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Comparison of Methods to Reduce Dioxin and Polychlorinated Biphenyls Contents in Fishmeal: Extraction and Enzymatic Treatments

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Methods to remove dioxins and polychlorinated biphenyls (PCBs) from fishmeal were investigated and compared. The tested methods include (i) lowering the fat content and simultaneously the level of toxic contaminants using either organic solvents or (ii) lowering the fat content using protease and (iii) removal of dioxins and PCBs using either oil extraction or (iv) breakdown of dioxin and PCBs using oxidoreductase. The results showed that the organic solvents tested (ethanol, isopropanol, and isohexane) were efficiently lowering the oil content of the fishmeal by 80%. However, the treated fishmeal has a low fat content and may contain traces of solvent. The protease alcalase was not as efficient as the solvent extraction and only removed approximately 30% of the oil but presented the advantage of being a mild process. Other proteases, alone or in combination with other enzymes, might give better yield if the reaction conditions are optimized. In contrast, extraction of dioxin and PCBs using olive oil or fish oil was very effective and resulted in 60−75% extraction of dioxin and PCBs, respectively, after a single extraction step. No preference for the oil type was observed. This method is very simple and quick and does not require an important investment for the fishmeal producer. It is expected that with optimization this method could be implemented at an industrial scale without too many difficulties. In contrast, the oxidoreductases tested did not result in a major degradation of dioxins and PCBs with only 10−15% degradation achieved. However, with the recent advancement in biotechnology, it is possible that future research will result in the development of enzymes that effectively degrade recalcitrant contaminants.

KEYWORDS: Fishmeal; dioxins; PCBs; laccase; peroxidase; extraction

INTRODUCTION

Dioxins and polychlorinated biphenyls (PCBs) have in the last decades received a great deal of attention due to their high toxicity (*1*, *2*). They have been claimed to contribute to a number of diseases (*3*, *4*). Short-term exposure is found to be responsible for skin disease while long-term exposure can result in impairment of the immune system, the nervous system, the endocrine system, and the reproductive functions. Chronic exposure of animals to dioxins can result in the development of numerous cancers (*5*). Dioxins and PCBs are ubiquitous in the environment and are mainly introduced into the environment through pollution from anthropogenic sources. They are lipohilic contaminants, and they accumulate in the lipid fractions of animal feeding on contaminated soils and sediments (*6*). Because of their extreme toxicity, their levels in food and feed stuff are strictly legislated and controlled. As they are not equally toxic, congeners have been weighted in relation to each other by determining a toxic equivalency factor (TEF). The total dioxin content in fishmeal is obtained by weighting each of the 17 measured concentrations of the congeners with their respective TEF values and summing them up to produce a toxic equivalency quantity for dioxins expressed in ng TEQ/kg. In Europe, the maximum authorized level for dioxins in food and feed is set by the European Union (EU) commission and is presently 1.25 ng TEQ/kg for fishmeal and 6 ng TEQ/kg for fish oils (*7*). Moreover, the EU Commission has introduced new combined maximum levels for dioxin and dioxin-like PCBs levels in fishmeal, applicable since November 2006 (*8*). These combined maxima are 4.5 ng TEQ/kg for fishmeal (3.25 ng TEQ/kg dioxin-like PCBs and 1.25 ng TEQ/kg dioxin) and 24 ng TEQ/kg for fish oil (18 ng TEQ/kg dioxin-like PCBs and 6 ng TEQ/kg dioxin). For fishmeal producers, these limits are severe burdens and reducing the level of contamination in fishmeal and fish oil is a challenge. Methods to remove dioxin from oils have been developed using solid-phase extraction (*9*, *10*) or short path distillation (*11*). However, no efficient and simple method or processing alternatives for removing dioxins and PCBs from fishmeal without impairing its nutritional value are available at the moment. A recent investigation demonstrated that it was possible to directly reduce the level of dioxin in

