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Removal of Lipids, Dioxins, and Polychlorinated Biphenyls during Production of Protein Isolates from Baltic Herring (*Clupea harengus*) Using pH-Shift Processes

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Dioxins and PCBs are toxic, lipophilic, and persistent substances that impose a serious health threat. A major risk of exposure to these toxic substances is consumption of fish from polluted waters, such as the Baltic Sea. The aim of this study was to investigate if pH-shift processing of Baltic herring with elevated toxicity levels could be used to produce a protein isolate with low fat content and, thereby, reduced dioxin and PCB levels. Both acid (pH 2.7) and alkaline (pH 11.2) pH-shift processing were investigated and resulted in efficient reduction of fat, dioxin, and PCB levels. A reduction of 70−80% per amount of protein was determined for all of these parameters. The amounts, and thus the removal, of lipids and dioxins (R^2 = 0.952) as well as lipids and PCBs (R^2 = 0.996) were highly correlated ($p < 0.01$). A mass balance of the alkaline pH-shift process showed that most of the fat and pollutants were found in the floating fat emulsion layer of the first centrifugation, followed by the pellet of the first centrifugation. These data show that the pH-shift protein isolation technique can be used to process herring with elevated dioxin and PCB levels and thereby increase the usage possibilities of such fish.

KEYWORDS: Herring; *Clupea harengus*; dioxin; PCDD; PCDF; PCB; acid or alkaline solubilization; pH shift; protein isolation; lipids; detoxification

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins (PCDDs), dibenzofurans (PCDFs), and polychlorinated biphenyls (PCBs) have gained a lot of attention due to their environmental persistence and subsequent toxic effects. Since these substances are both lipophilic and stable, they are problematic to remove (*1*). Moreover, they are extremely toxic. The dioxin-induced toxicity arises from a subset of these compounds (29 congeners out of 419). These molecules have a planar conformation and can thus bind into the aryl hydrocarbon receptor and cause a biological response. The binding affinity varies among them, and the concept of toxic equivalency factors (TEF) has been created to accommodate this. The individual factors are related to the most toxic congener, 2,3,7,8-TCDD (tetrachlorinated dibenzo-*p*-dioxin), which has been assigned a value of 1. This means that a single number can be calculated from the individual concentrations to describe dioxin-like toxicity, i.e., toxic equivalents (TEQ) (*2*). Dioxin exposure can lead to cancer and, e.g., impaired reproduction, endocrine functions, and endocrine development (*1*).

One of the most polluted seas of the world is the Baltic Sea. This is a very important fishing ground for many European countries, but fatty fish caught here often contain the toxic PCDDs and PCDFs (commonly called dioxins) and dioxin-like PCBs (DL-PCBs) above EU limitations and thus cannot be sold on the EU market (*3*, *4*). The current EU limitations for fish and fish-based food products are 4.0 pg of dioxin TEQ or 8.0 pg of dioxin and PCB TEQ per g fresh weight. These limits are under revision, originally to Dec 31, 2008, and might be lowered (*4*). Sweden and Finland have an exception from these dioxin and PCB limits until the end of 2011, and contaminated fish is allowed to be sold on the domestic market (*4*). This is because these countries have detailed recommendations on how often it is safe to eat certain fish. According to the Swedish recommendations, children and fertile women should not consume fish, e.g., Baltic herring, that may contain high dioxin or PCB levels more than 2−3 times per year (*5*).

There are regional and seasonal differences in the dioxin and PCB content of Baltic fish (*3*, *6*). However, overall there is no tendency for reduced contamination levels in fish any longer (*7*), despite harder regulations for emission of these pollutants into nature. Recent research has therefore been conducted to develop methods to reduce the levels of contaminants in the fish or fish products. This has successfully been achieved for fishmeal and fish oil. For fish meal, dioxins and DL-PCBs can be reduced by extractions with solvents (e.g., *n*-hexane) to decrease the fat content or with oil to remove fat-soluble compounds (*8*, *9*). In fish oil, these toxic compounds can be reduced using short path distillation (*10*) or solid-phase extraction (*11*−*13*). For whole fish, removal of the fish skin and normal cooking preparations can

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partially reduce the toxic levels due to reduced fat content (*3*, *14*). Other food preparation procedures such as hot smoking of fish could affect the amount of certain PCB congeners, but the results from two recent studies are not conclusive (*15*, *16*).

Protein isolation using pH-shift methods (also called acid or alkaline solubilization) is an alternative way to separate lipids from fish proteins, as has previously been shown in several studies (*17*−*19*). Furthermore, these processes generally allow the proteins to retain their functional properties such as gel formation, and thus they can be used in many food formulations (*17*, *19*). On the basis of the lipophilic nature of dioxins and PCBs and the ability of the pH-shift processing to remove lipids, we hypothesize that pH-shift protein isolation can be used to minimize the levels of dioxins and DL-PCBs. The hypothesis is further supported by recent data indicating that pH-shift processing can reduce the content of presumably lipophilic diarrheic shellfish poisoning toxins from mussels (*20*).

