Environmental Research
of the Federal Ministry for the Environment,
Nature Conservation and Nuclear Safety

Report

Project No. (FKZ) *3710 63 402 1*

Validierung der Testrichtlinie für den Bioakkumulations-Fütterungs-Test an Fischen, Analytik

Validation of a technical guidance document for biomagnification studies on fish, Analytics

by

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June 30, 2011

Abstract

A ring test program has been initiated to evaluate the draft revised OECD TG 305 guideline by measuring the BMFs of five hydrophobic compounds. As a first step analytical validation protocols were established in the participating laboratories. The protocols provided guidance to set up a diet preparation technique based on corn oil suspension or co-solvent spiking and to apply standardized analytical methods for extraction and analysis of spiked diets and fish samples. Ring test feed and fish samples loaded with five hydrophobic test items were provided by EMBSI to validate the chemical analysis carried out by the project partners.

This report describes the results of a fish dietary bioaccumulation study with rainbow trout (*Oncorhynchus mykiss*) which was carried out by Fraunhofer IME according to the draft revised OECD TG 305. The biomagnification potential of the test items - Hexachlorobenzene, Musk Xylene, o-Terphenyl, Methoxychlor and Benzo[a]pyrene - was determined by a kinetic approach.

Kurzbeschreibung

Ein Ringtest wurde mit fünf hydrophoben Substanzen durchgeführt, um die Messung von Biomagnifikationsfaktoren (BMF) auf Basis des Entwurfs der revidierten Richtlinie OECD 305 zu bewerten. Protokolle zur Validierung der Analytik wurden in den teilnehmenden Laboratorien etabliert. Die Protokolle enthielten alle Informationen zur Herstellung von Versuchsfuttermitteln auf Basis des Maisölsuspensions- oder Lösungsmittelverfahrens und definierten standardisierte Analysemethoden für die Extraktion und Messung von Testfuttermitteln und Versuchstierproben. Für die Validierung der chemischen Analyse, wurde allen Ringtestpartnern durch das EMBSI Futtermittel- und Fischproben zur Verfügung gestellt, die mit den fünf hydrophoben Testsubstanzen angereichert waren.

Dieser Bericht beschreibt die Ergebnisse einer Biomagnifikationsstudie mit Regenbogenforellen (*Oncorhynchus mykiss*), die im Fraunhofer Institut IME gemäß des Entwurfs der revidierten Richtlinie OECD 305 durchgeführt wurde. Das Biomagnifikationspotential der Testsubstanzen im Fisch – Hexachlorbenzol, Moschusxylol, o-Terphenyl, Methoxychlor und Benzo[a]pyrene – wurde aus der Kinetik der Auf- und Abnahme der einzelnen Substanzen bestimmt.

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List of Abbreviations

BaP	Benzo[a]pyrene
BCF	Bioconcentration factor
BMF	Biomagnification factor
CAS #	CAS registry number
EMBSI	ExxonMobil Biomedical Sciences, Inc.
GC-MS	Gas chromatography – Mass spectrometry
HCB	Hexachlorobenzene
IS	Internal standard
LOQ	Limit of quantification
m/z	Mass-to-charge ratio (in mass spectrometry)
MX	Musk Xylene (2,4,6-trinitro-5-tert-butyl-1,3-xylene)
OECD	Organisation for Economic Co-operation and Development
oTP	o-Terphenyl
RSD	Relative standard deviation
TG	Technical guideline

1 Background

The Organisation for Economic Co-operation and Development (OECD) is currently considering adoption of a test method for measuring the biomagnification factor (BMF) of poorly water soluble chemical compounds in fish using a novel dietary exposure technique. A ring test program has been initiated to evaluate the proposed guideline by measuring the BMF of five hydrophobic compounds. Because of the importance of the analytical methods employed, an analytical validation protocol has been developed to ensure that the preparation and analysis techniques employed are suitable for accurately measuring chemical concentrations in food and fish tissue. During the first part of the ring test program, the contributing laboratories analysed feed and fish samples loaded with the five test items according to the analytical validation protocol written by Eric Febbo and Mark Lampi from Exxon Mobil Biomedical Sciences, Inc. (EMBSI). The samples were provided by EMBSI to be analysed during the ring test. Feed samples were prepared by the participating labs in accordance with a standard protocol for diet preparation used by all partners. Alternatively, one of two spiking techniques using corn-oil or an organic carrier solvent as a vehicle for the test chemicals was applied.

As the final part of the ring test, a fish dietary bioaccumulation study was conducted by every participating lab to determine the elimination rate constant after chemical analysis of incurred fish for the five test compounds from rainbow trout (*Oncorhynchus mykiss*) tissue. The data collected in this study were used to derive the half-life (t1/2, from the elimination rate constant, kdepuration), the assimilation efficiency (α), the biomagnification factor (BMF) and the lipid-normalised biomagnification factor (BMFL) for the individual substances.

2 Objectives

The aim of this study was

- to prepare an experimental diet containing the five test items in defined concentrations using the solvent spiking protocol
- to carry out a fish dietary bioaccumulation study with rainbow trout according to the draft revised OECD TG 305
- to analyze the experimental diet and the fish samples collected during the feeding study
- to estimate the BMF and lipid normalized BMFL for each test item

3 Test items

The test items and internal standards were ordered from commercial supplier companies before the start of the study. Identity and purity of test item and reference items were not checked analytically by the test facility (chemical purities listed below are as stated by the supplier).

Table 1: Specification of the test items

CAS#	Compound		MW (g/mol)	MP (oC)	BP (oC)	Supplier/cat # (purity)
118-74-1	Hexachlorobenzene	solid	284.8	228	324	Aldrich, 17,105-0, (99%)
81-15-2	Musk Xylene (2,4,6-trinitro-5-tert-butyl-1,3-xylene)	solid	297.3	114		Dr. Ehrenstorfer GmbH, C15360000, (99.5%)
84-15-1	o-Terphenyl	solid	230.3	58	335	Aldrich, T280-0, (99%)
72-43-5	Methoxyclor	solid	345.7	83	346	Supelco, 49054 (analytical standard)
50-32-8	Benzo[a]pyrene	solid	252.3	177	496	Sigma, B1760, (≥96%)

4 Preparation of the test diet (solvent spiking)

4.1 Materials

- fish feed (Biomar, Inicio Plus, pellet size 0.8mm)
- pump spray bottle (ca. 100 mL)
- individual test compounds (5)
- acetone (J.T.Baker)
- 50 mL volumetric flask
- micro stir bar and stir plate
- amber bottle (ca. 150 mL)
- analytical balance (0.0001 g)

4.2 Method

The experimental diet was spiked with the five test items to reach a nominal concentration of 25mg/kg Hexachlorobenzene, 50mg/kg Musk Xylene, 50mg/kg o-Terphenyl, 100mg/kg Methoxychlor and 150mg/kg Benzo[a]pyrene. 40ml of acetone containing the five test items were sprayed portionwise on 100g of fish food with a pump spray bottle to achieve the required nominal dose levels. The food/test substance was constantly mixed during the spiking procedure in a stainless steel mixing bowl. The freshly-dosed

fish food was left in the bowl in a laboratory hood for two days (stirred occasionally) to allow the excess acetone to evaporate. The spiked diet was transferred into a brown glass bottle and kept frozen (-20°C) for four months due to the postponement of start of the experiment.

5 Fish dietary bioaccumulation study

The fish dietary bioaccumulation study with rainbow trout was carried out following the official ringtest protocol (see Annex 1).

6 Analysis

6.1 Spiked diet extraction and analysis

The experimental diet spiked with the five test compounds was extracted and analyzed according to the proposed EMBSI method. (see Annex 2 & 3). Samples were analyzed by GC/EI-MS.

6.2 Extraction and analysis of fish samples

All fish samples collected during the feeding study were processed as described in the EMBSI method and analyzed by GC/MS(see Annex 2 & 3).

6.3 Lipid extraction

Fish and feed samples collected during the feeding study for lipid analysis were extracted following the protocol described by Smedes 1999 (Analyst 124: 1711-1718) using non-chlorinated solvents.

6.4 Calculations

Test results were used to derive the elimination rate constant as a function of the total wet weight of the fish. In addition, the assimilation efficiency (α), the biomagnification factor (BMF) and its lipid-normalized value (BMF_L) were calculated (see Annex 1 for a detailed description).

7 Results and discussion

The results of the spiked feed analysis are presented in Table 2. The concentrations of all test items were slightly higher than the nominal concentrations. Only Methoxychlor showed a significantly higher concentration in the experimental diet with a recovery of almost 130%. The lipid content of the experimental diet was 15.3%.

The weight, length and lipid content of the control and test animals are presented in Tables 3 and 4. The average weight and lipid content of the animals is summarized in Table 5. Animals of both groups showed a significant growth increment during the biomagnification study lasting 41 days leading to an average final weight of around 13 g (start weight =

2 g). The growth rate of control and test animals during the depuration period is presented in Figures 1 and 2, respectively. No significant difference could be observed between the growth performances of both groups showing that the spiked diet had no harmful effect on the experimental animals. Animals of both groups were characterized by an increasing lipid content throughout the study with an average lipid content of 5.1 % at the onset of the experiment. Animals sampled at the end of the study showed a comparable average lipid content of 8.9 % and 8.8 % for control and test group, respectively. The results underline that the growth correction and lipid normalization of calculated biomagnification factors is required. The high growth increment and lipid accumulation suggests a less intensive feeding regime for future studies.

Table 2: Sample weight and quantification results [µg/mL] of feed samples

Test substances		**HCB**		**MX**		**oTP**		**Methoxychlor**		**BaP**	
Nominal conc. [mg/kg]		**25**		**50**		**50**		**100**		**150**	
Replicate	Sample weight [g]	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2
1	1.010	1.23		2.35		2.22		5.26		6.66	
2	1.101	1.31		2.45		2.32		5.70		6.94	
Analytical results [mg/kg]											
Test substances		**HCB**		**MX**		**oTP**		**Methoxychlor**		**BaP**	
Replicate	Sample weight [g]	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2	Inj. 1	Inj. 2
1	1.029	30.50	0.00	58.24	0.00	55.05	0.00	130.20	0.00	164.83	0.00
2	0.985	29.63	0.00	55.68	0.00	52.77	0.00	129.41	0.00	157.56	0.00
Recoveries [%]											
Replicate		**HCB**		**MX**		**oTP**		**Methoxychlor**		**BaP**	
1.00		121.98		116.49		110.10		130.20		109.88	
2		118.53		111.35		105.54		129.41		105.04	
	Mean (compound):	120.25		113.92		107.82		129.80		107.46	
	Standard deviation:	2.44		3.63		3.22		0.56		3.43	
	RSD [%]:	2.03		3.19		2.99		0.43		3.19	
Overall Mean:		115.9									

Test substances	**HCB**		**MX**		**oTP**		**Methoxychlor**		**BaP**	
Nominal conc. [mg/kg]	**25**		**50**		**50**		**100**		**150**	
(Overall) Standard deviation:	9.11									
(Overall) RSD [%]:	7.86									

GC/MS measuring, EI-SIM, Bruker/Varian 320-MS

Table 3: Weight, length and lipid content of control animals sampled during the biomagnifications study

Test stage						Concentrations (ug/g)				
	day	sample code	weight (g)	total length (mm)	LIPID (%)	HCB	MX	oTP	methoxychlor	BaP
uptake	0	1	1.8	5.4	4.8					
	0	2	2.2	5.5	5.1					
	0	3	1.7	5.2	4.8					
	0	4	1.9	5.6	5.9					
	0	5	1.7	5.3	5.1					
	0	6	2.2	5.6		0	0	0	0	0
	0	7	2.2	5.5		0	0	0	0	0
	0	8	1.8	5.5						
	0	9	1.9	5.3						
	0	10	2.2	5.5						
	3	24	2.8	5.9		0	0	0	0	0
	3	25	2.8	6		0	0	0	0	0
	3	26	3.0	6.3						
	3	27	2.7	6.1						
	3	28	2.5	5.8						
	13	34	3.7	6.8	7.7					
	13	35	3.8	7.1	7.1					
	13	36	3.2	6.8	6.9					
	13	37	3.9	7	7.2					
	13	38	3.2	6.7	7.1					
depuration	1	44	4.3	7.1		0	0	0	0	0
	1	45	3.3	6.5		0	0	0	0	0
	1	46	3.4	6.6						
	1	47	4.1	7.1						
	1	48	4.4	7.3						
	3	54	5.0	7.8		0	0	0	0	0
	3	55	4.8	7.5		0	0	0	0	0
	3	56	4.4	7.2						
	3	57	4.8	7.5						
	3	58	4.0	6.9						
	7	64	4.4	7.3		0	0	0	0	0
	7	65	5.6	7.8		0	0	0	0	0

Test stage						Concentrations (ug/g)				
	day	sample code	weight (g)	total length (mm)	LIPID (%)	HCB	MX	oTP	methoxychlor	BaP
	7	66	6.0	8.1						
	7	67	4.3	7.4						
	7	68	4.7	7.4						
	14	74	5.4	7.8		0	0	0	0	0
	14	75	9.0	9		0	0	0	0	0
	14	76	6.2	8						
	14	77	6.5	8						
	14	78	7.8	8.8						
	21	84	11.5	10		0	0	0	0	0
	21	85	8.8	9.1		0	0	0	0	0
	21	86	8.3	9						
	21	87	9.2	9.5						
	21	88	10.4	9.4						
	28	104	10.3	9.2		0	0	0	0	0
	28	105	13.4	10.4		0	0	0	0	0
	28	106	13.5	10.5						
	28	107	10.9	9.6						
	28	108	15.4	10.6						
*	28	94	13.6	10.5	8.8					
*	28	95	10.3	9.6	9.2					
*	28	96	17.9	11.5	9.4					
*	28	97	10.4	9.6	8.9					
*	28	98	12.6	10.1	8.4					

Table 4: Weight, length and lipid content of test animals sampled during the biomagnifications study

test stage						Concentrations (ug/g)				
	day	sample code	weight (g)	total length (mm)	LIPID (w/w)	HCB	MX	oTP	methoxychlor	BaP
uptake	0	6	2.2	5.6						
	0	7	2.2	5.5						
	0	8	1.8	5.5						
	0	9	1.9	5.3						
	0	10	2.2	5.5						
	3	29	2.1	5.7		1.5	3.7	2.5	3.5	1.3
	3	30	3.0	6.2		2.1	4.4	2.8	2.6	2.0
	3	31	2.8	6		1.4	3.3	2.3	1.9	1.1
	3	32	2.1	5.6		1.1	2.7	1.3	1.0	0.7
	3	33	2.5	5.9		1.4	3.4	2.4	1.8	1.3
	13	39	3.6	6.9	7.2					
	13	40	3.0	6.5	6.3					
	13	41	3.8	7	6.6					
	13	42	4.4	7.3	6.8					
	13	43	3.7	6.6	7.4					
depuration	1	49	4.5	7		6.1	11.5	8.8	10.0	1.4
	1	50	3.6	6.6		4.7	9.2	6.0	6.7	2.7
	1	51	3.8	6.6		5.2	10.3	7.1	8.3	1.8
	1	52	4.4	7.2		4.9	9.4	6.4	3.9	1.3
	1	53	3.7	6.6		3.5	6.8	3.2	2.9	2.1
	3	59	3.6	6.8		3.3	5.8	3.4	1.5	< LOQ
	3	60	4.6	7.5		4.4	7.7	4.2	7.1	< LOQ
	3	61	4.1	7.2		4.2	7.7	2.9	1.1	< LOQ
	3	62	4.7	7.6		5.2	9.8	4.5	7.2	< LOQ
	3	63	4.5	7.3		4.0	6.8	4.5	1.6	< LOQ
	7	69	4.9	7.4		2.6	4.2	2.6	0.5	< LOQ
	7	70	4.3	7.2		2.5	4.1	2.2	0.4	< LOQ
	7	71	5.8	8		3.4	5.2	3.0	4.4	< LOQ
	7	72	4.5	7.3		2.2	2.7	0.8	0.3	< LOQ
	7	73	4.4	7.2		3.0	4.0	1.8	1.3	< LOQ
	14	79	8.6	9		2.3	2.8	1.4	0.2	< LOQ

test stage						Concentrations (ug/g)				
	day	sample code	weight (g)	total length (mm)	LIPID (w/w)	HCB	MX	oTP	methoxychlor	BaP
	14	80	4.9	7.6		1.2	1.4	0.8	< LOQ	< LOQ
	14	81	7.2	8.8		2.5	3.1	0.4	0.2	< LOQ
	14	82	6.9	8.4		2.8	3.6	1.1	0.3	< LOQ
	14	83	6.9	8.3		2.3	2.9	0.9	1.3	< LOQ
	21	89	8.1	8.9		1.9	1.9	1.0	0.8	< LOQ
	21	90	12.5	10.1		1.4	1.3	1.0	0.5	< LOQ
	21	91	6.4	8.1		1.4	1.5	0.3	< LOQ	< LOQ
	21	92	11.6	10.1		1.4	1.3	1.1	0.3	< LOQ
	21	93	7.8	8.6		1.4	1.5	0.5	0.7	< LOQ
	28	109	17.1	11.3		1.2	1.1	0.3	0.3	< LOQ
	28	110	10.2	9.5		1.5	1.7	0.2	0.3	< LOQ
	28	111	14.6	10.5		0.8	0.6	< LOQ	0.1	< LOQ
	28	112	12.7	10.1		0.8	0.7	0.6	0.3	< LOQ
	28	113	11.2	9.6		1.2	1.0	< LOQ	< LOQ	< LOQ
*	28	99	10.3	9.5	9.4					
*	28	100	15.9	11	8.6					
*	28	101	17.1	11	8.1					
*	28	102	12.1	9.8	8.7					
*	28	103	10.3	9.5	9.2					

LOQ defined: 0,1 mg/kg FW, HCB, MX, oTP & Methoxychlor

LOQ defined: 0,01 mg/kg FW for BaP

Calibration: BaP: 0,3 bis 300 ng/mL

HCB, MX, oTP + Methoxychlor: 3,0 bis 3000 ng/mL

Table 5: Average mass and lipid content of control and test animals

day	average mass of control fish (g)	average lipid content of control fish (%)	average mass of test group fish (g)	average lipid content of test group fish (%)
up 0	2.02	5.1	1.91	5.1
up 3	2.47		2.77	
up 13	3.72	7.2	3.56	6.9
dep 1	3.99		3.86	
dep 3	4.31		4.6	
dep 7	4.78		5.01	
dep 14	6.91		6.97	
dep 21	9.28		9.63	
dep 28	13.16	8.9	12.68	8.8

Figure 1: Growth rate of control animals during the depuration period

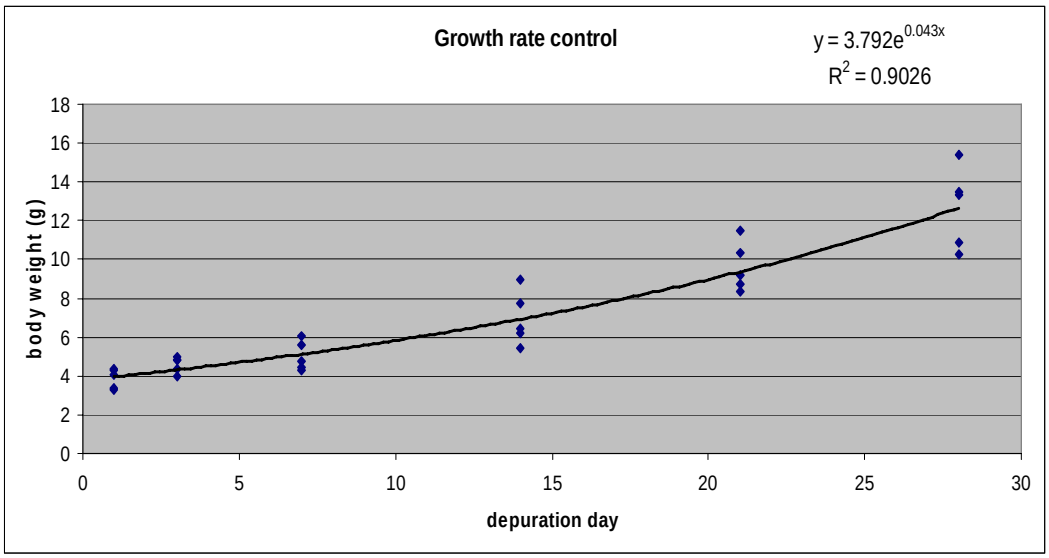
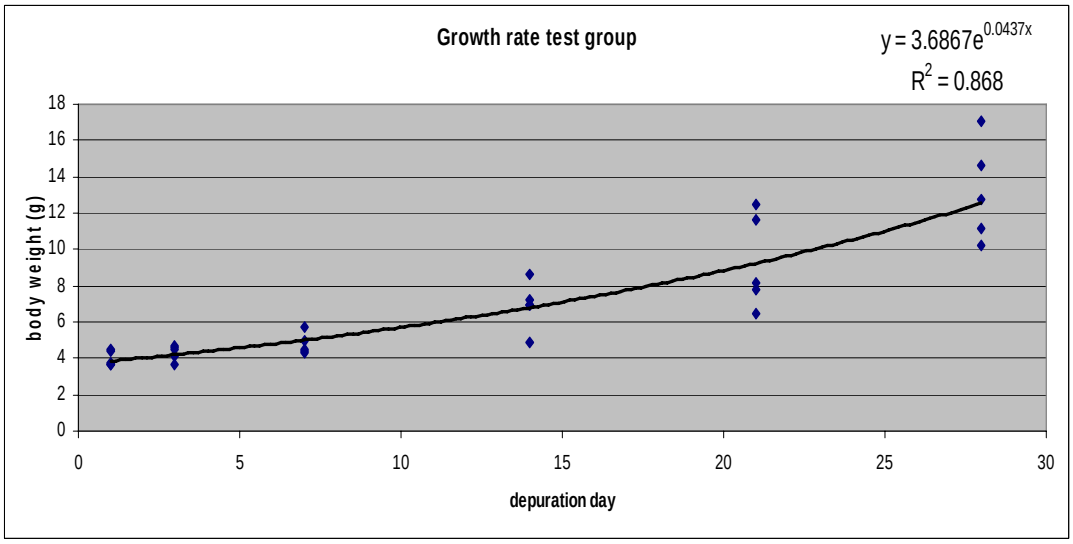


Figure 2: Growth rate of test animals during the depuration period



The data collected in this study were used to derive the half-life ($t_{1/2}$, from the elimination rate constant, $k_{depuration}$), the assimilation efficiency (α), the biomagnification factor (BMF) and the lipid-normalised biomagnification factor (BMF_L) for the individual substances. Calculated BMF and BMF_L values for all test items are presented in Table 6.

