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Optimisation of procedure for determination of polycyclic aromatic hydrocarbons and their derivatives in fish tissue; estimation of measurements uncertainty

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Additives/Contaminants:	PAH
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Table I. [Characterisation](#) of GPC systems used PAHs – co-extract separation

Deleted: Characterization

GEL	column dimensions (mm)	particle size/ pore size	mobile phase	flow rate of mobile phase (ml min ⁻¹) [#]	Code of procedure
Bio-Beads S-X3	500 x 8	200-400 mesh ~ (37 – 75 µm)	chloroform	0.6	GPC-BB-C
			EtAc-CyH (1:1, v/v) [*]	0.6	GPC-BB-EC
PL gel	500 x 7.5	10 µm/50 Å	chloroform	0.6	GPC-P-C
			EtAc-CyH (1:1, v/v) [*]	0.6	GPC-P-EC
Envirogel	450 x 19 (150 x 19 & 300 x 19)	15 µm/100 Å	chloroform	3	GPC-E-C

^{*} ethyl acetate-cyclohexane (1:1, v/v)[#] The same flow rate was used for similar column dimension (GPC-BB and GPC-P set-up)

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Table II. FLD settings for PAHs determination

PAHs	<i>Time window</i> (min)	<i>λ excitation</i> (nm)	<i>λ emission</i> (nm)
Naph, 1-MeNaph	0-1.0	217	338
Ace, Flt	1-10.6	250	341
DBT, Phe	10.6-12.1	240	360
Ant	12.1-14.0	248	405
Flt	14.0-16.0	232	445
Pyr	16.0-17.4	236	389
2-MeAnt	17.4-19.3	250	397
1-MePyr, B[a]A, Chr	19.3-21.9	270	401
5-MeChr, B[b]N[d]T	21.9-25.5	270	369
B[e]P, B[b]F, B[k]F, B[a]P	25.5-27.5	266	425
DB[ah]A, B[ghi]P	27.5-35.0	295	405
I[1,2,3-cd]P	35.0-38.7	248	484

Table III. Summary of results obtained by different GPC systems employed for separation efficiency of PAHs and co-extracts testing

Code of procedure	volume of PAHs fraction (ml)	volume of co-extracts fraction (ml)	Total time needed for fractionation (min)	“purity” of PAHs fraction*
GPC-BB-C	15.5 – 30 (14.5)	6 – 16 (10)	50	6 % co-elution of PAHs and lipids
GPC-BB-EC	15 – 45 (30)	6 – 18 (12)	75	8 % co-elution of PAHs and lipids
GPC-P-C	15 – 36 (21)	9 – 21 (12)	60	30 % co-elution of PAHs and lipids
GPC-P-EC	21 – 57 (36)	3 – 15 (12)	57	complete separation of PAHs and lipids
GPC-E-C	75 – 115 (40)	50 – 75 (25)	40	complete separation of PAHs and lipids

* purity of PAHs fraction is expressed as % of lipid fraction from total lipids loaded on column which is co-eluted with PAHs fraction

Table IV. Efficiency of different extraction solvents for isolation of PAHs from fish (n = 6; sonication was used as a testing extraction technique)

Analytes	Chloroform		Hexane - dichloromethane (1:1, v/v)		Hexane - acetone (1:1, v/v)	
	Relative efficiency (%) [*]	RSD (%)	Relative efficiency (%) [*]	RSD (%)	Analyte content (µg/kg) [#]	RSD (%)
<i>Naph</i>	86	25	87	20	4.30	28
<i>1-MeNaph</i>	78	13	56	18	1.89	12
<i>Ace</i>	87	8	81	16	1.25	7
<i>Fln</i>	119	12	93	13	11.92	8
<i>DBT</i>	56	14	55	27	21.32	7
<i>Phe</i>	62	19	62	27	11.61	6
<i>Ant</i>	80	22	97	21	2.82	7
<i>Flt</i>	70	20	62	16	1.52	6
<i>Pyr</i>	61	24	48	20	1.83	12
<i>2-MeAnt</i>	80	19	45	21	1.12	10
<i>1-MePyr</i>	57	15	48	18	0.38	12
<i>B[a]A</i>	98	23	71	20	0.21	11
<i>Chr</i>	74	19	74	20	0.21	7
<i>5-MeChr</i>	57	8	44	7	0.04	11
<i>B[b]N[d]T</i>	47	14	40	18	0.42	14
<i>B[e]P</i>	64	15	39	20	0.85	12
<i>B]b]F</i>	73	11	46	25	0.12	7
<i>B[k]F</i>	77	9	44	23	0.06	4
<i>B[a]P</i>	60	28	41	17	0.20	8
<i>DB[ah]A</i>	65	39	41	20	0.04	9
<i>B[ghi]P</i>	62	28	43	21	0.19	6
<i>I[cd]P</i>	60	10	44	12	0.15	8

* Hexane-acetone (1:1, v/v) value was set as 100 %

Content of PAHs was calculated on fresh weight (moisture of sample was 62.5 %).