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fishmeal using UV light exposure (*12*), but UV light also triggered oxidative reactions leading to rancidity. Because of the lipohilic properties of dioxins, the reduction of the fat content of fishmeal results in the concurrent reduction of the lipohilic contaminants. Lowering the fishmeal fat content either mechanically or chemically using organic solvents has been implemented successfully by the Danish fishmeal industries, but no scientific reports on the efficacy of these processes are available. Moreover, the reduction of the fat content results in fishmeal with altered physicochemical properties and poorer nutritional value and also requires manipulation of a large volume of inflammable solvent. In addition, these methods often necessitate drastic modifications of operating units for fishmeal producers. A recent preliminary report indicated that another type of extraction using oil as the extraction medium could be an alternative (*13*). The data from this pilot study indicate that extraction of the press cake with vegetable oil can result in a reduction of almost 90% of polychlorinated contaminants, but further research on the applicability and efficacy of the method is necessary.

Alternatively, proteases have been shown to efficiently facilitate the release of the lipids from protein matrices in fish waste (*14*). Extensive proteolysis with proteases results in protein cleavage, which can induce the release of the lipids from the membrane or the lipids bound to protein via a non-covalent bond, for example, hydrophobic interactions (*15*). Lipids are therefore released from the protein matrices, and this approach could be an alternative for the treatment of fishmeal in order to reduce its fat content and simultaneously its dioxin and PCBs levels. Enzymatic treatment is a mild procedure, which is environmentally friendly. However, enzymes are sometimes expensive, and after treatment with protease, the resulting fishmeal is highly hydrolyzed and sometimes presents a bitter taste. As an alternative to extraction treatments, direct degradation of dioxins and PCBs using microorganisms or enzymes has received some interest for the treatment of contaminated soils and sediments but has not been investigated for fishmeal. Previously, it was reported that some microorganisms are able to degrade a variety of recalcitrant environmental pollutants (*16*, *17*). Degradation of dioxin-like compounds in contaminated soils has been thoroughly investigated (for a review, see ref *18*). Recent investigations have shown that fungal laccases and peroxidases from white rot fungi can catalyze the degradation of organic pollutants (*19*, *20*), and these enzymes deserve further attention.

In the present study, extraction of dioxins and PCBs from fishmeal using organic solvents and oils was examined. The use of proteases to reduce fishmeal fat content and the ability of oxidoreductases to directly degrade dioxins and PCBs were also investigated. The investigation has been performed as a comparative study to evaluate how effective the different treatments are at reducing dioxins and PCBs contents in fishmeal. This investigation aimed at demonstrating that different possibilities for lowering the level of contaminants in fishmeal exist.

MATERIALS AND METHODS

Materials. Fishmeal samples (dioxin and PCBs contents of 4.89 and 2.43 ng TEQ/kg, respectively) and cleaned fish oil (dioxin and PCBs contents of 0.71 and 6.59 ng TEQ/kg, respectively) were obtained from the fishmeal producer FF-of-Denmark (Skagen, Denmark) and stored at 2 °C until use. Extra virgin olive oil (Coosur S.A., Madrid, Spain) (dioxin and PCBs contents of 0.0003 and 0.04 TEQ/kg, respectively) was purchased at the local supermarket. Laccase Novozym 51003, peroxidase (Novozym 51004) (both from genetically modified *Aspergillus* sp.), and alcalase (Alcalase 2.4 L) were a gift from Novozymes (Bagsværd, Denmark). Laccase Daiwa Y120 (from white rot fungus *Trametes* sp.) was a gift from Amamo Enzyme Europe Ltd. (Chipping Norton, United Kingdom). Analytical grade isopropanol was from Merck (Darmstad, Germany), and isohexane was from Fisher Scientific Ltd. (Loughborough, United Kingdom).

Dioxins and PCBs Analysis. Dioxin and PCBs levels of the fishmeal and oil were assessed by Eurofins Gfa (Münster, Germany), who determined the concentration of the toxic congeners in ng/kg as well as the toxic equivalent quantity (in ng TEQ/kg). Dioxin levels expressed in ng TEQ/kg were the sum of weighted congeners relative to 2,3,7,8-polychlorinated dibenzodioxin (PCDD). PCBs levels are also expressed in ng TEQ/kg as the sum of the 12 PCB congeners weighted relative to 2,3,7,8-PCDD. On the basis of the PCDD/F congeners and on 12 PCBs congeners concentrations, TEQ values were calculated according to the WHO model.