Table 6: Calculated BMF and lipid normalized BMF_L

	BMF	BMF_L
HCB	1.64	3.21
MX	0.496	0.973
oTP	0.208	0.408
Methoxychlor	0.051	0.103
BaP	0.001	0.002

HCB showed the highest BMF with 1.64 followed by MX and oTP with BMFs of 0.496 and 0.208, respectively. Methoxychlor and BaP show no biomagnification potential with BMF factors far below zero. The lipid normalization of all BMFs was leading to a two time increase of the estimated biomagnification factors reflecting the increase in fish lipid content during the study. The detailed results of this biomagnification approach are presented in an MS Excel file attached to this document.

8 Annex 1 – Protocol Fish, Dietary Bioaccumulation Study

8.1 INTRODUCTION

8.1.1 Objective

This study will be conducted to determine the elimination rate constant after chemical analysis of incurred fish for the five test compounds from rainbow trout (*Oncorhynchus mykiss*) tissue. The test substances will be administered to the test system via the diet.

The data collected in this study will be used subsequently to derive the half-life ($t_{1/2}$, from the elimination rate constant, $k_{depuration}$), the assimilation efficiency (α), the biomagnification factor (BMF) and the lipid-normalised biomagnification factor (BMF_L) for the individual substances. Calculation methods will be made available separately.

8.1.2 Testing Facility

[to be completed by laboratory]

8.1.3 Compliance

This study will be performed in compliance with the draft OECD Guideline 305[1].Additionally, general OECD Guidelines[2] regarding fish handling and husbandry procedures shall be observed.

8.1.4 Justification for Selection of Test System

Oncorhynchus mykiss is a common test species for freshwater toxicity studies. This study will form part of the ring test activities for the draft additional method, biomagnification in fish, being considered for inclusion in the OECD 305 test guideline.

8.1.5 Justification of Dosing Route

Potential environmental exposure of the test compounds is via feeding on lower trophic levels. Dietary bioaccumulation in fish is a meaningful measure of the potential for hydrophobic chemicals to undergo dietary biomagnification in the environment.

8.2 MATERIALS and METHODS

8.2.1 Test Substance Identification

Table 7: Five individual test compounds will be tested

Compound Name	CAS
hexachlorobenzene	118-74-1
musk xylene (2,4,6-trinitro-5-tert-butyl-1,3-xylene)	81-15-2
o-terphenyl	84-15-1
methoxychlor	72-43-5
benzo(a)pyrene	50-32-8

8.2.2 Carrier

Finfish Starter - Zeigler Bros., Inc., Gardners, PA or similar.

8.2.3 Dilution Water

Natural water or reconstituted moderately hard water can be used in the test, and should be obtained from an uncontaminated and uniform quality source in accordance with the existing OECD 305 test guideline. Test water should be characterized as far as possible, and total organic carbon and natural particle content remain as low as possible during the test. Source of the test water should be documented. The dilution water will be aerated prior to use. Concentrations of trace elements as recommended by OECD are attached at the end of this document.

8.2.4 Storage Conditions

Refrigeration (test feed)

Freezer (-80^{o}C) for sacrificed fish

8.2.5 Characterization of Test Material

The test substances' identity (chemical name and CAS number), purity and known impurities (along with concentrations) shall be documented. As a minimum, this may be based on information obtained from the manufacturer and incorporated into the study report.

8.2.6 Analysis of Substances in Feed

Samples of the test and control diets will be extracted and analyzed for the test compounds prior to initiation of the study and at the conclusion of the uptake phase. The methods of analysis will be included in the raw data and described in the final report. The method that was used on the

EMBSI-supplied feed samples in the pre-study recovery work should be followed (see Annex 2 – “Dietary ring test analytical (v2.1).doc” for EMBSI’s protocol). 3 samples should be randomly taken from the ca. 100g spiked batch of feed at test initiation, and 3 samples at the end of the uptake phase. Each sample must be analysed for all five test substances. Concentration results should not vary by more than 15% between the 3 samples taken at test start, and between the 3 samples taken at the end of the uptake phase. If variation is >15% between the 3 samples at test start, the feed batch should be remixed and reanalyzed before the test is begun. Mean measured concentrations for each of the five test substances should not vary more than 20% between test start and the end of uptake phase.

8.2.7 Sampling of Fish for Test Substance Analysis

Sampling intervals for test substance analysis includes one optional sampling point on uptake phase day 3 (used to estimate uptake rate) and are depuration phase days 1, 3, 7, 14, 21 and 28. To save analytical resources, it is possible to analyse only 2 of the 5 control fish at each sampling point for chemical concentrations; however the remaining 3 control fish must still be removed and their weight and length measured before storing (frozen). If any test substance is measured in the control fish, then it is necessary to analyse all five control fish for the test compounds. The method that was used on the EMBSI-supplied fish samples in the pre-study recovery work should be followed (see Annex 2; document “dietary ring test analytical (v2.1).doc” for EMBSI’s protocol).

8.2.8 Fish Lipid Analysis

Lipid content will be measured individually on 5 fish taken from the stock population at the beginning of the uptake phase, 5 fish from each of the treatment tanks at the end of the uptake phase and 5 fish taken at the end of the depuration phase. The method used to quantify the lipid will be documented in the raw data and the results included in the final report.

8.2.9 Solubility

Not applicable within the confines of the study design.

8.2.10 Test System

Juvenile *Oncorhynchus mykiss*

8.2.11 Supplier

The fish supplier will be documented in the raw data and final report.

8.2.12 Husbandry and Acclimation

The stock population will be acclimated in dilution water for at least one week at test temperature and fed throughout on a sufficient diet and of the same type to be used during the test. Any remaining fish from the stock population will be maintained for a reasonable time, so that they may be used for method development, training purposes, etc. Following that they will be euthanized according to the most appropriate and humane technique. An example of such a method is to use a tricaine methane sulfonate (MS 222) solution, prepared in laboratory dilution water (an MS222 concentration of 500 mg/L in laboratory dilution water pH buffered to 7.0-7.5 could be used).

Fish are held under static conditions using biological and mechanical filtration and are fed daily with Finfish Starter or similar.

The contents of the “clean” fish feed, especially lipid content, will be recorded and included in the test report.

8.2.13 Number and Sex

Proposed number: 118 (10 for initial weight assessment on arrival, 90 for study, 8 for spike recovery analysis, 10 for analytical method development).

Sex: Not Applicable

8.2.14 Age at Initiation of Exposure

Juveniles, actual age will be noted in the raw data and final report.

8.2.15 Test System Identification

Organisms will not be individually identified prior to or while in the test chambers. All test chambers will be labeled to show study number and concentration.

8.2.16 Selection

Organisms will be randomly selected from the stock population and transferred directly to the test chambers. A printout of the randomization schedule will be included in the raw data. Organisms should be within a weight range of 1-8 g at test start. Select fish of similar weight such that the smallest are no smaller than two-thirds of the weight of the largest. All should be of the same year-class and come from the same source. Since weight and age of a fish appear sometimes to have a significant effect on BCF values record these details accurately. Optionally, a sub-sample of the stock of fish can be weighed before the test in order to estimate the mean weight. NB no fish should be less than 1g in weight.

To ensure that quality organisms are used for the study, fish will be selected from a pool of organisms larger than that needed for the study. The study director or his designee determines organism suitability.

8.2.17 Feeding Rate

Fish will be fed at a level of approximately 3% of wet body weight per day. The amount of feed may be adjusted at each sampling point based on the weights of sacrificed fish to account for growth during the experiment to maintain a level of 3%. When adjusting the amount of feed required, the individual weights from test and control fish should be combined and averaged so that both test and control groups are fed the same quantity of feed. Initial feeding will be based on weight measurements of the stock population prior to the start of the test.

8.2.18 Test Feed

Feed containing each of the five test compounds spiked via a corn oil suspension of the test substances, solvent-based spiking of the test substances, or a combination of the two methods. It is fed to the treatment fish during the uptake phase of the study.

8.2.19 Control Feed

Feed treated in exactly the same manner as the test feed (i.e. including corn or fish oil and/or treated with a solvent), but containing no test substances should be fed to the control fish during the uptake phase of the study.

8.2.20 Clean Feed

Feed as supplied by the manufacturer, containing no test substance and not treated with solvent or corn/fish oil for spiking and fed to all fish (test and control groups) during the depuration phase of the study.

8.2.21 Contaminants

No known contaminants should be present in the test water or the feed at levels high enough to interfere with this study. The dilution water may be prepared from UV-sterilized, deionised well water.

8.3 EXPERIMENTAL PROCEDURE

8.3.1 Definitive Test Design

Table 8: Nominal concentration and number of organisms

GROUP	NOMINAL CONCENTRATION (μg/g)	NUMBER OF ORGANISMS
Control	0	45 (40**)
Treatment	375*	45 (40**)

* Total dietary concentration minimized to mitigate cumulative effects on fish and provide sufficient analytical sensitivity. The concentrations used are based on previous successful testing. ** If the test lab opts out of the sampling time point during uptake, fewer fish are required.

Table 9: Test substances and nominal concentrations

TEST SUBSTANCES	NOMINAL CONCENTRATION* (μg/g)
hexachlorobenzene	25
musk xylene (2,4,6-trinitro-5-tert-butyl-1,3-xylene)	50
o-terphenyl	50
methoxychlor	100
benzo(a)pyrene	150
Total Diet Concentration	375

*feed as supplied and used in spiking; concentrations therefore include the minimal quantity of moisture associated with supplied feed.

8.3.2 Preparation and Administration of Test Material

A flow-through system will be used to provide a sufficient volume of dilution water to the test tanks. The actual flow rate should be sufficient to supply at least five water replacements over a 24 hour period, and will be recorded in the raw data.

The appropriate amounts of the test substances will be added to the test feed to achieve nominal concentrations as per the definitive test design. A single diet will be prepared containing each of the five test compounds. It is important to note that the lipid content of the diet should not be artificially increased by the addition of large quantities of fish or corn oil. (the draft guideline says that “ideally the lipid content of feed should be 15 – 20% w/w”; however slightly lower lipid concentrations are not ruled out).

The test compounds will be dissolved or suspended in:

i) corn oil at a concentration that yields the approximate individual diet concentrations when 0.5 mL of corn oil suspension is added to a total of 100 g fish feed (0.5% corn oil in feed). The exact methods and procedures for dosing the feed will be documented and included in the raw data. Control feed will be prepared by adding 0.5 mL of corn oil containing no test compounds to 100 g of clean feed. (the method for preparing the spiked and control diets by this method is given in document “dietary ring test analytical (v2.1).doc” – Annex 2)

ii) an appropriate quantity of a suitable organic solvent (eg cyclohexane or acetone; 10 – 40 mL). Either an aliquot, or all (added in portions), of this solution is mixed with the appropriate mass of fish food to achieve the required nominal dose level. The food/test substance can be mixed in a stainless steel mixing bowl and the freshly-dosed fish food left in the bowl in a laboratory hood for two days (stirred occasionally) to allow the excess acetone to evaporate, or mixed in a rotary evaporator bulb with continuous rotation. The excess solvent can be “blown” off under a stream of air or nitrogen if necessary. Care must be taken to ensure that the test substance does not crystallise as the solvent is removed. The spiked diet should be stored under conditions that maintain stability of the test chemical within the feed mix (eg refrigeration) until use.

iii) a combination of the two methods described above: test substances in solution (hexane or acetone) are prepared and added to corn/fish oil and then the organic solvent is evaporated before mixing the oil with the feed. The feed is dried overnight to ensure no solvent remains. Concentrations of solutions of the test substances in solvent should be chosen such that the target concentrations in final feed are met by the addition of the solvent solution to the minimum quantity of fish/corn oil (ca 0.5 mL).

The lipid content of the spiked feed must be recorded (NB lipid content of test and control diets should be the same). The amount of treated (Test) and untreated (Control) diets will be measured daily and fed to the fish *ad lib* as one feeding. The daily dose of feed may be split into two feedings if the feed is not all eaten (ie still remaining when tanks are cleaned 30 minutes after feeding) or not taken up rapidly enough (NB this may have consequences for when analysis is carried out).

8.3.3 Test Chamber and Volume of Solution

A suitable set up of test and control chambers will be used, in accordance with the OECD 305 TG, ensuring appropriate fish loading rates.

An example laboratory set up is as follows: Test chambers may be 40 L glass aquaria with stainless steel standpipes that allow approximately 31 L of solution in the test chamber. Fresh dilution water will flow through

the test chambers at a target rate of 160 - 200 mL/minute, resulting in 7 - 9 water changes in a 24 hour period. The test chambers will be aerated throughout the study to ensure that adequate dissolved oxygen levels are maintained. Chambers will be covered to minimize contamination and/or evaporation.

During fish feeding, the flow will be turned off (and, if used, the standpipes capped) to prevent loss of feed. Once the fish are observed to have completed their feeding, the water flow is resumed (and, if used, the standpipes uncapped).

8.3.4 Exposure Duration

Uptake phase: 13 days

Depuration phase: 28 days

8.3.5 Physical Measurements

Range of acceptable test water temperatures: 13-17°C. Variation should be less than ± 2°C in any one tank (e.g. 15 ± 2°C)). Temperature should be measured daily, and preferably continuously in one tank.

Dissolved Oxygen (≥ 60% of air saturation) and pH. As a minimum these should be measured at test start and in the middle and at the end of the uptake phase, and once a week during the depuration phase.

Total organic carbon. This should be measured before the test start as part of the routine water quality assessment, but TOC measurement is not required during or after the test. (If laboratories routinely measure TOC during studies, then they can do so).

Diurnal light: ~16 hours light, ~8 hours dark.

A record of daily observations for any mortality, adverse effects and feeding behavior in both control and test group should be kept.

Environmental conditions should be monitored to provide a record of the continuous measurements for temperature and lighting in the test area.

8.3.6 Fish Sampling Procedure

Treatment fish will be fed for 13 days with feed containing the test compounds. Control fish will be fed for the same duration with feed treated in the same manner as the test feed, but containing no test compounds. In the event that signs of stress are observed, the uptake phase may be shortened; the ring test coordinators should be made aware and a memo will be added to the study file documenting the details. Fish samples will be collected from each tank according to the sampling schedule listed in this protocol. Sampling intervals include uptake phase day 3 (used to estimate uptake rate) and depuration phase days 1, 3, 7, 14, 21 and 28.

At each sampling period, 5 fish from each tank (control and treatment) will be sacrificed. Sampling should occur at roughly the same time of day for each sample during the depuration phase. Optionally, the guts may be removed from the test fish before analysis; if this is done, fish must still be treated in the same way as detailed below to ensure that each laboratory samples fish at the same time and that each fish receives a similar period of “clearing” prior to analysis in the case of sampling on day 3 of uptake and day 1 of depuration.

In order to ensure that gastro-intestinal tract is cleared of test or control diet, fish sampled on uptake day 3 will be treated as follows. Shortly after being fed their respective diets, 5 test and control fish will be transferred to separate smaller tanks containing clean water. Approximately 5 hours after being fed their day 3 diets, they will be fed clean feed. These fish will be sacrificed the following morning.

On uptake day 13, the remaining fish will also be fed clean food approximately 5 hours after their last treatment or control feeding. Five test and control fish will be sacrificed the following morning and correspond to day 1 depuration samples. On days when fish sampling is scheduled during the depuration phase, it shall occur just prior to the daily feeding.

All fish will be euthanized according to the most appropriate and humane method. An example is the use of a tricaine methane sulphonate (MS 222) solution; prepared in laboratory dilution water (an MS222 concentration of 500 mg/L in laboratory dilution water pH buffered to 7.0-7.5 could be used).

All fish will be treated humanely in accordance with National and OECD guidance. The study design and personnel training must be sufficient to minimize animal pain within the confines of the study objective.

8.3.7 Experimental Evaluation

Observations for mortality, any adverse effects and feeding behaviour will be performed and recorded daily. Additional observations may be performed. Fish are considered dead if there is no respiratory movement and no reaction to a slight mechanical stimulus can be detected. During observations, organisms will be examined for abnormal behaviour or coloration. In the event that signs of stress are observed, the uptake phase may be shortened; the ring test coordinators should be made aware and a memo will be added to the study file documenting the details. Any dead fish will be removed.

After completion of the study, the monitoring of environmental conditions will be discontinued. All remaining fish will be euthanized, weighed and measured (total length).

Table 10: Fish Sampling Schedule

Phase	Day	Analysis	Number of Fish Sampled
Uptake	0	Lipid	5 control [a]
Uptake	3 [b,c]	Test Substance Concentrations	5 control + 5 test
Uptake	13 [d]	Lipid	5 control + 5 test
Depuration	1	Test Substance Concentrations	5 control + 5 test
Depuration	3	Test Substance Concentrations	5 control + 5 test
Depuration	7	Test Substance Concentrations	5 control + 5 test
Depuration	14	Test Substance Concentrations	5 control + 5 test
Depuration	21	Test Substance Concentrations	5 control + 5 test
Depuration	28	Test Substance Concentrations	5 control + 5 test
Depuration	28	Lipid	5 control + 5 test

All fish should be individually weighed and length (total) measured prior to analysis.

a. Fish taken from same lot just prior to initiation. Not included as study fish.

b. OPTIONAL - Used to estimate uptake rate.

c. Test fish fed spiked diet in the morning of the designated day. Approximately 5 hours after test diet feeding, fed clean feed. Fish sacrificed the following morning.

d. Test fish fed spiked diet in the morning of the designated day. Approximately 5 hours after test diet feeding, fed clean feed. Fish sacrificed the following morning corresponding to depuration day 1.

8.3.8 Cleaning

To maintain good hygiene, uneaten food (if uneaten food is observed it will be documented) and faeces will be siphoned from the test chambers shortly after feeding (within 30 minutes to 1 hour). Care will be taken not to injure the test organisms.

8.3.9 Organism Loading

Loading will be in accordance with the OECD 305 TG (0.1 - 1.0 g of fish (wet weight) per litre of solution per day based on the flow through conditions).

8.3.10 Length / Weight of Test System

Length and weight measurements of a sub-sample of the stock fish will be recorded prior to the start of the study in order to estimate a mean weight. The 5 fish used for lipid determination can be used for this purpose as part of the ten stock fish. Individual length and weight

measurements will also be recorded on fish removed at each sampling period. Total length will be measured.

8.3.11 Conditions for Validity

The test is acceptable if:

- Temperature variation is less than ± 2 ºC in treatment or control groups.
- Concentration of dissolved oxygen does not fall below 60% of the air saturation value.
- The concentration of the test substance in fish food is kept constant over the feeding period within a range of ± 20%.
- Concentrations of test chemical are not detected, or are present only at typical trace levels, in un-spiked food or control fish tissues relative to treated samples.
- A high degree of homogeneity of substance in feed must be demonstrated in preliminary analytical work on the spiked diet; concentrations for the same substance between the 3 samples must not vary more than +/- 15%.
- Mortality or other adverse effects/disease in both control and test group fish should be ≤10% at the end of the test Average growth in both test and control groups should to be similar.

8.3.12 Calculations

Test results are used to derive the elimination rate constant as a function of the total wet weight of the fish. The Excel spreadsheet for the ring test allows tabulation of test data, and automates the calculations described below. In addition, the assimilation efficiency (α), the biomagnification factor (BMF) and its lipid-normalised value (BMF_L) will be calculated. A memorandum outlining the calculations will be provided at a later date.

Basic data analysis and calculations are as follows:

- Weight dataIndividual fish weights and lengths are tabulated separately for test and control groups during the uptake and depuration phases.
- Weight and length data are converted to natural logs and plotted vs. day, separately for test and control.
- A linear least squares correlation is calculated for the ln(fish weight) vs. day for both test and control (individual data not daily means) using standard statistical procedures.

- The variances in the slopes of the test and control lines are calculated and used to evaluate the statistical significance of the difference in the two slopes (growth rates) using the student t-test. If there is no significant difference, the test and control data are pooled and an overall fish growth rate for the study (k_{growth}) calculated as the overall slope of the linear correlation. If statistical differences are observed growth rates for control and treated fish are reported separately, and the growth rate for the treated fish is used in subsequent calculations.
- Test chemical concentration in fish dataIndividual fish test substance residue measurements expressed in terms of concentration (w/w) are tabulated for test and control fish for individual sample times.
- The individual fish concentration data for the depuration period are converted to their natural logarithms and plotted versus time (day). If a visual inspection of the plot shows obvious outliers, a statistically valid outlier test may be applied to remove spurious data points.
- A linear least squares correlation is calculated for the ln(concentration) vs. depuration day data. The slope and intercept (day 0 of depuration) of the line are reported as the overall elimination rate ($k_{overall}$) and time zero concentration ($C_{0,depuration}$).
- The variances in the slope and intercept of the line are calculated using standard statistical procedures and the 90% confidence intervals around these results evaluated.

Lipid normalisation The mean lipid fraction (w/w) in the fish and the food are calculated.

8.4 REPORTS

After termination of the study, a final report will be prepared containing select information acquired during the study. As a minimum the report shall contain:

- In-life / feeding observations.
- Test substance concentrations measurements in spiked diets for each analysis.
- Food type employed and feeding rates during uptake phase, any deviations from once-a-day feeding.
- Tabulated fish individual weight and length data and calculations for test and control groups, derived growth rate(s) and 95% confidence interval(s), plots of growth data.
- Indication of any treatment related effects on fish growth.

- Complete description of all chemical and lipid analysis procedures employed including quantitation limits, variability and recovery.

