FRACTIONATION OF PERSISTENT ORGANIC POLLUTANTS IN FISH OIL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY EQUIPPED WITH A 2-(1-PYRENYL)ETHYL SILICA COLUMN

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Introduction

Analytical procedures for the analysis of persistent organic pollutants (POPs) usually require a fractionation step to separate the different families of analytes. In this study, an overall method for the fractionation of polychlorinated-*p*-dibenzodioxins and polychlorinated dibenzofurans (PCDD/Fs), marker and dioxin-like polychlorinated biphenyls (PCBs), polybrominated diphenylethers (PBDEs) and organochlorine pesticides (OCPs) by high performance liquid chromatography equipped with a 2-(1-pyrenyl)ethyl silica column (HPLC-PYE) was developed. PYE columns have the ability to separate compounds by their planarity/aromaticity. This property has been used in the past for the fractionation of PCDD/Fs and PCBs [1], or some PBDEs and PCBs [2]. It is useful for the separation of marker PCBs and OCPs (usually found in environmental samples in higher levels) from target analytes, such as PCDD/Fs, dioxin-like PCBs or PBDEs, avoiding with this fractionation step instrumental interferences. This separation has been used as a step of fish oil analysis. This oil is being widely used in the human and animal diet as a nutritive complement for its high content in omega-3 fatty acids. However, this oil can present high levels of pollutants, because POPs tend to bioaccumulate in fatty tissues and fishes have also a very low metabolization capability for these compounds [3]. The whole method for the analysis of POPs in fish oil with HPLC-PYE fractionation has been validated.

Materials and methods

Hexane and toluene were supplied by Baker (Deventer, Holland). PCDD/Fs and PCBs standards and labelled solutions were from Wellington Laboratories (Ontario, Canada). PBDEs standards and labelled solutions were from Cambridge Isotope Laboratories inc. (New Haven, USA). Organochlorine pesticides mixture was acquired from Dr. Ehrenstorfer GmH (Ausburg, Germany). For the validation of the method, two 20 ml bottles of cod liver oil were acquired from the Norwegian Institute of Public Health Reference Laboratory (Nydalen, Norway). The HPLC system consisted of a HP-1050 chromatograph (Hewlett-Packard, Palo Alto, USA), equipped with a 300 µl injection loop and a 250 x 4.6 mm Cosmosil 5-PYE HPLC column, particle size 5 µm (Nacalai Tesque, Japan). Instrumental determination was carried out by high resolution gas chromatography with micro-electron capture detector (HRGC-µECD, Agilent Technologies 6890N, Santa Clara, USA) for OCPs and marker PCBs, and high resolution gas chromatography-high resolution mass spectroscopy (HRGC-HRMS, AutoSpec-Ultima, Micromass, Manchester, UK) for PCDD/Fs, dioxin-like PCBs and PBDEs.

For the study of the HPLC-PYE fractionation, a mixture of PCDD/Fs, PCBs, PBDEs and OCPs (Table 1) was prepared, including the congeners of study recommended by US Environment Protection Agency [4,5]. 150 µl of this solution were directly injected in the HPLC, with a flow programme consisting of 1 ml/min of hexane during the first 15 minutes, followed by 1 ml/min of toluene for the next 30 minutes. The fractions collected are shown in Table 2. Fractions were analyzed by HRGC-ECD to determine the retention time of each pollutant in the PYE column. The chromatograph was fitted with a ZB-5 silica capillary column (60 m x 0.25 mm i.d.. 0.25 µm) from Phenomenex Zebron (California, USA). The samples were injected (2 µl) on splitless mode (1 min) with a 7683B autosampler (Agilent Technologies, Santa Clara, USA) into an injector at 300ºC. Carrier gas was helium at 1 ml/min flow. Temperature programme was 100ºC (held for 1 min), increased at 20ºC/min to 195ºC, increased at 0.5ºC to 210ºC, increased at 5ºC/min to 280ºC (held for 5 min), increased at 5ºC/min to 320ºC (held for 10 min). In addition, a chromatographic method was developed for PBDEs determination, using a DB-5 silica capillary column (15 m x 0.25 mm i.d.. 0.25 µm) from J&W Scientific (California, USA). Carrier gas was helium and temperature was 100ºC (held for 3 min) to 300ºC at 10ºC/min (held for 37 min).

