



Detection of tropomyosin and determination of proteins in crustacean oils



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ARTICLE INFO

Article history:

Received 19 December 2012
Received in revised form 15 February 2013
Accepted 20 February 2013
Available online 14 March 2013

Keywords:

Antarctic krill
Calanus finmarchicus
Oils
Proteins
Tropomyosin
Western blotting
Amino acid analysis

ABSTRACT

Tropomyosin is known to be the main allergen in crustaceans and the objective of this study was to investigate if this protein could be detected in commercial crustacean oils from Antarctic krill (*Euphausia superba*) and the zooplankton *Calanus finmarchicus*. We also examined the possibility of determining the protein content in the oils by direct amino acid analysis. Western blotting showed that a commercial antibody against shrimp tropomyosin cross-reacted with a protein of similar size in Antarctic krill and *C. finmarchicus*. The protein tentatively identified as tropomyosin, was also detected in krill oil products, but not in oils from *C. finmarchicus*. The acetone–heptane method used for extracting proteins in the oils is however not optimal. Other extraction methods should therefore be considered when investigating the presence of allergenic proteins in oils. Direct amino acid analysis on oils should be further explored as a method for determining the total amount of proteins present.

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1. Introduction

Crustaceans are one of eight foods or food groups that are thought to account for more than 90% of all immunoglobulin E food allergies world-wide (Hidalgo & Zamora, 2006). The myofibrillar protein tropomyosin (TM) is a cross-reacting allergen among crustaceans and has been shown to be the main allergen in species like shrimp, lobster, crab, and Antarctic krill (Nakano, Yoshinuma, & Yamada, 2008; Reese, Ayuso, & Lehrer, 1999). As reviewed by Hidalgo and Zamora (2006) proteins and peptides may be found in many crude and refined plant oils and several studies have shown that allergenic proteins may be found in oils extracted from peanuts (Ramazzotti et al., 2008), soybeans (Errahali et al., 2002), sunflower seeds (Zitouni et al., 2000), and gourmet nuts (Teuber, Brown, & Haapanen, 1997). Very few studies have however been carried out on the allergenicity of fish oils (Mark, Beaty, & Slavin, 2008) and to the best of our knowledge, the presence of allergens in oils from crustaceans has not been reported.

There has been a large increase in the demand for fish oils containing the unique long-chain polyunsaturated ω -3 fatty acids (*n*-3 LC-PUFA). The limited amount of fish oils available has led to extensive search for alternative sources of oils rich in *n*-3 LC-PUFA. These fatty acids in the fish oil originate from marine phytoplank-

ton and one possibility is to harvest resources lower in the marine food web such as smaller crustaceans (Miller, Nichols, & Carter, 2008). Antarctic krill (*Euphausia superba*) and *Calanus finmarchicus* which are small shrimp-like crustaceans present in large amounts in the South and the North Atlantic Oceans, respectively (Greene et al., 2003; Nicol, Foster, & Kawaguchi, 2012), are currently harvested for the production of oils rich in *n*-3 LC-PUFA and astaxanthin. The oils are available on the nutraceutical markets and so far most studies on health effects have been carried out with krill oils (Eilertsen et al., 2012; Grimstad et al., 2012; Piscitelli et al., 2011; Tou, Jaczynski, & Chen, 2007).

When oil is extracted from a vegetable or animal source some proteins and peptides pass to the oil. The amount present can, however, be strongly reduced by refining the oil (Crevel, Kerkhoff, & Koning, 2000; Rigby et al., 2011). Oils extracted from crustacean species like Antarctic krill and *C. finmarchicus* are usually not refined to any extent since they contain desirable components like phospholipids and/or astaxanthin which are lost during traditional refining steps (Gunstone, 2004). Crustacean oils may therefore contain proteins that could cause reactions in people with crustacean allergies.

There is apparently no established method for extracting proteinous materials present in oils (Martín-Hernández, Bénet, & Obert, 2008). The proteins are commonly extracted with different aqueous buffers (Crevel et al., 2000; Ramazzotti et al., 2008) or precipitated using acetone or acetone together with hexane (Hidalgo, Alaiz, & Zamora, 2001; Martín-Hernández et al., 2008). When

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determining the protein content in oils, amino acid analysis of extracted proteins is often recommended instead of using colorimetric methods (Hidalgo et al., 2001; Martín-Hernández et al., 2008; Ramazzotti et al., 2008).