8.5 RECORDS

All appropriate materials, methods and experimental measurements required in this protocol will be recorded and documented in the raw data. Any changes, additions or revisions of this protocol must be approved by the ring test coordinator and OECD drafting team. These changes will be documented in writing, including the date and the justification for the change. The protocol, final report, raw data or computer generated listings of raw data, and supporting documentation will be maintained in the Archives of the testing facility.

8.6 REFERENCES

[1]Bioconcentration and Bioaccumulation in Fish: Aqueous and Dietary Exposure. OECD Guidelines for Testing of Chemicals. Guideline 305, Draft: 22.03.2010

[2]Bioconcentration: Flow-through Fish Test. OECD Guidelines for Testing of Chemicals. Section 3: Degradation and Accumulation, Guideline 305, adopted June 14, 1996.

9 Annex 2 - Analytical Validation Protocol (Dietary BCF Ring Test - OECD TG 305)

9.1 Background

OECD is currently considering adoption of a test method for measuring the bioconcentration factor (BCF) of poorly water soluble chemical compounds in fish using a novel dietary exposure technique. A ring test program has been initiated to evaluate the proposed guideline by measuring the BCFs of five (5) hydrophobic compounds. Because of the importance of the analytical methods employed, an analytical validation protocol has been developed. This will permit the laboratories participating in the ring test to confirm that the preparation and analysis techniques employed are suitable for accurately measuring dietary BCFs.

9.2 Objectives

This analytical validation protocol is intended to provide the following:

- a procedure for preparing spiked diet using a corn oil suspension.
- an analytical method for extraction and analysis of the five test compounds in spiked diet.
- an analytical method for the extraction and analysis of the five compounds in fish.
- a method for gravimetrically measuring the fish lipid content using accelerated solvent extraction (ASE).

9.3 Analytical Methods

EMBSI will provide suggested analytical methods for determination of test compound concentrations in diet and fish, and measurement of fish lipid content. The participating laboratories may apply these methods or utilize methods based on their respective capabilities and experience.

9.4 Analytical Validation Samples

EMBSI will provide the participating laboratories with a series of spiked diet samples, incurred fish samples and control fish samples. These samples will permit the participating laboratories to validate their analytical methodologies. Results of these sample analysis will be submitted for tabulation. The results of the validation analyses will be summarized and shared with the participating labs.

9.5 Outline of Validation Sequence

Phase 1 Diet Preparation and Analysis

Establish method for extraction and analysis of spiked diet

Analyze spiked diet supplied by EMBSI

Prepare and analyze own spiked diet

Phase 2 Fish Analysis

- Establish method for extraction and analysis of fish
- Analyze incurred fish supplied by EMBSI

Phase 3 Lipid Analysis

Measure lipid content of control fish supplied by EMBSI

Proposed Dates:

January 21-29, 2010

EMBSI ships spiked diet samples to participating labs.

February 1- March 30, 2010

Participating labs:

- establish analytical method for test compounds in diet
- analyze spiked diet sent by EMBSI
- establish own diet preparation technique

  (via corn oil suspension or co-solvent).

March 15-30, 2010

Report results of spiked diet analysis.

March 25-31, 2010

EMBSI ships incurred fish samples to participating labs for test compound analysis and lipid determination (fish samples will be sent to labs upon receipt of diet analysis results).

March 31 - May 21, 2010

Participating labs:

- establish analytical method for test compounds in fish
- analyze incurred fish sent by EMBSI
- establish method for fish lipid determination
- analyze fish sent by EMBSI for lipid content

May 25, 2010

Report results

9.6 Test Compounds

The test compounds are listed below along with basic physical and chemical parameters and suggested suppliers.

Table 11 Test compound with basic physical and chemical parameters

CAS#	Compound		MW (g/mol)	MP (°C)	BP (°C)	Supplier/cat # (purity)
118-74-1	Hexachlorobenzene	solid	284.8	228	324	Aldrich, 17,105-0, (99%)
81-15-2	Musk Xylene (2,4,6-trinitro-5-tert-butyl-1,3-xylene)	solid	297.3	114		City Chemical, B1116, (98%)
84-15-1	o-Terphenyl	solid	230.3	58	335	Aldrich, T2800, (99%)
72-43-5	Methoxyclor	solid	345.7	83	346	TCI, M1736, (>95%)
50-32-8	Benzo[a]pyrene	solid	252.3	177	496	Sigma, B1760, (96%)

9.7 Preparation of Spiked Diet using Corn Oil Suspension

9.7.1 Materials needed:

- fish feed (Finfish Starter #1, Zeigler Bros, Inc. Gardeners, PA or equivalent)
- wide bore syringe or pipette capable of dispensing 0.5 mL
- individual test compounds (5)
- corn oil (Sigma Aldrich)
- 5.0 mL volumetric flask
- amber bottle (ca. 150 mL)
- micro stir bar
- stir plate
- analytical balance (0.0001 g)
- mechanical tumbler
- **Method:**Combine 0. 10 g of each test compound in a 5.0 mL volumetric flask
- Bring to volume with corn oil. Resulting concentration of each compound is 20 mg/mL (2% w/v)
- Add micro-stir bar and stopper

- Mix rapidly on stir plate overnight to suspend/emulsify solid compounds in corn oil. There may be partial solubilization of the compounds in corn oil.
- Place 50 g of unspiked fish feed in amber glass bottle.
- Add 0.5 mL of corn oil suspension using a wide bore syringe or pipette.
- Add an additional 50 g of fish diet and cap bottle. The concentration of each compound in the diet will be 100 µg/g.
- Shake the bottle by hand to homogenize the corn oil suspension throughout the fish feed.
- Place the bottle containing the spiked diet on a mechanical tumbler and tumble slowly overnight.
- The spiked diet should be refrigerated (2-4^{o}C) when not in use and the bottle be covered with foil if not stored in brown or amber glass.

9.8 Spiked Diet Extraction and Analysis

9.8.1 Materials needed:

- U.S. EPA semi-volatile (SV) internal standard solution (1.0 mL solution of 2000 µg/mL each of d_4 1,4-dichlorobenzene, d_4 -naphthalene, d_{10}- acenaphthene, d_{10}- phenanthrene, d_{12}-chrysene, d_{12}-perylene)
- individual test compounds (5)
- extraction solvent (1:1 acetone/methylene chloride)
- glass extraction bottles (ca 30 mL) with Teflon lined septa caps
- mechanical shaker
- analytical balance (0.0001 g)
- ultrasonic bath
- assorted volumetric flasks (10, 25 mL) and pipettes
- GC-MS system capable of selected ion monitoring (SIM)

30m x 0.25 mm id capillary column with 0.25u DB-5MS (or equivalent) with guard column

9.8.2 Diet Extraction

- Weigh out 1.0000 g of spiked diet into 30 mL extraction vial. Record weight

- Add 25.0 mL extraction solvent. Crimp seal vial with Teflon faced septum
- Shake extraction bottles vigorously by hand for one minute
- Sonicate in ultrasonic bath for 30 minutes
- Shake extraction bottles vigorously by hand for one minute
- Agitate on mechanical shaker for 60 minutes
- Shake extraction bottles vigorously by hand for one minute
- Permit contents of vial to settle for 60 minutes
- Transfer 1.0 mL of the extract to an amber GC autosampler vial and crimp seal
- Add 10 μL of a 400 μg/mL SV internal standard solution. Analyze by GC-MS
- Transfer 4-5 mL of the remaining extract to an amber vial and store in freezer (-20°C)

9.8.3 Diet Extract Analysis

Extracts are analyzed by GC-MS in the single ion recoding (SIR) mode. One half (0.5) microliter splitless injections are made. The column flow is 0.9 mL/min (constant). The GC temperature program is 50°C for 1 minute ramped to 210°C (2 minute hold) at 10°C/min then to 320°C at 15°C min.

Table 12 Ions monitored and approximate retention times

Compound	ion	Retention time (min)
d4 1,4-dichlorobenzene [IS1]	150	6.3
d4 -naphthalene, [IS2]	136	8.9
d10- acenaphthene [IS3]	164	13.0
Hexachlorobenzene	284	15.8
d10-phenanthrene [IS4]	188	16.5
Musk Xylene	282	17.3
o-Terphenyl	230	17.7
Methoxychlor	227	23.4
d12-chrysene [IS5]	240	23.5
Benzo[a]pyrene	252	25.8
d12- perylene [IS6]	264	25.9

A three level standard curve is prepared at concentrations of approximately 0.8, 2 and 8 μg/mL of each of the five test compounds along with a constant internal standard concentration of 4 μg/mL.

9.8.4 Spiked Diet Reporting

The individual test compound concentrations for each of five (5) replicate analyses of spiked fish diet supplied by EMBSI along with the mean, standard deviation (SD) and relative standard deviation (RSD). Additionally, the results of analysis of at least two (2) control diet samples (containing no test compounds) should be reported.

9.9 Fish Extraction and Analysis

9.9.1 Materials needed

- U.S. EPA semi-volatile (SV) internal standard solution (1.0 mL solution of 2000 µg/mL each of d_4 1,4-dichlorobenzene, d_4 - naphthalene, d_{10}- acenaphthene, d_{10}- phenanthrene, d_{12}-chrysene, d_{12}-perylene)
- individual test compounds (5)
- extraction solvent (1:1 acetone / methylene chloride)
- Dionex Accelerated Solvent Extractor (ASE) 350 or equivalent pressurized fluid extractor
- mL stainless steel ASE extraction cells with cellulose filters
- Hydromatrix or equivalent drying reagent
- Metal spatulas
- analytical balance (0.0001 g)
- heating block with nitrogen evaporator.
- assorted volumetric flasks (10, 25 mL) and pipettes
- 25 mL graduated cylinders
- Silica solid phase extraction (SPE) cartridges (Waters 2g/12mL) or equivalent
- SPE manifold
- 15 mL graduated d glass centrifuge tubes with ground glass stopper.
- GC-MS system capable of selected ion monitoring (SIM)
- 30m x 0.25 mm id capillary column with 0.25u DB-5MS (or equivalent) with guard column

9.9.2 Fish Extraction

- Use the fish weights recorded on each sample vial for calculations. This represents the wet weight recorded at time of sampling
- Place one whole fish in glass beaker along with 3 g of pre-extracted Hydromatrix and homogenize mixture with metal spatula

- Place the beakers in a laboratory fume hood and permit to dry overnight with occasional mixing
- Transfer the contents of the beakers to 10 mL ASE cells containing cellulose filters. Rinse each beaker with 5 mL of 1:1 acetone / methylene chloride and add this to the respective ASE cells. Add additional Hydromatrix as necessary to minimize cell dead volume

Cap the ASE cells and extract using the following conditions:

- Heat Time: 5 min
- Solvent: 50% Acetone, 50% MeCl
- Static Time: 5 min
- Percent Flush: 30
- Temperature: 120°C
- Purge Time: 60 sec
- Pressure: 1500 psi
- Cycles: 2

Each extract generates approximately 15-20mL of solvent collected in 40 mL vials.

- Transfer the extract to 25 mL graduated cylinders and adjust volume to 15 mL under a gentle stream of nitrogen
- Return the extract to its original vial and cap with foil lined or new septum cap
- Refrigerate the raw fish extract (2-5^{o}C)

9.9.3 Fish Extract Clean-Up

- Allow raw fish extract to reach room temperature
- Condition Silica SPE (2g/12mL) cartridges with 8 mL of 1:1 acetone / methylene chloride followed by 8 mL residue hexane
- Dry cartridges by applying vacuum
- Place 15 mL centrifuge collection tubes in manifold and close valve for each SPE cartridge
- Fill SPE cartridge with 8 mL hexane. Add 0.5 mL of raw fish extract
- Open SPE valves and collect cartridge eluent
- Elute cartridges with additional 2 x 2 mL of 1:1 methylene chloride / hexane and collect
- Apply vacuum to collect residual solvent from cartridges. Total solvent collected from each SPE cleaned-up extract is 12-13 mL

- Add 5uL of a 16 µg/mL SV internal standard solution to each cleaned-up extract
- Transfer centrifuge tubes to heating block set at 50°C and evaporate under a gentle stream of nitrogen to a final volume of 0.5mL
- Transfer aliquots of the final extract to amber GC autosampler vials with inserts
- Store extracts in freezer (-20°C) pending analysis

9.9.4 Fish Extract Analysis

Cleaned-up fish extracts are analyzed by GC-MS using the same conditions describe for analysis of the diet extracts with the following changes. The injection volume is increased to two microliters and the MS multiplier voltage is increased as necessary to achieve sufficient sensitivity. A series of standards at a minimum of 5 concentrations levels covering the range of approximately 3 to 3200 ng/mL is analyzed. Each standard contains 160 ng/mL of the SV internal standard.

Note: Each laboratory should demonstrate satisfactory recoveries of the five test compounds using their own spiked samples in fish matrix using the extraction and analysis procedure they employ. This validation should be performed according to each laboratory's own Standard Operating Procedures.

9.9.5 Incurred Fish Reporting

The individual test compound concentrations for each of the eight (8) incurred fish samples supplied by EMBSI along with the mean, standard deviation (SD) and relative standard deviation (RSD). Concentrations should be reported in µg/g (ppm) wet weight using the fish weights provided. Additionally, the results of analysis of at least two (2) control fish (not fed spiked diet) should be reported.

9.10 Gravimetric Fish Lipid Determination Using Accelerated Solvent Extraction

9.10.1 Materials needed

- Dionex Accelerated Solvent Extractor (ASE) or equivalent pressurized fluid extractor
- mL ASE extraction cells with cellulose fibers
- Hydromatrix drying reagent
- hexane
- glass beakers
- 40 mL VOA vials for extract collection amber bottle (ca. 150 mL)

- metal spatulas
- laboratory fume hood
- analytical balance (0.0001g)
- drying oven

9.10.2 ASE extraction conditions

- Temperature: 125°C
- Pressure: Default value (1500 psi)
- Pre-heat Time: 0 min
- Static Time: 5 min
- Heat Time: 6 min
- Purge Time: 60 seconds
- Solvent: n-Hexane (100%)
- Purge Time: 60 seconds
- Extraction Cycles: 2

9.10.3 ASE extraction

- Weigh out one individual fish with a weight of approximately 2-4g into a glass beaker. Record exact weight of fish to at least four decimal places.
- Add 3 g Hydromatrix and mince thoroughly with a metal spatula.
- Place beakers containing fish-Hydromatrix mixture in fume hood and allow to dry overnight.
- Mince the samples several additional times to ensure thorough drying.
- Quantitatively transfer the dried samples to 10 mL ASE extraction cells containing cellulose filters.
- Extract the samples for lipids using the ASE condition listed above.
- With each batch of fish lipid samples, a single blank sample should be extracted containing only 3 g of Hydromatrix.
- Evaporate the hexane solvent extracts to dryness under a gentle stream of nitrogen at 65^{0}C.
- Dry extracts at 105^{o}C to a constant weight..
- Calculate lipid content using the following formula:

9.10.4 Calculations

$$\frac{\text{finals as l li is is as ls lans is}}{\text{ini ials as ls is}} \times 100\text{s} = \%\ \text{Lipid Content (wet-weight basis)}$$

9.10.5 Fish Lipid Reporting

The individual lipid content for each of the five (5) control fish samples supplied by EMBSI along with the mean, standard deviation (SD) and relative standard deviation (RSD) should be reported.

10 Annex 3 - Analytical Report

10.1 Preface

The analytical methods for the “Determination of the organic test compounds in fish and spiked diet using GC-MS” are based on the 'Analytical validation protocol, version 2.1' (Annex 2).

10.2 Chemicals, reagents and analytical equipment

10.2.1 Chemicals and reagents

Table 13: Test compounds / analytical standards (see CoA's in chapter 10.10)

Compound name	acronym / abbreviation	CAS-RN	Supplier / catalog no.	Purity / Lot no.
Hexachlorobenzene	HCB	118-74-1	Aldrich / 171050	99.76 % / 81222006
Musk xylene[1]	MX	81-15-2	Dr. Ehrenstorfer GmbH / C 15360000	99.5 % / 70615
o-Terphenyl	oTP	84-15-1	Aldrich / T2800	99.9 % / 04403JH-399
Methoxychlor[2]	DMDT[3]	72-43-5	Supelco / 49054	99.9 % LB69763
Benzo[a]pyrene	BaP	50-32-8	Sigma / B1760	98 % / 129K1892

Semi-volatile internal standard mixture (IS-mix), Ultra scientific (distributor: LGC Standards GmbH), product no. ISM-560 (see CoA in chapter 10.10.6)

IS working solution (dilution of the IS-mix in a toluene) containing the used/active internal standards Phenanthrene-D_{10} (PHE-D10), Chrysene-D_{12} (CHR-D12) and Perylene-D_{12} (PER-D12) in concentrations of 4.0 µg/mL each (EMBSI method: 400 µg/mL)

- Acetone, 'Baker ultra resi-analyzed', Article No. 9254 (J T. Baker)
- Dichloromethane (Methylene chloride, MeCl), 'Baker ultra resi-analyzed', Article No. 9264 (J T. Baker)
- Toluene, 'Baker ultra resi-analyzed', Article No. 9336 (J.T. Baker)

[1] IUPAC: 1-tert.-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene

[2] IUPAC: 1,1,1-trichloro-2,2-bis(4-methoxyphenyl)ethane

[3] Synonym (trade name) for Methoxychlor derived from Dimethoxy-D(D)T

- Extraction solvent: acetone/MeCl (1+1, v+v)
- Sodium sulfate (Na_2SO_4), anhydrous, GR for analysis, baked out at 500 °C for 2 hours (Merck no. 1.06649.1000)

10.2.2 Analytical equipment

- Assorted volumetric flasks (glass) and standard labware products (e.g. glass beakers and aluminium foil)
- Microman pipettes, M25, M50 and M250 (Gilson Medical Electronics)
- Autosampler vials, 1.8 mL, glass, with screw caps (sealed with Teflon®-coated butyl gummy septa (WiCom)
- GC-MS system Bruker/Varian TQ 320-MS
- GC columns: see chapter 10.4 and 10.4.3

Additional equipment for diet analysis

- 40mL sample/collection vials (1^{st} class, EPA, screw thread), clear glass, with screw caps and Teflon®-lined septa (VWR international)
- Horizontal (mechanical) shaker LS 20 (Gerhard)
- Ultrasonic bath Sonorex Digital 10P (Bandelin)
- Centrifuge Heraeus Megafuge 1.0R (Thermo Electron Corp.)

Additional equipment for fish analysis

- Pressurized Liquid Extractor (PLE), Dionex ASE-200
- Extraction cells, 22 and 33 mL stainless steel cells (Dionex)
- Cellulose filters (Whatman), size 1.98 cm (Dionex P/N 049458 Rev. 05 D28 filter)
- 60mL sample/collection vials (1^{st} class, EPA, screw thread), clear glass, with screw caps and Teflon®-lined septa (VWR international)
- Drying reagent 1 (fish): Chem Tube-Hydromatrix, Varian Part #: 198003
  (baked out at 500 °C for 2 hours)
- Drying reagent 2 (fish extract): Na_2SO_4, (baked out at 500 °C for 2 hours)
- Nitrogen evaporator 1: TurboVap® II concentration workstation (Zymark)
- 50 mL Zymark concentration vessels (for Turbo Vap II) with screw caps GL32 and Teflon® lined sealing disks (Schott)
- Nitrogen evaporator 2: IME homemade (for small volumes/ sample vials)

- Volumetric flasks (volume fixing fish extracts): 50mL / 100 / 200 mL
- Solid phase extraction (SPE) manifold: SPE vacuum apparatus spe-12G (Baker)
- SPE cartridges: Sep-Pak® Vac 12cc (2g) Silica (Waters, part no. WAT036920)

10.3 Sample preparation

10.3.1 Extraction and analysis of spiked diet

The **bold highlighted text passages** in the following method listing indicate the changes to the proposed EMBSI method. The main modification was the final centrifugation of the diet extract and the subsequent dilution with toluene at a ratio of 1:100 prior to GC-MS analysis.

- Weigh out **approx. 1 g** of spiked diet into 40 mL extraction vial, record **exact** weight.
- Add 25.0 mL extraction solvent. Crimp seal vial with Teflon faced septum.
- Shake extraction bottles vigorously by hand for one minute.
- Sonicate in ultrasonic bath for 30 minutes, **cool with crushed ice**.
- Shake extraction bottles vigorously by hand for one minute.
- Agitate on mechanical shaker for 60 minutes.
- Shake extraction bottles vigorously by hand for one minute.
- **Centrifugate the extraction vial for 5 min at 1500 rpm (without application of the brakes).**
- Transfer 10.0 µL of the clear extract and 10.0 µL of the IS working solution into an autosampler vial.
- **Add 1 mL toluene, mix thoroughly by hand and seal with a crimp cap.**
- Mix thoroughly by hand and analyze by GC-MS.
- Transfer 4 – 5 mL of the remaining extract to an amber vial and store as an extract retain in a freezer.

10.4 GC-MS measurement

The diluted extracts were analyzed by GC-MS in the single ion monitoring (SIM) mode. One microliter splitless injections were made; the column flow was constant 1.0 mL/min. The further instrument settings are listed in the following tables.