In this study, the amounts of dioxins and PCBs in Baltic herring (*Clupea harengus*) have been investigated before and after acid and alkaline pH-shift processing. Process modifications to further decrease the fat content have also been investigated. Moreover, a mass balance has been performed in order to evaluate the distribution of fat, dioxins, and PCBs between the different subfractions from alkaline pH-shift processing.

MATERIALS AND METHODS

Materials. Herring (*C. harengus*), kindly provided by Gävlefisk AB, was caught in southern Bothnian Bay, Baltic Sea, January 2008. The herring was stored on ice during transport to the laboratory where it was immediately processed. The time from catch to processing was approximately 48 h.

Sodium hydroxide, hydrochloric acid (37%), calcium chloride, and sodium carbonate of purity p.a. were from Scharlau (Barcelona, Spain). Citric acid, puriss p.a., and bovine serum albumin were from Sigma-Aldrich (Seelze, Germany). Sodium potassium tartarate was obtained from ICN Biomedicals (Aurora, OH) and SDS from MP Biomedicals (Solon, OH). Cupric sulfate pentahydrate, puriss p.a., was obtained from Fluka (Buchs, Switzerland). Chloroform and methanol of HPLC grades were purchased from Labscan (Gliwice, Poland), and ethanol (95%) was from Kemetyl (Haninge, Sweden). The olive oil was bought in a local store and was of the brand X-tra, manufactured in Belgium for Coop Sweden (Stockholm, Sweden).

For the dioxin and PCB analysis, *n*-hexane, acetone, dichloromethane, methanol, and toluene were of the highest purity (glass distilled) from Fluka (Buchs, Switzerland). Celite 545 and tetradecane (olefin free, puriss p.a.) was also from Fluka (Buchs, Switzerland). Anhydrous sodium sulfate, sulfuric acid, and anhydrous diethyl ether of purity p.a. were obtained from Merck Chemicals (Darmstadt, Germany), and potassium hydroxide, puriss p.a., was from Eka Chemicals (Sweden). Ethanol (spectrographic grade 99.5) was from Kemetyl (Haninge, Sweden), and carbon AX21 was obtained from Andersson Development Co. (Adrian, MT).

Methods. *Sample Preparation*. The whole herring was manually gutted and cut into smaller pieces. These herring pieces, including skin and bones, were then minced in a food grinder (Kitchen Aid, S. Joseph, MI), first using a grid with holes of 10 mm diameter and thereafter using a grid with 5 mm diameter. The herring and herring mince were kept on ice, and the grinding process was performed in a walk-in cold room (6 °C). The minced herring was stored at −80 °C in plastic LDPE zip-lock bags (VWR, Stockholm, Sweden) for up to 5 months.

Acid and Alkaline pH-Shift Processing. The herring mince was thawed under running cool water, and during all subsequent processing the mince was kept in a beaker on crushed ice. One hundred grams of herring mince was mixed with 900 mL of ice-cold distilled water and homogenized twice for 30 s using an Ultra Turrax T18 basic homogenizer (IKA, Taquara, RJ, Brazil). The pH of the homogenate was adjusted to either 11.2 (alkaline pH-shift method) or 2.7 (acid pH-shift method) using 2 M NaOH or 2 M HCl during constant manual stirring until the pH was stable. The pH was monitored with a calibrated Hamilton double pore electrode (Bonaduz, Switzerland) coupled with a pH meter (MeterLab PHM210; Radiometer Analytical S.A., Villeurbanne Cedex, France). The muscle homogenates were centrifuged at 8000*g* in a precooled (4 °C) Avanti centrifuge, J-20 XP (Beckman Coulter, Fullerton, CA), for 20 min. The solubilized proteins in the supernatant were collected and separated from the pellet and the floating fat layer by filtering through three layers of cotton gauze (AKLA AB, Danderyd, Sweden). The floating fat layer and pellet were separately weighed and stored at −80 °C in plastic zip-lock bags until further analysis. Following solubilization testing at different pHs, pH 5.4 (alkaline pH-shift method) or pH 6.2 (acid pH-shift method) was chosen for precipitation of the proteins. The pH was adjusted using 2 M HCl or 2 M NaOH. A second centrifugation was performed, and the pellet, referred to as protein isolate, was collected, weighed, and stored at −80 °C in plastic zip-lock bags until further analysis.