Enzyme Activity Measurement. Peroxidase and laccases activities were measured using a 2,2′-azino-bis(3-ethylbenzthiazoline-6-solfonic acid) (BTS) liquid substrate system (Sigma Saint Louis, MO). The specific activity of alcalase 2.4 L was given as 2.4 Anson units (AU) per gram.

Oil Content. The oil content of the fishmeal was determined gravimetrically using chloroform and methanol according to the protocol of Bligh and Dyer but using a 40% reduction of the amount of solvent (*21*).

Fatty Acid Composition. Fatty acids in the Bligh and Dyer extract were transesterified to methyl ester using a base-catalyzed transesterification followed by a boron trifluoride-catalyzed esterification according to the AOCS method (*22*). An HP 5890 gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector was used for separation of the fatty acid methyl esters. The column used was an Omegawax 320 fused silica capillary (0.32 mm $\times$ 30 m $\times$ 0.25 μm) (Supelfo, Bellefonte, PA). The fatty acids concentration was calculated using methyl tricosanoate (C23:0) as the standard.

Lipid Class. Lipid extracts were separated into lipid classes using solid-phase extraction [Sep-Pak Vac 3 cm^3 columns (Waters, Milford, MA)] and according to the protocol described by Kim and Salem (*23*). The column was equilibrated with heptane and, after sample loading, was eluted with a chloroform:2-propanol mixture (4:1) to obtain the neutral lipid fraction (NL); thereafter, 2% acetic acid in diethyl ether was applied to obtain the free fatty acid fraction (FFA), and finally, methanol was applied to extract the phospholipids (PLs). Calculation of the percentage of FFA, PL, and NL extracted was performed by setting the initial content of the fishmeal FFA, PL, and NL to 100%.

Lipid Oxidation. Peroxide values were measured on the lipid extract by colorimetric determination using the ferric thiocyanate assay as described by Shantha and Decker (*24*).

Extraction. *Extraction Using Solvent.* Fishmeal samples of 5 g were extracted with 40 mL of solvent, i.e., ethanol, isohexane, or isopropanol, using an ultra turrax homogenisator. After 30 s of homogenization, 10 mL of solvent was added and homogenization proceeded for another 30 s. Subsequently, the homogenate was centrifuged at 2800 rpm for 10 min and filtered on paper. The oil content in the organic phase was determined gravimetrically. The oil content was calculated, and the fatty acid composition was determined. The percentage of extracted oil was determined by comparison with the initial oil content determined using Bligh and Dyer and setting it to 100%. The fishmeal extracted with ethanol was further extracted using the Bligh and Dyer protocol to determine the non-extracted oil content.

Extraction Using Alcalase. Fishmeal samples of 50 g were incubated at 60 °C for 1 h either without alcalase (control sample) or with inactivated alcalase (boiled for 15 min) or with 1 or 10% (w/w) alcalase. For fishmeal containing the enzymes, the pH was adjusted and maintained at 9 using NaOH (1 M), which is the optimum pH for alcalase activity. For the control sample (fishmeal alone), the pH was not adjusted but the sample was incubated at 60 °C for 1 h similarly to the samples containing the enzyme. To stop the reaction, inactivation of the enzymes was performed by readjusting the pH to 6 in the samples containing alcalase (for both inactivated and activated). After centrifugation, the oil content was determined in the treated fishmeal using

Table 1. Percentage of Oil, NL, FFA, and PLs Extracted from Fishmeal Using Solvents with Different Properties[a]

	ethanol C_2H_6O	isohexane C_6H_{14}	isopropanol C_3H_8O
oil (%)	84.6 (±1.3)	66.1 (±5.5)	71.1 (±6.1)
NL (%)	89.9 (±0.4)	87.6 (±5.1)	90.1 (±6.8)
FFA (%)	65.9 (±1.3)	58.2 (±5.5)	60.0 (±6.1)
PL (%)	59.0 (±3.9)	29.5 (±2.1)	41.4 (±10)
explosion limit (%)	3.3–19	1.2–7	2–12
LD_{50} rats (g/kg)	7.1	NA	5.0
boiling point (°C)	78.5	60.0	82.4

