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Analysis of persistent halogenated hydrocarbons in fish feeds containing fish oil and other alternative lipid sources

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ABSTRACT

A trade-off exists between beneficial n-3 long-chain polyunsaturated acids and toxic persistent halogenated hydrocarbons (PHHs), both of which primarily originate from fish oil commonly used in fish feeds. Alternative lipid sources are being investigated for use in fish feeds to reduce harmful contaminant accumulation, hence, research is needed to evaluate PHHs in fish feeds with various lipid compositions. An analytical method was developed for PHHs including nine organochlorine insecticides (OCPs), 26 polychlorinated biphenyls (PCBs) and seven polybrominated diphenyl ethers (PBDEs) in fish feeds with differing proportions of fish oils and alternative lipid sources by GC-ECD after accelerated solvent extraction, gel permeation chromatography (GPC), and sulfuric acid cleanup. The GPC removed the majority of the neutral lipids and sulfuric acid treatment effectively destroyed the polar lipids. Thus, the combination of the two methods removed approximately 99.7% of the lipids in the extracts. The method detection limits were less than 5 ng/g dry weight (dw) for most PHHs, while recoveries were 75–118%, 67–105%, 69–92%, 63–100% and 94–144% with relative standard deviations of 0.2–39%, 0.3–20%, 0.5–12%, 1.5–18% and 1.5–15% for PHHs in five types of fish feeds made from different lipid sources. Although the source of lipid showed no impact on cleanup efficiency and the developed method worked well for all feeds, fish feeds with 100% fish oil contained background PHHs and more interference than feeds containing alternative lipids.

1. Introduction

Both nutritional and medical communities have recommended incorporating n-3 long-chain polyunsaturated fatty acids (LC-PUFA), particularly eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), into the human diet to reduce the risk of cardiovascular disease and seafood has been suggested as an effective way of increasing intake of n-3 LC-PUFA [1–3]. Unfortunately, persistent halogenated hydrocarbons (PHHs) including organochlorinated pesticides (OCPs), polychlorinated biphenyls (PCBs), and polybrominated diphenyl ethers (PBDEs) are also associated with fish (particularly oily fish) because of their hydrophobicity. As a result, bioaccumulation of those harmful contaminants may occur in humans through fish consumption intended to increase intake of healthy n-3 LC-PUFA [4–11].

The contribution of farm-raised fish to total seafood consumption has been increasing in recent years [12,13]. Currently, roughly half of the seafood consumed globally is farm-raised, a share that is projected to increase dramatically in the future [14]. However, aquaculture will not be able to fill the growing 'seafood gap' without commensurate growth within the aquafeeds industry. Traditionally aquafeeds were largely comprised of marine-derived feedstuff such as fish meal and fish oil (FO), but the rising price of these ingredients (currently $1600–1800 USD/MT) has created substantial economic incentive to spare marine-derived ingredients with terrestrially derived alternatives. Meanwhile, studies have indicated higher levels of PHHs in farm-raised fish in comparison to wild-caught fish, which some have argued poses greater risk to humans who consumed farm-raised fish [7,8,15]. Marine-derived feedstuffs, are considered the major source of contaminants in farm-raised fish [7,8,16,17]. Therefore, it is expected that fish raised on alternative ingredients would accumulate lower levels of contaminants in their fillet, and techniques have been developed to reduce PHHs in farm-raised fish by replacing FO with other terrestrial formulations [18,19]. Thus, there are both economic and food safety concerns which incentivize FO sparing in aquafeeds. However, the content of beneficial LC-PUFA declines in the resultant aquaculture products after changing feeds to the terrestrial alternatives [20]. Reducing contaminants in FO through active carbon treatment and distillation has also been proposed [21,22], but these processes are not widely considered to be economically viable within the aquaculture feed manufacturing sector. Recent research

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showed fish tissues mirror recent nutritional history, and by using 'finishing feeds' with high LC-PUFA content prior to harvest, the nutritional value of fish raised on alternative lipid-based diets could be restored [23–26]. In this way, exposure of farm-raised fish to unwanted contaminants in FO would be minimized, while maintaining the beneficial composition of the end product.

To facilitate the application of alternative lipids and finishing feeds in aquaculture, it is necessary to evaluate concentrations of toxic PHHs in fish feeds composed with lipids from different sources. Methods analyzing PHHs in fish feeds usually involve extraction, removal of co-extracted lipids and/or other interference and selective determination by gas chromatography (GC) [16,17]. Although previous studies have reported detection of PHHs in fish feeds [16,17], and various analytical methods have been developed to analyze PHHs in fish tissue or oils [27–33], few studies have addressed the influence of lipid source and composition on performance of analytical procedures of PHHs in fish feeds.

There is considerable and growing interest in analyzing the effect of FO sparing on the composition and contaminant burden of aquafeeds and the resultant farm-raised seafood products. Accordingly, the objective of the present study was to develop and validate an analytical method for quantifying PHHs including OCPs, PCBs, and PBDEs in fish feeds containing differing proportions of FO and alternative lipid sources. The analytes were determined by GC-electron capture detector (ECD) after accelerated solvent extraction (ASE), gel permeation chromatography (GPC), and sulfuric acid cleanup. The influence of lipid composition in various fish feeds on analyzing the PHHs was also evaluated.