10.4.1 Instrument settings of the GC/MS system in diet analysis

GC parameter

Gas chromatograph: Bruker/Varian 450-GC

Carrier gas: Helium 5.0 (purified with a Varian CP-GasClean filter, type GC/MS),
Carrier gas flow: Constant column flow mode at 1.0 mL/min (EFC[4] controlled)

Capillary: Varian factor FOUR™ capillary column, 30 m x 0.25 mm ID,
0.25 µm VF-5ms with 10 m x 0.25mm ID EZ-Guard

Oven temperature: 1.0 min 90°C isothermal, rate 1: 25°C/min to 210°C,
rate 2: 5°C/min to 223°C, rate 3: 25°C/min to 320°C,
6.0 min 320°C isotherm, total run time: 18.28 min

Injector: Varian 1177 equipped with 'Siltek® deactivated' glass frit liner
in 'split/splitless' mode at 300°C

Autosampler: CTC Analytics CombiPAL

Injection: 1 µl toluene, Inject speed: 50 µL/sec
Wash solvent 1: toluene, Wash solvent 2: toluene

MS parameter

Mass spectrometer: Bruker/Varian TripleQuad GC-MS/MS system 320-MS

Ionization: EI @ 70 eV, Polarity: positive ions

Scan mode: Centroid, Multiplier: EDR5

SIM width: 0.7 amu total, Filament current: 50 µA

Transferline temperature: 280°C, Source temperature: 250°C

Manifold temperature: 40°C, Q1/Q3 peak width: as calibrated

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4 EFC = Electronic flow control

5 EDR = Extended dynamic range

Table 14: Aquisition list / SIM parameter

Segment	Compound / acronym	Analyt/ IS	Acquisition time [min]	Retention time, tR [min]	Quantification (SIM) ion [m/z]	Dwell time [sec]
1	HCB	A1	6.5 – 7.5	7.12	283.7 285.7	0.15 0.15
2	MX PHE-D10	A2 IS1	7.5 – 8.25	8.02 7.72	282.0 188.1	0.15 0.20
3	oTP	A3	8.25 – 10.0	8.39	215.1 230.1	0.15 0.15
4	DMDT CHR-D12	A4 IS2	10.0 – 12.5	11.65 11.73	227.1 240.1	0.10 0.15
5	BaP PER-D12	A5 IS3	12.5 – 14.0	13.38 13.46	252.0 264.1	0.20 0.20

10.4.2 Extraction of fish samples

Extraction of fish samples and clean-up of the raw extract

The **bold highlighted text passages** in the following method listing indicate the changes to the proposed EMBSI method. The modifications were:

- For PLE extraction instead of the stated 10 mL ASE cells, 22 or 33 mL cells were used; overlarge fishes are extracted in two steps.
- The ASE extracts were dried by adding sodium sulfate; the volumes of the extracts are fixed in volumetric flasks to defined volumes.
- A solvent changing to acetone/MeCl was executed prior to clean-up to enable the performance of the clean-up step analogous to the EMBSI method.
- Prior to GC-MS analysis the solvent was changed to pure toluene.

Fish extraction

- Place one whole fish in a glass beaker (record **the actual fish weight**) along with **4.5 to 8 g** of pre-extracted Hydromatrix
- chop and homogenize mixture with a metal spatula
- **seal beaker with a piece of aluminium foil**

Remark 1: Fishes above circa 10 g fresh weight are dried and extracted in two steps/portions due to their large size;
Remark 2: Fish wet weights are recorded at time of sampling as well.

- Place the beakers in a laboratory fume hood and permit to dry overnight with occasional mixing

- Transfer the contents of the beakers to **22 or 33 mL** ASE cells containing cellulose filters. Rinse each beaker with extraction solvent **twofold and add the solvent** to the respective ASE cells. Add additional Hydromatrix as necessary to minimize cell dead volume.
  Cap the ASE cells and extract using the following conditions:
  Heat time: 6 min (not adjustable), Solvent: Extraction solvent
  Static time: 10 min, Percent flush: 100 (2 x 50 %)
  Temperature: 120 C, Purge time: 150 sec
  Pressure: 10.5 mPa (105 bar), Cycles:2

Each extraction generates approximately 30 - 35 mL of solvent using 22 mL ASE cells or approx. 55 mL using 33 mL ASE cells

- collect extracts in 60 mL sample vials
- Extract drying and volume fixing
- Add 2.5 g or 3.5 g Na2SO4 to the ASE extract(s)
- shake thoroughly by hand for 0.5 min
- centrifuge 2 min at 1200 rpm and decant the extract into 50 / 100 / 200mL volumetric flask[6],
- wash Na_2SO_4 two times with the extraction solvent (with centrifugation)
- fill the volumetric flask to ring mark with extraction solvent
- Store until clean-up in a refrigerator at approx. 4°C

Fish extract clean-up

Allow raw fish extract to reach room temperature.

- Prearrangement:
  Pipet exact 1.5 mL of the raw extracts into 1.8mL auto sampler vials, evaporate the extraction solvent to approx. 50 µL by a gentle stream of nitrogen (IME evaporator) and reconstitute the residues with 500 µL extraction solvent.
- Condition Silica Sep-Pak® SPE cartridges with 8 mL of extraction solvent followed by 8 mL residue hexane.
- Dry cartridges by applying vacuum.
- Place 50 mL Zymark concentration vessels into the SPE manifold and close valve for each SPE cartridge.

[6] Volumetric flask of 50 mL if 22 mL ASE cells are used; 100 mL if 33 mL cells are used; 200 mL only if extraction is performed in two ASE steps.

- Fill SPE cartridge with 8 mL hexane and add 0.5 mL of raw fish extract.
- Open SPE valves and collect cartridge eluent.
- Elute cartridges with additional 2 x 2 mL of 1:1 MeCl / hexane and collect cartridge eluent.
- Apply vacuum to collect residual solvent from cartridges. Total solvent collected from each SPE cleaned-up extract is 12 - 13 mL.
- **Add 20 µL of the IS working solution (conc. 4.0 µg/mL in toluene)** to each cleaned-up extract.
- Transfer Zymark vessels to the TurboVap® II concentration workstation and evaporate under a gentle stream of nitrogen at a water bath temperature of 45°C to a volume of 0.5 mL.
- **Transfer the concentrates to GC autosampler vials and evaporate to almost dryness under a gentle stream of N_2 using the IME evaporator and reconstitute the residues in 500 µL toluene.**
- Store extracts in freezer (-20°C) pending analysis.

10.4.3 GC-MS measurement

The cleaned-up fish extracts are analyzed by GC-MS comparable to the diet extracts. The instrument settings are listed blow:

Instrument settings of the GC/MS system in fish analysis:

GC parameter

Gas chromatograph: **Bruker/Varian 450-GC**

Carrier gas: Helium 5.0 (purified with a Varian CP-GasClean filter, type GC/MS),
Carrier gas flow: Constant column flow mode at 1.0 mL/min (EFC controlled)

Capillary: Varian factorFOUR™ capillary column, 30 m x 0.25 mm ID,
0.10 µm VF-5ms

Oven temperature: 1.5 min 90 °C isothermal, rate 1: 25 °C/min to 260 °C,
rate 2: 5°C/min to 274°C, rate 3: 25°C/min to 310°C,
6.0 min 310°C isotherm, total run time: 18.54 min

Injector: Varian 1177 equipped with 'Siltek® deactivated' glass frit liner in 'split/splitless' mode at 300°C

Autosampler: CTC Analytics CombiPAL

Injection: 1 µl toluene, Inject speed: 50 µL/sec
Wash solvent 1: toluene, Wash solvent 2: toluene

MS parameter I General settings

Mass spectrometer: Bruker/Varian TripleQuad GC-MS/MS system 320-MS

Ionization: EI @ 70 eV, Polarity: positive ions
Scan mode: Centroid, Multiplier: EDR
SIM width: 0.7 amu total, Filament current: 50 µA
Transferline temperature: 280°C, Source temperature: 250°C
Manifold temperature: 40°C, Q1/Q3 peak width: as calibrated)

MS parameter II:Acquisition list / SIM parameter

Table 15: Acquisition list / SIM parameter

Segment	Compound / acronym	Analyt/ IS	Acquisition time [min]	Retention time, tR [min]	Quantification (SIM) ion [m/z]	Dwell time [sec]
1	HCB	A1	5.80 – 6.40	6.20	283.7 + 285.7	0.10 / 0.10
2	PHE-D10	IS1	6.40 – 6.70	6.57	188.0	0.15
3	MX	A2	6.70 – 6.87	6.80	282.0	0.15
4	oTP	A3	6.87 – 7.50	6.97	215.0 + 230.1	0.10 / 0.10
5	DMDT CHR-D12	A4 IS2	7.50 – 10.75	9.23 9.24	227.1 240.1	0.10 0.10
6	BaP PER-D12	A5 IS3	10.75 – 12.5	11.26 11.40	252.0 264.1	0.15 0.15

10.5 Calibration, Quantification and Calculation of the analytical results

10.5.1 Solutions of the analytes and the internal standards

Stock solutions of the test compounds were prepared at nominal concentrations of 1 mg/mL in pure toluene. The stock solutions were prepared by precisely weighing and solving the substances in volumetric flasks.

The used U.S. EPA semi-volatile (SV) internal standard solution (IS-mix) contained the 'active' internal standards PHE-D10, CHR-D12 and PER-D12 in concentrations of 2000 ug/mL each.
The IS working solution with concentrations of 4.0 µg/mL was prepared by diluting the IS-mix in the ratio of 1:500 with pure toluene.

10.5.2 Preparation of the calibration standards

Calibration standards for diet analysis

Seven 'calibration standards' were prepared in toluene following the sample preparation procedure in diet analysis. This means that different volumes of the analyte stock solutions were pipetted together in 40mL

samples vials (glass extraction bottles) and were diluted with 25 mL extraction solvent, see pipetting plan below[7].

Table 16: Preparation of the calibration standards for diet analysis

No. of the calibration standard	'Nominal' volumes of each analyte stock solutions [µL]	'Nominal' dilution factor	Target analyte concentrations [µg/mL]	Concentration ratio (= conc. analyte/ conc. IS *)
1	20.0	1:1250	0.80	0.200
2	37.5	1:666.6	1.50	0.375
3	62.5	1:400	2.50	0.625
4	100	1:250	4.00	1.0
5	150	1:166.6	6.00	1.5
6	200	1:125	8.00	2.0
7	250	1:100	10.0	2.5

*) = Abscissa entry of the calibration function

The prepared calibration standards were measured as described in chapter 10.4 after diluting 10 µL of the calibration solutions with 10 µL of the IS working solution (conc. 4.0 µg/mL of each IS) and 1 mL toluene in 1.8 mL autosampler vials.

The injected analyte concentrations were therefore 0.008 to 0.10 µg/mL, the concentration of the IS was constant 0.040 µg/mL in all solutions.

10.5.3 Calibration standards for fish analysis

In addition to the stock solutions an analyte intermediate solution was prepared in toluene by diluting the stock solutions in the ratios of 'nominal' 1:25 respective 1:250 together in one volumetric flask. The target analyte concentrations were exact 40.0 µg/mL for HCB, MX, oTP and DMDT and only 4.00 µg/mL for BaP.

Again seven 'calibration standards' were prepared by diluting different aliquots of the analyte intermediate solution in volumetric flasks with toluene, see pipetting plan in Table 17.

[7] Calibration standards 3 to 7: The toluene was evaporated to approx. 100 µL with a gentle stream of nitrogen prior to dilution with extraction solvent.

Table 17: Preparation of the calibration standards for fish analysis

No. of the calibration standard	Volume of the analyte intermediate solution [µL]	Volume of the volumetric flask [mL]	Target analyte concentrations [ng/mL] *)	Concentration ratio (= conc. analyte/ conc. IS **)
1	7.50	100	3.00 / 0.30	0.01875 / 0.001875
2	12.5	50.0	10.0 / 1.00	0.0625 / 0.00625
3	18.75	25.0	30.0 / 3.00	0.1875 / 0.01875
4	50.0	20.0	100 / 10.0	0.625 / 0.0625
5	75.0	10.0	300 / 30.0	1.875 / 0.1875
6	250	10.0	1000 / 100	6.25 / 0.625
7	375	5.00	3000 / 300	18.75 / 1.875

*) HCB, MX, oTP, DMDT / BaP **) = Abscissa entry of the calibration function

Aliquots of 500 µL of the prepared calibration standards were transferred into 1.8 mL autosampler vials and were then spiked with 20 µL of the IS working solution (conc. 4.0 µg/mL of each IS) analogous to the sample preparation procedure in fish analysis.
GC-MS measurements were done as described in chapter 10.4.3, the concentration of the IS in the injection solutions were 160 ng/mL.

10.5.4 Creating of the calibration functions

The calibration solutions were measured by GC-MS according to the instructions given in analytical guidance's. The basic calibration functions were set up by plotting the calculated peak area ratio (PAR, peak area analyte / peak area IS) against the used 'analyte concentrations'. With the calibration data linear or quadratic regression analyses were carried out.

To plot the calibration function the used Varian Saturn® GC/MS Workstation software even use the concentration ratio (concentration of the analyte / concentration of the IS) as abscissa entry (see concentration ratios in Table 16 and Table 17).

10.5.5 Quantification and Calculation of the analytical results

The quantification data ($C_{GC/MS}$) were generated by processing (integration) the chromatographic raw data and by subsequent calculation of the quantification results using the basic calibration functions.

10.5.6 Calculation of the analytical results in diet analysis

The concentrations of the test compounds in diet (C_D, in units of µg/g) were calculated by equation (1):

$$C_D = \frac{C_{GC/MS} \cdot V_E}{m_{Diet}} \quad (1)$$

C_D = Concentration of the test compounds in diet [µg/g]

$C_{GC/MS}$ = Conc. of the test compounds in the diet extract measured by GC/MS [µg/mL]

V_E = Volume of the used extraction solvent [(25) mL]

m_{Diet} = Mass of the weighed diet [(1) g]

10.5.7 Calculation of the analytical results in fish analysis

The concentrations of the test compounds in fish (C_F, in units of µg/g) were calculated using equation (2):

$$C_F = \frac{C_{GC/MS} \cdot V_{E,t} \cdot V_{Sp}}{m_{Fish} \cdot V_{Cu} \cdot 1000} \quad (2)$$

C_F = Concentration of the test compounds in fish [µg/g]

$C_{GC/MS}$ = Conc. of the test compounds in the fish extract measured by GC/MS [ng/mL]

$V_{E,t}$ = Total volume of the received fish extract [(50, 100 or 200) mL]

V_{Sp} = Volume at the end of the sample preparation [(0.5) mL]

V_{Cu} = Volume of the ASE extract used in the clean-up procedure [(1.5) mL]

m_{Fish} = Mass of the weighed fish [g]

1000 = Conversion factor [ng → µg]

10.6 Validation of the analytical method

10.6.1 Preliminary remarks

The validation of the analytical method for the “Determination of the test compounds in fish by GC-MS” was performed by so-called 'fortification experiments', the fortified concentration was adjusted to fifty times the limits of quantification (LOQ).
For this purpose fish samples were spiked with the test compounds and were afterwards prepared and analyzed as described in chapter 10.3. Finally the obtained quantification results were processed statistically

and were compared to facts listed in the EU guidance documents SANCO/825/00 rev. 7 (17/03/2004)[8] and SANCO/3029/99 rev. 4 (11/07/2000)[9].

The Limits of Quantification (LOQs) were fixed to 0.1 µg/g fresh weight for HCB, MX and oTP and to 0.01 µg/g fresh weight for DMDT and BaP.
The LOQs were calculated by inserting the lowest calibration concentration ($C_{GC/MS}$ = 3.0/0.3 ng/mL) as well as the 'standard sample preparation parameters [10] in formula (2) and by subsequent multiplication by an 'expansion factor' of 4.0.

10.6.2 Fortification procedure

Each of the five fortification samples/replicates contained the test compounds HCB, MX and oTP in concentrations of 5.0 µg/g as well as DMDT and BaP on a reduced conc. level of only 0.5 µg/g fresh weight.

The fortification samples were prepared on June 22, 2010 as described in chapter 0 using four zebra fishes with total weights of approx. 2 g. The fish/hydromatrix mixtures were spiked after storing overnight with a 'fortification solution' directly in the ASE cells; the 'fortification solution was prepared in toluene and contained the analytes in conc. of 40 and 4.0 µg/mL, respectively (HCB, MX, oTP / DMDT, BaP).

After a short aging the samples were prepared and measured twofold as described in the analytical guidance.
In addition to the five 'test compound' replicates an untreated control sample (blank sample) was measured.

10.7 Results

10.7.1 Calibration functions

The analyses of fish diet and the fishes by itself were executed at different times using varied GC/MS settings and different calibration ranges, thus two different calibration experiments were executed.

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[8] SANCO/825/00 rev. 7 (17/03/2004): European Commission, Directorate General Health and Consumer Protection: Guidance document on residue analytical methods.

[9] SANCO/3029/99 rev.4 (11/07/2000): European Commission. Residues: Guidance for generating and reporting methods of analysis in support of pre-registration data requirements for Annex II (part A, Section 4) and Annex III (part A, Section 5) of Directive 91/414. Working document.

[10] The 'standard sample preparation parameters' used are $V_{E,t}$ = 50 mL, V_{Sp} = 0.5 mL, V_{Cu} = 1.5 mL and m_{Fish} = 2 g.

10.7.2 Diet analysis

The calibration range was set up from 0.8 to 10 µg/mL and was spread over seven calibration solutions (cp. Table 16).

The calibration function measured for HCB in diet analysis is shown in Figure 3. The calibration function was calculated by linear regression analysis to:

- PAR = 0.2732 * (c_{Cal}) + 0.0011 r^2 = 0.9996

PAR = Peak area ratio (peak area analyte / peak area internal standard)

c_{Cal} = Analyte concentration of the calibration solutions

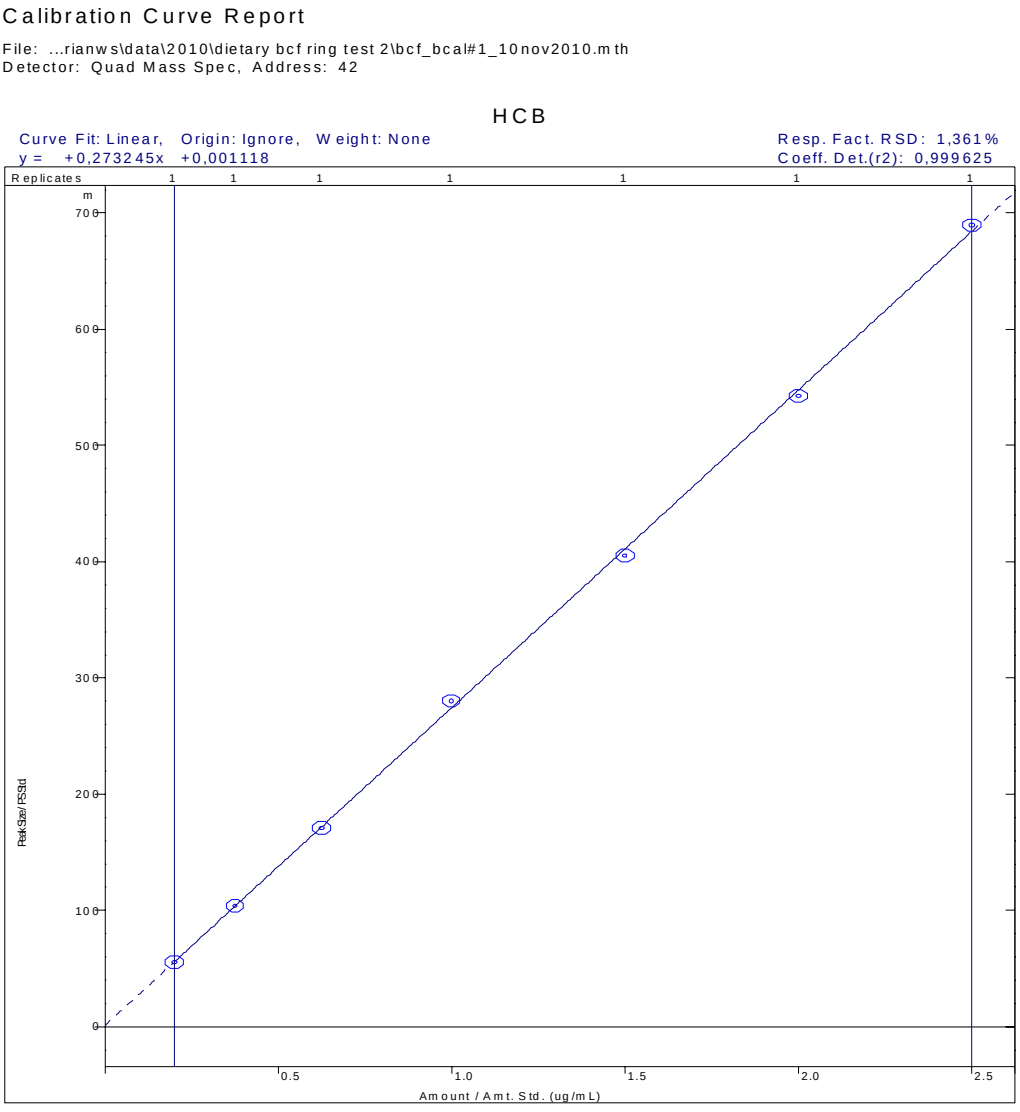


Figure 3: Basic calibration function of Hexachlorobenzene measured in diet analysis

The calibration functions of the further test compounds are shown in annex 10.11.1.

10.7.3 Fish analysis

The calibration in fish analysis was again spread over seven calibration levels and ranged from 0.30 to 300 ng/mL for BaP and 3.0 to 3000 ng/mL for the other test compounds HCB, MX, oTP and DMDT (cp. Table 17).