Table V. Efficiency of different extraction techniques for isolation of PAHs from fish (n = 6)

Analytes	Sonication		Saponification		Soxhlet extraction		
	Relative efficiency (%) *	RSD (%)	Relative efficiency (%) *	RSD (%)	Analyte content ($\mu\text{g}/\text{kg}$) #	RSD (%)	Recovery (%)
<i>Naph</i>	106	24	101	16	4.05	54	70
<i>1-MeNaph</i>	99	15	80	18	1.89	22	71
<i>Ace</i>	98	10	102	19	1.28	15	85
<i>Fln</i>	96	5	87	9	12.41	10	98
<i>DBT</i>	100	6	103	5	21.32	14	106
<i>Phe</i>	105	5	106	23	11.06	2	107
<i>Ant</i>	103	3	109	12	2.74	4	104
<i>Flt</i>	102	9	104	7	1.49	4	88
<i>Pyr</i>	63	8	63	8	2.91	4	86
<i>2-MeAnt</i>	97	12	99	16	1.15	5	103
<i>1-MePyr</i>	81	10	70	9	0.47	12	85
<i>B[a]A</i>	70	12	62	9	0.30	2	105
<i>Chr</i>	53	9	48	20	0.40	6	78
<i>5-MeChr</i>	48	15	40	7	0.08	14	82
<i>B[b]N[d]T</i>	44	15	29	4	0.96	13	108
<i>B[e]P</i>	68	10	54	3	1.25	15	84
<i>B]b]F</i>	49	12	35	16	0.24	4	100
<i>B[k]F</i>	45	10	30	18	0.15	4	85
<i>B[a]P</i>	30	8	25	3	0.66	5	84
<i>DB[ah]A</i>	104	5	109	6	0.05	5	83
<i>B[ghi]P</i>	97	5	95	7	0.18	6	80
<i>I[cd]P</i>	51	7	51	12	0.30	5	83

* Soxhlet extraction value was set as 100 %

Content of PAHs was calculated on fresh weight (moisture of sample was 62.5 %).

Table VI. Uncertainties of optimised analytical procedure obtained by "top-down" approach (%)

<i>Analytes</i>	u_{REC}	u_{RSD}	u_{std}	u	U
<i>Phe</i>	2.0	14.1	0.5	14.2	28.3
<i>Ant</i>	3.8	9.2	0.7	9.8	19.6
<i>Flt</i>	4.3	17.1	0.4	17.5	35.1
<i>Pyr</i>	4.1	9.0	0.4	9.9	29.8
<i>B[a]A</i>	1.6	11.5	0.3	11.6	23.2
<i>Chr</i>	6.3	9.8	0.3	11.7	23.3
<i>B[b]F</i>	4.0	17.3	0.3	17.8	35.5
<i>B[k]F</i>	4.2	21.9	0.4	22.3	44.6
<i>B[a]P</i>	4.6	12.1	0.5	12.9	25.8
<i>DB[ah]A</i>	5.0	17.1	1.8	17.8	35.6
<i>B[ghi]P</i>	5.8	13.7	1.0	14.9	29.8
<i>I[cd]P</i>	4.8	14.2	0.6	15.0	30.0

Captions:

u_{REC}	uncertainty of recovery of analytical procedure, Eq. (3)
u_{RSD}	repeatability of the procedure (n=6), Eq. (4)
u_{std}	uncertainty of purity of the standard solution, Eq. (5)
u	total uncertainty - for calculation see Eq. (1)
U	expanded uncertainty - for calculation see Eq. (2); coverage factor $k = 2$

Table VII. Uncertainties (%) of optimised analytical procedure obtained by "bottom-up" approach

Source of uncertainty	Component of uncertainty	Contribution to the total uncertainty of PAHs measurement (%)											
		Phe	Ant	Flt	Pyr	B[a]A	Chr	B[b]F	B[k]F	B[a]P	DB[ah]A	B[ghi]P	I[cd]P
Weighting of sample	Repeatability (n=10)	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
	Accuracy of balance	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005	0.00005
	u₁	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Extraction	Repeatability (n=6)	14.1	9.2	17.1	9.0	11.5	9.8	17.3	21.9	12.1	17.1	13.7	14.2
	Recovery (n=6)	2.0	3.8	4.3	4.1	1.6	6.3	4.0	4.2	4.6	5.0	5.8	4.8
	u₂	14.2	9.8	17.5	9.9	11.6	11.6	17.8	22.3	12.8	17.7	14.9	15.0
RVO 1	Repeatability (n=5)	13	8	17	9	7	7	7	8	10	7	7	9
	Recovery (n=5)	2.0	4.0	2.9	1.4	1.4	0.6	2.0	1.4	2.3	0.6	1.2	0.6
	u₃	13.2	9.0	17.2	8.6	6.7	6.5	7.3	8.1	10.3	7.0	7.1	9.0
Make-up to 10 ml (chloroform)	Repeatability (n=10)	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1	1.1
	Calibration of volumetric flask	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14	0.14
	Temperature volume expansion of solvent (for 22±2°C)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
	u₄	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13	1.13
GPC	Repeatability (n=5)	8.1	7.6	7.7	8.3	6.2	5.8	6	7.1	9.2	5.8	5.9	8
	Recovery (n=5)	4.9	4.0	0.1	0.2	1.4	1.4	1.4	1.5	1.6	1.6	1.3	1.6
	u₅	9.5	8.6	7.7	8.3	6.4	6.0	6.2	7.9	9.3	6.5	6.0	8.5
RVO 2	Repeatability (n=5)	8.3	7.1	7.3	6.9	6.2	5.8	5.4	7.2	9.1	5.9	4.8	8.0
	Recovery (n=5)	1.4	4.0	1.2	0.9	0.6	0.5	0.1	1.1	0.5	0.9	0.2	0.3
	u₆	8.4	8.2	7.4	7.0	6.2	5.8	5.4	7.3	9.1	6.0	4.8	8.0
Transferring into 0.5 ml acetonitrile	Repeatability (n=10)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Calibration of syringe	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12	0.12
	u₇	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
HPLC/FLD	Concentration of standards - u_{8a}	0.5	0.7	0.4	0.4	0.3	0.3	0.3	0.4	0.5	1.8	1	0.6
	Dilution of standard working solution - u_{8b}	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
	Repeatability of injection and the response of the detector (n=10) - u_{8c}	2.6	2.2	2	1.9	1.7	1.7	1.5	1.5	1.5	1.6	1.5	1.5
	u₈	3.05	2.31	2.04	1.94	1.73	1.73	1.53	1.56	1.58	2.41	1.81	1.62
Total uncertainty of the procedure - u		11.9	6.2	16.2	7.6	10.0	10.3	17.0	21.4	9.3	17.0	14.2	12.8
Expanded uncertainty of the procedure - U		23.9	12.4	32.4	15.2	20.0	20.6	34.0	42.8	18.6	34.0	28.4	25.6