[a] The initial fishmeal content in oil, NL, FFA, and PL was set to 100%. Results are presented as the mean of at least duplicate measurements (± standard deviation). NA, no available data in the literature.

the Bligh and Dyer method described previously and subsequently using the sum of the fatty acids obtained from the fatty acid profile determination. The oil in the water phase was also extracted using (3×) 50 mL of chloroform.

Extraction Using Oil. Fishmeal samples of 50 g were mixed with an equal amount (w:w) of fish oil or olive oil. The mixture was stirred for 30 min or 24 h at room temperature. After centrifugation at 12,500 rpm for 15 min, the oil was filtered and further analyzed for dioxin and PCBs content and fatty acid composition.

Enzymatic Degradation. Fishmeal samples of 200 g in 300 mL of deionized water were mixed with 10% enzyme (v:v) and incubated in a water bath at 60 °C for 24 h under magnetic stirring. Laccase I refers to Laccase Novozym 51003 while laccase II refers to Laccase Daiwa Y120. Peroxidase was incubated in the presence of 0.1 mM hydrogen peroxide. Thereafter, the fishmeal samples were freeze-dried prior to further analysis. Controls without enzyme were also run in parallel. Additionally, a control containing $FeCl_3$ (8 mM) and 1% hydrogen peroxide was also performed in order to evaluate the potential of oxidative reaction at breaking down dioxins and PCBs.

RESULTS

Extraction Using Solvents. Extraction of the fishmeal fat was performed using different organic solvents. Isohexane was chosen because it is currently used by a Danish fishmeal industry (*25*) while isopropanol and ethanol were selected as they have often been used to prepare fish protein concentrates (*26*, *27*). These solvents have different polarities and different properties, but all solvents tested are able to remove more than 50% of the oil initially present in fishmeal (**Table 1**). Ethanol gave better results (85%) than the other two solvents, isohexane being the least efficient with only 66% oil extracted. After separation of the lipids into classes, it was found that isohexane gave a poorer extraction for all lipid classes (**Table 1**). Isohexane was especially less efficient at extracting PLs with only 30% of the PLs extracted as compared to ethanol that was able to extract 60% of the initial PL content in the fishmeal used in the experiment. Overall, it was observed that ethanol was the best extraction solvent. After the ethanol extraction, further extraction of the fishmeal using chloroform and methanol (Bligh and Dyer protocol) indicated that only 20 g oil/kg fishmeal remained in the fishmeal (data not shown). The fishmeals used in the experiment contained on average 100 g oil/kg fishmeal, and this means that the oil content was reduced by a factor of 5; consequently, the dioxin and PCBs levels were also reduced by the same factor, assuming that the contaminant concentration is proportional to the oil content. No data are available regarding the affinity of the dioxin and PCBs contaminants for the different lipid classes (NL, FFAs, or PLs) or even the sterols, but further investigations should be performed to elucidate this issue.

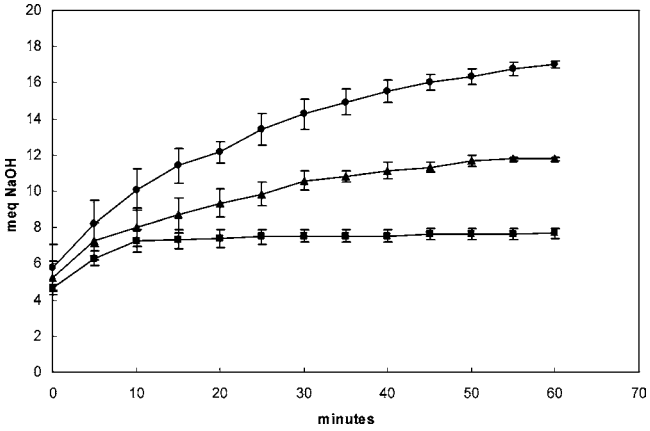

Figure 1. Titration of acidity using NaOH (1 M) during treatment of fishmeal with 1% alcalase (▲), 10% alcalase (●), and inactivated alcalase (■).