The calibration function for HCB measured in fish analysis is shown in Figure 4. The calibration function was calculated by quadratic regression analysis to:

- $PAR = 0.00044 * (c_{Cal})^2 + 0.1470 * (c_{Cal}) + 0.0002$ $r^2 = 1.0000$

PAR = Peak area ratio (peak area analyte / peak area internal standard)
c_{Cal} = Analyte concentration of the calibration solutions

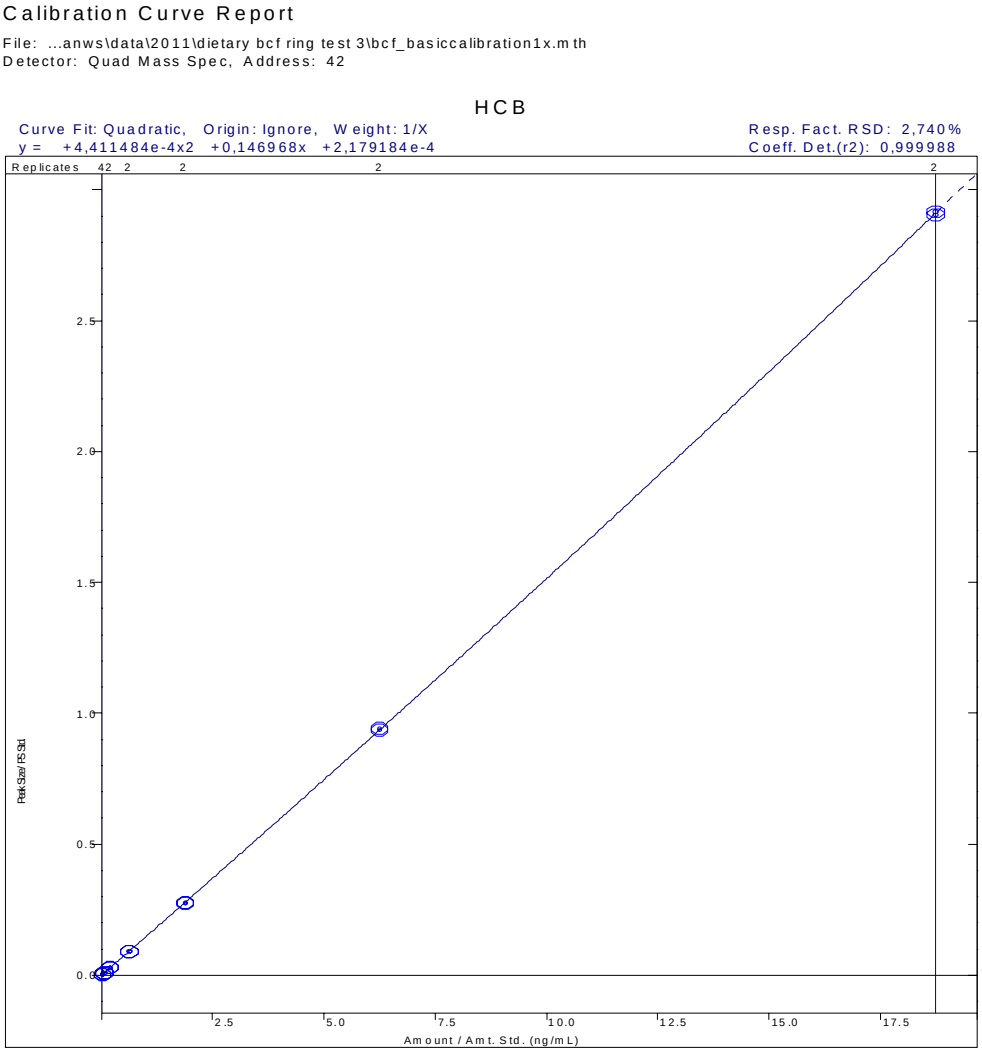


Figure 4: Basic calibration function of Hexachlorobenzene measured in fish analysis

The calibration functions of the further test compounds are shown in annex 10.11.2.

10.7.4 Method validation

The analytical method for the determination of the test compounds in fish matrix has been validated in accordance with the EU guidance documents SANCO/3029/99 and SANCO/825/00 on one fortification level.

The validation results were calculated using the quantification ($C_{GC/MS}$) data of the prepared fortification samples. The concentrations of the test compounds in spiked fish (C_F) were calculated using equation (2), the analytical results and are summarized in Table 18.
The nominal concentrations of the test compounds in fish were 5.0 µg/g for HCB, MX and oTP and 0.5 µg/g fresh weight for DMDT and BaP.

Table 18: Summarized quantification ($C_{GC/MS}$) data and analytical results (C_F) of the validation experiment

Test compound	Replicate 1		Replicate 2		Replicate 3		Replicate 4		Replicate 5	
	CGC/MS *) [ng/mL]	CF [µg/g]	CGC/MS *) [ng/mL]	CF [µg/g]	CGC/MS *) [ng/mL]	CF [µg/g]	CGC/MS *) [ng/mL]	CF [µg/g]	CGC/MS *) [ng/mL]	CF [µg/g]
HCB	524	4.36	538	4.48	533	4.44	537	4	536	4.47
MX	610	5.08	666	5.55	633	5.28	646	5.38	640	5.33
oTP	614	5.11	610	5.09	611	5.09	598	4.98	606	5.05
DMDT	67.2	0.56	71.3	0.59	58.5	0.49	60.1	0.50	60.1	0.50
BaP	59.0	0.49	58.9	0.49	57.6	0.48	56.8	0.47	57.6	0.48

*) Mean of two injections

10.7.5 Accuracy (Recovery)

The accuracy of the validated method is reported as the mean recovery ± relative standard deviation (RSD).

The percent recovery rates of the analytes (%R) were calculated as follows:

$$\%R = \frac{C_F \cdot 100\%}{F} \quad (3)$$

%R = Recovery rate of the analyte [%]

C_F = Concentrations of the test compounds in (spiked) fish [µg/g]

F = Fortification level of the analytes [µg/g]

The recovery rates obtained during method validation as well as their means, standard deviations (s) and relative standard deviations (RSD) are summarized in Table 19.

Table 19: Recovery rates, means, standard deviations (s) and RSD values obtained during method validation

Test comp.	Recovery [%] Replicates 1 - 5					Mean recovery	s	RSD
	1	2	3	4	5	[%]	[%]	[%]
HCB	87.3	89.6	88.9	89.6	89.3	88.9	0.98	1.1
MX	101.6	111.1	105.5	107.6	106.7	106.5	3.43	3.2
oTP	102.3	101.7	101.9	99.7	100.9	101.3	1.04	1.0
DMDT	111.9	118.8	97.6	100.1	100.1	105.7	9.20	8.7
BaP	98.3	98.1	95.9	94.7	96.0	96.6	1.57	1.6

The mean recoveries are close to 100 % and prove the accuracy of the analytical method.

10.7.6 Repeatability (Precision)

The precision of the validated method is reported in Table 19 as the relative standard deviation (RSD) of the repeatability for each test compound.
The mean RSDs from 1.0 % to 8.7 % prove the repeatability of the analytical method for the determination of the test compounds in fish.

10.7.7 Specificity and Blanks

The GC-EI/MS system used is capable to determine the test compound in the worked-up injections solution without interference of matrix compounds. The specificity of the method is shown by GC-MS a chromatogram of an ‘untreated’ control sample of the investigated fish matrix, see chromatogram in Figure 10; in the prepared blank sample no analyte could be measured.

10.7.8 Linearity

The working range of the basic calibration in diet analysis covered a narrow concentration range from 0.80 to 10 ng/mL (cp. Table 16). The coefficients (r^2) calculated by linear regression of all calibrations were very close to 1; therefore the linearity of the calibration functions was accepted.

The calibrations in fish analysis included concentrations of 3 orders of magnitude. Due to this wide spread calibration quadratic functions were obtained for all test compounds. Therefore the quantifications were done using quadratic regression functions.

Matrix effects

The obtained recovery rates close to 100 % for the investigated matrix associated with the adequate precision, suggest that no matrix effect was present.

Applicability of the method

The statement concerning the applicability of the validated method is based on the obtained validation data and the compliance with guidelines, see below given line-up:

Validation parameter: Criteria according to SANCO regulations compliance

Recovery: Mean recovery 70 – 110 % (ideally 80 – 100 %) yes

Repeatability: 5 Determinations per fortification level, RSD < 20 % yes

Blanks < 30 % of the LOQ yes

Linearity: Narrow calibration range (diet analysis) yes
Enlarged calibration (fish analysis) no

Specificity: No interference of the analytes to matrix compounds (matrix effect) yes

The validated method is applicable to determine the test compounds in fish with no specified limitations.

10.8 Results of the analyzed samples

10.8.1 Fish diet

The spiked fish diet was analyzed in two replicates on November 10, 2010. The diet sample was stored at a temperature of -25 °C in a freezer until analysis. After thawing the sample vessel was homogenized on a roller mixer for 30 minutes, two subsamples were analyzed afterwards as described in chapter 10.3.1.
The analytical results were calculated analogous to equation (1), and are summarized in

Table 20 together with derived statistical values.

Table 20: Analyzed concentrations (C_D) of the test compounds in diet [µg/g] and derived statistical data

Result parameters		Test compounds				
		HCB	MX	oTP	DMDT	BaP
		Nominal conc. [µg/g]				
		25	50	50	100	150
CGC/MS [µg/mL]	replicate 1 *)	1.23	2.35	2.22	5.26	6.66
	replicate 2 **)	1.31	2,45	2.32	5.70	6.94
CD [µg/g]	replicate 1	30.5	58.2	55.0	130.2	164.8
	replicate 2	29.6	55.7	52.8	129.4	157.6
CD [µg/g], mean		30.1	57.0	53.9	130	161
Mean Recovery [%]		120.3	113.9	107.8	129.8	107.5
Standard deviation, s [%]		2.4	3.6	3.2	0.56	3.4
RSD [%]		2.0	3.2	3.0	0.43	3.2
Overall mean [%]		115.9				
Overall RSD [%]		7.9				

*) 1.010 g, **) 1.101 g

10.8.2 Fish samples

The fish samples were stored as well in a freezer until analysis, after thawing they were prepared and analyzed as described in chapter 10.4.2. The analytical results were calculated analogous equation (2); the results and further sample data (changed during sample preparation) are summarized in

Table 21.

Additionally to the listed fish samples 15 controls and 3 procedural blanks were processed; the results for all test compounds in all controls were “< LOQ”.
The LOQs in fish analysis were fixed to 0.1 µg/g fresh weight for HCB, MX, oTP and DMDT and to 0.01 µg/g fresh weight for BaP.

The following parameters were fixed for all samples during sample preparation:

- Volume at the end of the sample preparation, VSp = 0.5 mL
- Volume of the ASE extract used for clean-up, VCu = 1.5 mL.

Table 21: Analyzed concentrations (C_F) of the test compounds in fish [µg/g] and further sample data

Test stage	Sampling day (2011)	Sample (fish) weight	VE,t *)	Concentrations (CF) of the test compounds in fish				
				HCB	MX	oTP	DMDT	BaP
		[g]	[mL]	[µg/g]	[µg/g]	[µg/g]	[µg/g]	[µg/g]
Uptake	5 (Jan. 29)	2.00 **)	50	1.50	3.72	2.53	3.50	1.26
		2.88 **)	50	2.12	4.35	2.79	2.64	2.00
		2.72 **)	50	1.43	3.32	2.30	1.88	1.07
		2.01 **)	50	1.11	2.71	1.35	0.98	0.72
		2.34 **)	50	1.44	3.40	2.45	1.84	1.31
Depuration	1 (Feb. 08)	4.30	100	6.07	11.51	8.79	9.98	1.39
		3.47	100	4.67	9.23	6.00	6.69	2.66
		3.69	100	5.23	10.29	7.05	8.29	1.77
		4.25	100	4.93	9.35	6.45	3.90	1.26
		3.50	100	3.48	6.79	3.20	2.93	2.07
Depuration	3 (Feb. 10)	3.40	100	3.30	5.82	3.43	1.48	< LOQ
		4.42	100	4.40	7.73	4.25	7.08	< LOQ
		3.96	100	4.22	7.74	2.87	1.06	< LOQ
		4.52	100	5.21	9.83	4.52	7.19	< LOQ
		4.33	100	4.00	6.82	4.54	1.59	< LOQ
Depuration	7 (Feb. 14)	4.66	100	2.58	4.15	2.62	0.55	< LOQ
		4.15	100	2.46	4.05	2.18	0.43	< LOQ
		5.52	100	3.37	5.16	3.04	4.45	< LOQ
		4.29	100	2.16	2.71	0.81	0.29	< LOQ
		3.92	100	3.00	4.03	1.76	1.31	< LOQ
Depuration	14 (Feb. 21)	8.31	100	2.30	2.78	1.41	0.24	< LOQ
		4.63	100	1.20	1.43	0.77	< LOQ	< LOQ
		6.72	100	2.47	3.10	0.37	0.18	< LOQ
		6.49	100	2.84	3.58	1.07	0.27	< LOQ
		6.49	100	2.32	2.91	0.90	1.33	< LOQ
Depuration	21 (Feb. 28)	7.84	100	1.90	1.93	1.00	0.81	< LOQ
		11.68 ***)	200	1.42	1.26	1.01	0.50	< LOQ
		6.16	100	1.42	1.45	0.28	< LOQ	< LOQ
		10.98 ***)	200	1.39	1.34	1.14	0.26	< LOQ
		7.45	100	1.42	1.51	0.50	0.69	< LOQ
Depuration	28 (March 07)	16.08 ***)	200	1.18	1.06	0.25	0.32	< LOQ

Test stage	Sampling day (2011)	Sample (fish) weight	VE,t *)	Concentrations (CF) of the test compounds in fish				
				HCB	MX	oTP	DMDT	BaP
		[g]	[mL]	[µg/g]	[µg/g]	[µg/g]	[µg/g]	[µg/g]
		9.57 ***)	200	1.50	1.67	0.20	0.34	< LOQ
		14.88 ***)	200	0.82	0.62	< LOQ	0.14	< LOQ
		11.83 ***)	200	0.81	0.73	0.63	0.29	< LOQ
		10.97 ***)	200	1.21	0.99	< LOQ	< LOQ	< LOQ

*) $V_{E,t}$ = Total volume of the fish extract

**) 22mL ASE cells were used (in all other cases (fish samples) 33mL ASE cells were used)

***) ASE extraction was performed in two steps (in two 33mL ASE cells)

10.9 Representative chromatograms

10.9.1 Diet analysis

GC-MS chromatograms of standards and a fish diet extract are shown in Figure 5 to Figure 7.

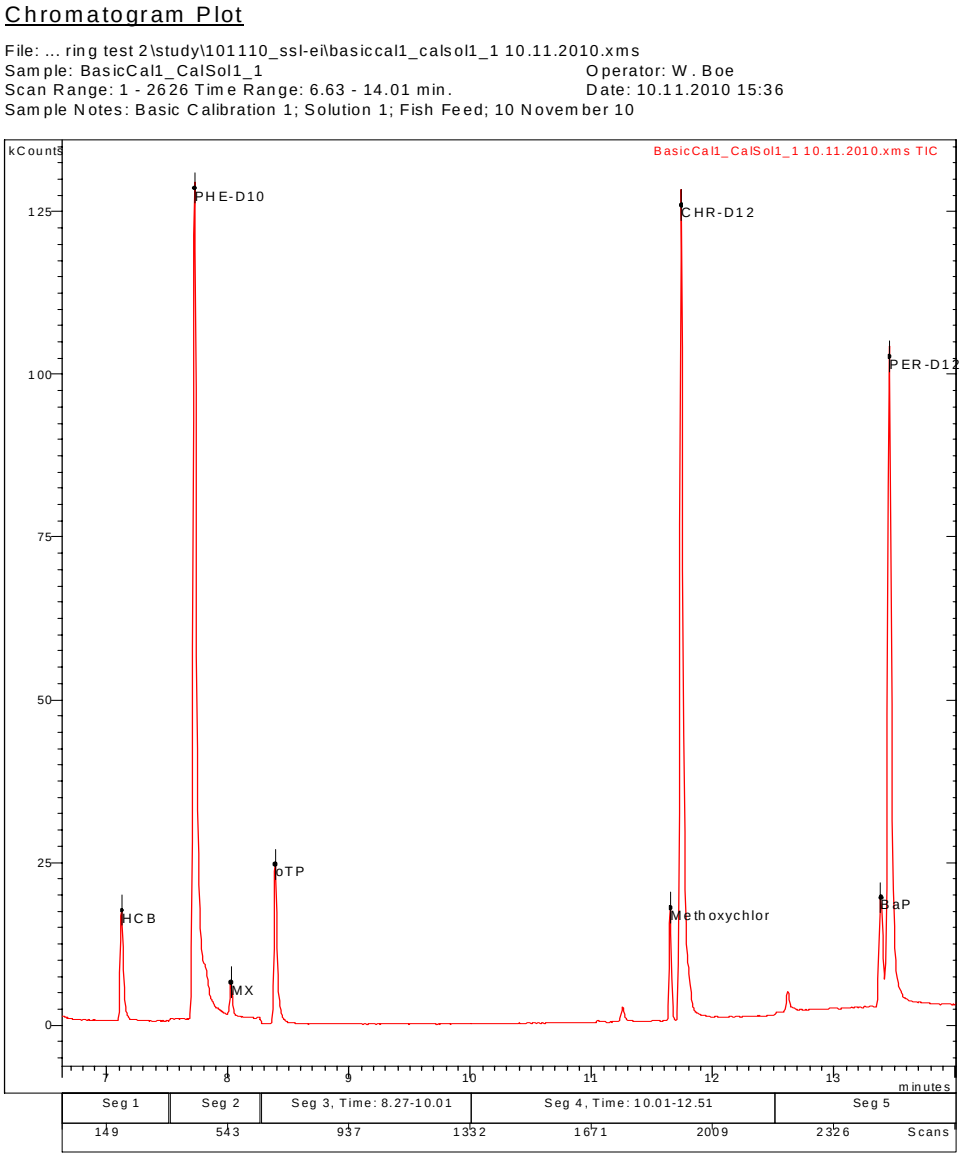


Figure 5: Chromatogram of calibration solution level 1, analyte conc. injected: 8.0 ng/mL

Chromatogram Plot

File: ... ring test 2\study\101110_ssl-ei\basiccal1_calsol7_1 10.11.2010.xms
Sample: BasicCal1_CalSol7_1 Operator: W. Boe
Scan Range: 1 - 2620 Time Range: 6.63 - 14.01 min. Date: 10.11.2010 18:20
Sample Notes: Basic Calibration 1; Solution 7; Fish Feed; 10 November 10

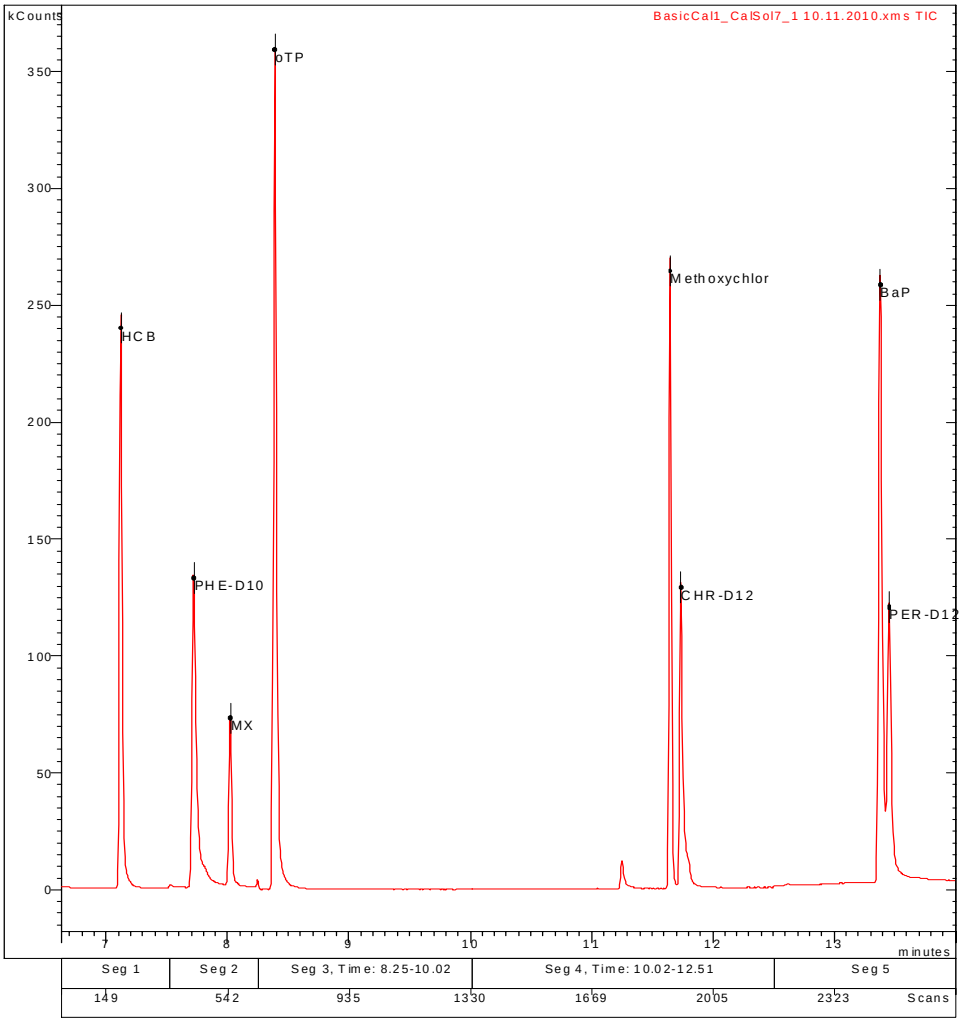


Figure 6: Chromatogram of calibration solution level 7, analyte conc. injected: 100 ng/mL

Chromatogram Plot

File: ...ry bcf ring test 2\study\101110_ssl-ei\ff_extract1_1 10.11.2010.xms
Sample: FF_Extract1_1 Operator: W. Boe
Scan Range: 1 - 2620 Time Range: 6.63 - 14.00 min. Date: 10.11.2010 14:26
Sample Notes: Fish Feed; Test; Extraction Solution 1

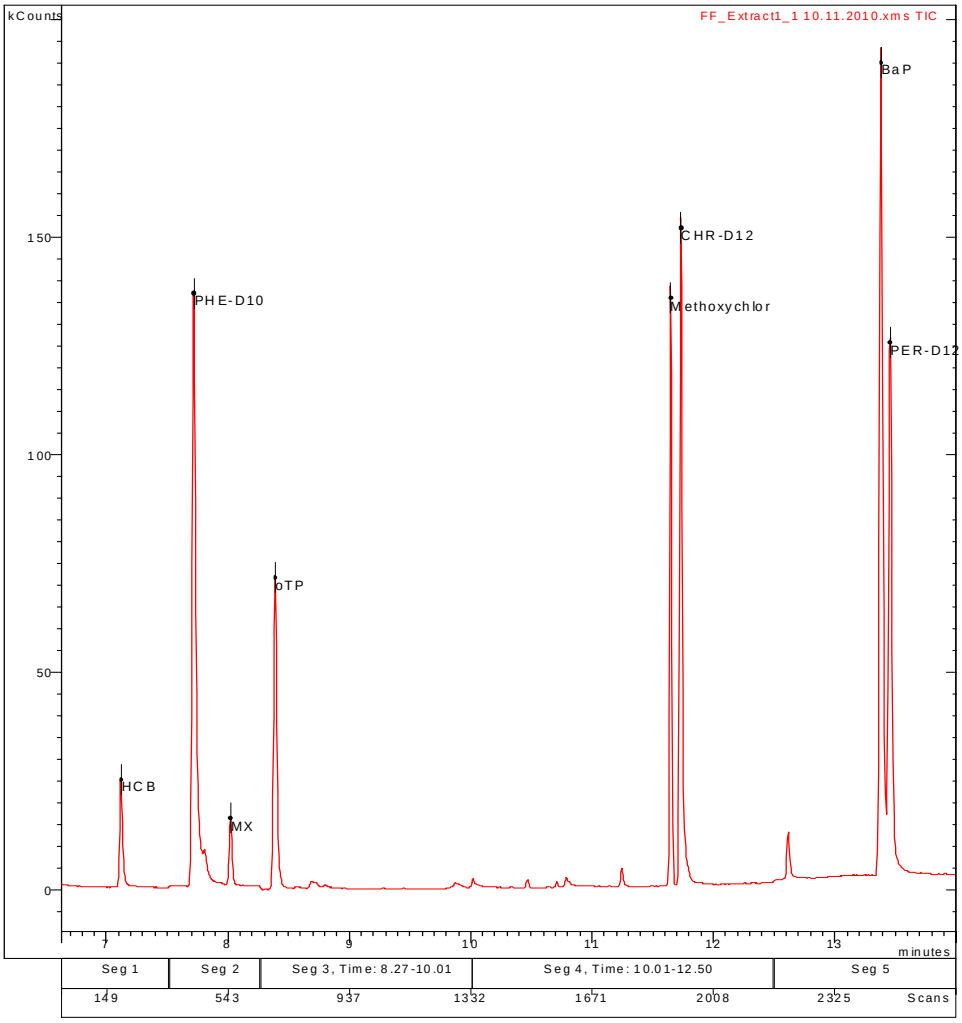


Figure 7: **Chromatogram of the diet extract 'replicate 1'**

10.9.2 Fish analysis

GC-MS chromatograms of standards and processed fish samples are shown in Figure 8 to Figure 13.