Table VIII captions:

u₁₋₈	total uncertainties of individual steps of the procedure – see Figure 4
u_{8a-8c}	total uncertainties of individual operations related to HPLC/FLD
u	total uncertainty of the procedure, calculated according to Eq. (1)
U	expanded uncertainty of the procedure (see Eq. (2), coverage factor $k = 2$)
RVO 1	evaporation of extraction solvent before transferring to chloroform
RVO 2	evaporation of solvent (chloroform) from purified extract (GPC fraction)

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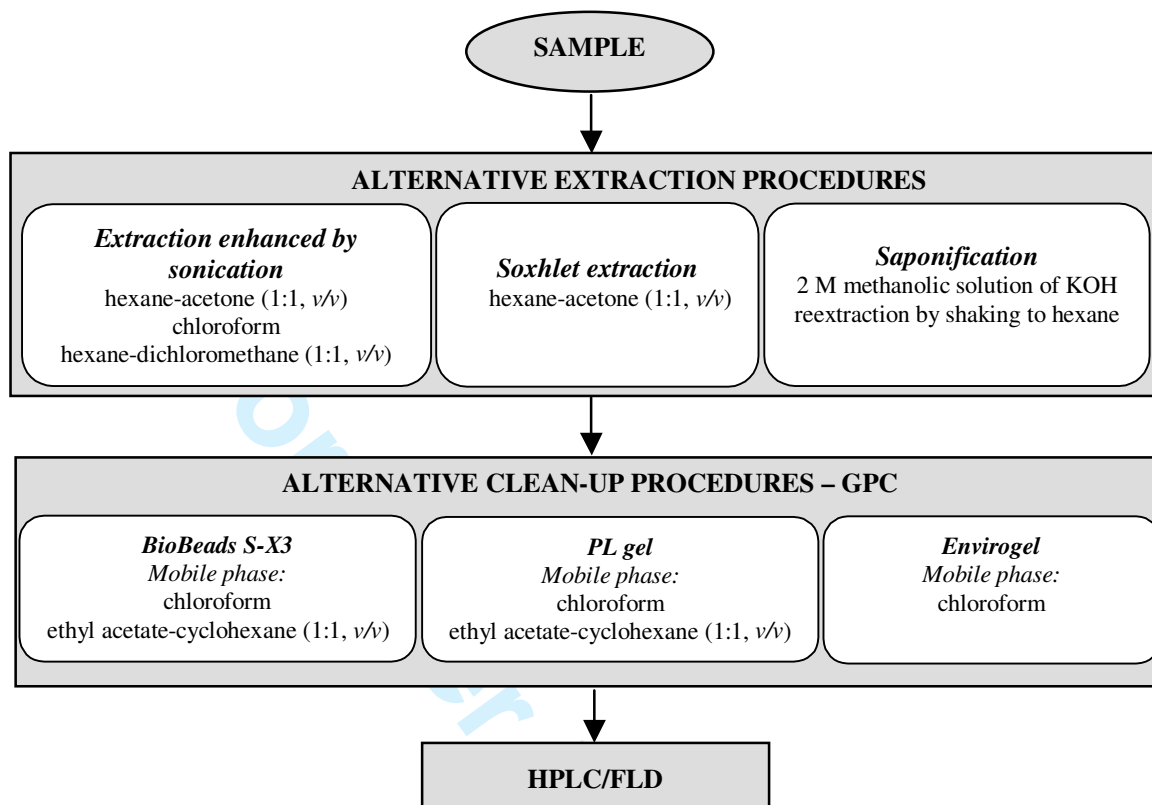