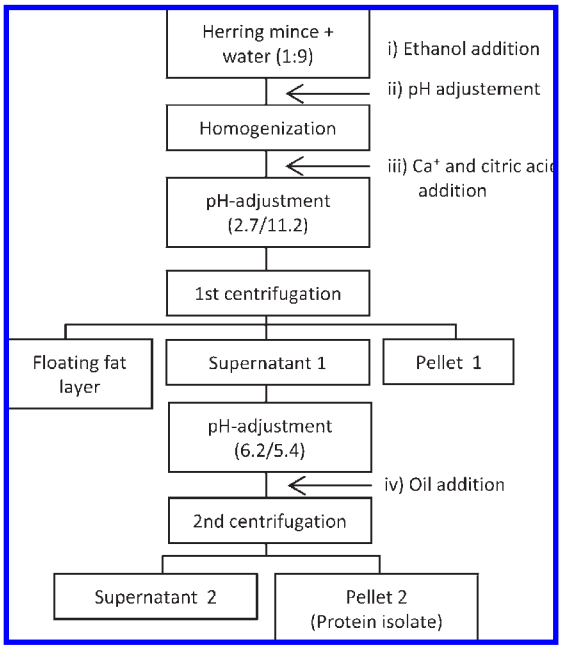

Figure 1. Overview of the pH-shift protein isolation process, indicating with (i)−(iv) the parts of the process that were changed in the four different modifications. All modifications were made to the alkaline version of the process.

Modifications of the Alkaline Process. In order to further decrease the fat/dioxin/PCB-levels of the protein isolate, several different modifications were made to the alkaline pH-shift method. All modifications were made in at least duplicates and analyzed for total fat, water, and protein content. The protein isolate with the lowest fat content from each process modification was then analyzed once for fat/dioxin/PCB levels. After evaluation of these results, a second protein isolate from the most promising modification was then analyzed for fat/dioxin/PCB levels. The modifications tested were (i) ethanol addition, (ii) homogenization at the isoelectric point (p*I*) of the herring proteins (pH 5.4), (iii) calcium chloride and citric acid addition, and (iv) addition of oil (**Figure 1**). In (i), the fish mince was mixed with nine times 5.0% ethanol (final concentration) instead of distilled water. In (ii), the herring homogenate was adjusted to pH 5.2 using 0.2 M HCl before homogenization with nine times water (reaching a pH of 5.7 after homogenization). In (iii), the addition of calcium chloride and citric acid to the homogenized fish and water was made according to Liang and Hultin (*21*). After homogenization 5 M citric acid was added to a concentration of 5 mM and incubated for 30 min. Then 8 M $CaCl_2$ was added to a final concentration of 8 mM Ca^{+}. The final liquid volume was 900 mL. In (iv), the addition of 30% olive oil (w/w) was made before the second centrifugation. The oil was mixed in by magnetic stirring for 30 min. The oil floating up during the second centrifugation was then decanted off.

Moisture Content. The moisture content were determined by drying 2 g samples at 105 °C until constant weight (overnight) using an Electrolux 939 oven (Electrolux, Stockholm, Sweden). The analysis

Table 1. Fat, Protein, and Moisture Content of Herring Mince and Protein Isolates[a]

sample	moisture (%)	fat % (wet wt)	protein % (wet wt)	fat % (dry wt)	protein % (dry wt)	fat % (protein wt)
herring mince (n = 2)	77.9 ± 0.2	7.8 ± 0.2 a	11 ± 1.5	35 ± 1.1 a	49 ± 6.7	71 ± 2.2 a
alkali-made isolate (n = 4)	88.7 ± 0.6	1.1 ± 0.3 b	7.2 ± 1.1	10 ± 2.4 b	64 ± 12	16 ± 5.7 b
acid-made isolate (n = 3)	89.3 ± 0.4	1.2 ± 0.2 b	7.7 ± 1.2	11 ± 1.8 b	72 ± 15	16 ± 5.1 b
alkali-made isolate with 5% ethanol (n = 2)	89.8 ± 0.1	0.54 ± 0.05 b	5.6 ± 0.49	5.2 ± 0.45 b	55 ± 4.4	10 ± 0.06 b

[a] a and b indicate significant differences (p = 0.001).

was performed on freshly produced protein isolates, i.e., not frozen after production.

Protein Analysis. The protein content was analyzed using the method of Lowry et al. (*22*) as modified by Markwell et al. (*23*). A Cary50 BIO UV−visible spectrophotometer (Varian Australia Pty Ltd., Victoria, Australia) was used for absorbance measurements, and the standard curve was based on bovine serum albumin (Sigma-Aldrich) using a concentration range of 10−100 μg of protein/mL.

Total Fat Content. The total fat content was measured gravimetrically as a part of the dioxin and PCB analysis. As a preanalysis and control to this method, the total fat was also measured according to Lee et al. (*24*) as modified by Undeland et al. (*25*). For the herring mince and the floating fat layer, a chloroform to methanol ratio of 2:1 (v/v) was used, while for all other samples the ratio 1:1 (v/v) was used. Only the total fat content as determined during the dioxin and PCB analysis is presented. Results are expressed as percent on a wet weight, dry weight, and protein basis.

