of digested muscle, at both types of fish studied. Comparing both procedures, it became clear that LP protocol is more sensitive, efficient and accurate. It offers innovative characteristics like being more handy and easier to use even for unskilled personnel, such as fish markets and factories workers, than CODEX. Besides increasing comfort and usability, this novel procedure reduces costs and test times. This fact leads to a huge reduction of the expenses and time dedicated to quality and safety controls at industries, without variation on results reliability. These kinds of improvements are extremely significant, also for research centers, to make faster progresses in specific aspects of the parasites and the public health preventing programs.

Conflict of interest statement

The authors declare no conflict of interest.

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Tables

Table 1

Equivalences between different units used for presenting proteolytic activities in commercial pepsins (T.K. Langdon, 2009).

ENZYMATIC EQUIVALENCES	UNITS LEGEND
3000 FCC = 3000 NF / NFU = 0,5 U/mg Ph Eur = 0,5 U/mg FIP	FCC (FOOD CHEMICALS CODEX) NF / NFU (NATIONAL FORMULARY) Ph Eur (EUROPEAN PHARMACOPEIA) FIP (INTERNATIONAL PHARMACEUTICAL FEDERATION)

Table 2

Comparison among 3 different commercial pepsins (each one with its own enzymatic activity), in 500 ml water and 2.5 g pepsin (at concentration of 0.5%), in an acid solution (pH=1.5) with HCl, at 0.063M. Six assays were carried out using each pepsin, with muscular samples of 25 g of lean (*Merluccius merluccius*) and fatty (*Trachurus trachurus*) fresh fish.

PEPSIN NAME	ENZYMATIC ACTIVITY	FISH MUSCLE (g)	WEIGHT/VOLUME RATIO	DIGESTION TIME (minutes)	DIGESTION TEMPERATURE (°C)	FISH SPECIES	DIGESTIONS (N)	RESULTING MUSCLE RESIDUE Mean (g) ± SD	DIGESTED MUSCLE Mean (%) ± SD	COMMERCIAL REFERENCE
Pepsin 1	800-2,500 U/mg protein	25	1:20	30	37°C	<i>Merluccius merluccius</i>	6	1,048 ± 0.18	95.809 ± 0.7	Sigma Aldrich 10333P7000-100G
						<i>Trachurus trachurus</i>	6	4.933 ± 1.04	80.270 ± 4.15	
Pepsin 2	2000 FIP U/g	25	1:20	30	37°C	<i>Merluccius merluccius</i>	6	0,820 ± 0.2	96.719 ± 0.78	Merck 10 1.07190-1000G
						<i>Trachurus trachurus</i>	6	3.180 ± 0.71	87.281 ± 2.83	
Pepsin 3	660U Ph Eur/ml	25	1:20	30	37°C	<i>Merluccius merluccius</i>	6	0,085 ± 0.02	99.649 ± 0.12	Panreac (Liquid) 88331764081214-5lt
						<i>Trachurus trachurus</i>	6	1.337 ± 0.45	94.652 ± 1.82	

Table 3

Comparison among 2 different commercial pepsins (at different concentration each one; enzymatic activities have been equaled at 5000U FIP), in 500 ml of acid solution (pH=1.5) with HCl at 0.063M. Six assays were carried out using each pepsin, with muscular samples of 25 g of lean fresh fish (*Merluccius merluccius*) and fatty (*Trachurus trachurus*) fresh fish.

PEPSIN NAME	ENZYMATIC ACTIVITY	ENZYMATIC ACTIVITY (FIP)	PEPSIN DOSE (g)	FISH MUSCLE (g)	WEIGHT/ VOLUME RATIO	DIGESTION TIME (minutes)	DIGESTION TEMPERATURE (°C)	FISH SPECIES	DIGESTIONS (N)	RESULTING MUSCLE RESIDUE Mean (g) ± SD	DIGESTED MUSCLE Mean (%) ± SD
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								<i>Trachurus trachurus</i>	6	1.838 ± 0.9	92.647 ± 3.61
Pepsin 3	660U Ph Eur/ml	543 FIP U/g	9.2	25	1:20	30	37°C	<i>Merluccius merluccius</i>	6	0.785 ± 0.19	96.86 ± 0.4
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Resulting muscle residues (g) and digested muscle (%) means, comparing the Liquid Pepsin (LP) protocol (new digestion assay using the selected liquid pepsin, or pepsin 2, at concentration of 0.5% in 2000 ml water), to CODEX STAN 244-2004 protocol (using the recommended powdered pepsin or pepsin 1, at concentration of 0.5% in 1000 ml water). Both digestions were carried out in an acid solution (pH=1.5) with HCl, at 0.063M. A total of 240 assays with samples of 200 g of fish were carried out; 120 for each method (30 assays were performed for fresh, and 30 for frozen lean fish belonging to *Merluccius merluccius*, and the same number for fatty fish (*Trachurus trachurus*).