Table 2. Effect of Alcalase Treatment (pH 9; Temperature, 60 °C; and Reaction Time, 60 min) on the Release of Oil from Fishmeal[a]

	fishmeal control	inactivated alcalase	1% alcalase	10% alcalase
(A) oil % in solid phase Bligh and Dyer	9.18 (±0.37)	6.59 (±0.33)	9.03 (±0.25)	10.01 (±0.03)
(B) oil % in water phase chloroform	1.86 (±0.10)	3.39 (±0.25)	2.49 (±0.31)	3.16 (±0.29)
(C) oil % in solid phase FAME	6.94 (±0.42)	6.08 (±0.63)	5.45 (±0.22)	4.60 (±0.08)

[a] (A) Oil content after extraction in the solid phase using the Bligh and Dyer protocol, (B) oil content in the water phase after extraction using chloroform, and (C) oil content in the solid phase after extraction using Bligh and Dyer and obtained from the fatty acid profiles using GC-FID (FAME). Inactivated alcalase, boiling for 15 min before reaction. Fishmeal control sample, fishmeal without pH adjustment to 9.

Extraction Using Alcalase. In contrast to oil extraction using solvent, a mild procedure was investigated using the protease alcalase on fishmeal in order to facilitate oil release from the protein fraction. Titration of acidity using NaOH (1M) was performed in the presence of inactivated alcalase or 1 and 10% alcalase, and the results showed that treatment with alcalase induces changes in the acidity of the fishmeal substrate (**Figure 1**) as a consequence of protein hydrolysis (*28*). The level of hydrolysis was found to be proportional to the amount of enzyme with 10% generating more acidity in the fishmeal substrate than 1% alcalase. Evaluation of the oil content after enzymatic treatment in the fishmeal (solid phase) by gravimetry using the Bligh and Dyer protocol and in the water phase using chloroform was performed, and the results are presented in **Table 2**. Surprisingly, using the traditional Bligh and Dyer extraction, it was found that the level of oil in the fishmeal after alcalase treatment was lowest with inactivated alcalase (6.59%), indicating that the pH shift from 6 to 9 allowed more efficient release of the oil in the water phase. This was further supported by the data obtained from the control sample (fishmeal) where the pH was not initially adjusted to 9 and where the oil in the water phase was low (1.86%) as compared to the samples that underwent the pH shift. However, a higher oil content in the fishmeal treated with 1 and 10% alcalase was observed as compared to the inactivated enzyme (6.59%) when

Table 3. Dioxin Profile of Fishmeal, Olive Oil, and Fish Oil before and after 30 min or 24 h of Extraction at Room Temperature

	initial			olive oil		fish oil	
PCDDs (ng/kg)	fishmeal	olive oil	fish oil	30 min	24 h	30 min	24 h
2378-tetraCDD	0.39	ND	0.07	0.21	0.21	0.23	0.27
12378-pentaCDD	0.79	ND	0.11	0.45	0.54	0.44	0.57
123478-hexaCDD	0.15	ND	0.06	0.10	0.15	0.10	0.15
123678-hexaCDD	0.68	ND	0.10	0.46	0.47	0.49	0.60
123789-hexaCDD	0.17	ND	0.06	0.11	0.12	0.12	0.12
1234678-heptaCDD	0.59	ND	0.40	0.51	0.74	0.41	0.44
octaCDD	1.07	2.93	0.99	2.65	2.86	<0.99	<0.99
ng TEQ/kg[a]	4.89	0.0003	0.71	2.55	2.95	3.22	3.45

[a] Coefficient of variation (CV), 12%.

determined using the Bligh and Dyer protocol. It is likely that hydrolysis of the fishmeal by alcalase released hydrophobic amino acids and peptides, which might be extracted simultaneously with the oil during the Bligh and Dyer extraction and resulted in over-estimation of the oil content determined by gravimetry. The data suggest that the Bligh and Dyer method was not suitable for highly hydrolyzed matrices. The lipid content obtained after analysis of the fatty acid profile and using an internal standard for quantification indicates a fat content of 7% in fishmeal while treatment with inactivated alcalase results in a fat content of 6% and treatment with active alcalase revealed approximately 5 to 4.5% fat in fishmeal. Consequently and on the basis of the obtained reduction in fat content, the level of contaminant is estimated to be reduced to 20−30% using alcalase treatment.