Figure 1. Procedure flow chart

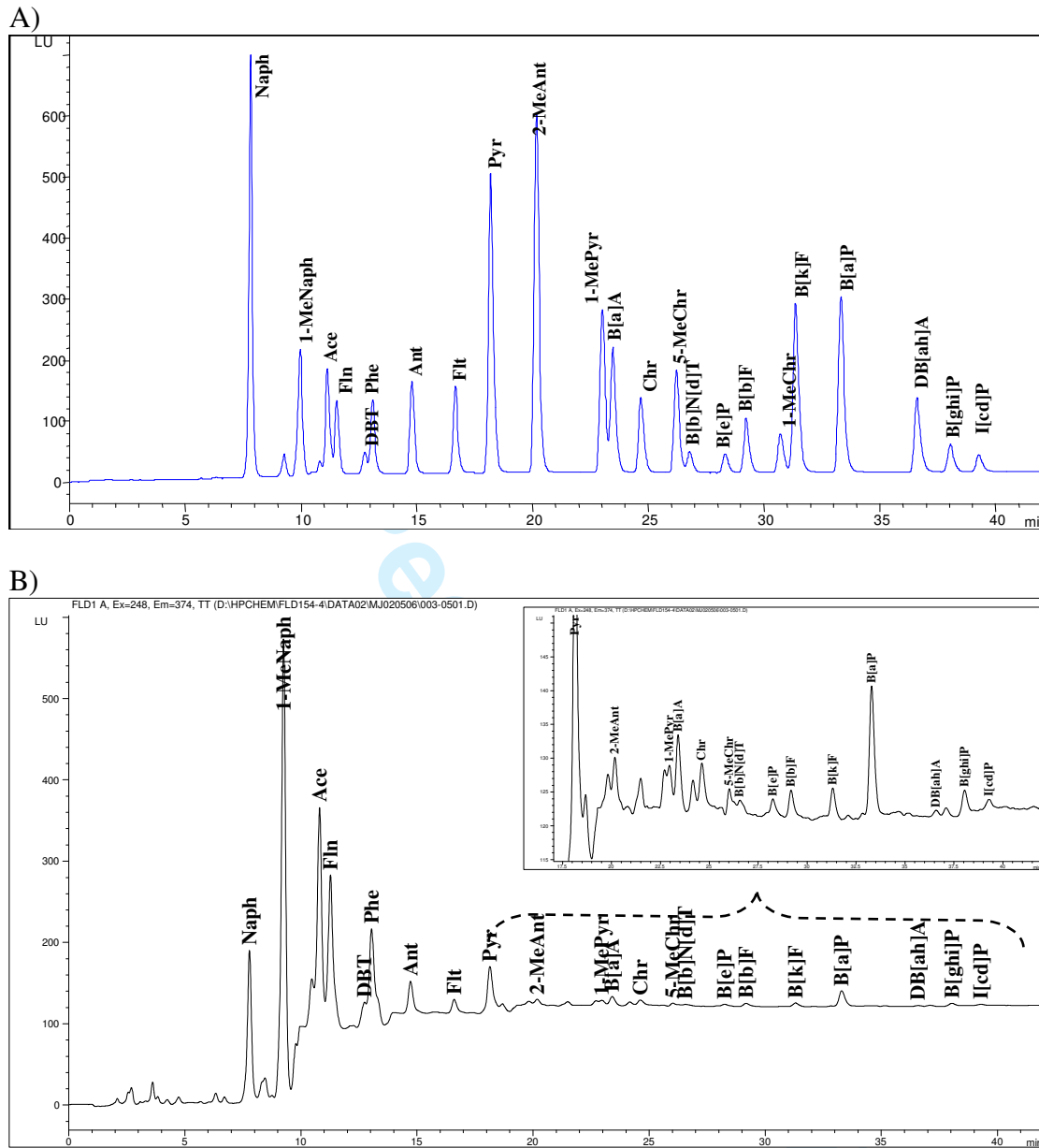


Figure 2. Illustration of HPLD/FLD analysis
 A) Standard mixture (PAHs in range 3-83 ng ml⁻¹)
 B) Purified fish extract (80 mg of matrix - equivalent corresponding to 20 µl injected)