2. Experimental

2.1. Chemicals

In the present study, three classes of PHHs were analyzed in five types of fish feeds with various lipid sources and composition. The target analytes were selected based on their environmental relevance, high bioaccumulation potential, and having previously been detected in fish feeds and tissues. The target compounds included nine OCPs (α-, β-, γ- and δ-BHCs, α- and γ-chlordanes, and *p,p′*-DDE, *p,p′*-DDD and *p,p′*-DDT), 26 PCBs (CB-18, 28, 29, 44, 50, 52, 66, 70, 77, 87, 101, 104, 105, 118, 126,128, 153, 170, 180, 183, 187, 188, 195, 201, 206 and 209), and seven PBDEs (BDE-28, 47, 99, 100, 153, 154 and 183). The stock solutions of the chemical standards were purchased from AccuStandard (New Haven, CT, USA) and diluted with hexane before use. Two surrogates, 4-4′-dibromooctafluorobiphenyl (DBOFB) and 2,2′,3,4,4′,5,5′,6-octachlorobiphenyl (PCB203), were added to the samples before extraction and used to quantify the performance of the developed method. The DBOFB was purchased from Supelco (Bellefonte, PA, USA), while the PCB 203 was from AccuStandard.

Pesticide grade solvents including acetone, dichloromethane, and hexane, and anhydrous Na_2SO_4, sea sand, silica gel, and concentrated H_2SO_4 were purchased from Fisher Scientific (Pittsburgh, PA, USA). Anhydrous Na_2SO_4 was baked at 450 °C for 4 h, while silica gel was baked at 130 °C overnight prior to use.

2.2. Fish feed preparation and sample spiking

Fish feeds used in the present study were prepared in the Fisheries and Illinois Aquaculture Center, Southern Illinois University at Carbondale, IL, USA and contained various proportions of FO (Omega Protein, Houston, TX, USA), coconut oil (CO; Jungle Products, Inc, Edison, NJ, USA), and palm oil (PO; Jungle Products, Inc, Edison, NJ, USA). These two alternative oils were selected because of their fatty acid composition, specifically the abundance of saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) found in these oils. In previous research [26,34], we demonstrated that alternative lipids, including CO and PO, minimize the loss of LC-PUFA from fish tissues and/or increase the responsiveness of fish tissues to LC-PUFA restoration during finishing. Hence, to maximize the relevance of the method development described in the present manuscript, we focused on feed formulations commonly used in our aquaculture nutrition research. Other ingredients were also included in fish feeds, including sodium phosphate monobasic, calcium phosphate dibasic, choline chloride, carboxymethyl cellulose, vitamins and mineral premixes, soybean meal, wheat bran and corn gluten meal, and they were purchased from Fisher Scientific and local stores.

Table 1
Fatty acid composition of total lipid extracted from fish feeds.[a]

Fatty acid(s)	FO	CO-50	CO-75	PO-50	PO-75
	g/100 g Fatty Acid Methyl Esters (FAME)				
8:0	0.0	0.7	2.2	0.0	0.0
10:0	0.0	2.2	3.6	0.0	0.0
12:0	0.2	20.9	30.0	0.2	0.2
14:0	7.8	11.9	13.4	4.4	3.0
16:0	19.5	15.1	13.0	28.4	31.6
18:0	3.9	3.5	3.4	4.4	4.5
SFA[b]	31.4	54.4	65.6	37.4	39.3
16:1n-7	10.2	5.7	3.5	5.4	3.4
18:1n-7	3.1	1.8	1.2	1.9	1.5
18:1n-9	9.7	8.6	7.9	23.5	29.5
20:1n-9	0.9	0.5	0.3	0.5	0.4
MUFA[c]	23.9	16.6	12.8	31.4	34.7
16:2n-4	1.3	0.7	0.4	0.7	0.4
16:3n-4	1.5	0.8	0.5	0.8	0.5
18:2n-6	10.0	9.2	9.2	12.5	13.9
20:4n-6	1.1	0.7	0.5	0.6	0.4
n-6[d]	11.2	9.9	9.7	13.1	14.4
18:3n-3	2.1	1.4	1.1	1.5	1.2
18:4n-3	2.6	1.6	0.8	1.3	1.1
20:5n-3	12.6	7.2	4.5	6.8	4.2
22:5n-3	2.3	1.3	0.8	1.2	0.8
22:6n-3	11.0	6.2	3.8	5.9	3.5
n-3[e]	30.7	17.6	11.0	16.6	10.7
n-3:n-6	2.8	1.8	1.1	1.3	0.7
PUFA[f]	44.7	29.0	21.6	31.2	26.0
MC-PUFA[g]	14.8	12.2	11.1	15.3	16.2
LC-PUFA[h]	27.1	15.3	9.6	14.5	8.9

[a] Data are presented as means of triplicate samples.
[b] Saturated fatty acids—sum of all fatty acids without double bonds.
[c] Monounsaturated fatty acids—sum of all fatty acids with a single double bond.
[d] Sum of all n-6 fatty acids.
[e] Sum of all n-3 fatty acids.
[f] Polyunsaturated fatty acids—sum of all fatty acids with ≥2 double bonds.
[g] Medium-chain PUFA—sum of all PUFA with chain length of 18 carbon atoms.
[h] Long-chain PUFA—sum of all fatty acids with chain length ≥20 carbon atoms and double bonds ≥3.