The main objective of this study was to investigate if TM could be detected in proteins extracted from commercial crustacean oil samples with the use of Western blotting. In addition, we also examined if the protein content in the oils could be estimated by direct amino acid analysis without prior extraction of proteins.

2. Materials and methods

2.1. Samples

Krill meal and Superba™ Krill Oil (KO) from Antarctic krill were provided by Aker Biomarine AS, Norway. Commercially available Superba™ Krill Oil capsules (KOc) were obtained from a local supermarket. Calanus® Oil (CO) from *C. finmarchicus*, Calanus® Oil capsules (COc) and whole frozen *C. finmarchicus* were supplied by Calanus AS, Norway. Fresh boiled shrimp (*Pandalus borealis*) were obtained locally and the muscle was recovered manually.

2.2. Extraction of proteins from oils

Proteins in KO and CO samples were extracted using a modified version of the acetone-hexane method described by Martín-Hernández et al. (2008). To 25 g of oil 62.5 ml cold acetone–heptane (AH, 1:1 w/w) was added. The mixture was shaken vigorously every 10 min for 1 h at 0 °C. It was then left on ice overnight in a refrigerator and subsequently centrifuged at 4000g and 4 °C for 20 min. The supernatant was discarded and the precipitate washed 6 times with vigorous shaking before centrifugation. Calanus oil samples were first washed with 30 ml AH followed by washing with 10 ml acetone-methanol (AM, 1:1 w/w) and subsequently 5 ml AH. The mixtures were centrifuged at 4000g and 4 °C for 20 min and the supernatant discarded between each wash. The final three washings steps were performed with 5 ml of AH, AM, and AH, respectively. The mixtures were centrifuged at 15000g and 4 °C for 20 min and the supernatant discarded after each wash. Krill oil samples followed the same procedure, however due to large amounts of polar lipids (phospholipids) the precipitate was washed with AM, AH, and AM in the first three washing steps followed by AM, AH, and AM as the final 3 washes. The pellets were left to air dry and samples intended for SDS-PAGE were dissolved in 500 µl sample buffer (25% glycerol, 2% SDS, 0.1% bromophenol blue, 14.4 mmol 2-mercaptoethanol, 60 mM Tris/HCl, pH 6.8). Separate protein samples intended for amino acid analysis were extracted in the same manner.

Oils in the commercial capsules were obtained by using a syringe. Each oil sample (10 g) was submitted to the same protein extraction procedure as described above using the same oil to solvent ratios. The final pellets were air dried and dissolved in 200 µl electrophoresis sample buffer.

As positive controls, 25 g of CO were spiked with 50, 200, 500, and 1000 µg bovine serum albumin (BSA, Standard, Pierce BCA Protein Assay Kit, Thermo Scientific, Rockford, IL, USA). The proteins in the oils were extracted as described and dissolved in 500 µl sample buffer. As a second control, BSA was dissolved directly in sample buffer to a concentration of 1 mg/ml.

2.3. Extraction of proteins from krill meal, *C. finmarchicus* and shrimp muscle

The proteins present in krill meal, frozen *C. finmarchicus* and shrimp muscle were extracted using the trichloroacetic acid

(TCA)-acetone method of Méchin, Damerval, and Michel (2007) with minor modifications. A TCA-acetone extraction solution (10% TCA, 0.07% 2-mercaptoethanol dissolved in cold acetone) was made fresh and kept at –20 °C before each extraction. The acetone washing solution (0.07% 2-mercaptoethanol in cold acetone) was also stored at –20 °C prior to use.

Raw frozen *C. finmarchicus* (0.4 g) was added 3.6 ml of ice-cold extraction solution and homogenised with a T25 Ultra-Turrax (IKA Laboratory and Analytical Equipment, Staufen, Germany) for 30 s while kept on ice. The sample was stored at –20 °C for 2 h before centrifugation at 20000g and 4 °C for 15 min. The supernatant was removed and the pellet suspended in 1.8 ml cold acetone washing solution. The sample was kept at –20 °C overnight before centrifugation and removal of supernatant. The washing step was repeated twice with an incubation time of 1 h at –20 °C before centrifugation. After the final washing step the pellet was dried under nitrogen. The sample was added 1 ml of 10% SDS and heated at 90 °C for 10 min before being diluted 1 + 1 in SDS electrophoresis sample buffer. Samples of krill meal and shrimp muscle were extracted in the same manner. The krill meal sample was added 3 ml of 10% SDS due to the high protein concentration and heated at 90 °C for 10 min before being diluted 1 + 1 in SDS electrophoresis sample buffer.