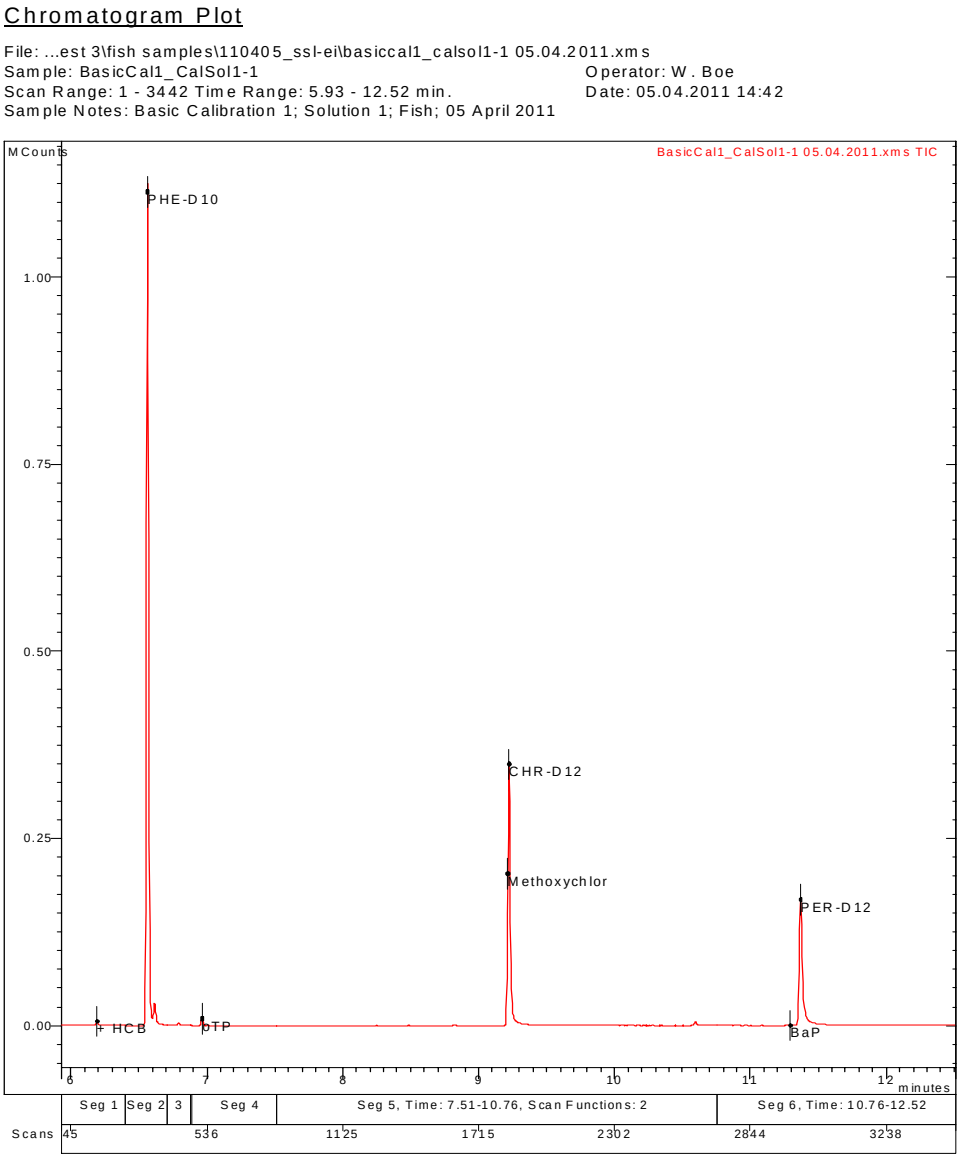


Figure 8: Chromatogram of calibration solution level (fish) 1,
analyte conc. injected: 0.3 ng/mL (BaP) respective 3.0 ng/mL (HCB, MX, oTP and Methoxychlor)

Chromatogram Plot

File: ...est 3\fish samples\110405_ssl-ei\basiccal1_calsol7-2 05.04.2011.xms
Sample: BasicCal1_CalSol7-2 Operator: W. Boe
Scan Range: 1 - 3424 Time Range: 5.93 - 12.50 min. Date: 05.04.2011 19:41
Sample Notes: Basic Calibration 1; Solution 7; Fish; 05 April 2011

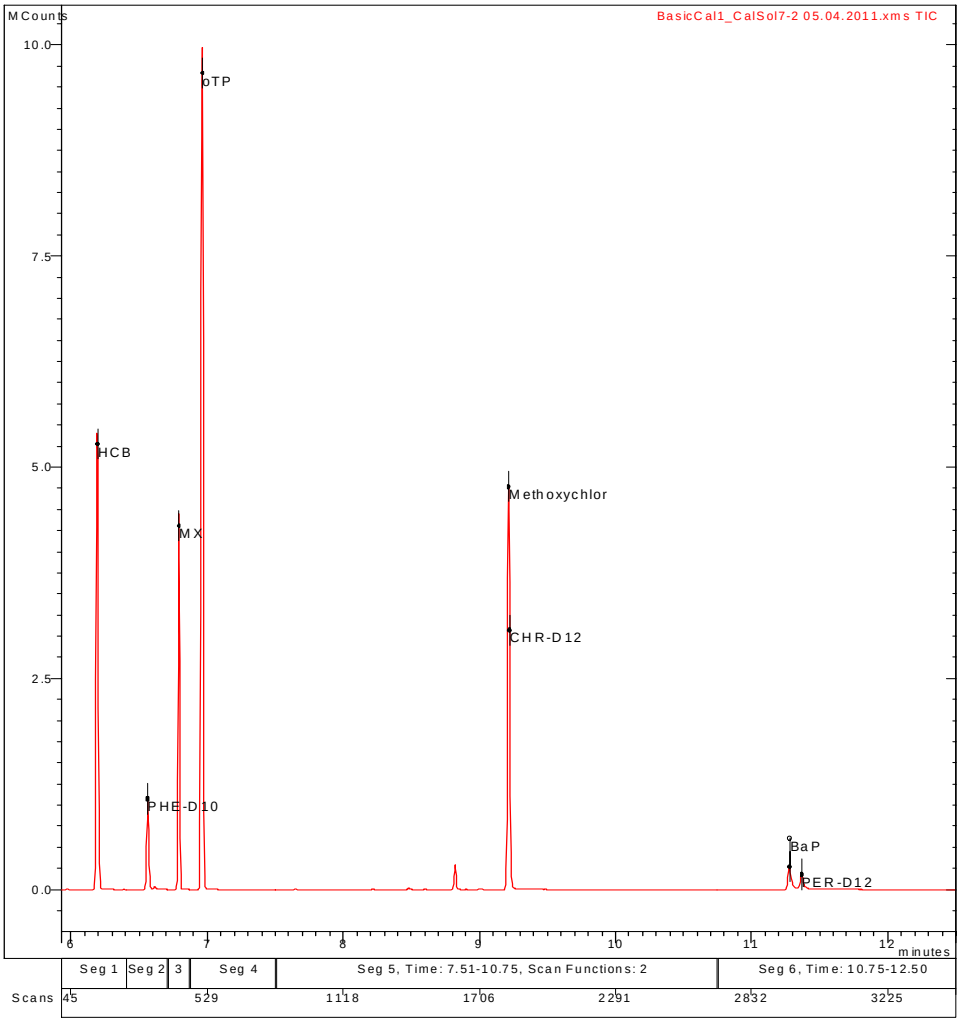


Figure 9: Chromatogram of calibration solution level (fish) 7,
analyte conc. injected: 300 ng/mL (BaP) respective 3 000 ng/mL (HCB, MX, oTP and Methoxychlor)

Chromatogram Plot

File: ...g test 3\fish samples\110406_ssl-ei\s24_fish_contr-1 06.04.2011.xms
Sample: S24_Fish_Contr-1 Operator: W. Boe
Scan Range: 1 - 3441 Time Range: 5.93 - 12.51 min. Date: 06.04.2011 12:56
Sample Notes: Sample 24; Fish; Control; Sampling Date: Jan. 29, 2011; Extracti

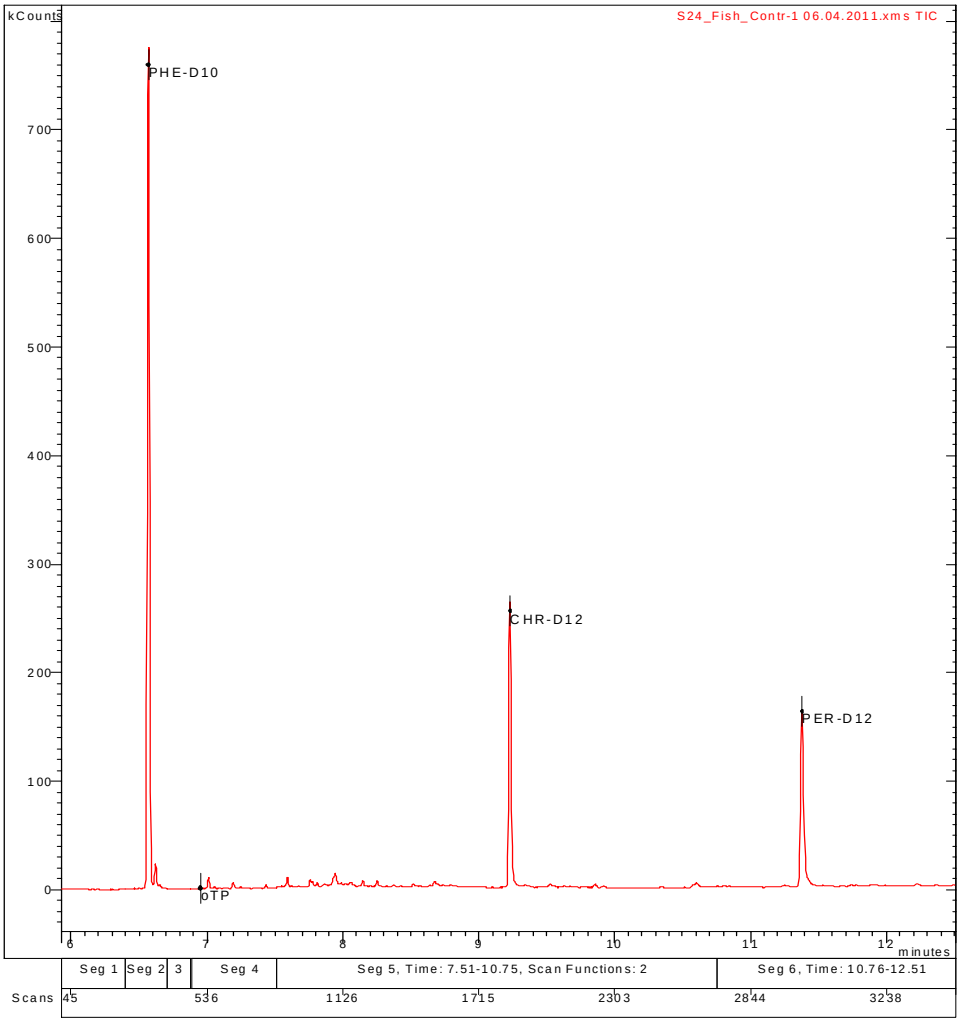


Figure 10: **Chromatogram of a processed fish extract (control, uptake day 5)**

Chromatogram Plot

File: ...est 3\fish samples\110406_ssl-ei\s30_fish_ringtest-1 06.04.2011.xms
Sample: S30_Fish_Ringtest-1 Operator: W. Boe
Scan Range: 1 - 3425 Time Range: 5.93 - 12.50 min. Date: 06.04.2011 18:17
Sample Notes: Sample 30; Fish; Ringtest; Sampling Date: Jan. 29, 2011; Extract

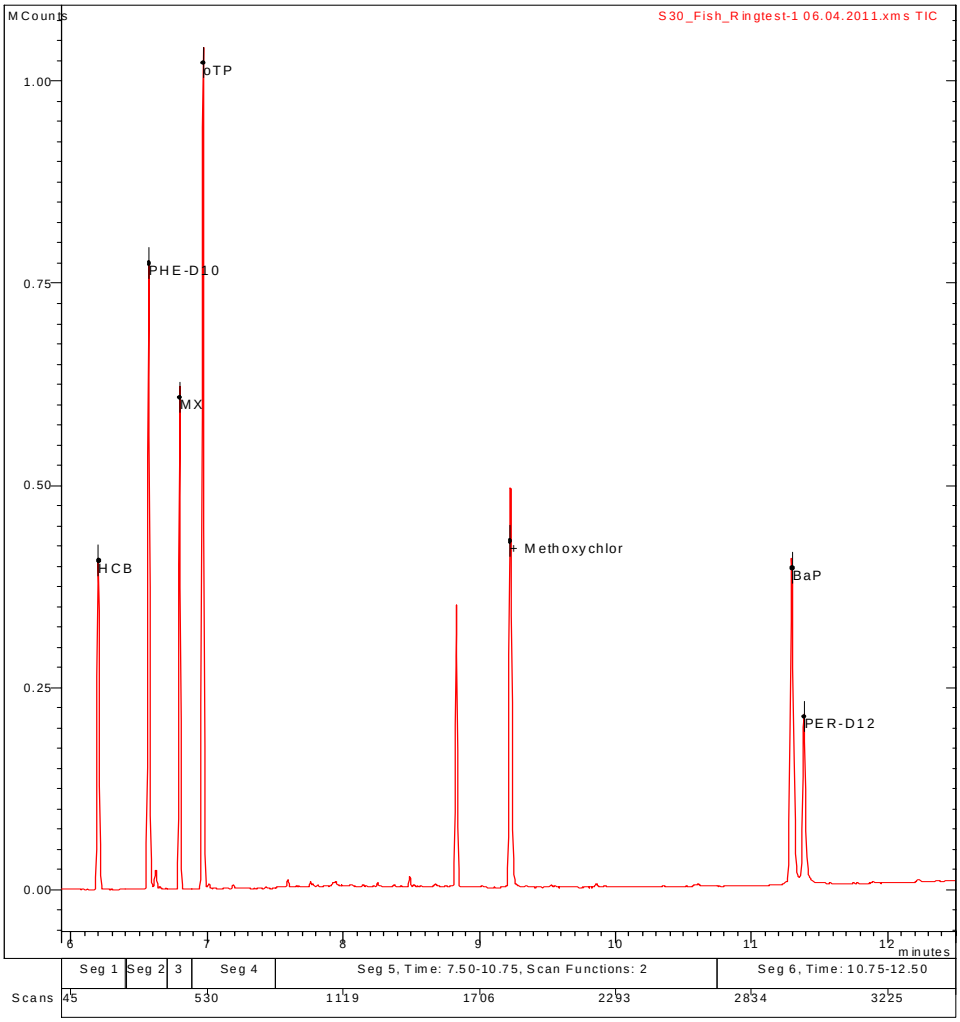


Figure 11: **Chromatogram of a processed fish extract (uptake day 5)**

Chromatogram Plot

File: ...est 3\fish samples\110406_ssl-ei\s49_fish_ringtest-1 06.04.2011.xms
Sample: S49_Fish_Ringtest-1 Operator: W. Boe
Scan Range: 1 - 3427 Time Range: 5.93 - 12.50 min. Date: 06.04.2011 19:49
Sample Notes: Sample 49; Fish; Ringtest; Sampling Date: Feb. 08, 2011; Extract

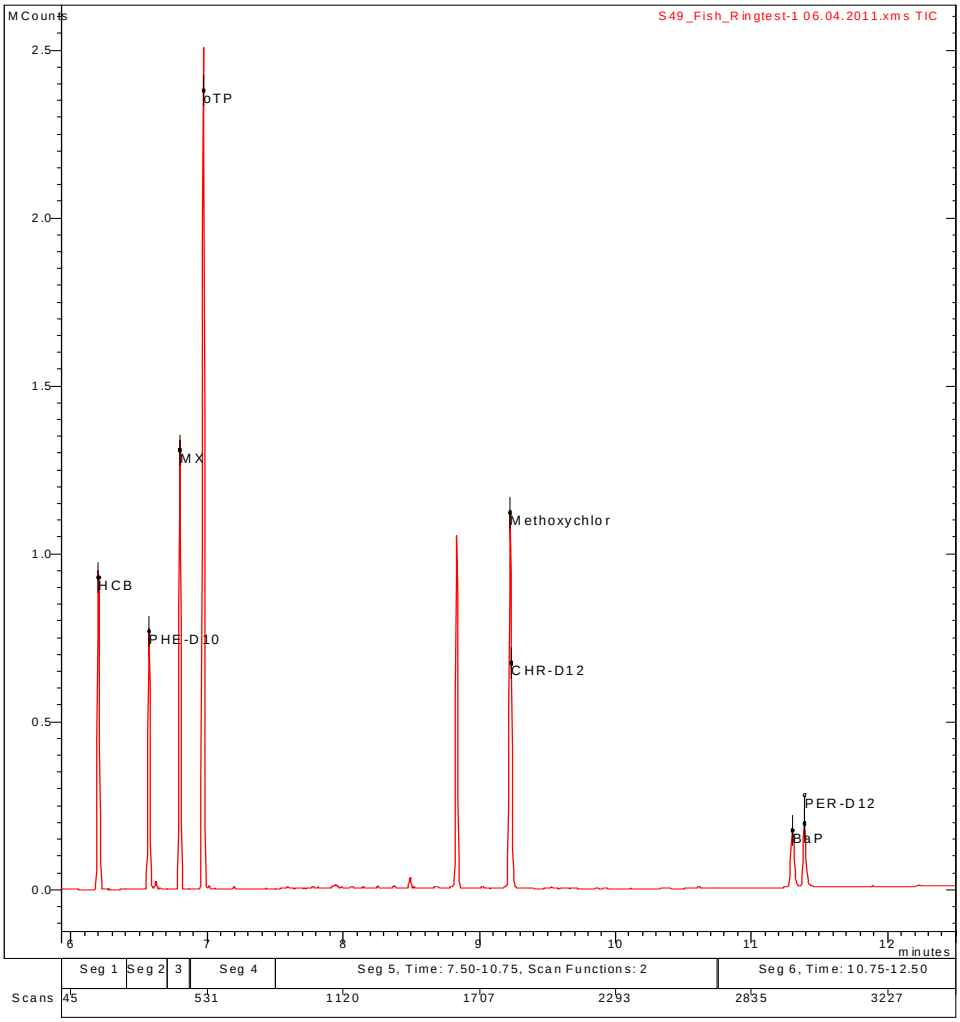


Figure 12: **Chromatogram of a processed fish extract (depuration day 1)**

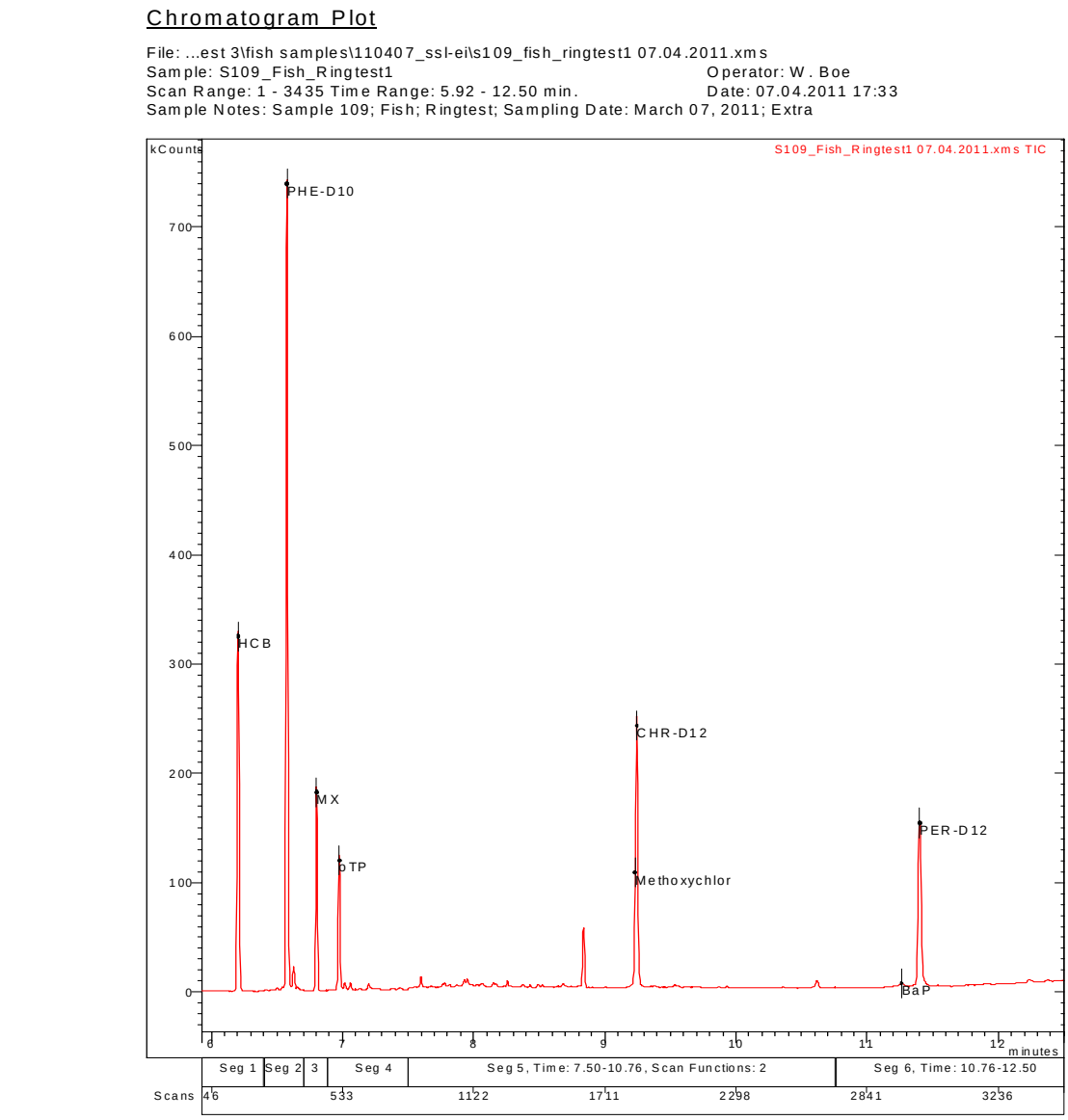


Figure 13: **Chromatogram of a processed fish extract (depuration day 28)**

10.10 Certificates of Analysis

10.10.1 Certificate of Analysis of the analytical standard Hexachlorobenzene, page 1 of 1

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Hexachlorobenzene, 99%
Product Number	171050
Product Brand	ALDRICH
CAS Number	118-74-1
Molecular Formula	C_6Cl_6
Molecular Weight	284.78