Extraction Using Oils. The initial levels of contamination for the fishmeal and the oil used in the extraction experiment are presented in **Table 3**. The fishmeal used had a high level of contamination with a dioxin TEQ in ng/kg equal to 4.89. In contrast, the oil used had a low level of contamination. It is noteworthy to observe that olive oil contained a high level of the octachlorinated congeners (OCDD) but a very low TEQ due to the low toxicity factor attributed to OCDD. Extraction was performed at room temperature for 30 min or 24 h, and the results are presented in **Table 3**. It is demonstrated that extraction of the fishmeal using oil did result in substantial partitioning of the dioxin into the oily phase. Enrichment of the oily phase in dioxin was observed for all congeners and for both oil types. Using a 1:1 fishmeal to fish oil ratio, more than 50% of all congeners were extracted and no preference for any of the congeners was observed. The affinity of the congeners for the oil was independent of the type of oil used. In contrast to dioxin extraction, which represented more than 50%, the extraction of long-chain fatty acids EPA/DHA (eicosadecanoic acid and decosahexanoic acid, respectively) in vegetable oil and of linoleic acid (C18:2, n-6) in fish oil seemed to be less efficient (**Table 4**). Increasing the time for dioxin extraction only resulted in a minor increase in the level of dioxin in the oils, indicating that equilibrium and partitioning of the contaminant in the oil are fast processes. A second extraction of the fishmeal with fish oil or olive oil under the same conditions resulted in further extraction of the dioxin congeners (data not shown). Investigation of the partitioning of the PCBs in the oil was also performed, and it was revealed that not only dioxins but also PCBs were efficiently extracted using this method. The results for extraction using olive oil are shown (**Table 5**). As observed for dioxin, increasing the time of reaction did not significantly affect the extraction yield. A slightly higher yield of extraction was observed for PCBs than for dioxin, with 75 and 60%, respectively, after 24 h.

Table 4. Content of Linoleic Acid and EPA and DHA in Olive Oil and Fish Oil before and after Extraction of Fishmeal for 24 h[a]

	mg/kg		
	linoleic acid	EPA	DHA
olive oil	795 (±7)	0 (±0)	0 (±0)
olive oil, 24 h	690 (±14)	35 (±7)	70 (±0)
fish oil	105 (±7)	775 (±63)	1090 (±98)
fish oil, 24 h	120 (±0)	870 (±40)	1205 (±50)

[a] The fatty acid content of the oils was obtained from fatty acid analysis profiles using GC-FID (FAME).

Table 5. PCBs Profile of Initial Fishmeal and Olive Oil and Olive Oil after Extraction of Fishmeal for 30 min and 24 h at Room Temperature

		olive oil		
	fishmeal	initial	30 min	24 h
	non-*ortho*-PCBs (ng/kg)			
PCB 77	53	3.2	37.6	34.1
PCB 81	1.1	1.4	1.4	1.4
PCB 126	18.0	0.7	13.1	13.6
PCB 169	4.1	1.4	3.3	3.0
	mono-*ortho*-PCBs (ng/kg)			
PCB 105	937	78.1	610	668
PCB 114	24.3	2.8	71.7	93.7
PCB 118	2660	241	2010	1980
PCB 123	23.4	2.8	23.5	32.4
PCB 158	320	7.6	285	248
PCB 157	73.8	2.8	57.1	54.8
PCB 167	181	6.7	142	139
PCB 189	29.8	5.3	24.3	32.8
ng TEQ/kg[a]	2.43	0.04	1.81	1.87

[a] Coefficient of variation (CV), 12%.