2.4. Determination of protein by amino acid analysis

The protein content in KO and CO and in the protein pellets extracted from the oils by the AH method were determined by amino acids analysis. KO (200 mg) and CO (600 mg) were diluted to 1.1 ml with distilled water and 0.1 ml of 20 mM norleucine (internal standard) was added. The protein pellets extracted from 25 g of oils were added 1.0 ml distilled water and 0.2 ml of 20 mM norleucine. Finally, in all samples, 1.2 ml of 12 M hydrochloric acid (HCl) was added and the mixture was flushed with N₂-gas for 15 s before hydrolysis at 110 °C for 24 h (Moore & Stein, 1963). Aliquots of the supernatants were dried under N₂-gas and dissolved in a 0.2 M lithium citrate buffer (pH 2.2) prior to analysis. The analysis of amino acids was performed using a Biochrom 30 amino acid analyser (Biochrom Co, Cambridge, UK) and the UV signals were analysed by Chromeleon software (Dionex, Sunnyvale, CA, USA) and compared with A9906 physiological amino acids (Sigma Chemicals Co, St. Louis, MO, USA). Protein content in the samples was calculated as the sums of the individual molecular weight of the amino acid residues after the deduction of the molecular weight of water according to recommendations by FAO (2003).

2.5. SDS-PAGE and Western blotting

The extracted proteins dissolved in sample buffer were heated to 90 °C for 10 min and analysed by SDS-PAGE performed basically according to Laemmli (1970) using 4–12% NuPAGE® Novex® Bis-Tris polyacrylamide gels in an XCell SureLock™ Mini-Cell (Life Technologies Corporation, Carlsbad, CA, USA) with MES-buffer, 200 V for 45 min at room temperature. SeeBlue® Plus2 Pre-Stained Standard (Life Technologies) was used as a molecular weight marker. The gel was stained with Coomassie blue (SimplyBlue™ Safe-Stain, Life Technologies Corporation, Carlsbad, CA, USA).

The protein content in the SDS-PAGE and Western blotting samples originating from the oils, including oils from the capsules, were based on the amino acid analysis of the protein pellets extracted from the KO and CO as described above. The protein content in the electrophoresis samples extracted from krill meal and *C. finmarchicus* was estimated on basis of amino acid analysis of krill meal and *C. finmarchicus*. In the shrimp muscle sample, the protein content was based on dry weight of the extracted pellet.

Western blotting (PVDF-membrane) were performed basically as described by Towbin, Staehelin, and Gordon (1979) by using iBlot™ Dry-Blotting System (Life Technologies Corporation, Carlsbad, CA, USA) and performed according to the manufacturer's instructions. MagicMark™ XP Western Protein Standard (Life Technologies Corporation, Carlsbad, CA, USA) was used as a molecular weight marker. Immediately after blotting, the membrane was washed 2 × 5 min at room temperature in TBS buffer (20 mM Tris-HCl, pH 7.5, containing 500 mM NaCl) and blocked with 25 ml TBS containing 0.05% Tween 20 and 5% fat-free milk for 2 h (blocking buffer). The membrane was then incubated at 4 °C overnight with primary antibody, polyclonal shrimp TM antibody (catalog number: orb11347) produced in mouse (Biorbyt Limited, Cambridge, UK), diluted 1:1000 in blocking buffer. The membrane was subsequently washed 3 × 5 min in TBS containing 0.05% Tween (TTBS), followed by incubation for 1 h at room temperature with a secondary antibody conjugated to horseradish peroxidase (goat anti-mouse IgG-HRP, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at a dilution of 1:30000 in TTBS. The membrane was then washed 3 × 5 min in TTBS followed by 5 min in MilliQ water. Subsequently, the membrane was incubated with 0.75 ml Supersignal West Dura Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and developed using Kodak Molecular Imaging (Carestream Health, Onex Corp., Toronto, Canada).