The method for the analysis of PCDD/Fs, PCBs, PBDEs and OCPs in fish oil using HPLC-PYE fractionation was validated. For this purpose, three 6-g samples of cod liver oil were spiked with PCDD/Fs, dioxin-like PCBs and PBDEs $^{13}C_{12}$ labelled standard solutions. After a multilayer silica column purification eluted with 400 ml of hexane, extracts were evaporated to 2.5 g in conic tubes. 0.2 g aliquots (8%) were taken from the samples and syringe standard (1,2,3,4-tetrachloronaphtalene) was added. They were injected in HRGC-ECD for marker PCBs and OCPs quantification. In this case, fractionation was not necessary since they are usually found in the environment in higher levels. Remaining samples were evaporated to 150 µl and injected in the HPLC-PYE. Fractions were taken from 4,5 to 15 and from 15 to 35. Second and third fractions were evaporated to 15 µl with a nitrogen stream and corresponding PCDD/Fs, dioxin-like PCBs and PBDEs syringe standards were added. Fractions were injected in HRGC-HRMS for PCDD/Fs, dioxin-like PCBs and PBDEs quantification. Mass spectrometer was operating in EI ionization (35eV) at 10000 resolving power.

Results and discussion

The analysis of the obtained HPLC-PYE fractions showed that the bulk of marker PCBs could be obtained between minutes 3 and 4,5. OCPs were collected during minutes 3 and 9 except for β-, δ- and ε - HCH, β -endosulfan and 4,4'-DDD (even though most of them would be previously retained or degraded in the purification column). Planar PCBs were obtained between minutes 5.5 and 13. PBDEs were eluted between minutes 4.5 and 9, with the exception of BDE-209. PCDD/Fs were obtained only when toluene was used as mobile phase (altogether with BDE-209 and some OCPs) and between minutes 15 and 35. With the data obtained (Table 1), one feasible fractionation for POPs analysis can be taking 3 different fractions: With the first one (from min 0 to 4.5) the bulk of OCPs and marker PCBs can be separated from the other analytes, as they are usually found in environmental samples in higher levels interfering with other compounds. In the second fraction (from min 4.5 to 15) dioxin-like PCBs and PBDEs (except BDE-209) are obtained. In the last fraction (from min 15 to 35) PCDD/Fs and BDE-209 are obtained. Fractionation between minutes 15 and 20 has been studied as an attempt to collect BDE-209 and PCDD/F in different fractions, but this was not possible as BDE-209 and Tetra-CDD/F co-elute during minute 19.

When HPLC-PYE fractionation was plotted vs. retention time of the pollutants in HRGC-ECD (60 m column), it could be observed that PCDD/Fs were the most retained compounds in both PYE and GC column. This is because their higher planarity induce a more effective electron donor-acceptor complex with the pyrenyl stationary phase [6] (HPLC-PYE) and have low volatility (HRGC-ECD). PBDEs (in Figure 1 only represented the congeners observed with the 60 m column method) had higher retention times in GC, especially those congeners with the highest bromination degree due to their difficult volatilization. For this reason, their analysis in a 15 m column was considered. Their retention in the PYE column was similar to dioxin-like PCBs. The only exception was BDE-209. This congener had higher retention time in HRGC, even with the 15m column, due to his extremely difficult vaporization (its molecular weight is over 959). It is also more retained in PYE column because of its bigger size, being eluted altogether with PCDD/Fs, when toluene is the mobile phase. Marker PCBs had lower retention than dioxin-like PCBs, because their chlorine(s) in *orto* position makes them less planar [6]. Despite of this, all PCBs could be eluted with Hexane. Finally, organochlorine pesticides have a very disperse retention time due to their different structures. It is remarkable the big retention in PYE column of β -, δ - and ε- HCH and β -endosulfan.

For the method validation of POPs analysis in fish oil, the results of the analysis of PCDD/Fs, marker PCBs, dioxin-like PCBs, PBDEs, ΣDDT and hexachlorobenzene (HCB) are shown in Table 3. The method has shown a good precision (with RSD values almost always lower than 5%) and accuracy (almost always lower than 10%) with the real concentration of each POP in the reference fish oil.

The results obtained show that HPLC-PYE fractionation is a suitable step in the POPs analysis, capable to separate in different fractions most of the congeners of OCPs and marker PCBs, dioxin-like PCBs and PBDEs, and PCDD/Fs. This step allows having less interferences in the instrumental determination of each family of pollutants. The validated method for POPs analysis in fish oil has proven to be robust, with high precision and accuracy.