Five types of fish feeds were generated by mixing different ratios of FO, CO, and PO, while the remaining ingredients were kept constant among all feeds. Fish feed (1 kg) contained 200 g of fish meal, 140 g of corn gluten meal, 203.8 g of wheat bran, 300 g of soybean meal, 20 g of carboxy methyl cellulose, 15 g of sodium phosphate monobasic, 15 g of calcium phosphate dibasic, 6 g of choline chloride, 1 g of mineral premix, 1.2 g of vitamin premix, and 98 g of oil mixture. The oil mixtures included 100% FO (FO-100), 50% FO and 50% CO (CO-50), 25% FO and 75% CO (CO-75), 50% FO and 50% PO (PO-50), and 25% FO and 75% PO (PO-75). To prepare the feed, the dry macro ingredients (mass ≥ 20 g/kg of feeds) were weighed, combined in a 18.9 L bucket, and mixed thoroughly using a cutter-mixer (Model CM450, Hobart Corporation, Troy, Ohio). Depending on the lipid sources of the desired feed, an oil mixture with appropriate percents of FO and/or its alternatives were added and further

Table 2
Percent of neutral lipids in total lipids (neutral lipids and polar lipids) in accelerated solvent extraction (ASE) extracts of fish feeds made from different lipid sources without cleanup or with gel permeation chromatography (GPC) and/or sulfuric acid treatment (Acid).[a]

	Percent of neutral lipid in total lipid in extract (%)				
	FO	CO-50	CO-75	PO-50	PO-75
ASE extract	91.3	86.6	91.5	96.1	90.3
GPC cleaned extract	34.6	49.6	29.9	45.6	29.3
GPC and acid cleaned extract	50.0	50.0	43.6[b]	77.8[b]	61.7

[a] Values represent means of duplicate samples.
[b] Data were from one sample because in another sample the percent mass balance was extremely high (>200%) which was a results of low lipid contents.

mixed. The remaining micro ingredients were weighed, dissolved in water and then blended with the dry ingredients in the cutter-mixer. The combined ingredients were thoroughly mixed and the mixture was pelletized using a Cabela's No. 32 commercial grade electric grinder (Sidney, NE, USA) twice to form intact pellet strands. After being placed on a drying tray, the strands of feed were crumbled by hand to appropriate sizes and dehydrated at 100 °C in a Harvest Saver R-5A (Eugene, Oregon, USA).

Fish oil was spiked in the positive control with the PHHs at various concentrations by directly mixing an appropriate amount of a mixture of PHH standards into the oil with acetone as the carrier. The oil was stirred for 24 h to facilitate mixing of the analytes and to allow for evaporation of the solvent. The oil was then incorporated into the feed and the feed was dried in a drying oven for 24 h at 100 °C, which allowed additional time for evaporation of any remaining solvent.

Table 3
Fatty acid composition of neutral lipid extracted from fish feeds.[a]

Fatty acid(s)	FO	CO-50	CO-75	PO-50	PO-75
	g/100 g Fatty Acid Methyl Esters (FAME)				
8:0	0.0	2.6	3.0	0.0	0.0
10:0	0.0	3.6	5.3	0.0	0.0
12:0	0.1	25.1	36.0	0.2	0.2
14:0	8.4	12.3	14.1	4.6	3.4
16:0	18.7	13.3	11.1	28.6	30.5
18:0	3.5	3.1	2.9	4.0	4.4
SFA[b]	30.6	60.0	72.3	37.5	38.5
16:1n-7	10.5	5.0	2.8	5.4	4.2
18:1n-7	3.1	1.6	0.9	1.9	1.7
18:1n-9	9.5	7.8	7.0	23.8	27.1
20:1n-9	0.9	0.5	0.3	0.5	0.4
MUFA[c]	24.0	14.8	11.0	31.7	33.5
16:2n-4	1.3	0.6	0.3	0.7	0.4
16:3n-4	1.7	0.8	0.4	0.8	0.5
18:2n-6	8.7	7.2	6.7	11.5	15.2
20:4n-6	1.2	0.6	0.3	0.6	0.5
n-6[d]	9.9	7.8	7.1	12.1	15.8
18:3n-3	2.1	1.2	0.8	1.4	1.4
18:4n-3	3.0	1.4	0.7	1.5	0.7
20:5n-3	13.5	6.6	3.6	7.0	4.5
22:5n-3	2.4	1.2	0.6	1.2	0.8
22:6n-3	11.6	5.8	3.1	6.1	3.8
n-3[e]	32.6	16.1	8.9	17.2	11.3
n-3:n-6	3.3	2.1	1.3	1.4	0.7
PUFA[f]	45.4	25.2	16.7	30.9	28.0
MC-PUFA[g]	13.8	9.8	8.3	14.4	17.4
LC-PUFA[h]	28.6	14.1	7.7	15.0	9.7

[a] Data are presented as means of duplicate samples.
[b] Saturated fatty acids—sum of all fatty acids without double bonds.
[c] Monounsaturated fatty acids—sum of all fatty acids with a single double bond.
[d] Sum of all n-6 fatty acids.
[e] Sum of all n-3 fatty acids.
[f] Polyunsaturated fatty acids—sum of all fatty acids with ≥2 double bonds.
[g] Medium-chain PUFA—sum of all PUFA with chain length of 18 carbon atoms.
[h] Long-chain PUFA—sum of all fatty acids with chain length ≥20 carbon atoms and double bonds ≥3.

Table 4
The instrumental detection limits (IDL, ng/mL) on both columns (DB-608 and Rtx-1614), method detection limits (MDL, ng/g dw) and limits of quantification (LOQ, ng/g dw) for the persistent halogenated hydrocarbons in fish feeds including 100% fish oil. na = not available.