3. Results and discussion

3.1. Protein content in crustacean oils

The proteins present in the oil samples were extracted using a modified version of the AH method described by Martín-Hernández et al. (2008). It is known that lipids may interfere with SDS-PAGE (Rigby et al., 2011) and the precipitated protein pellets were therefore washed six instead of three times with organic solvents. This extensive washing procedure may however have resulted in loss of peptides and proteins from the precipitate. To investigate possible loss during the extraction procedure, the protein content was determined by direct amino acid analysis of the oils. The results showed that KO and CO contained 5.68 and 0.18 mg protein/g oil, respectively (Table 1). The analysis of the protein pellets indicated that the KO contained 0.19 mg/g while the CO contained 0.02 mg/g. This shows that only 3.3% of proteins in KO and 12% of the proteins in CO were recovered in the precipitates. In addition to loss of proteins during the washing steps, inefficient precipitation may also have contributed to the low yield. Recent results suggest that mixing oils with petroleum ether and then extracting the proteins with bicarbonate or borate buffers improves the recovery (Rigby et al., 2011). The results presented by Rigby et al. (2011) suggest that low temperature acetone precipitation with several washing steps may result in underestimation of protein levels in oils. The reported protein content in crude vegetable oils varies substantially probably both due to different extraction and protein determination methods. Crevel et al. (2000) reported the presence of 0.1–0.3 mg protein/ml crude peanut and sunflower oil, while Martín-Hernández et al. (2008) found 1.32 mg/kg peanut oil and 3.28–11.1 mg/kg rapeseed oils. Unre-

finned peanut, walnut and almond oils have been reported to contain 0.010, 0.016 and 0.062 mg/ml, respectively (Teuber et al., 1997). To avoid inaccurate results due to low recovery of intact and degraded proteins during extraction, the possibility of quantitative determination of proteins by direct amino acid analysis on the oils should be investigated further. The protein content should then be adjusted for the possible presence of free amino acids in the oils.

3.2. SDS-PAGE and Western blotting

The results in Fig. 1(A) show that several protein bands can be seen in the samples extracted from *C. finmarchicus* (lane 2), krill meal (lane 5) and shrimp muscle (lane 8). The Western blotting analysis (B) of the same samples showed that the primary antibody produced against shrimp TM reacted with a protein with a molecular weight of about 38 kDa, corresponding to that of TM (Nakano et al., 2008; Reese et al., 1999). Based on the known allergenic cross-reactivity of TM in different crustacean species it is reasonable to assume that this protein band in the *C. finmarchicus* and Antarctic krill samples is TM. The antibody has apparently a stronger affinity for the *C. finmarchicus* TM than the Antarctic krill protein. In the shrimp muscle sample, the TM antibody also showed weak cross-reactivity with protein bands at approximately 40 and 45 kDa. The immunoreactive 45 kDa band can also be seen in the *C. finmarchicus* (lane 2) and krill meal (lane 5) samples. The identities of these bands are uncertain. In the KO samples (lanes 6 and 7), the protein band tentatively identified as TM (~38 kDa) can be detected. The antibody reacted however more strongly with the protein band at ~45 kDa. No cross-reactivity between the TM antibody and proteins extracted from the CO samples could be detected (lanes 3 and 4). The reason for this could be that the proteins in the CO samples apparently are more degraded compared to the proteins in the KO samples (Fig. 1(A)). In the CO samples, the Coomassie staining indicated the presence of only poorly resolved low molecular weight proteins (<10 kDa). The poor resolution may be due to residual lipids present in the samples (Rigby et al., 2011). It is well known that Antarctic krill (Tou et al., 2007) and *C. finmarchicus* (Bergvik, Overrein, Bantle, Evjemo, & Rustad, 2012) are very susceptible to autolytic degradation unless endogenous enzymes are rapidly inactivated post mortem. Antarctic krill harvested by Aker Biomarine is immediately processed onboard the vessels by boiling and drying to krill meal to avoid autolysis. Krill oil for human consumption is subsequently extracted from the meal by ethanol (Aker Biomarine Antarctic AS, 2011). The harvested *C. finmarchicus* are immediately frozen on board and freeze-stored in standard freezing facilities before being processed to oil and meal (Pedersen, Vang, & Olsen, 2013). Other details about the production process of CO are not available. It has been reported that hydrolytic enzymes in *C. finmarchicus* are active during the frozen storage (Bergvik et al., 2012). It is apparent from the SDS-PAGE that the proteins recovered from the CO are more degraded than the protein extracted from the frozen *C. finmarchicus* (Fig. 1(A)). It is known that proteolytic degradation of allergenic proteins will reduce the allergenicity (Shimakura, Tonomura, Hamada, Nagashima, & Shiomi, 2005).