Determination of Fat, Dioxins, and Dioxin-like PCBs. The analysis of the dioxins and PCBs was performed with the isotope dilution technique based on the standard method SS-EN 1948-3, differing slightly in some aspects, e.g., how the ^{13}C-labeled standards were divided between internal and recovery standard.

All solid samples (i.e., herring mince, pellet, floating layer, and protein isolate) were thawed at room temperature and mixed with 300 g of Na_2SO_4 in a homogenizer 1094 (Tecator, Höganäs, Sweden). The Na_2SO_4 was previously activated at 550 °C for 48 h. The mixture was filled into glass columns, and an internal standard (containing all compounds to be analyzed isotopically labeled with ^{13}C) was added. The column was extracted with 300 mL of *n*-hexane:acetone, ratio 5:2 (v/v), and 300 mL of *n*-hexane:diethyl ether, ratio 9:1 (v/v). The extracts were evaporated in a Büchi RE111 rotavapor (Buchi Laboratiums-Technik, Flawil, Switzerland) at 30 °C until constant weight and weighed to determine the amount of fat in the sample.

An internal standard was added to the liquid samples (i.e., the supernatant of the second centrifugation), and the samples were mixed with 100 mL of dichloromethane in a separation funnel. The lower dichloromethane phase was collected and the procedure repeated another three times. The extracts were evaporated and weighed as described above.

To remove the fat, the samples were redissolved in a small amount of chloroform and applied to an open silica column. The silica pretreatment consisted of washing twice with ethanol and twice with dichloromethane and heating at 130 °C for 24 h before impregnation with acid (H_2SO_4) or base (KOH). The 35 mm column consisted of (from the bottom) glass wool, 10 mL of KOH−silica, 5 mL of neutral silica, 20 mL of 40% H_2SO_4−silica, 10 mL of 20% H_2SO_4−silica, 20 mL of neutral silica, and 5 mL of Na_2SO_4 (pretreated as described above). Before use, the silica column was washed with 100 mL (2 column heights) of *n*-hexane. The column was extracted with 200 mL of *n*-hexane, and 50 μL of tetradecane was then added to the extract as a so-called keeper to avoid loss of analytes during evaporation. The samples were evaporated as described above.

To fractionate the dioxins and the ortho-PCBs, the sample was redissolved in a small amount of *n*-hexane and applied to a glass column with 0.5 g of carbon sorbent at the center, supported on both sides with glass wool. The sorbent consisted of a mix of active carbon (AX-21, 7.9%) and diatomaceous earth (Celite 545, 92.1%). Before use, the column was washed with 4 mL of dichloromethane:methanol:toluene at a ratio of 15:4:1 (v/v/v), 1 mL of dichloroethane:*n*-hexane, ratio 1:1 (v/v), and 5 mL of *n*-hexane. The ortho-PCB congeners were extracted using 40 mL of dichloromethane:*n*-hexane (1:1, v/v). The column was then turned upside down, and PCDD/F and the four non-ortho-PCBs (PCBs 77, 81, 126, and 169) were eluted using 40 mL of toluene. The dioxin fractions were concentrated, and recovery standard was added. The PCB fraction was purified on a second mini-silica column, as described above, before concentration and recovery standard addition.

Analysis of 2,3,7,8-chlorinated PCDD/F, WHO-PCBs (PCBs for which toxic equivalency factors, TEFs, exist) and seven indicator PCBs (PCBs 28, 52, 101, 118, 138, 153, and 180) were made, the latter as an indication of the total amount of PCBs. For the analysis, a gas chromatograph (GC) coupled to a high-resolution mass spectrometer (HRMS) was used. The GC was an Agilent 6890N (Agilent Technologies Inc., Santa Clara, CA) equipped with a GC Pal autosampler (CTC Analytics AG, Zwingen, Switzerland). The GC separation column used was 60 m, 0.25 mm diameter with 0.25 μm film thickness of the type J&W DB-5 or DB-5 ms (Agilent Technologies Inc., Santa Clara, CA), coupled to the HRMS (Waters Autospec Ultima, Milford, MA) operating at a mass resolution (R) of 10000. The ionization technique utilized was electron impact, and for enhanced sensitivity selective ion registration (SIR) was performed. The instrument was controlled by the software package Masslynx 4.0 (Waters, Milford, MA) including the Quanlynx application used for quantification of the results. The amounts of DL-PCB and dioxins were converted to their toxic equivalents (TEQ) (*1*), according to the equivalency factors (TEF) for dioxin-like toxicity updated in 2005 (*2*).