Class	Analyte	IDL		MDL	LOQ
		DB-608	Rtx-1614		
Organochlorine insecticides (OCs)	α-BHC	0.8	0.6	3.4	11
	β-BHC	2.3	2.0	4.1	13
	γ-BHC	1.0	0.7	1.9	6.1
	δ-BHC	0.8	1.2	3.0	9.6
	α-chlordane	1.1	1.4	3.0	9.6
	γ-chlordane	1.0	1.3	3.5	11
	DDE	0.5	1.1	4.8	15
	DDD	1.7	2.8	4.5	14
	DDT	1.0	9.1	10	32
Polychlorinated biphenyls (PCBs)	CB-18	9.5	4.2	2.4	7.6
	CB-28	2.1	2.3	2.3	7.3
	CB-29	4.3	2.5	4.7	15
	CB-44	3.2	3.4	4.1	13
	CB-50	2.1	2.4	1.5	4.8
	CB-52	4.5	4.4	5.4	17
	CB-66	2.5	3.0	2.7	8.6
	CB-70	4.7	4.4	4.2	13
	CB-77	4.6	7.0	4.4	14
	CB-87	3.1	3.4	3.0	9.6
	CB-101	3.1	3.0	7.1	23
	CB-104	2.1	3.4	2.8	8.9
	CB-105	1.9	3.6	3.6	12
	CB-118	2.3	3.6	4.3	14
	CB-126	1.0	3.0	4.7	15
	CB-128	2.0	1.9	3.7	12
	CB-138	1.0	1.8	4.4	14
	CB-153	2.2	3.6	5.1	16
	CB-170	0.3	1.1	4.0	13
	CB-180	1.3	1.3	4.2	13
	CB-187	1.0	1.7	3.6	12
	CB-188	2.7	4.1	4.0	13
	CB-195	0.3	0.9	3.0	9.6
	CB-201	1.8	1.8	3.2	10
	CB-206	0.2	0.5	3.0	9.6
	CB-209	0.2	0.7	3.3	10
Polybrominated diphenyl ethers (PBDEs)	BDE-28	2.1	3.7	3.5	11
	BDE-47	0.4	2.0	4.4	14
	BDE-99	0.3	0.7	3.5	11
	BDE-100	0.3	1.4	3.7	12
	BDE-153	0.7	1.1	4.6	15
	BDE-154	4.2	0.9	3.7	12
	BDE-183	na	1.6	3.1	9.9

2.3. Extraction and cleanup procedures

The PHHs were extracted from fish feeds using a Dionex 2000 ASE (Sunnyvale, CA, USA). The extraction was conducted following a previously developed ASE method [35]. Each pelletized feed was ground and homogenized, and approximately 3 g of the ground feed and 1 g of silica gel were loaded into an ASE cell. Silica gel was used as a normal phase dispersion agent for in-line cleanup of the extracts by trapping lipids and other polar interference inside the ASE cell [35]. Then clean sand was added to the cell to fill the void and the two surrogates (DBOFB and PCB-203) were added. The sample was extracted using a mixture of dichloromethane and acetone (1:1, v/v) at 100 °C and 2000 psi for two static cycles of 5 min. Silica gel was used as an in-line matrix-retaining material. The extract was concentrated and solvent exchanged to 2 mL of dichloromethane using a TurboVap II evaporator (Zymark, Hopkington, MA, USA), and filtered through a 0.2 μm Whatman GD/X filter (Florham Park, NJ, USA) prior to cleanup.

Table 5
Percent recovery (PR) and relative standard deviation (RSD) of the spiked persistent halogenated hydrocarbons from the five types of fish feeds spiked at a concentration of 33 ng/g dw.

Analyte	FO-100		CO-50		CO-75		PO-50		PO-75	
	PR (%)	RSD (%)	PR (%)	RSD (%)	PR (%)	RSD (%)	PR (%)	RSD (%)	PR (%)	RSD (%)
α-BHC	76	4.7	69	2.2	71	7.9	65	7.2	96	4.1
β-BHC	80	4.7	72	10	79	4.3	69	7.5	104	5.2
γ-BHC	74	4.3	67	2.2	69	8.3	63	7.4	94	4.0
δ-BHC	81	4.0	70	2.7	72	6.5	67	8.2	102	4.0
α-chlordane	88	5.1	74	2.5	75	5.7	70	7.1	105	2.1
γ-chlordane	86	3.8	76	1.7	78	6.8	71	8.0	110	1.6
DDE	95	4.6	78	4.5	78	5.4	74	6.7	106	4.0
DDD	94	2.5	81	5.6	79	4.7	75	6.9	111	1.5
DDT	96	3.1	84	8.4	83	4.7	74	5.8	114	2.8
CB-18	86	4.9	88	11	87	3.3	86	7.2	111	4.4
CB-28	91	0.2	92	6.7	87	7.8	88	1.5	127	1.6
CB-29	89	3.9	94	5.7	91	1.8	92	7.7	118	14
CB-44	75	39	89	0.3	83	2.2	86	8.3	118	6.8
CB-50	87	6.6	94	8.6	87	2.2	78	7.7	123	8.9
CB-52	99	4.8	89	3.2	81	1.2	87	8.8	124	3.2
CB-66	97	5.4	93	3.4	87	2.3	92	6.4	130	2.0
CB-70	94	5.3	81	13	90	5.9	86	18	120	15
CB-77	98	0.4	92	5.4	83	3.0	87	7.2	124	2.6
CB-87	100	5.1	99	4.4	92	2.8	98	6.9	137	2.4
CB-101	111	3.8	102	8.9	90	3.6	101	8.1	144	6.8
CB-104	90	1.7	84	5.0	79	0.6	81	8.9	115	1.6
CB-105	88	1.6	89	5.3	81	2.2	84	9.0	121	4.7
CB-118	104	3.4	98	3.8	90	2.5	91	11	132	2.9
CB-126	93	2.8	95	11	85	4.7	83	6.2	124	3.9
CB-128	86	1.8	89	6.6	80	3.3	83	7.6	120	2.7
CB-138	110	1.7	102	6.5	91	4.9	92	9.0	129	4.2
CB-153	118	3.1	105	6.2	92	3.2	98	8.3	132	2.0
CB-170	86	2.2	91	8.4	81	4.1	86	5.9	121	4.0
CB-180	88	2.3	92	8.4	81	3.9	86	5.8	120	4.0
CB-187	101	3.0	92	7.5	82	2.0	82	4.9	120	4.6
CB-188	99	15	93	20	76	2.8	76	8.6	110	5.7
CB-195	85	1.7	91	8.5	81	3.9	86	5.6	119	4.2
CB-201	84	2.1	89	8.2	80	3.4	82	5.7	116	3.9
CB-206	92	2.2	94	8.7	82	4.3	88	5.2	121	4.6
CB-209	94	2.4	96	8.6	85	4.5	90	5.0	123	4.2
BDE-28	91	3.3	91	5.2	85	0.5	85	5.5	124	1.4
BDE-47	96	3.8	98	6.6	87	4.0	81	9.7	131	2.6
BDE-99	92	1.9	79	7.5	83	12	69	3.3	114	12
BDE-100	86	2.3	92	9.2	83	4.2	65	2.3	122	4.3
BDE-153	109	6.6	88	6.9	89	11	78	5.3	118	3.2
BDE-154	86	3.0	86	18	78	11	65	4.4	113	9.0
BDE-183	102	8.3	80	4.9	85	1.3	75	7.5	121	3.2