Table 1. Persistent organic pollutants studied

Compound	RT in 60 m. column (min)	PYE fraction	Compound	RT in 60 m. column (min)	PYE fraction
α-HCH	16.8	H5-6	1,2,3,6,7,8-HxCDF	56.6	T17
Hexaclorobenzene	17.4	H9	2,3,4,6,7,8-HxCDF	57.4	T17
β -HCH	18.3	T16	1,2,3,7,8,9-HxCDF	58.5	T17
γ-HCH	18.8	H6	1,2,3,4,6,7,8-HpCDF	60.9	T18
δ -HCH	20.4	T16	1,2,3,4,7,8,9-HpCDF	62.7	T17
ε-HCH	21.3	T16	OCDF	66.4	T19
PCB-28	23.1	H5	2,3,7,8-TCDD	47.1	T16
Heptachlor	24.7	H4	1,2,3,7,8-PeCDD	52.5	T17
PCB-52	26.4	H5	1,2,3,4,7,8-HxCDD	57.6	T17
Aldrin	28.4	H4	1,2,3,6,7,8-HxCDD	57.7	T17
Isodrin	31.9	H5	1,2,3,7,8,9-HxCDD	58.2	T17
Heptachlor-exo-epoxide	33.3	H5	1,2,3,4,6,7,8-HpCDD	62.2	T17
oxy-Chlordane	33.3	H5	OCDD	66.2	T18
Heptachlor-endo-epoxide	33.8	H6-7	PCB-81	40.4	H8-9
trans-Chlordane	36.5	H8	PCB-77	41.4	H9
2,4'-DDE	37.1	H5	PCB-105	45.5	H7
PCB-101	37.4	H5	PCB-114	44.4	H6-7
α-Endosulfan	38.1	H5	PCB-118	43.5	H6-7
cis-Chlordane	38.4	H5-6	PCB-123	43.4	H6-7
4,4'-DDE	40.6	H5	PCB-126	47.7	H11
Dieldrin	40.8	H5-6	PCB-156	50.1	H7-8
2,4'-DDD	41.4	H7	PCB-157	50.5	H8
Endrin	42.7	H7	PCB-167	48.8	H7
beta-Endosulfan	43.5	T16	PCB-169	52.3	H13
4,4'-DDD	44.2	H11	PCB-170	52.8	H7
2,4'-DDT	44.4	H5	PCB-180	51.1	H6
PCB-153	45.2	H5-6	PCB-189	54.6	H8-9
4,4'-DDT	46.8	H7	BDE-28	43.7 (14.4*)	H5-6
PCB-138	47.1	H6	BDE-47	51.3 (16.5*)	H7
Methoxychlor	50.1	H7-8	BDE-99	58.2 (18.4*)	H7
PCB-180	51.1	H6	BDE-100	58.8 (18.0*)	H7
Mirex	52.8	H4-5	BDE-153	- (20.1*)	H8
2,3,7,8-TCDF	46.2	T16	BDE-154	62.0 (19.6*)	H7
1,2,3,7,8-PeCDF	51.1	T16-17	BDE-183	- (21.8*)	H9
2,3,4,7,8-PeCDF	52.0	T17	BDE-209	-(33.1*)	T16
1,2,3,4,7,8-HxCDF	56.4	T17			

*retention times determined in a 15 m column

Table 2. HPLC-PYE fractions

Minute	Eluent	Fraction name	Minute	Eluent	Fraction name
0-1	Hexane	H1	11-12	Hexane	H12
1-2	Hexane	H2	12-13	Hexane	H13
2-3	Hexane	H3	13-14	Hexane	H14
3-4	Hexane	H4	14-15	Hexane	H15
4-5	Hexane	H5	15-20	Toluene	T16
5-6	Hexane	H6	20-25	Toluene	T17
6-7	Hexane	H7	25-30	Toluene	T18
7-8	Hexane	H8	30-35	Toluene	T19
8-9	Hexane	H9	35-40	Toluene	T20
9-10	Hexane	H10	40-45	Toluene	T21
10-11	Hexane	H11			