TEST	**LOT 81222006 RESULTS**
QC Acceptance date	20-JAN-1999
APPEARANCE - COLOUR	WHITE
APPEARANCE - STATE	POWDER
FT-IR SPECTROSCOPY - FTIR SPECTRUM	CONFORMS TO REFERENCE
FT-IR SPECTROSCOPY - REFERENCE	1(1), 1016B
MELTING POINT	227.9-228.5 DEG C
GAS CHROMATOGRAPHY - AVERAGE PURITY	99.76%

10.10.2 Certificate of Analysis of the analytical standard Musk xylene, page 1 of 2

Certificate of Analysis

Dr. Ehrenstorfer

Reference Materials for Residue Analysis

Product Identification

15360000 Musk xylene

CA 1-tert.-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene

IUPAC 1-tert.-Butyl-3,5-dimethyl-2,4,6-trinitrobenzene

Formula C12H15N3O6

Mol.Weight 297.27

CAS No. 81-15-2

Expiry Date 03.07.2011

Lot Number 70615

Store at 20 °C ±4 °C

Please note: The expiry date is valid under recommended storage conditions only.

Toxicological Data

R Code 02-40-50/53

S Code 02-36/37/39-60-61

LD50 (Rats female/male in mg/kg) >10000

Physical Data

Phase	crystalline solid	Vapour pressure N/A at °C
Color	colourless	Solubility in water N/A g/l at °C
Melt.Range	105,1 °C	Boiling Range (lit.)

Analytical Data

Detection: GC/MSD
Column: DB-5, 60 m, ID 0.25 mm
Inj.-Vol.: 1,00 µl
Flow: 1,0 ml/min
Ret.-Time: **16,23 min.**

Method Details:
Injector: 280° C
Start Temperature: 60° C for 5 min
End Temperature: 280° C for 1 min
Gradient: 15° C/min

Detection: HPLC/DAD
Column: ReproSil-Pur ODS3 5µ 250x3
Inj.-Vol.: 10,00 µl
Flow: 1,0 ml/min
Ret.-Time: **3,86 min.**

Method Details:
Acetonitrile:H2O 4:1

Identity: UV, MS
Comment

Water Content 0,0 % Determined by Karl-Fischer Titration
Det. Purity 99,5 % Tolerance/Uncertainty +/- 0,5 %

The uncertainty/tolerance of this standard is calculated in accordance with the EURACHEM/CITAC Guide - Quantifying Uncertainty in Analytical Measurement - Second Edition. The uncertainty given is the expanded combined uncertainty and represents an estimated standard deviation equal to the positive square root of the total variance of the uncertainty of components. The expanded uncertainty is U which is Uc(y)*K, where K is the coverage factor at the 95% confidence level (K=2). The expanded uncertainty is based on the combination of uncertainties associated with each individual operation involved in the preparation of this product.

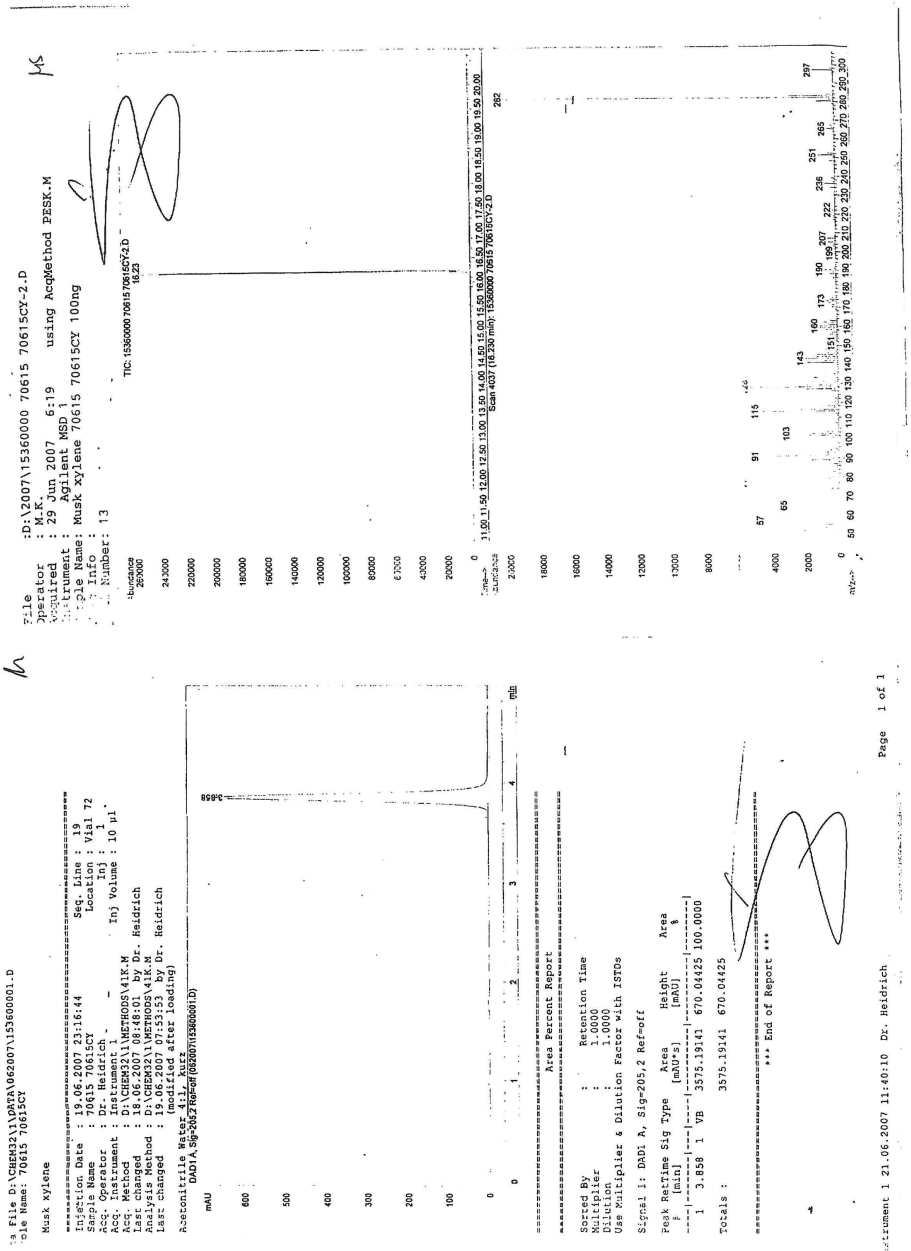
Certified on 03.07.2007
by Dr. J. Heidrich

Authorized copy from ... 29 ... 2009

Labor Dr. Ehrenstorfer-Schäfers · Bgm.-Schlosser-Str. 6 A · 86199 Augsburg · Germany
Phone +49 821 906080 Fax +49 821 9060888 info@analytical-standards.com

ISO 9001

Certificate of Analysis of the analytical standard Musk xylene, page 2/2



10.10.3 Certificate of Analysis of the analytical standard o-Terphenyl, page 1 of 1

Certificate of Analysis

SIGMA-ALDRICH

Product Name	o-Terphenyl, 99%
Product Number	T2800
Product Brand	ALDRICH
CAS Number	84-15-1
Molecular Formula	$C_6H_5C_6H_4C_6H_5$
Molecular Weight	230.30

TEST	SPECIFICATION	LOT 04403JH-399 RESULTS
APPEARANCE	WHITE CRYSTALLINE POWDER AND/OR CHUNKS	WHITE POWDER AND CHUNKS
INFRARED SPECTRUM		CONFORMS TO STRUCTURE.
GAS LIQUID CHROMATOGRAPHY	98.5% (MINIMUM)	99.9%
QUALITY CONTROL ACCEPTANCE DATE		JULY 2007

Barbara Rajzer, Supervisor
Quality Control
Milwaukee, Wisconsin USA

10.10.4 Certificate of Analysis of the analytical standard Methoxychlor, page 1 of 1

Certificate of Analysis

DESCRIPTION: Methoxychlor
SYNONYM: MARLATE
MFG. DATE: Sep 2009
CATALOG NO.: 49054 (1)
LOT NO.: LB69763 EXP. DATE: Sep 2012

CAS NUMBER: 72-43-5 MOLECULAR FORMULA: C16H15Cl3O2
MOLECULAR WEIGHT: 346

PHYSICAL PROPERTIES	ASSAY	
FTIR	Matches: SUPELCO	Lib. No.: USER1 191
GC - Mass Spec	Matches: NIST	Lib. No.: 73611
Purity (2)	99.9% (a)	

Note: Supelco guarantees the purity of this chemical standard +/- 0.5% deviation prior to the expiration date shown on the label. This guarantee is exclusive of any contamination caused by the customer.

(1) This product is packaged from R427190 Lot number LB66558.
(2) Determined by GC-FID unless otherwise noted.
(a) HPLC UV-254nm

Elwood Doughty
QA Manager

Supelco warrants that its products conform to the information contained in this publication. Purchaser must determine the suitability of the product for its particular use. Please see the latest catalog or order invoice and packing slip for additional terms and conditions of sale.

SUPELCO
Analytical
595 North Harrison Road
Bellefonte, PA 16823-0048 USA
Phone (814) 359-3441

10.10.5 Certificate of Analysis of the analytical standard Benzo[a]pyrene, page 1 of 1

Certificate of Analysis

SIGMA-ALDRICH

Product Name	Benzo[a]pyrene, ≥96% (HPLC)
Product Number	B1760
Product Brand	SIGMA
CAS Number	50-32-8
Molecular Formula	$C_{20}H_{12}$
Molecular Weight	252.31

TEST	**SPECIFICATION**	**LOT 129K1892 RESULTS**
Appearance (Color)	Yellow to Yellow-Green to Brow	yellow
	Light Yellow to Yellow-Green to Yellow-brown	
Appearance (Form)	Powder	Powder
Solubility (Color)	Light Yellow to Yellow-Green	Yellow-Green
	Light Yellow to Yellow-Green, Yellow-Brown,or Yellow-Orange	
Solubility (Turbidity)	Clear	Clear
	50 mg/mL, CHCl3	
Purity (HPLC)	≥96 %	98 %
Recommended Retest Period	----------	----------
	4 years	
Specification Date:		JAN 2010
Date of QC Release:		JAN 2010
Recommended Retest Date:		DEC 2013
Print Date:		JAN 22 2010

Rodney Burbach, Manager
Quality Control
St. Louis, Missouri USA

10.10.6 Certificate of Analysis of the semi-volatile internal standard mixture (IS-mix), page 1 of 1

ULTRA SCIENTIFIC
Analytical Solutions

Certificate of Analysis

Semi-Volatiles Internal Standard Mixture

Product	ISM-560			**Page:**	1 of 1
Lot Number:	CF-1611	**Lot Issue**	May-2009	**Expiration Date:**	May-2012

This Certified Reference Material (CRM) was manufactured and verified in accordance with ULTRA's ISO 9001 registered quality system, and the analyte concentrations were verified by our ISO 17025 accredited laboratory. The true value and uncertainty value at the 95% confidence level for each analyte, determined gravimetrically, is listed below.

Analyte	CAS#	Analyte Lot	True Value
acenaphthene-d10	015067-26-2	PR-17277	2004 ± 10 µg/mL
chrysene-d12	001719-03-5	PR-19587	2004 ± 10 µg/mL
1,4-dichlorobenzene-d4	003855-82-1	PR-18488	2004 ± 10 µg/mL
naphthalene-d8	001146-65-2	H336BP17	2004 ± 10 µg/mL
perylene-d12	001520-96-3	PR-16756	2004 ± 10 µg/mL
phenanthrene-d10	001517-22-2	PR-17315	2004 ± 10 µg/mL

Matrix: methylene chloride (dichloromethane)

ULTRA uses balances calibrated with weights traceable to NIST in compliance with ANSI/NCSL Z-540-1 and ISO 9001, and calibrated Class A glassware in the manufacturing of these standards.

A2LA ACCREDITED
ISO 17025:2005 Accredited A2LA Cert. No. 0851.01

ISO 9001:2000 Registered TUV USA, Inc. Cert. No. 06-1004

250 Smith Street, North Kingstown, RI 02852 USA
401-294-9400 Fax: 401-295-2330
www.ultrasci.com

See Reverse For Additional Information

William J. Leary
Quality Assurance Manager

10.11 Annex 2.1 Calibration functions

10.11.1 Diet analysis

All calibration functions in diet analysis were calculated by linear regression analysis; the individual functions are shown in the header information of the corresponding 'Calibration Curve Reports' in Figure 14 to Figure 17.