The cleanup process was composed of two steps including high performance GPC and sulfuric acid cleanup. The GPC was conducted on an Agilent 1100 high performance liquid chromatograph (Agilent Technologies, Palo Alto, CA, USA). The extract was introduced into the GPC using a Rheodyne 7225 injector with a 500 μL sample loop (Cotati, CA, USA), separated using a 300 mm × 19 mm Envirogel GPC column coupled with a 5 mm × 19 mm guard column (Waters, Milford, MA, USA), and the fraction between 8 and 12 min was collected by a Foxy Jr™ Fraction collector (ISCO Inc., Lincoln, NE, USA). Dichloromethane was used as the mobile phase and the flow rate was set at 5 mL/min. With higher molecular weights, most lipids were eluted from the GPC column before 7 min, while the OCs eluted at 8.5–12 min and the PCBs and PBDEs eluted at 11–12 min. Hence, the fraction between 8 and 12 min was collected. To reduce the method detection limits (MDL), maximize the efficiency of lipid removal during the GPC process and avoid overloading the column, two injections were conducted for each sample. The two GPC collections were combined, evaporated to near dryness and reconstituted to 1 mL of hexane.

The remaining lipid and other interference left in the GPC-cleaned extract were further removed by concentrated H_2SO_4. After adding 1 mL of concentrated H_2SO_4 to the extract, the solution was vortexed for 5 min to ensure sufficient contact between the extract and the acid. Then, the solution was centrifuged for 5 min at 4400 rpm for better separation of the two phases. After removing the hexane layer, the acid layer was washed with 1 mL aliquots of hexane three times. All hexane solutions were combined, passed through an anhydrous Na_2SO_4 column, and concentrated to 0.5 mL using a Pierce Model 1878 ReactiVap™ (Rockford, IL, USA).

2.4. Instrumental analysis

The cleaned extracts were analyzed on an Agilent 6890 GC-ECD equipped with 7683 autosampler (Agilent Technologies, Palo Alto, CA). For analysis, 2.0 μL of the sample was injected into the GC using a pulsed splitless injector at a pulsed pressure of 50 psi. Two columns, a DB-608 (30 m × 0.32 mm × 0.50 μm) (Agilent Technologies) and a Rtx-1614 (30 m × 0.25 mm × 0.10 μm) (Restek, Bellefonte, PA, USA) were used to confirm the analytical results. Helium and nitrogen were used as the carrier and the makeup gases, respectively. The flow rates of the carrier gas for the DB-608 and the RTX-1614 columns were set at 2.0 and 1.7 mL/min, respectively. For the DB-608 column, the oven temperature was set at 100 °C, heated to 250 °C at 10 °C/min, then to 280 °C at 3 °C/min and held at this temperature for 20 min. For the RTX-1614 column, the oven temperature started at 100 °C, then ramped at 15 °C/min to 190 °C, then at 6 °C/min to 214 °C, and finally at 20 °C/min to 280 °C and held for 6 min at 280 °C. Identification of target analytes