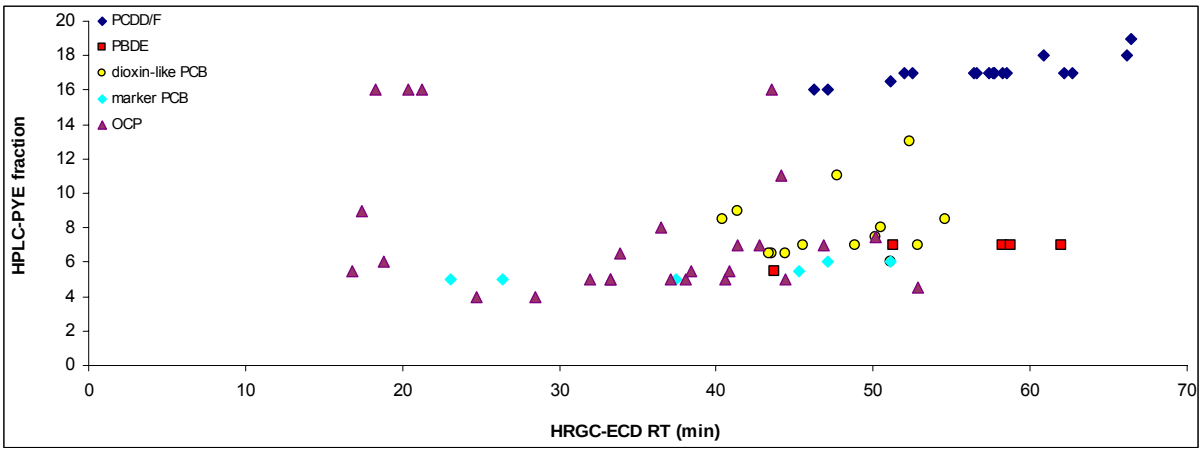

Figure 1. HRGC-ECD retention time vs. HPLC-PYE fractionation

Table 3. Method validation results

Compound	Avg. Conc. in samples (ng/g)	% RSD	% Accuracy (R-X)/R*100	Compound	Avg. Conc. in samples (ng/g)	% RSD	% Accuracy (R-X)/R*100
Hexaclorobenzene	14.3 x 10E3	0.62	-	1,2,3,4,7,8-HxCDD	0.04	49.5	11.7
PCB-28	2.38 x 10E3	2.57	3.43	1,2,3,6,7,8-HxCDD	0.45	1.46	2.45
PCB-52	6.31 x 10E3	2.48	5.92	1,2,3,7,8,9-HxCDD	0.10	7.19	4.11
2,4'-DDE	1.72 x 10E3	1.31	-	1,2,3,4,6,7,8-HpCDD	0.20	4.64	2.85
PCB-101	11.1 x 10E3	1.51	8.20	OCDD	0.20	4.12	37.5
4,4'-DDE	61.0 x 10E3	1.85	-	PCB-81	3.93	0.21	6.35
2,4'-DDD	9.82 x 10E3	6.94	-	PCB-77	89.7	1.48	9.38
4,4'-DDD	13.0 x 10E3	10.7	-	PCB-105	6.35 x 10E3	0.14	11.8
2,4'-DDT	24.7 x 10E3	7.22	-	PCB-114	368	1.26	0.17
PCB-153	30.3 x 10E3	1.61	5.74	PCB-118	16.2 x 10E3	2.00	0.72
4,4'-DDT	15.5 x 10E3	2.20	-	PCB-123	238	2.48	3.99
PCB-138	25.4 x 10E3	1.98	4.34	PCB-126	80.2	0.74	1.53
PCB-180	8.46 x 10E3	0.40	5.68	PCB-156	1.99 x 10E3	0.71	10.0
2,3,7,8-TCDF	7.19	1.59	1.34	PCB-157	545	1.00	5.36
1,2,3,7,8-PeCDF	1.12	1.03	6.95	PCB-167	1.06 x 10E3	1.00	2.16
2,3,4,7,8-PeCDF	1.17	4.13	6.42	PCB-169	17.3	3.00	1.77
1,2,3,4,7,8-HxCDF	0.26	2.03	2.36	PCB-189	153	0.87	5.97
1,2,3,6,7,8-HxCDF	0.44	1.00	0.92	BDE-28	602	0.21	2.04
2,3,4,6,7,8-HxCDF	0.42	2.30	1.58	BDE-47	11.3 x 10E3	0.93	9.32
1,2,3,7,8,9-HxCDF	0.00	-	-	BDE-99	282	1.15	5.81
1,2,3,4,6,7,8-HpCDF	0.16	2.34	1.76	BDE-100	1.65 x 10E3	0.79	8.28
1,2,3,4,7,8,9-HpCDF	0.03	14.8	35.9	BDE-153	43.6	4.85	1.40
OCDF	0.03	18.9	67.3	BDE-154	763	0.84	9.45
2,3,7,8-TCDD	0.36	0.26	3.74	BDE-183	5.63	4.44	4.56
1,2,3,7,8-PeCDD	0.18	0.14	0.29	BDE-209	30.9	25.3	62.7

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