Enzymatic Degradation. Peroxidases and laccase were investigated for their abilities to degrade dioxins and PCBs, and results are presented in **Table 6**. Both enzymes are oxidoreductases, but in contrast to laccase, peroxidase requires the presence of hydrogen peroxide to be active. Both enzymes showed only little activity toward the degradation of dioxins and PCBs ranging from 11 to 15% for dioxin degradation and from 7 to 18% for PCBs. Surprisingly, the inactivated enzymes also showed some minor degradation of dioxins and PCBs. For inactivated peroxidases, the presence of hydrogen peroxide might trigger oxidative reactions as observed by the increase in peroxide values. Similarly, degradation using laccases is performed at low pH, which increases iron solubility and might induce degradation via oxidative reactions. To further investigate the ability of oxidative reactions to degrade dioxins and PCBs, iron and hydrogen peroxide were added to the fishmeal. The results confirmed that oxidative reactions can participate in the degradation of dioxins and PCBs with a preference for degradation of PCBs. Both laccases tested gave the most interesting results, but with only 14−15 and 14−18% degradation for dioxin and PCBs, respectively. However, it is still quite unclear how much the enzyme itself or side reactions contributed to the degradation of the contaminants.

DISCUSSION

This investigation aimed at pointing out new directions in the removal of dioxins and PCBs in contaminated fishmeal and also compared the different methods. From the obtained results,

Table 6. Effect of Oxidoreductase on Fishmeal Oxidation, Dioxin, and PCBs Profiles[a]

	fishmeal control	peroxidase inactivated + H_2O_2	10% peroxidase	laccase inactivated	10% laccase I	10% laccase II	H_2O_2/Fe^{3+}
pH	6.3	6.3	6.3	4.5	4.5	4.5	6.3
oil (%)	13.2	12.5	12.9	12.8	11.6	12.9	13.3
PV	4.0	5.3	4.0	6.2	7.0	7.3	15.6
dioxins (%)	100	92	87	89	86	85	94
PCBs (%)	100	96	93	85	84	82	85

[a] Dioxins and PCBs represent the contaminants (ng TEQ/kg) content of fishmeal expressed in % of the initial content of contaminants.

it is clear that extraction using organic solvent is a good solution as it was possible to extract most of the lipids from fishmeal. However, the resulting fishmeal was low in fish oil content and might contain some traces of solvent and therefore might be a less-appealing product for the customers. Isohexane is used today by the industry to remove dioxins as an additional step in the fishmeal production (*25*), but it was found that the alcohols gave better results with extraction of more PLs and more FFAs. Indeed, PLs are more polar and require polar solvents to disrupt hydrogen bonds or electrostatic forces while NLs are extracted with relatively non-polar solvents. Others have also reported that a more polar solvent was better at extracting FFAs and diglycerides (*29*). A study using mixed solvent reported that it gave good lipid recovery (*30*) and also reduced protein denaturation (*31*), but this was not investigated here. In the present study, the extraction step was applied on the final product and it is possible that applying the extraction as an additional step during the production of fishmeal would give different results. However, an earlier study performed on sardine indicated that extraction performed on cooked fish protein gave better lipid yield as compared to uncooked fish (*27*). One of the advantages of using alcohols over isohexane is that they are less explosive than isohexane and possibly less toxic (**Table 1**). Supercritical solvent extraction and accelerated solvent extraction have received some attention for the analysis of dioxin content in food and feed, but similar methods cannot be used by fishmeal producers because they are destructive (*32*). However, it is possible that milder extraction conditions and appropriate solvent mixtures will be available in the future for the food and feed industry.