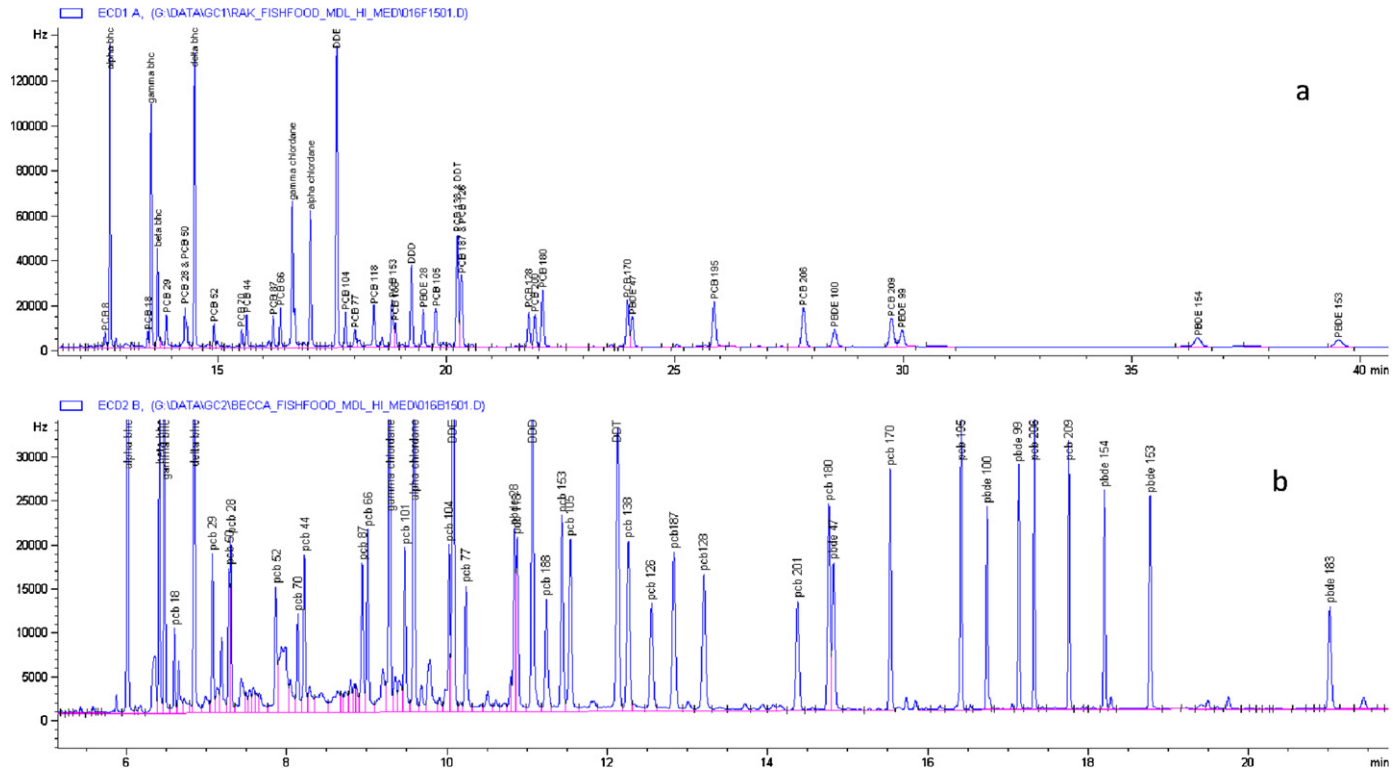

Fig. 1. Chromatograms of the target analytes spiked at 33 ng/g dw in fish feed made by 100% fish oil on DB-608 column (a) and RTX-1614 column (b).

was established using a retention time window of 1% with confirmation on the two columns. Six external calibration standards containing 5, 10, 50, 100, 250 and 500 ng/mL of each analyte and surrogate were used for quantification, and the calibration curves were linear within the concentration ranges.

Additionally, representative samples from each phase of the extraction and cleanup procedure were separated into neutral and polar lipid classes using SPE procedures described by Trushenski et al. [26], while lipid samples were analyzed to determine fatty acid composition by GC-flame ionization detector according to procedures described by Lane et al. [24].

2.5. Data analysis

The developed method was validated by calculating the percent recovery, relative standard deviation (RSD), instrumental detection limits (IDLs), method detection limits (MDLs) and limits of quantification (LOQs) of each PHH in the various fish feeds. Analyte recovery was the ratio of the concentrations of the detected PHHs in feeds compared to their spiked concentrations, and was expressed as a percentage. While recovery indicated the accuracy of the method, RSD was a measure of the precision of the method. All feed samples were analyzed using four replicates with the exception of the FO-100 fish feed sample spiked at the lowest concentration. This treatment was processed using seven replicates and used to calculate the MDLs and the LOQs.

The IDLs evaluated the instrumental performance and was calculated using the GC results for the injection of the calibration standards. The IDL was the ratio of three times the instrumental noise and the slope of the calibration curve of the analytes. On the other hand, the MDL was defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration was greater than zero [36] and it may vary as a function of the type of matrix sampled and the efficiency of the sample preparation procedures. The MDLs and LOQs were computed from seven replicates of the FO-100 fish feed spiked at 7.5, 12.5 and 12.5 ng/g dw for OCPs, PCBs, and PBDEs, respectively, and were calculated as follows: $MDL = st_{(0.99,n-1)}$ and $LOQ = 10s$. Where, s was the standard deviation of the seven replicate measurements and $t_{(0.99,n-1)} = 3.14$ was the t-distribution value taken at a confidence level of 0.99 and degrees of freedom of six.

3. Results and discussion

3.1. Influence of lipid composition on cleanup efficiency

The fatty acid compositions of total lipid extracted from the feeds were shown in Table 1. In general, FO-based feeds contained greater n-3 LC-PUFA than feeds supplemented with CO or PO, which were composed of greater amounts of SFA and MUFA. These results are consistent with our previous work evaluating these lipids [34], and unsurprising given that over 90% of total fatty acids in CO are SFA, the composition of PO is roughly 50% SFA and 40% MUFA and neither alternative lipid contains LC-PUFA (unpublished data). The effects of fish oil replacement on dietary fatty acid profile observed in the present study were also consistent with the well-established effects of fish oil sparing on the composition of aquafeeds [37–39].