The two weak protein bands at ~100 kDa present in the samples extracted from COc and KOc (Fig. 1(A), lanes 4 and 7) are most likely subunits of collagen originating from the capsule wall.

Extraction of proteins in oil samples by the AH method was further investigated by spiking the CO with different amounts of BSA. The results in Fig. 2(A) show that BSA can readily be detected in lane 4 where the oil had been spiked with 8 µg BSA/g oil before extraction. Based on this amount added to the oil, the maximum theoretical amount of BSA applied in lane 4 was 2 µg BSA. In lanes 5 and 6 the theoretical values of BSA were 5 and 10 µg, respec-

Table 1

Protein content in the Superba™ krill oil and the Calanus® oil and in the protein pellet extracted from the oils by the acetone–heptane method. The protein content was based on amino acid analysis and expressed as mg/g oil.

	Protein pellet	Oil
Superba™ krill oil	0.19 ± 0.01 mg/g	5.68 ± 0.20 mg/g
Calanus® oil	0.02 ± 0.01 mg/g	0.18 ± 0.00 mg/g

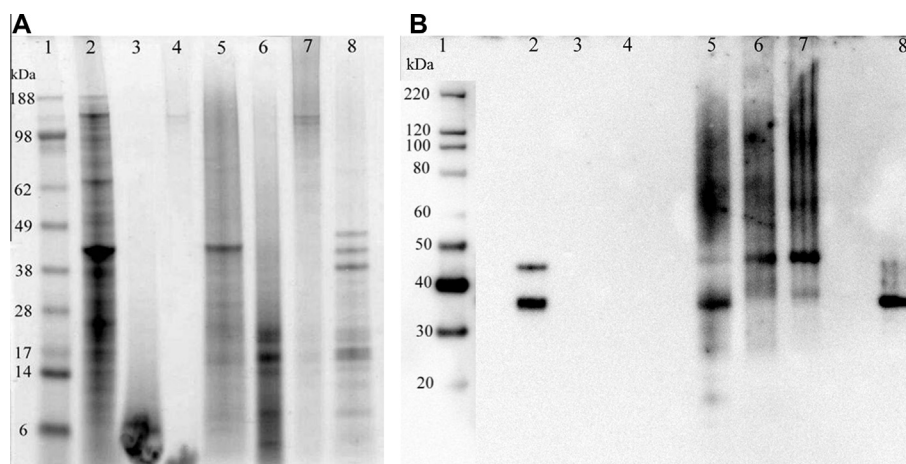


Fig. 1. SDS-PAGE analysis (A) and Western blot analysis (B) of proteins extracted from *C. finmarchicus*, Calanus® Oil and Calanus® Oil capsules are shown in lanes 2, 3, and 4, respectively. Proteins extracted from Antarctic krill meal, Superba™ Krill Oil and Superba™ Krill Oil capsules are shown in lanes 5, 6 and 7. Lane 8: Proteins extracted from shrimp muscle. SeeBlue® Plus2 Pre-Stained Protein Standard (A) and MagicMarker™ XP Western Protein Standard (B) were used as molecular weight markers (lane 1). In SDS-PAGE analysis (A) the amount of protein applied per lane with the corresponding amount of oil in brackets were as follows; 35 µg, 5 µg (0.25 g oil) and 6 µg (0.3 g oil) in lanes 2, 3 and 4, respectively. Lanes 5, 6 and 7; 131 µg, 48 µg (0.25 g oil) and 29 µg (0.15 g oil) in that order. In lane 8, approximately 200 µg shrimp protein was applied. In Western blotting analysis (B) the amount of protein applied per lane with the corresponding amount of oil in brackets were as follows; 3 µg, 5 µg (0.25 g oil) and 2 µg (0.1 g oil) in lanes 2, 3 and 4, respectively. Lanes 5, 6 and 7; 66 µg, 34 µg (0.18 g oil) and 34 µg (0.18 g oil) in that order. In lane 8, approximately 5 µg shrimp protein was applied.