Statistical Analysis. Data were analyzed by Excel (Microsoft Corp.). Differences between starting material, acid-made isolate, alkali-made isolate, and alkali-made isolate with ethanol were analyzed using ANOVA single factor analysis. Pairwise comparisons were performed using the t test, assuming equal variance. Each treatment was repeated at least three times (n = 3), if not otherwise stated. In the case n = 2 (max − min)/2 is reported instead of SD. Correlation analyses were made using two-tailed Pearson correlation with n = 19. Principal component analysis (PCA) has also been performed, using SIMCA P+ 12.0 (Umetrics AB, Umeå, Sweden).

RESULTS

Removal of Lipids, Dioxins, and PCBs. Data presented in **Table 1** show that pH-shift processing of minced gutted herring resulted in protein isolates with significantly (p = 0.001) reduced fat content. Because of parallel changes in several constituents during processing, these data are expressed on wet weight, dry weight, and protein bases. The data show that the protein isolates both had a higher protein (p < 0.01, dry weight) and lower fat content (p < 0.001) than the starting material. No difference regarding the total fat removal was seen between the acid and alkaline pH-shift methods.

The addition of 5% ethanol to the alkaline pH-shift process was the most successful of the modifications tested (**Figure 1**) to further reduce the fat/DL-PCBs/dioxins. The results are presented together with the original methods. On a wet weight basis, the ethanol addition increased the fat removal (p < 0.05) but also resulted in an isolate with somewhat lower protein content (**Table 1**). However, the net effect of the ethanol addition was an isolate with a slightly lower fat to protein ratio compared to the normal alkali-made isolate (p < 0.10). The two modifications "homogenization at *pI*", and "calcium chloride and citric acid addition" (*21*) did not further decrease the amount of fat while addition of oil led to an somewhat increased fat content.

The dioxin (**Table 2**), DL-PCB (**Table 3**), and I-PCB (data not shown) contents were 70−80% lower in the protein isolates

Table 2. Dioxin Content of Herring Mince and Protein Isolates[a]

sample	dioxin (pg/g, wet wt)	dioxin TEQ[b] (pg/g, wet wt)	dioxin (pg/g, dry wt)	dioxin (pg/g, protein wt)
herring mince ($n = 2$)	24 ± 0.97 a	6.3 ± 0.24 a	108 ± 4.4 a	218 ± 9 a
alkali-made isolate ($n = 4$)	4.8 ± 1.6 b	1.1 ± 0.18 b	42.1 ± 12 b	69 ± 27 b
acid-made isolate ($n = 3$)	4.6 ± 0.67 b	1.2 ± 0.04 b	43.1 ± 5.5 b	62 ± 18 b
alkali-made isolate with 5% ethanol ($n = 2$)	2.9 ± 0.07 b	0.59 ± 0.05 b	28.0 ± 2.8 b	51 ± 1 b

[a] a and b indicate significant differences ($p = 0.001$). [b] Using WHO (2005) TEFs (*2*).

Table 3. Dioxin-like PCB Content of Herring Mince and Protein Isolates[a]

sample	DL-PCB (ng/g, wet wt)	PCB TEQ[b] (pg/g, wet wt)	DL-PCB (ng/g, dry wt)	DL-PCB (ng/g, protein wt)
herring mince ($n = 2$)	10.1 ± 0.84 a	3.7 ± 0.13 a	46 ± 3.8 a	92 ± 7.7 a
alkali-made isolate ($n = 4$)	1.3 ± 0.40 b	0.48 ± 0.15 b	12 ± 3.0 b	19 ± 7.2 b
acid-made isolate ($n = 3$)	1.6 ± 0.26 b	0.60 ± 0.10 b	15 ± 2.0 b	22 ± 6.8 b
alkali-made isolate with 5% ethanol ($n = 2$)	0.82 ± 0.07 b	0.29 ± 0.04 b	8.0 ± 0.6 b	15 ± 0.1 b

[a] a and b indicate significant differences ($p = 0.001$). [b] Using WHO (2005) TEFs (*2*).

compared to in the starting material ($p < 0.001$). This material had levels of dioxins, and combined dioxins and DL-PCBs, above the EU limits (*4*) whereas the corresponding contents in the protein isolates were well below this limit. When the 5% ethanol addition was applied, there was a trend ($p < 0.10$) to a further reduced dioxin and DL-PCB content compared to the normal alkali-made protein isolate on wet weight and dry weight basis but not on a protein basis. The three other modifications of the alkali-made isolates did not further decrease the contaminant level. The oil addition though led to a protein isolate with a somewhat lower amount of contaminants per gram of fat.

Mass Balance and Correlation between Fat and Dioxin Removal. The mass balance of the alkaline pH-shift processing showed that most of the fat, dioxins, and DL-PCBs were found in the floating fat emulsion layer (**Figure 2**). Approximately 30% of the contaminants were found in the pellet of the first centrifugation. Very little fat and contaminants were detected in the supernatant of the second centrifugation. In relative numbers, 15−20% of the fat and contaminants from the herring mince was detected in the protein isolates. The amounts of fat, dioxins, and DL-PCBs in the various samples were closely correlated. This is demonstrated in the mass balance (**Figure 2**) and even more in the correlation matrix (**Table 4**).