A two-step cleanup including GPC and concentrated H_2SO_4 treatment was conducted for the extracts of the fish feeds after ASE extraction. The cleanup efficiencies of the two techniques were calculated by dividing the amounts of lipids removed by the cleanup step to the original amounts of lipids in ASE extracts. Results showed the majority of lipids in the ASE extracts of the five types of fish feeds (96.40, 95.70, 96.69, 96.89 and 96.73% for FO, CO-50, CO-75, PO-50, and PO-75, respectively) were removed by the GPC cleanup. However, the residue lipids in the extracts may still interfere with GC analysis of the PHHs. Furthermore, the injected residue lipids may condense on the liner of the GC injector

or the front end of the GC column, which may induce degradation of certain compounds, like DDT to DDD. Thus, the GPC-cleaned extracts were further treated with concentrated H_2SO_4 to remove the residue lipids and other labile compounds and an additional 3% of lipids were removed, which resulted in the lipid removal efficiency of 99.55, 99.84, 99.62, 99.80 and 99.55% for the five types of fish feeds, FO, CO-50, CO-75, PO-50, and PO-75, respectively. It indicated that only ~3.8 μg of lipids were injected into the GC/ECD when an injection volume of 2 μL was used compared to 1176 or 41 μg for injection without cleanup or cleanup with GPC alone, respectively.

The lipids in the extracts including the ASE extract, GPC-cleaned extract, and GPC and H_2SO_4 treated extract, were separated into neutral lipid and polar lipid components using a SPE cartridge. The percent of neutral lipids in the total lipids (neutral and polar lipids) for the extracts were shown in Table 2. Most lipids were neutral in the ASE extract with an average of 91.2% neutral lipid in the total lipid for all the feeds and the values dropped to 37.8% after GPC cleanup, which suggested the majority of lipids removed by GPC were neutral. Additionally, the composition of these neutral lipid extracts (Table 3) was broadly similar to the feed total lipid profile (Table 1). Conversely, the average percent of neutral lipid increased to 53.3% when the GPC-cleaned extracts were further treated with concentrated H_2SO_4 and it showed that the acid treatment was more effective in removing polar lipids. Thus, the combination of two cleanup techniques improved the cleanup efficiency by removing both neutral and polar lipids.

In summary, although the lipid class and fatty acid compositions varied among the five types of feeds (Tables 1 and 3), the newly developed two-step cleanup method worked equally well at removing lipids from the fish feeds composed of oil from different sources.

3.2. Method validation

Method validation included evaluation of sensitivity, accuracy and precision of the developed method for fish feeds with different lipid compositions. The cleaned extracts were analyzed on GC-ECD with confirmation of the analytical results from two columns with different polarity. As shown in Table 4, the IDLs were all lower than 5 ng/mL, which was the lowest calibration standard used in the present study. The calibration curves for all PHHs were linear in the range of 5–500 ng/mL. Because of its low volatility, BDE-183 was not eluted out from the DB-608 column within a reasonable time. Hence, the IDL for BDE-183 was only reported on one column (Table 4). Without a confirmation column for BDE-183, a false positive may have occurred during GC-EDC detection. Sensitivity for the method was determined by not only the instrumental performance, but also the effectiveness of removal of the matrix components from the extracts during the sample preparation process. As discussed earlier, the lipid removal efficiency was similar among the fish feeds, thus only fish feed composed with 100% FO was used to compute the MDL and the LOQs. As shown in Table 4, the MDLs for most analytes were lower than 5 ng/g dw and ranged from 1.5 to 4.8 ng/g dw. The MDLs for DDT, CB-52, CB-101, and CB-153 (10.0, 5.4, 7.1 and 5.1 ng/g dw, respectively) were higher than 5 ng/g dw and the higher MDLs possibly resulted from trace amounts of the native compounds in the blank FO. Previous studies [4,5,28–30,40,41] reported the existence of trace PHHs in fish oils. Nácher-Mestre et al. [28] reported PCBs and DDTs existed in fish oil at concentrations of 0.6–6.5 and 8.8–16.8 ng/g fresh weight, respectively. In the present study, the five un-spiked fish feeds were analyzed along with blank samples with sand only. No target compounds were detected in the blank sand sample, but trace amounts of OCPs and PCBs were noted in the five feeds. Conversely, no native PBDEs were detected in any feeds although BDE-183 was measured without confirmation from a second column. The feed FO-100 contained the greatest concentrations of contaminants compared to other feeds. In FO-100, CB-101, 138 and 153 were detected at 8.5, 6.4, and 8.4 ng/g dw, respectively, while DDE was the only OCPs found at greater than 5 ng/g dw (5.2 ng/g dw). Meanwhile, 5.9 ng/g dw CB-101 was also measured in PO-50. DDT and CB-52 were also detected in all the feeds, but the concentrations were all below 5 ng/g dw. The levels of PHHs were lower than those in the previous study [28]. The oils were added at 9.8% in the fish feeds used in the present study, thus concentrations of native PHHs in feeds were diluted.

Percentage recoveries and relative standard deviations (RSDs) indicated accuracy and precision of an analytical method, respectively. Table 5 presented analytical results of the three classes of PHHs spiked at 33 ng/g dw in the five fish feeds and samples were analyzed using four replicates. The chromatograms of the target compounds were presented in Fig. 1. Recoveries of OCs, PCBs and PBDEs were 74–96%, 75–118% and 86–109% in fish feed containing 100% FO with the corresponding RSDs of 2.5–5.1%, 0.2–15% and 1.9–8.3%, respectively, with an exception of a RSD of 39% for CB-44.