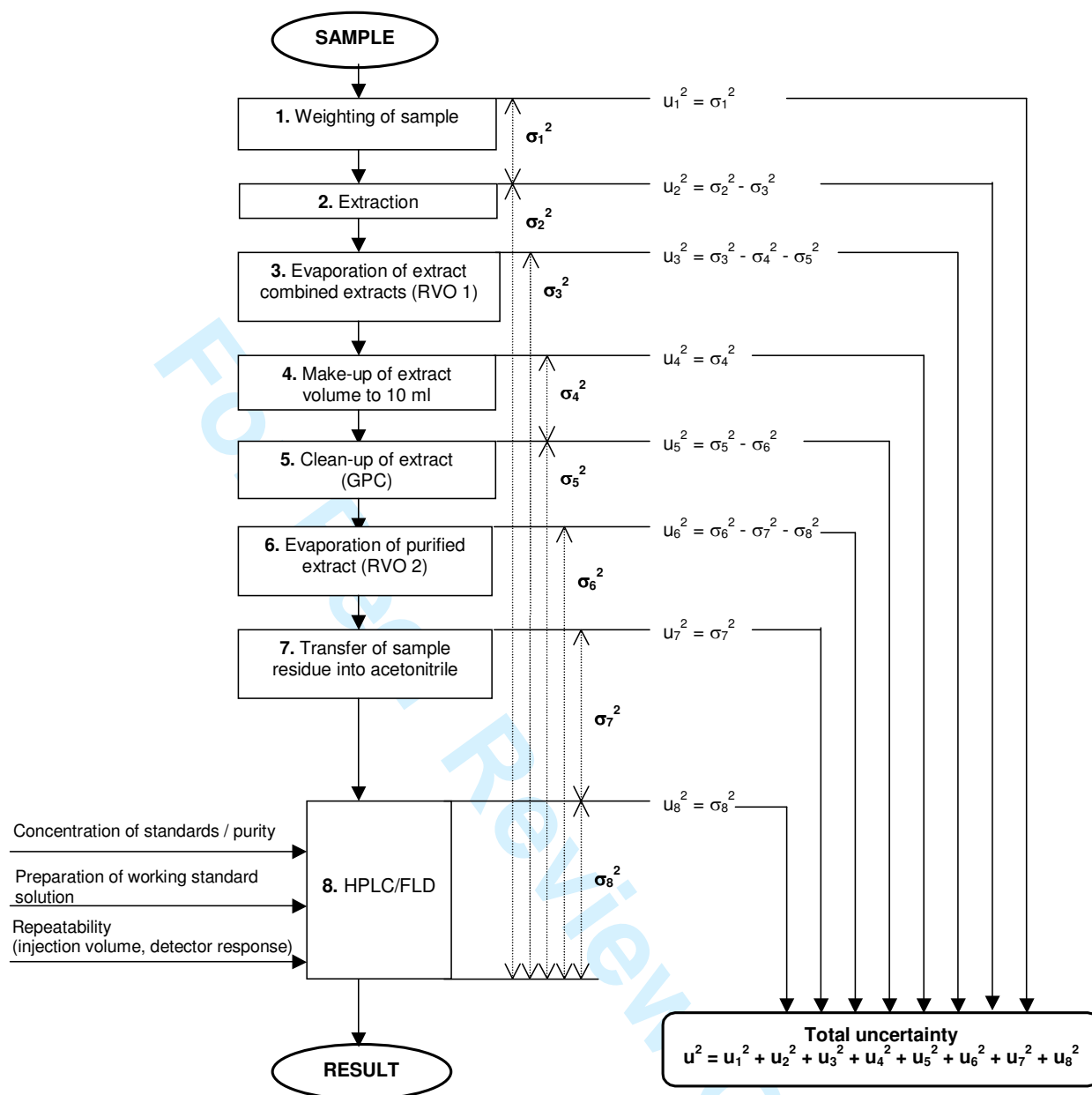


Figure 3. Flow-chart of the total uncertainty calculation

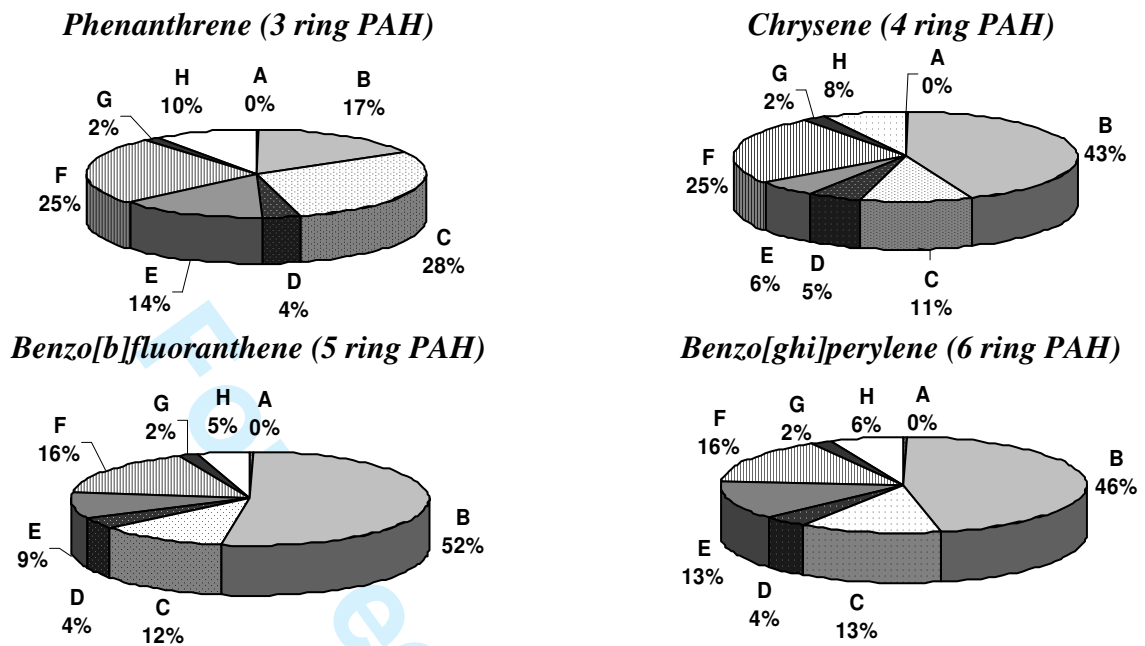


Figure 4. Contributions of partial uncertainties of the individual steps of procedure to the total uncertainty (A – weighting of sample, B – extraction, C – RVO 1, D – make-up to 10 ml, E – GPC, F – RVO 2, G – transferring into acetonitrile, H - HPLC)