Figures 3 and **4** illustrate the DL-PCB and dioxin congener profiles of two representative samples, herring mince and an acid-made protein isolate. Comparison of the raw data revealed that all analyzed samples (e.g., herring mince, floating fat layer, and protein isolate) had a very similar congener profile, and furthermore no significant differences could be seen between the dioxin-PCB TEQ ratios. The dioxin congener 23478-PeCDF gave the highest contribution to the total amount of dioxins. This congener, having a WHO (2005) TEF of 0.5, was also the congener that contributed most to the dioxin TEQ value. The PCB 118 gave the highest contribution to the total amount of PCBs. However, it contributed little to the TEQ value due to a TEF of only 0.00003, while PCB 126, due to its higher TEF of 0.1, contributed most to the PCB TEQ value.

DISCUSSION

The aim of this study was to investigate if pH-shift processing can be used to remove dioxins and DL-PCBs from contaminated herring during the production of protein isolates. These results show that pH-shift processing indeed is a good method for this purpose as the isolated proteins had more than 70% lower dioxin and DL-PCB TEQ values per unit of protein than the herring mince.

Analysis of the herring mince used in this study showed that it had a fat content that is normal for winter-caught Baltic herring, 8% (*26*). In addition, the dioxin TEQ value (>6 pg/g) and the DL-PCB TEQ value (3−3.5 pg/g) were normal for herring from the southern Baltic Sea (*6*, *27*). Moreover, 2,3,4,7,8-PeCDF was the dioxin congener and PCB 126 the PCB congener that contributed most to the toxicity of the herring, which is in agreement with several other studies on Baltic herring (*3*, *28*, *29*). Thus, the herring used in this study is representative of Baltic herring, and the results should therefore be valid not only for this stock. The pH-shift processing yielded a protein isolate with lowered fat content, as previously shown for other raw materials (*17*, *18*). In this study, a reduction was seen for all dioxin and DL-PCB congeners, and therefore, the pH-shift process can most likely be used on contaminated fish from different fishing grounds (i.e., with different dioxin and DL-PCB congener profiles) as well as for different fish species. It might also be used on other contaminated muscle materials than fish.

Acid vs Alkaline pH-Shift Processing. In this study, the acid and alkaline pH-shift processes performed equally well regarding dioxin and DL-PCB removal. They also removed fat to the same extent. The latter results differ from a previous report for herring light muscle, where the acid process gave an isolate with lower fat content than the alkaline one (*17*). The differences could be due to the more complex nature of the material used here, including larger amounts of neutral fat deposits. In a review by Nolsøe and Undeland (*19*), it is reported that the ranking order between the acid and alkaline method regarding fat removal differs between different materials.

The alkaline version of the pH-shift method generally results in isolates with less rancidity problems (*19*), a very important aspect especially when working with dark muscle fish, which is why the alkaline method was chosen for attempts to further minimize the fat of the protein isolates. Ethanol was added in the process since it has previously been shown to be a good solvent for fat, dioxins, and PCBs (*8*). The addition used here revealed a trend ($p < 0.10$) to reduced fat and contaminant levels on dry weight basis. However, it may have several consequences. First, the protein isolates will contain some ethanol, in this case 5%, which affects the isolates further usage. Second, the ethanol might cause conformational changes and denaturation of the proteins (*30*, *31*), leading to impaired yield and changed functional properties for the isolated proteins. Indeed, in a small prestudy (data not shown), the highest ethanol concentration (20%) yielded proteins that to high degree could not be solubilized by a pH change to 11.2, and the proteins were collected in the pellet after the first

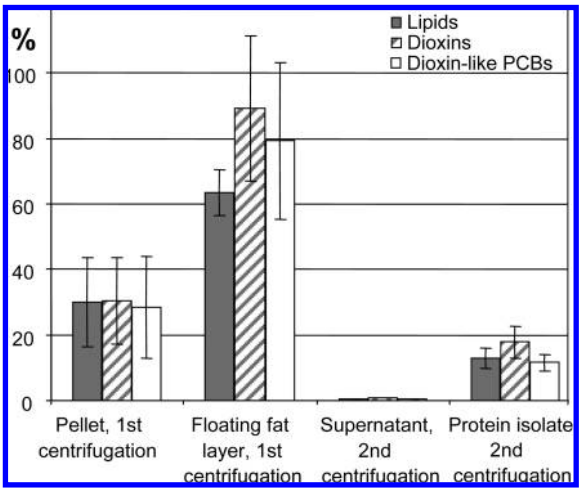

Figure 2. Distribution of lipids, dioxins, and dioxin-like PCBs in subfractions from the alkaline pH-shift process. One hundred percent is the amount of lipids, dioxins, or DL-PCBs found in the fish mince. $n = 3$, except in the case of the supernatant of the second centrifugation where $n = 2$. Where $n = 3$, error bars show standard deviation (SD), and in the case $n = 2$ (max − min)/2 is reported instead of SD.

centrifugation. Taking these aspects into account, ethanol addition has to be carefully considered, and further studies are therefore needed to investigate if the advantages of ethanol addition are larger than potential side effects.