Table 6
Percent recovery (PR) of spiked persistent halogenated hydrocarbons (PHHs) including organochlorine insecticides (OCs), polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) at different concentrations from fish feed including 100% fish oil.

	A		B		C	
	PR (%)	RSD (%)	PR (%)	RSD (%)	PR (%)	RSD (%)
α-BHC	77	12	64	1.6	76	4.7
β-BHC	91	13	70	0.3	80	4.7
γ-BHC	94	6.1	63	0.8	74	4.3
δ-BHC	142	7.2	66	4.2	81	4.0
α-chlordane	115	7.9	82	8.1	88	5.1
γ-chlordane	86	12	73	8.1	86	3.8
DDE	83	18	93	16	95	4.6
DDD	81	18	91	15	94	2.5
DDT	185	20	84	12	96	3.1
CB-18	102	4.1	87	28	86	4.9
CB-28	115	3.6	94	7.0	91	0.2
CB-29	109	7.7	75	33	89	3.9
CB-44	112	6.2	83	2.8	75	39
CB-50	112	2.5	88	13	87	6.6
CB-52	68	14	90	8.8	99	4.8
CB-66	94	4.8	103	11	97	5.4
CB-70	99	7.2	79	12	94	5.3
CB-77	120	7.1	108	19	98	0.4
CB-87	100	5.1	100	9.8	100	5.1
CB-101	111	11	122	7.4	111	3.8
CB-104	83	6.0	96	20	90	1.7
CB-105	93	7.1	87	14	88	1.6
CB-118	79	8.4	74	4.8	104	3.4
CB-126	95	8.7	87	10	93	2.8
CB-128	89	7.5	84	17	86	1.8
CB-138	77	11	124	14	110	1.7
CB-153	81	11	132	17	118	3.1
CB-170	86	8.3	78	18	86	2.2
CB-180	85	9.0	90	23	88	2.3
CB-187	82	8.2	93	16	101	3.0
CB-188	152	8.4	63	15	99	15
CB-195	96	5.7	80	25	85	1.7
CB-201	89	6.4	81	20	84	2.1
CB-206	93	6.0	89	24	92	2.2
CB-209	94	6.6	86	22	94	2.4
BDE-28	98	5.5	89	11	91	3.3
BDE-47	90	7.5	93	29	96	3.8
BDE-99	112	5.8	75	18	92	1.9
BDE-100	100	6.2	78	24	86	2.3
BDE-153	133	6.3	79	22	109	6.6
BDE-154	125	5.8	69	26	86	3.0
BDE-183	152	3.9	74	22	102	8.3

A: 7.5 ng/g dry weight (dw) for OCs and 12 ng/g dw for PCBs and PBDEs; B: 17 ng/g dw for all PHHs; C: 33 ng/g dw for all PHHs. RSD = relative standard deviation.

Similar results were achieved for the PHHs in the remaining four types of fish feeds. Recoveries of all PHHs ranged from 66.7–105%, 69–92%, 63–101% and 94–144% for feeds CO-50, CO-75, PO-50, and PO-75, respectively. The RSDs were 0.3–20%, 0.5–12%, 1.5–18% and 1.4–15% for all PHHs in feeds CO-50, CO-75, PO-50, and PO-75, respectively. The results suggested the composition of lipid in the fish feeds does not affect analysis of PHHs in feeds and the present method could be used for fish feeds containing different types of lipid sources with acceptable accuracy and precision.

The influence of analyte concentrations on performance of the analytical procedures were also validated by analyzing PHHs spiked into FO-100 fish feed at differing concentrations (7.5 (OCs) and 12 (PCBs and PBDEs), 17 (PHHs) and 33 (PHHs) ng/g dw) and results were shown in Table 6. High recoveries of 142%, 185%, 151% and 152% were noted for δ-BHC, DDT, CB-188, and BDE-183 in feed spiked at the lowest concentrations (7.5 ng/g dw for δ-BHC and DDT, whereas 12.5 ng/g dw for CB-188 and BDE-183). The presence of their native counterparts at trace levels in the fish oil may be the reason for the high recoveries for δ-BHC and DDT, while interference by other co-eluted contaminants may have elevated recovery of BDE-183 due to no confirmation information from a second column for this analyte. However, high recovery of CB-188 was unknown because it was not detected in the blank samples. Recoveries for the remaining PHHs ranged from 68 to 133% at this spiking level and the RSDs were 2.5–20% for all PHHs. In comparison, results were more accurate for PHHs spiked at the two higher concentrations. Recoveries for all PHHs were 63–132% and 74–118% with RSDs of 0.3–32% and 0.2–39% for PHHs spiked at 17 and 33 ng/g dw, respectively. Results indicated the developed method can be used for samples in a wide concentration range, but the presence of several native contaminants in raw fish oil may decrease the analytical sensitivity for those compounds.

4. Conclusion

Fish feeds prepared from fish oil and its alternatives were analyzed by GC-ECD after matrix-dispersion ASE extraction and two-step cleanup of GPC and sulfuric acid treatment. Although lipid composition was different in the various types of feeds, the developed method was equally effective at removing both neutral and polar lipids and analyzing PHHs in feeds at different concentrations. Therefore, the present method could be used for fish feeds containing different types of lipid sources with acceptable accuracy and precision.

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