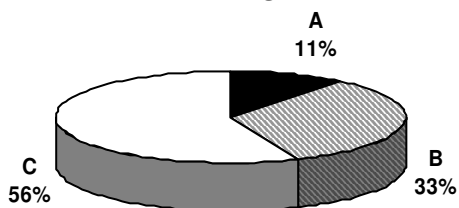
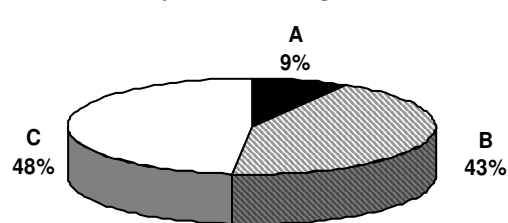
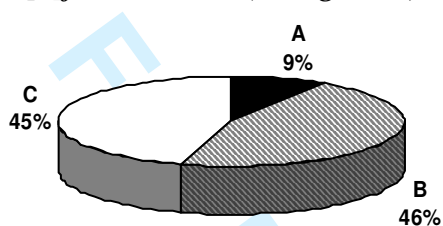
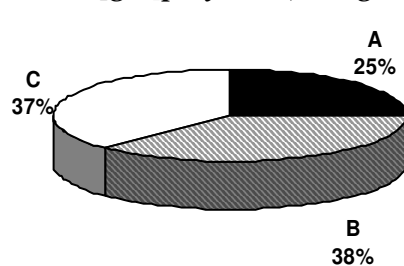
Phenanthrene (3 ring PAH)*Chrysene (4 ring PAH)**Benzo[b]fluoranthene (5 ring PAH)**Benzo[ghi]perylene (6 ring PAH)*

Figure 5. Contributions of partial uncertainties to the total uncertainty of HPLC/FLD step (A – concentration of standard solution, B – diluting of working standard solution, C – repeatability)

Optimisation of procedure for determination of polycyclic aromatic hydrocarbons and their derivatives in fish tissue; estimation of measurements uncertainty

Abstract

Three alternative procedures were employed for isolation of polycyclic aromatic hydrocarbons (PAHs; 15 of 16 US EPA priority pollutants and benzo[*e*]pyrene), their methyl-derivatives and sulphur analogues from fish tissue: (i) Soxhlet extraction, (ii) batch extraction enhanced by sonication and (iii) saponification of sample followed by reextraction of analytes into hexane. Soxhlet extraction using hexane-acetone (1:1, *v/v*) was found as the most efficient extraction technique, with analyte recoveries in the range 70-108%. Within optimisation of clean-up step, several types of gel permeation chromatography (GPC) systems were tested: two types of polystyrene divinylbenzene copolymer gels (PSDVB), both “soft” gel type (Bio-Beads S-X3) and “rigid” gels type (PL gel and Envirogel) in combination with various mobile phases were compared. Bio-Beads S-X3 and mobile phase chloroform were found as the most appropriate for purifying of crude extracts prior to the final determinative step. High performance liquid chromatography with fluorimetric detection (HPLC/FLD) was used for identification and quantification of PAHs in purified fish extracts. The uncertainties of PAHs measurements were estimated employing two alternative approaches. Both provided similar results: the expanded uncertainties obtained for individual PAHs by “top-down” approach were in the range 9-53 %, their values resulting from application of “bottom-up” were in the range 16-52 %.

Keywords: polycyclic aromatic hydrocarbons (PAHs), uncertainty, clean-up, gel permeation chromatography (GPC), extraction, fish tissue

Introduction

Polycyclic aromatic hydrocarbons (PAHs) represent a group of ubiquitous environmental contaminants originating from various emission sources. Their control in the environment and in the food chain is required due to the mutagenic and carcinogenic potential of some PAHs, exhibited in vertebrates. During the recent decade, many studies concerning with monitoring of PAHs inputs (these may result from industry incineration, transport, uncontrolled spills, surface runoff and/or atmospheric deposition), their fluxes and fate, particularly in aquatic environment, have been initiated (Hellou et al. 2005, Varanasi 1989, Koh et al. 2004, Ross et al. 2004, Shi et al. 2004, Reeves et al. 2004, Chen et al. 2004, Grimalt et al. 2004, Oost et al. 2003). It should be noted that the list of 16 US EPA PAHs is different from that established recently by European Union (EU, 2005).

Exposure of habitant biota to PAHs is often assessed through measurement of these hazardous chemicals and/or PAHs metabolites in their tissues. Since the concentrations of target analytes are typically very low, fine tuning of analytical procedure is needed to generate reliable data. Within the optimisation of analytical process, the availability of reference materials with certified concentrations of target analytes incurred in relevant matrix is of a great importance for accuracy control. Unfortunately, with very few exceptions, appropriate certified reference materials (CRMs) enabling analytical quality control (AQC) in monitoring studies concerned with occurrence of PAHs in various biotic matrices, are not commercially available.