Oil (olive, fish, and soybean) has previously been used to extract fat-soluble contaminants from fish meal (*8*, *9*). However, our results showed no effect of addition of oil, which could be a result of the amount of added oil (30% added) or the extraction temperature (~4 °C). In the study by Baron et al. (*8*) room temperature was used for the extraction, and 60−75% of the dioxins and DL-PCBs could then be removed with 100% added oil. In their study, an extraction time of 30 min, the same as in the current study, was found to be as efficient for dioxin and DL-PCB removal as 24 h (*8*). In the study by Oterhals et al. (*9*) extraction with 300% added oil was performed at 87 °C for 1 h, and then as much as 97% of the contaminants were removed. Hence, it is possible that an increased extraction temperature can increase the contaminant removal. However, it is not suitable to implement oil extraction into the pH-shift process at temperatures above those where the proteins start to denature. Also, the temperature effect on endogenous proteolytic enzymes has to be considered, as increased temperature has been shown to increase proteolytic activity and impair the functionality of the proteins (*32*).

Mass Balance and Correlation between Fat and Dioxin. The fat, dioxin, and DL-PCB contents were all highly correlated between the samples (i.e., starting material; floating layer of first centrifugation; pellet of first centrifugation, supernatant of second centrifugation, alkali-made protein isolate, acid-made protein isolate, and alkali-made protein isolate with 5% ethanol). These results were expected since dioxins and DL-PCBs are highly lipophilic. Hence, it was expected to find most of the contaminants in the floating fat layer and pellet after the first centrifugation since these subfractions contain large amounts of the lipids. According to theory, neutral lipids, having a density lower than water, are to be found mainly in the floating fat emulsion layer while membrane lipids are to be found in the pellet of the first centrifugation (*33*). Since it has been hypothesized that the coplanar dioxins to a higher degree should be found in the membranes than the DL-PCBs (*9*), it was expected to find different congener distribution in the different samples; e.g., more

Table 4. Pearson Correlation between Fat, Dioxin, and Dioxin-like PCB Content[a]

	fat	dioxin	DL-PCB
fat	1		
dioxin	0.952	1	
DL−PCB	0.996	0.961	1

[a] Two-tailed correlation is used. $N = 19$, and the samples used are the starting material, the different subfractions of the pH-shift analysis (see **Figure 1**), and the resulting protein isolates (alkali-made, acid-made, and alkali-made with 5% ethanol). The correlations are significant at the $p = 0.01$ level.

dioxins in the pellet of the first centrifugation and more DL-PCBs in the floating fat layer of the first centrifugation. However, no such differences were seen. Furthermore, no significant differences in dioxin−PCB TEQ ratio could be seen between the sample types. The reason that no significant differences are seen could possibly be due to an incomplete separation of fat classes between the floating layer and pellet of the first centrifugation. Further studies are needed to investigate whether dioxin and PCB congeners discriminate between membrane lipids and neutral lipids.

Investigation of the mass balance (**Figure 2**) shows that the total sum of dioxins, DL-PCBs, and lipids in the various layers obtained during the process exceeded 100%, being respectively 138%, 120% and 107%. The different subfractions analyzed vary largely in their composition (i.e., lipid, water, protein, and ash content). The methods applied to quantify lipids/lipid-soluble contaminants might therefore yield somewhat different results. It should also be noted that the analytical error when determining these trace contaminants is in the order of 10−20%. However, these overestimations, in particular of dioxins, do not impair the results. To the contrary, a possibly further lowered toxicity of the isolates than the reported would strengthen the results. Since the amount of fat and contaminants vary largely between the different subfractions (**Figure 2**), the relative distribution, i.e., the most fat and contaminants in the floating layer and the least in the water fraction of the second centrifugation, can be regarded as true. This is also in agreement with a previous study on herring light muscle (*17*).

The very high correlation between fat and contaminant levels (**Table 4**) implies that any method efficient in lowering the fat content would efficiently reduce the dioxins and DL-PCB levels. Since the fat, dioxin, and DL-PCB levels are so highly correlated, further optimization of contaminant removal can be done by monitoring the fat content only, with the exception for methods where oil is added in the process. This would reduce the cost since analyses of dioxin and DL-PCB levels would be minimized.