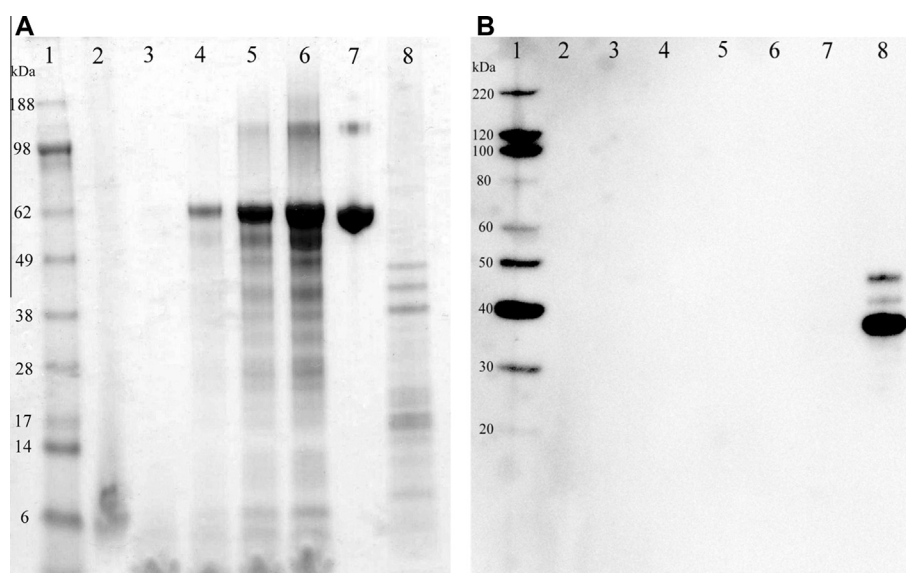


Fig. 2. SDS-PAGE analysis (A) and Western blot analysis (B) of proteins extracted from Calanus® oil as well as Calanus® oil spiked with bovine serum albumin (BSA). In lanes 2–6, 5 µg protein equivalent to 0.25 g of oil were added. The theoretical amount of BSA in lanes 3, 4, 5, and 6 were 0.5 µg, 2.0 µg, 5 µg, and 10 µg, respectively. In lane 7, 3 µg BSA was applied. In lane 8, approximately 200 µg (A) and 5 µg (B) protein from shrimp muscle were applied. SeeBlue® Plus2 Pre-Stained Protein Standard (A) and MagicMarker™ XP Western Protein Standard (B) were used as molecular weight markers (lane 1).

tively. The intensity of the BSA band in lane 7 where 3 µg BSA had been directly dissolved in the sample buffer, resembles the intensity of the BSA band in lane 6. This suggests that approximately 1/3 of the BSA added to the oil was recovered by the AH extraction method. This demonstrated the inefficiency of the AH extraction method to recover proteins from oils. The results shown in Fig. 2(A) suggest that spiking of oil with BSA leads to co-precipitation of proteins with molecular weights <62 kDa present in the oil. These proteins were not detected in the protein pellet without the addition of BSA to the oil (lane 2) or in the lane where BSA had been directly added to the sample buffer (lane 7). This result prompted us to carry out Western blotting on these samples to investigate if TM could be detected among the co-precipitated pro-

teins (Fig. 2(B)). The TM antibody did not detect any immunoreactive proteins in these samples (lanes 3–6) nor in sample where BSA had been directly applied to electrophoresis (lane 7). As a control, proteins extracted from shrimp muscle were also included in this experiment (lane 8) and the same 3 immunoreactive proteins as seen Fig. 1(B) were found.

The present work shows that a commercial antibody directed against shrimp muscle TM cross-reacts with a protein assumed to be TM in *C. finmarchicus* and Antarctic krill. The results demonstrated that TM could be detected in proteins extracted from commercial KO, but not in oil products made from *C. finmarchicus*. The AH method used for extracting proteins from these oils is however not optimal. Other extraction methods should therefore be evalu-

ated for analysis of the possible presence of allergenic proteins. Direct amino acid analysis on oils should be further investigated as a method for determining the total amount of proteins present.

It is well recognised that the threshold dose for inducing allergic symptoms may vary greatly between individuals. The results in our study underline the importance of labeling crustacean oil products with the recommendation that people with known crustacean allergies should avoid such products.

Acknowledgement

The authors would like to thank Aker Biomarine AS for providing Superba™ Krill Oil and Krill meal, and Calanus AS for providing Calanus® Oil, Calanus® Oil capsules as well as whole frozen *C. finmarchicus*.

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