Similarly to analysis of other organic pollutants, extraction of PAHs from respective matrix represents a critical step in their determination scheme. Various techniques have been applied for isolation of these substances from biotic and/or abiotic samples. Particularly

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2
3 1 Soxhlet extraction (Jaouen-Madoulet et al. 2000, Frankenius et al. 2001), batch extraction
4 2 enhanced by sonication (Wenzel et al. 1998, Wenzel et al. 1997) and alkaline saponification
5 3 followed by re-extraction (Chen et al. 1997) represent common methods in routine
6 4 laboratories. The use of supercritical fluid extraction (SFE) has also been described in some
7 5 studies (Taylor 1996, Luque de Castro et al. 1994, Librando et al. 2004), however, its routine
8 6 use for processing of biotic samples is limited, probably due to strong dependence of optimal
9 7 parameters setting on sample composition (fat, moisture) what is the cause of a rather low
10 8 robustness of SFE-based procedures. At present, a growing interest in alternative extraction
11 9 techniques such as pressurised liquid extraction (PLE) (Wenzel et al. 1998, Dean 1996, Saim
12 10 Nor'ashikin et al. 1997, Schantz et al. 1997, Lopez-Avila et al. 1997) and microwave assisted
13 11 extraction (MAE) (Saim Nor'ashikin et al. 1997, Lopez-Avila et al. 1997, Leray et al. 1995,
14 12 Saim et al. 1997, Budzinski et al. 1996) can be noticed. Organic solvents such as methanol,
15 13 acetone (Knulst et al. 1995, Franzaring et al. 1992), chloroform (Cejpek et al. 1995,
16 14 Tomaniová et al. 2000, Tomaniová et al. 1998), dichloromethane (Howsam et al. 2000,
17 15 Marvin et al. 1999, Gratz et al. 2000), hexane (Weiss et al. 2000) and cyclohexane (Brouwer
18 16 et al. 1994) or mixtures of them, e.g. hexane-acetone (Shu et al. 2000, Budzinski et al. 1999,
19 17 Chee et al. 1996, Lopez-Avila et al. 1995), dichloromethane-acetone (Popp et al. 1997,
20 18 Fischer et al. 1997, Tao et al. 2002), hexane-acetone-toluene (Berset et al. 1999), hexane-
21 19 dichloromethane (Hubert et al. 2001) are typically used for performance of extraction step.

22 20 When analysing complex samples such as plants, biota or foodstuffs, many other matrix
23 21 components are unavoidably co-isolated together with target analytes. Oils, waxes, essential
24 22 oils and natural pigments like carotenoids and chlorophylls are the most typical matrix
25 23 components occurring in extracts obtained from plant samples. In animal tissues, lipids
26 24 represent the major class of co-extracts. Effective removing of these substances, which can
27 25 adversely affect identification and/or quantification of PAHs, is the crucial prerequisite for
28 26 obtaining credible results. However, not only good separation of target analytes from co-
29 27 extracts but also the overall throughput of clean-up step (which is largely dependent on the
30 28 possibility of its automatisation) is important criterion considered when choosing an optimal
31 29 procedure. At present, gel permeation chromatography (GPC) employing fractionation
32 30 according to differences in the molecular size (or more exactly effective molecular volume) of
33 31 impurities and target contaminants together with adsorption chromatography based on
34 32 differences in polarity/chemical structure of sample components, represent nowadays
35 33 dominating purification techniques applied in processing of primary extracts in PAHs
36 34 analysis. GPC procedure may employ several types of gels for purification of primary
37 35 extracts, e.g. styrene divinylbenzene copolymer gels Bio-Beads S-X3 (Kaupp et al. 1996,
38 36 Czuczwa et al. 1989), S-X12 (Fernández et al. 1988), XAD-2 (Codina et al. 1994), PL gel
39 37 (Schantz et al. 1997) and Phenogel (Nyman et al. 1993). Elution of sample components is
40 38 usually carried out by different elution solvents, e.g. toluene (Kaupp et al. 1996),
41 39 dichloromethane (Schantz et al. 1997), mixture of dichloromethane-cyclohexane (Kaupp et al.
42 40 1996) and ethyl acetate-cyclohexane (Czuczwa et al. 1989). As regards the adsorption
43 41 chromatography, the use of following sorbent/eluent combinations was reported in literature:
44 42 (i) silica with cyclohexane (Tao et al. 2002, Karl et al. 1996, Popp et al. 1997), hexane (Chee
45 43 et al. 1996, Berset et al. 1999, Falcón et al. 1996), pentane (Budzinski et al. 1999), (ii) Florisil
46 44 with hexane (Wenzel et al. 1997), dichloromethane (Wang et al. 1999) and their mixtures
47 45 (Chen et al. 1997) and (iii) alumina with dichloromethane (Marvin et al. 1999) and mixtures
48 46 of pentane-dichloromethane (Jaouen-Madoulet et al. 2000).