Previous studies using addition of calcium chloride and citric acid (*18*, *21*) or chitosan (*34*) during pH-shift processing show a decrease in the amount of fat, especially membrane phospholipids. Therefore, it is possible that these modified pH-shift methods could be even more efficient in removing dioxins and DL-PCBs from contaminated fish than the unmodified method. Indeed, addition of citric acid and calcium chloride to the acid pH-shift method has been successfully applied to remove diarrheic shellfish poisoning toxins from blue mussels (*20*). However, in the present study, the addition of calcium chloride and citric acid did not improve the efficiency of fat or contaminant removal. The addition was done according to a previous study on herring fillets (*21*) and not optimized for gutted whole herring which was used here. The larger amounts of bones, and thereby calcium in the gutted herring mince, suggest that the method might need to be modified, taking the high calcium levels into account. It is also possible that other factors present in whole gutted herring, but

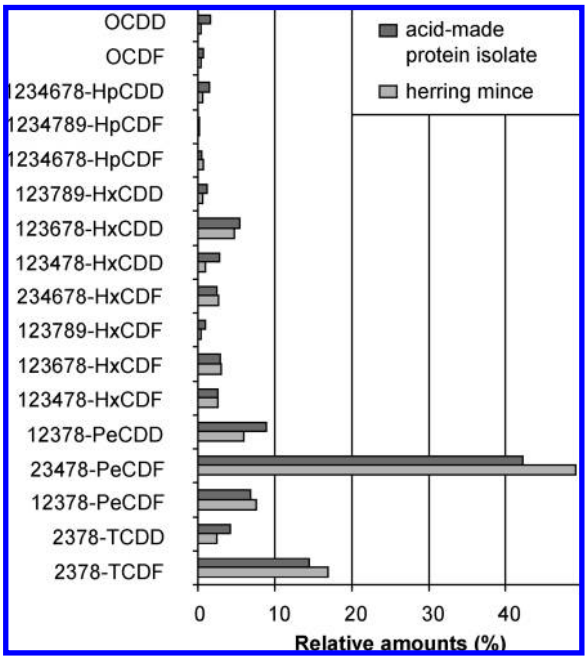

Figure 3. Two typical congener profiles for dioxins in acid-made protein isolate and minced herring.

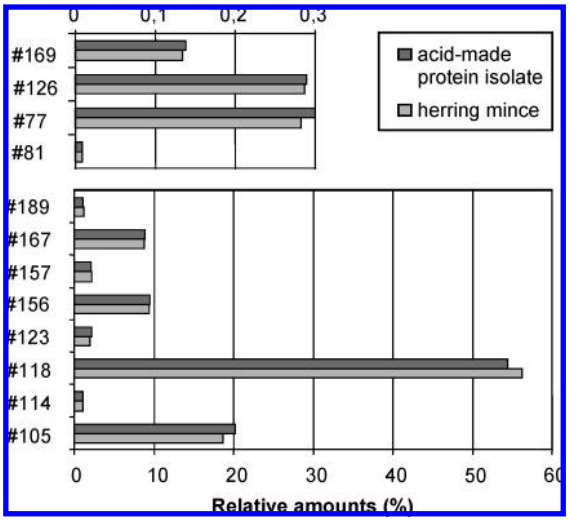

Figure 4. Two typical congener profiles for dioxin-like PCBs and ortho-PCBs in acid-made protein isolate and minced herring.

not herring fillets, could influence the citric acid and calcium chloride mediated fat removal.

The lipid contents (dry weight) of the protein isolates produced (**Figure 2**) were similar as was previously reported after pH-shift processing of herring fillets (*35*). Lower lipid contents have been obtained for other, in particular leaner, materials. Lower lipid contents have also been obtained with higher centrifugation forces, something that is difficult to achieve industrially, and with additions of calcium chloride and citric acid (*18*, *21*) or chitosan (*34*). The latter methods though lower the lipid content at the cost of lowered protein yield. Liang et al. stated that the chitosan addition presumably is too costly and time-consuming to be used industrially (*34*). Since further reduction of lipids and contaminants appears to come at the cost of lower yield and/or possibly a more costly process, the original process may be preferred.

The data reported show that the unmodified acid and alkaline pH-shift methods are able to reduce lipids, dioxins, and DL-PCBs with about 70−80% per amount of protein. The reduction of dioxins and DL-PCBs shown here is sufficient to lower the toxicity well below EU regulation limits for fish-based foods (*4*). Taken together with the very low market value for small pelagic fish like Baltic herring, and the restrictions in its use due to its elevated levels of dioxins, we believe there are great potentials with pH-shift processing of this raw material. It should also be stressed that the protein isolates open up for development of new and innovative food products since they are free of bones and also allow for other components (e.g., aroma, stabilizers) to easily be mixed in.

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Supporting Information Available: Tables of the fat, dioxin, and PCB content for the protein isolates made with the three modifications: (ii) homogenization at p*I*, (iii) citric acid and Ca^{2+} addition, and (iv) oil addition. This material is available free of charge via the Internet at http://pubs.acs.org.

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