DIGESTION METHOD	PEPSIN NAME	ENZYMATIC ACTIVITY	FISH MUSCLE (g)	STOMACHER TIME (minutes)	DIGESTION TEMPERATURE (°C)	WEIGHT/ VOLUME RATIO	FISH SPECIES	DIGESTIONS (N)	FISH SAMPLE TYPE	DIGESTION TIME (minutes)	RESULTING MUSCLE RESIDUE Mean (g) ± SD	DIGESTED MUSCLE Mean (%) ± SD
CODEX PROTOCOL (CODEX STAN 244-2004)	Pepsin 2	2000 FIP U/g	200	-	37°C	1:5	<i>Merluccius merluccius</i>	30	FRESH	20	125.2 ± 14.91	37.38 ± 7.45
								30	FROZEN	15	126.7 ± 11.22	36.63 ± 5.61
							<i>Trachurus trachurus</i>	30	FRESH	45	32.9 ± 5.33	83.52 ± 2.67
								30	FROZEN	45	36.48 ± 4.61	81.76 ± 2.3
LIQUID PEPSIN (LP) PROTOCOL	Pepsin 3	660U Ph Eur/ml	200	4	37°C	1:10	<i>Merluccius merluccius</i>	30	FRESH	20	0.653 ± 0.328	99.67 ± 0.16
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Figures Captions

Fig. 1. SDS-page silver staining profile obtained from the two selected commercial pepsins assayed. Low molecular weight standard (14-97 kDa) from GE Healthcare was used as pattern. Additional bands with lower molecular weight than pepsin were obtained at one of them. Black arrow: pepsin band.

Fig. 2. Resulting digestions after examining and controlling the viability of the larvae. (A) Ten anisakid larvae after CODEX digestion protocol of frozen *Merluccius merluccius*. Black arrowhead: anisakid larval. (B) Ten anisakid larvae after LP (Liquid Pepsin) digestion protocol of frozen *Merluccius merluccius*. (C) Sequence of two pictures showing live anisakid larvae moving after CODEX digestion protocol of fresh *Merluccius merluccius*. (D) Sequence of four pictures showing live anisakid larvae moving after LP (Liquid Pepsin) digestion protocol of fresh *Merluccius merluccius*.

Optimization of the pepsin digestion method for *anisakis* inspection in the fish industry

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Figure 1
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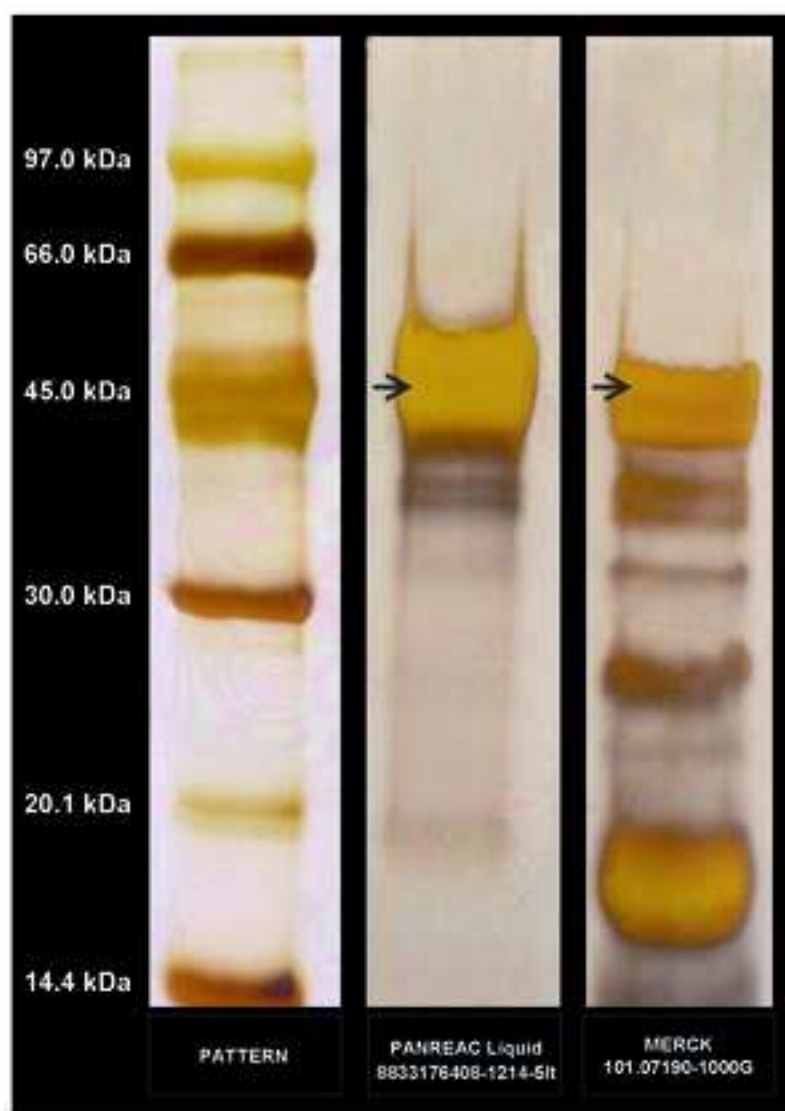


Figure 2
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