The alcalase experiment showed that it is possible to hydrolyze fishmeal and liberate the lipids from the fishmeal protein matrix. However, some of the problems associated with such a process are that the properties of the protein are changed as a result of hydrolysis. Reducing the protein length is reported to result in a decrease in functionality (*33*). Other studies on fish waste have evaluated the use of a combination of different proteases or addition of mixtures of proteases and lipases, and this could be investigated further on fishmeal (*34*, *35*). Furthermore, in the investigation, the enzyme/substrate ratio and the conditions of hydrolysis were not optimized and it is likely that higher oil yield may be obtained. It is observed that alone the alkaline treatment of the fishmeal resulted in a 10−12% decrease in the oil content while enzymatic treatment is shown to reduce the oil content by 20−30%, which is assumed to give a corresponding decrease in toxic contaminants. The experiment was performed directly on the fishmeal, but if applied during processing of the fish, enzymatic treatment may liberate more oil. In contrast to the solvent extraction process, the enzymatic treatment has several advantages: It requires no organic solvent, and it is quick and uses a low temperature and low-cost commercial proteases. However, more extensive investigations are needed in order to apply the process at an industrial scale. Interestingly, the present finding also indicates that determination of the oil content using classical solvent extraction is not appropriate for highly hydrolyzed matrices. A recent investigation also reported a higher yield for lipid after enzyme treatment of sardine viscera and argued that lipid extraction using classical extraction was not complete (*36*). In contrast, it is believed that extraction with organic solvent with highly hydrolyzed matrices results in overestimation of the oil content using classical extraction. Further investigation of extraction protocols is needed to further elucidate this issue.

Investigation of oil as an extraction medium gave promising results; it was fast and very effective at extracting all lipohilic contaminants. This process gave approximately a 60−75% reduction in the dioxin and PCBs content, as estimated from the amount extracted in the oil phase, and it showed no preference for any of the oil type used. Results for a single extraction were shown, but a second extraction lowered the contaminant content even further (data not shown). The process was applied on the final products, but others have shown that extraction of the press cake using olive oil gave slightly better results with a reduction of 89% for both contaminants (*13*). The interesting advantage of this process is that it can be applied as an integrated part in the production. Thus, the cleaned oil obtained from processing of fish into fishmeal can, after cleaning, be reinjected in the production line in order to remove the contaminants from the produced fishmeal. A main challenge will be to remove the excess oil introduced in the process; therefore, the recovery of the oil needs to be optimized. It is also noteworthy that the resulting fishmeal might have a higher level of fat and might be less stable to oxidative degradation, but this remains to be investigated. Surprisingly, the extraction of dioxin and contaminant in the oil was more effective than the extraction of the fatty acids (**Table 4**) as also reported by others (*13*).

The oxidoreductases gave very limited degradation of dioxin and PCBs. In this study, approximately 10% of dioxins were degraded by the tested peroxidase and 7% for PCBs after 24 h. It is possible that a longer incubation time would give higher degradation yields. However, a screening study for activity of peroxidase from fungi revealed that the most active strain was degrading between 15 and 20% of the TCDD in 10 days and this indicates that degradation is a very slow process (*37*). Investigation with laccase gave similar yields and almost identical degradations for both PCBs and dioxins after 24 h. Degradation of organic contaminant via oxidative reactions has previously been reported by a number of studies (*38*), and how much the enzyme is contributing to the degradation itself is difficult to assess. From the control experiment, it is revealed that the combined effect of iron and pH is critical in the degradation of contaminants via non-enzymatic reactions. Others have also shown that the pH of the reaction media influences

the electrostatic properties of the protein surface and can influence the enzymatic reaction (*39*). In agreement with our findings, Mino (*40*) reported little degradation of dioxins at pH 6.3 with iron and hydrogen peroxide but a significant increase with lower pH values. It has also been reported that increasing ferrous iron resulted in an increase in the degradation of contaminants (*41*). From the obtained results, it is clear that enzymes do represent a potential for the degradation of contaminants, but possible toxicity of metabolites has to be carefully investigated. It is likely that engineered enzymes designed to degrade recalcitrant contaminants effectively will be developed and available for food and feed industries in the future.

This study provides evidence that methods are available for removing dioxins and PCBs from fishmeal; some are simple and fast, and others need to be further investigated. All investigated methods have their advantages and disadvantages, but the most promising method seems to be the extraction with oil if it can be implemented into the existing production line. Direct enzymatic degradation of contaminants is not yet optimal, but it is possible that in the near future enzyme technology will represent a realistic option.

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