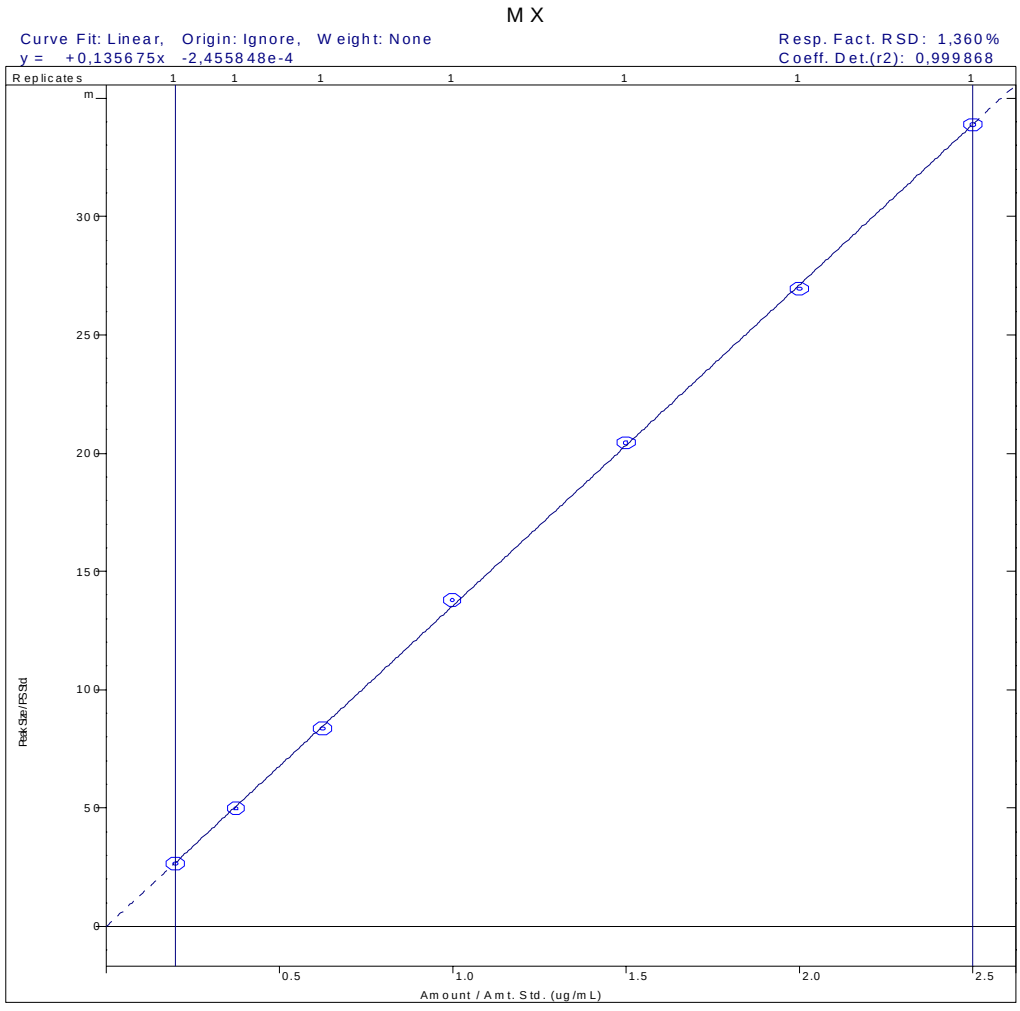


Figure 14: Basic calibration function of Musk xylene measured in diet analysis

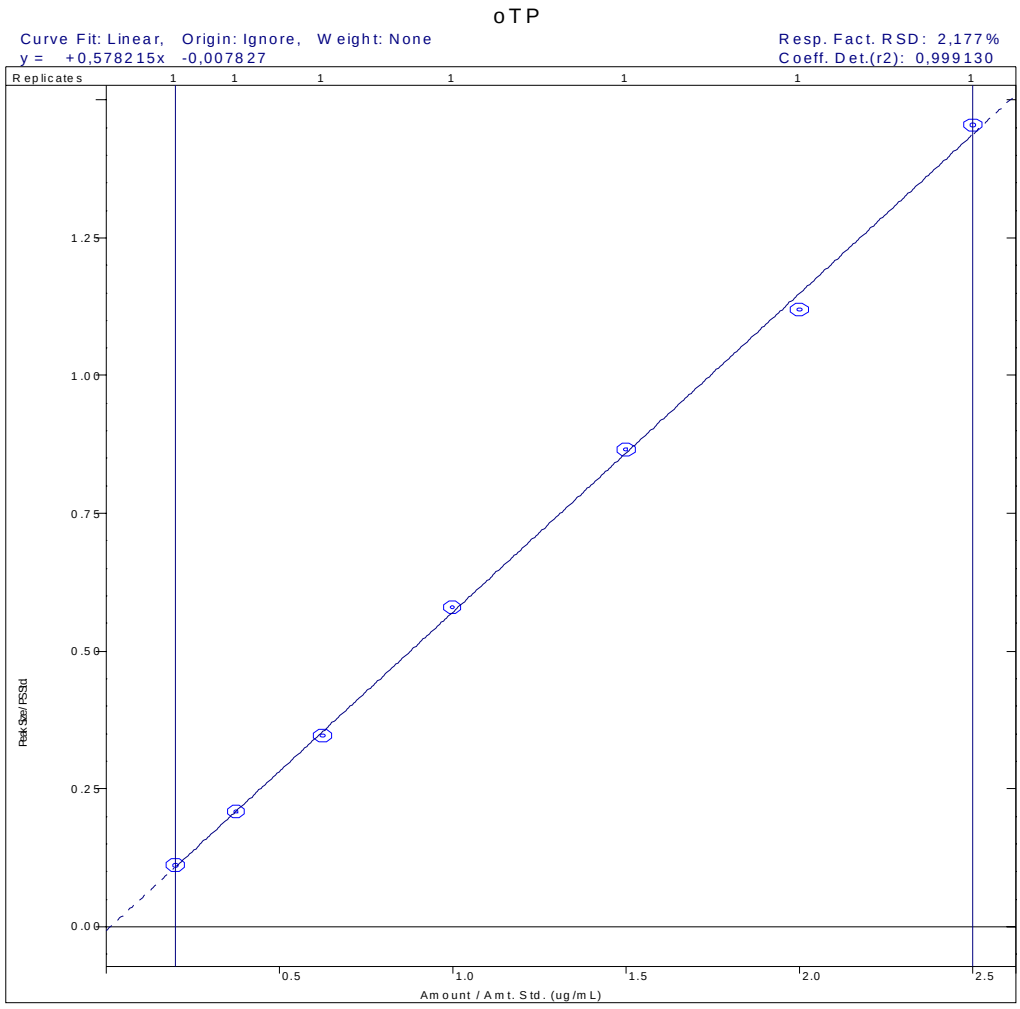


Figure 15: Basic calibration function of o-Terphenyl measured in diet analysis

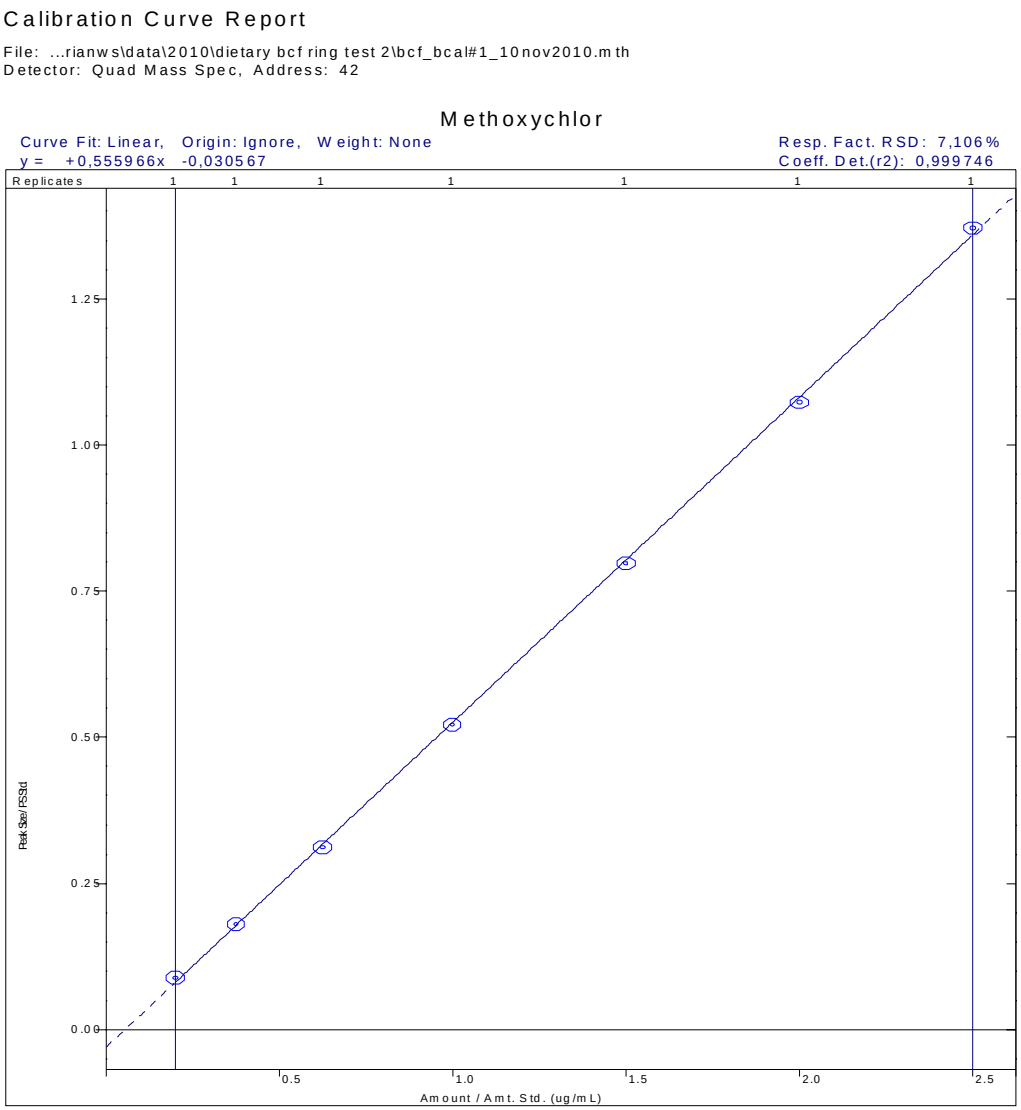


Figure 16: Basic calibration function of Methoxychlor (DMDT) measured in diet analysis

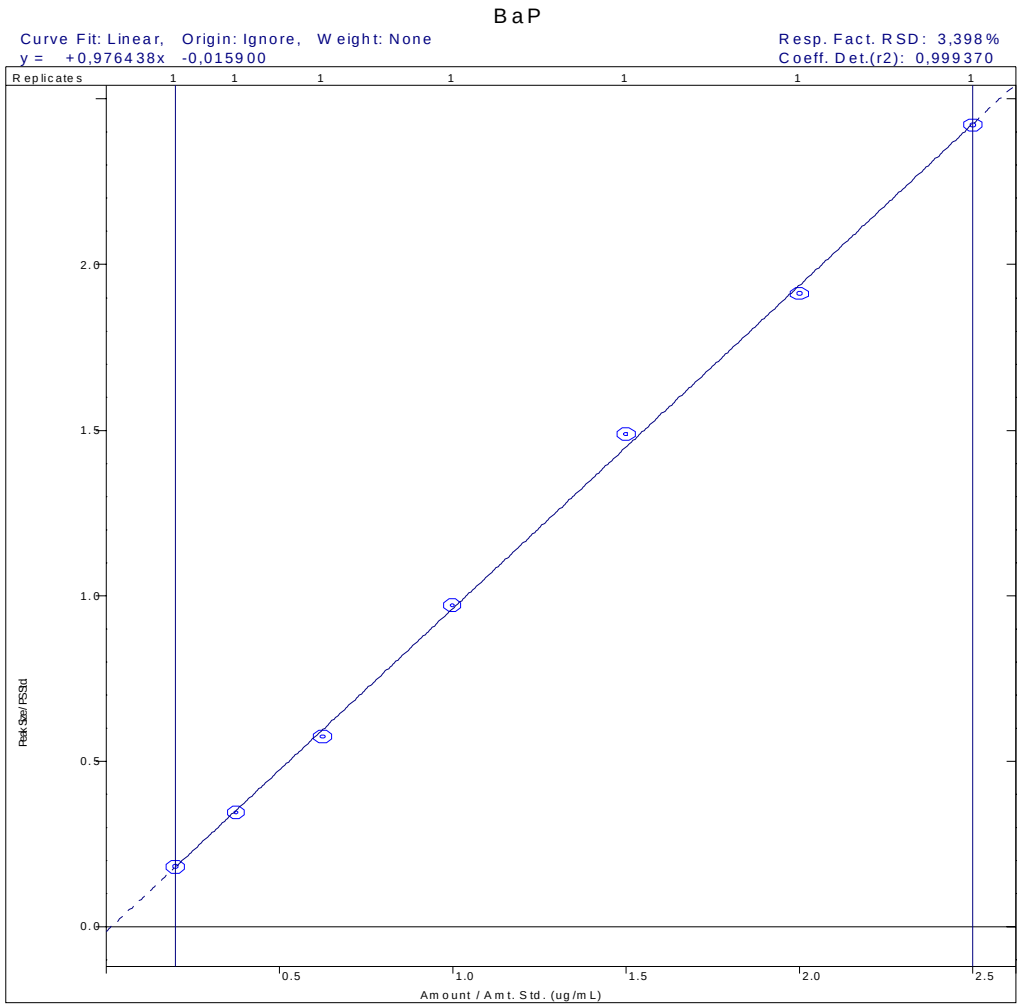


Figure 17: Basic calibration function of Benzo[a]pyrene measured in diet analysis

10.11.2 Fish analysis

Due to the broad calibration range (factor 1000, cp. Table 17) all calibration functions in fish analysis were calculated by quadratic regression analysis; the individual functions are shown in the header information of the corresponding 'Calibration Curve Reports' in Figure 18 to Figure 21.

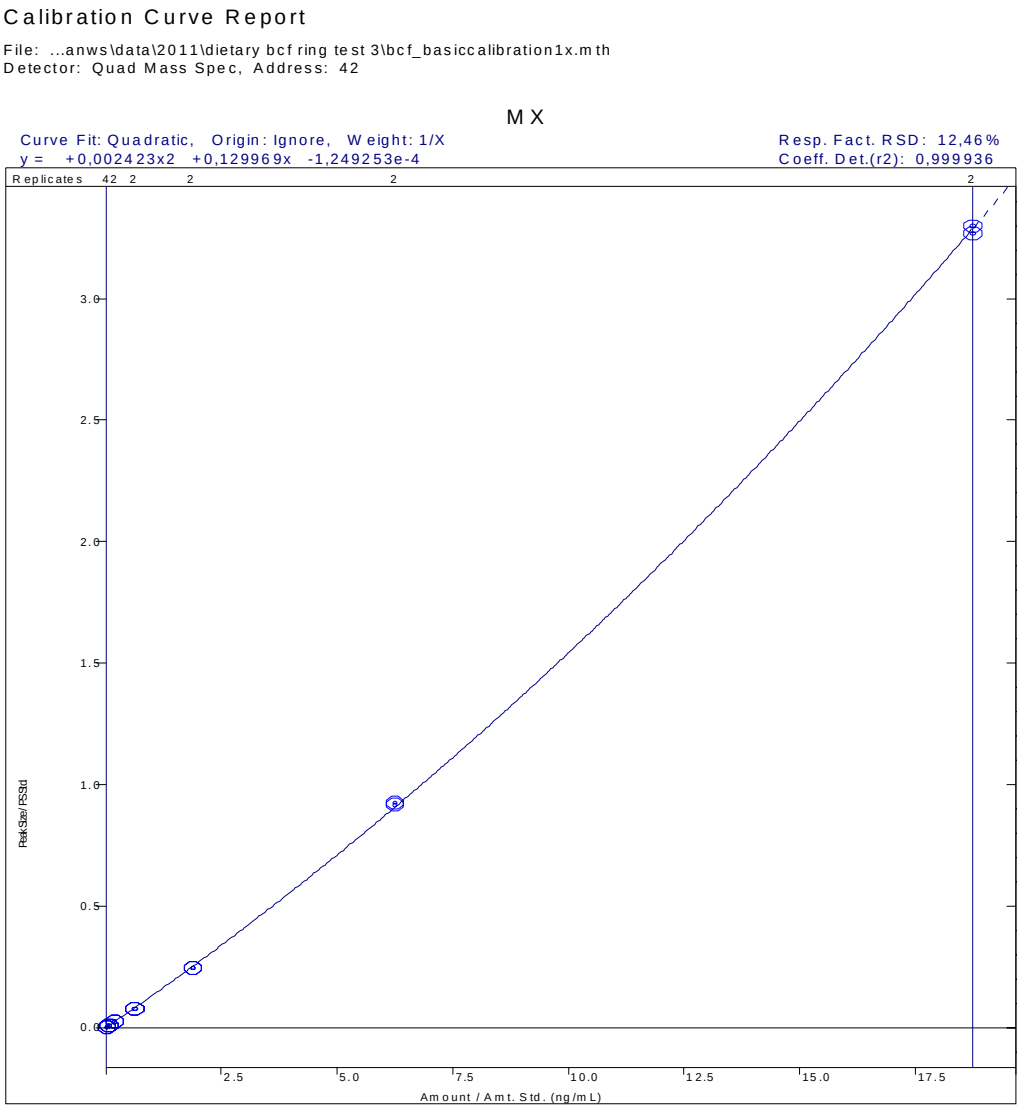


Figure 18: Basic calibration function of Musk xylene measured in fish analysis

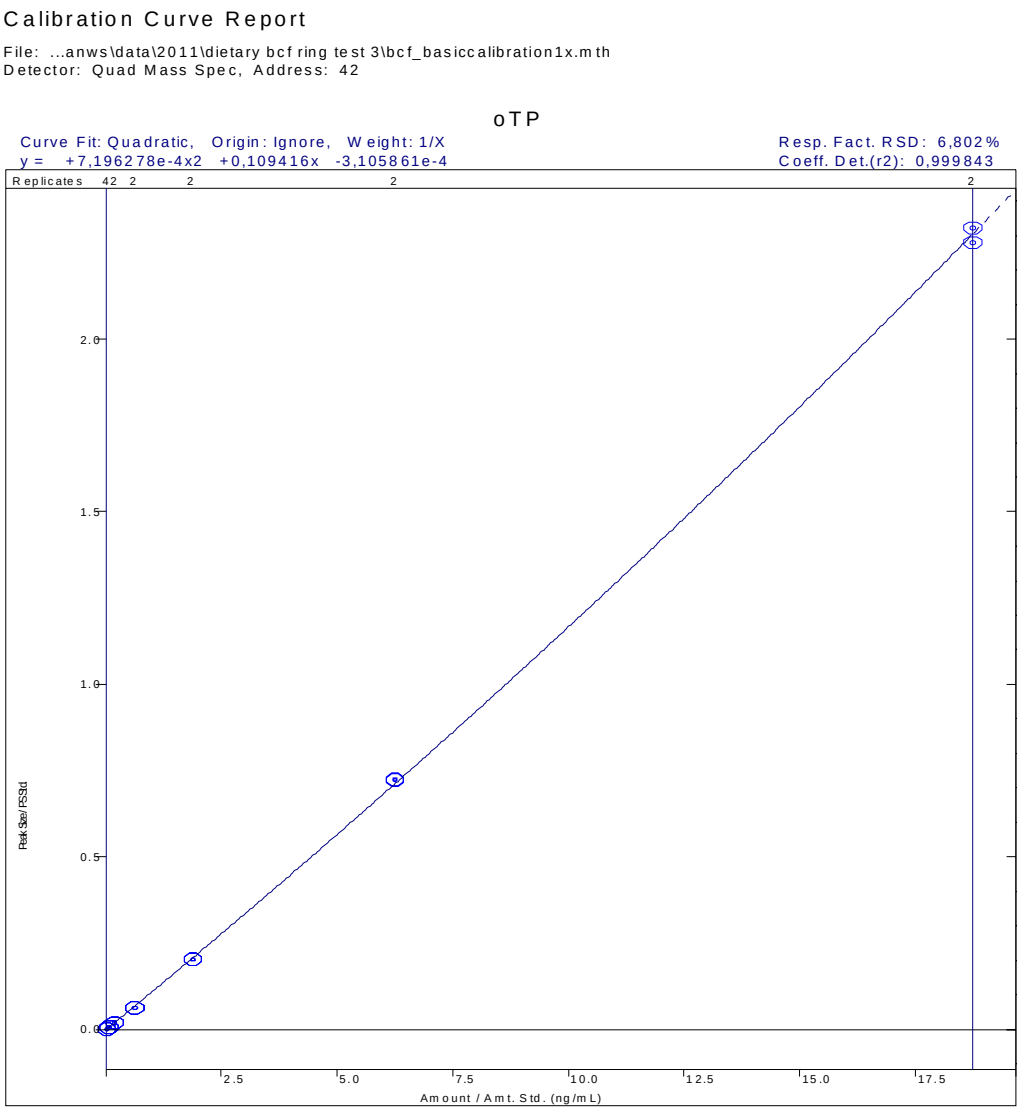


Figure 19: Basic calibration function of o-Terphenyl measured in fish analysis

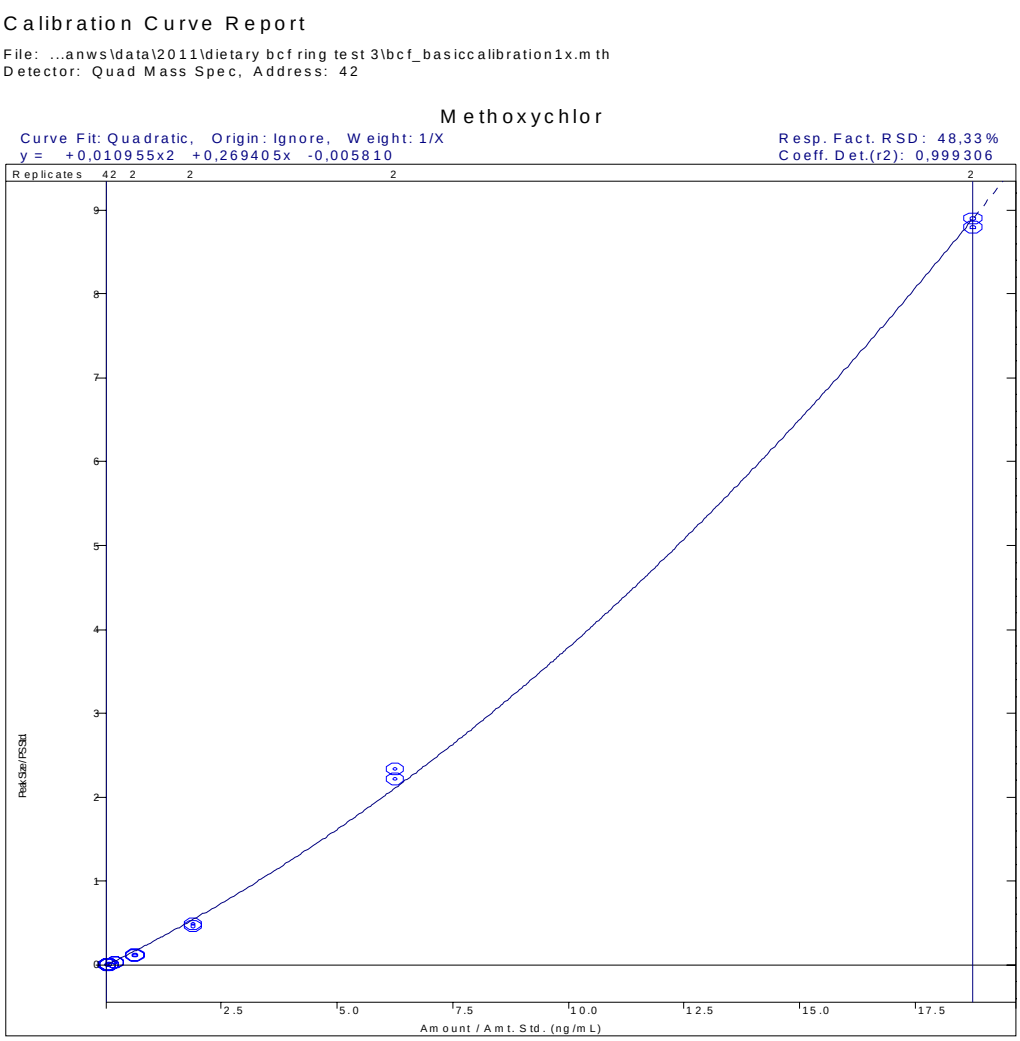


Figure 20: Basic calibration function of Methoxychlor (DMDT) measured in fish analysis

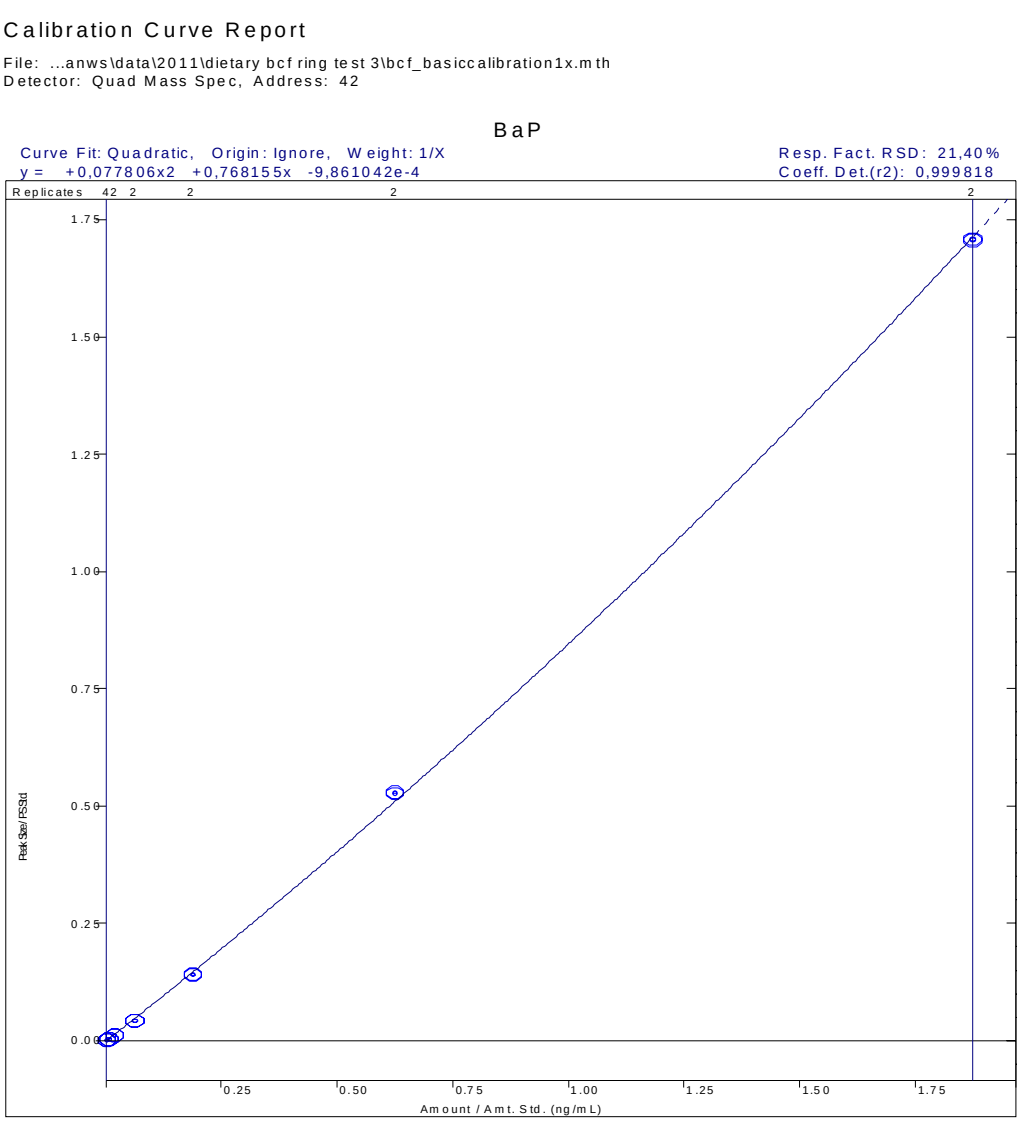


Figure 21: Basic calibration function of Benzo[a]pyrene measured in fish analysis