49 47 As regards determinative step, both gas chromatography coupled with mass spectrometry
50 48 (GC/MS) and reversed phase high performance liquid chromatography employing
51 49 fluorimetric detector (HPLC/FLD) are used for quantification (Jacob de Boer et al. 2003).
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3 1 Nowadays several dedicated HPLC as well as GC columns are available at the market for
4 2 separation of complex PAH mixtures.

5 3 Since no measurement is entirely free of random and/or systemic errors, uncertainties of
6 4 results unavoidably exist. According to Eurachem Guide (Ellison et al. 2000) uncertainty is
7 5 defined as a “parameter, associated with the result of a measurement, that characterizes the
8 6 dispersion of the values that could reasonably be attributed to the measurand”. In practice,
9 7 information on uncertainty is needed in test report whenever (i) it is relevant to the validity or
10 8 application of the test results; (ii) a client so requires for particular purpose; (iii) the
11 9 uncertainty may affect compliance to a specification limit. Especially the last requirement for
12 10 uncertainty declaration is important in PAHs analysis, results of which are dedicated for
13 11 decision-making process. Supposing e.g. hygienic limits established for toxic chemicals
14 12 occurring in specified (environmental/food) matrices is to be controlled, then uncertainty
15 13 value has an obvious implication for the interpretation of the analytical results (compliance -
16 14 non compliance). Detailed analysis of uncertainty sources can also provide identification of
17 15 critical points in analytical flow-chart. Identification of uncertainty source is an important
18 16 stimulus for further improvements of method performance and, consequently, for achieving
19 17 the better quality of generated data.

20 18 Uncertainty can be expressed as a *standard uncertainty*. The relationship between the
21 19 combined standard uncertainty $u_c(y)$ of y and the uncertainty of the independent parameters
22 20 x_1, \dots, x_n on which it depends is characterised by following equation:

$$23 \quad 21 \quad u_c(y(x_1, x_2, \dots)) = \sqrt{\sum_{i=1, n} (\partial y / \partial x_i)^2 u(x_i)^2} = \sqrt{\sum_{i=1, n} u(y, x_i)^2} \quad (1)$$

24 22 where $y(x_1, \dots, x_n)$ is a function of parameters x_1, \dots, x_n , $\partial y / \partial x_i$ is the partial differential of y with
25 23 respect to x_i and $u(y, x_i)$ is the uncertainty y derived from the uncertainty x_i (Ellison et al.
26 24 2000).

27 25 In analytical chemistry an *expanded uncertainty* (U) is commonly used rather than
28 26 standard uncertainty. Its value defines an interval within which the value of the measurand
29 27 lies with a known level of confidence. U is obtained by multiplying the combined standard
30 28 uncertainty (u) by a coverage factor (k):

$$31 \quad 29 \quad U = k \cdot u \quad (2)$$

32 30 A coverage factor, k , is typically in the range from 2 to 3. The choice of this factor is based on
33 31 a level of confidence required for particular purpose. For example, for an approximate level of
34 32 confidence of 95 %, $k = 2$.

35 33 Several concepts were developed for the estimation of uncertainties related to results.
36 34 Nevertheless, in practice two main different approaches to uncertainty estimation are used –
37 35 “bottom-up” described in EURACHEM/CITAC document (Ellison et al. 2000) and “top-
38 36 down” recommended in the recently published ISO 21748:2004 (ISO/TS 21748 2004) which
39 37 gives a guidance for the estimation of measurement uncertainty using the data obtained from
40 38 interlaboratory studies performed in an accordance with ISO 5725-2 (determination of
41 39 repeatability and reproducibility of a standard measurement method) (ISO 5725-2 1994).
42 40 Currently, under discussion there is a draft ISO standard (ISO/CD 15753, 2004), which
43 41 describes the interlaboratory validated method for determination of 15 US EPA PAHs in
44 42 animal and vegetable oils from where data on reproducibility could be taken (provided that
45 43 this particular standardised procedure is used for sample analysis)

46 44 Alternative concepts aimed at practical and understandable way of measurement uncertainty
47 45 calculations, based mainly on already existing quality control and validation data, namely the
48 46 use of certified reference materials (CRM), participation in interlaboratory comparisons
49 47 (proficiency testing) and recovery tests are described in the Handbook for calculation of
50 48 measurement uncertainty in environmental laboratories (Magnusson et al. 2003).

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3 1 The presented study concerning PAHs analysis was focused on following tasks (i) critical
4 2 assessment of extraction efficiencies of most conceivable isolation techniques represented by
5 3 Soxhlet extraction, batch extraction enhanced by sonication and saponification of sample
6 4 followed by re-extraction; (ii) to find a suitable experimental configuration of GPC for
7 5 purifying of crude extracts within determination of PAHs and some of their derivatives in fish
8 6 samples; (iii) estimation of uncertainties of optimised methods frequently employed in food
9 7 analysis (using “bottom-up” and “top-down” approaches) with respect to their practical use.
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12 8 13 9 **Materials and methods**

14 10 15 11 *Experimental materials*

16 12
17 13 The sample of smoked fish (mackerel) used for optimisation of analytical procedure was
18 14 obtained from the common market of the Czech Republic. Before homogenization in a
19 15 blender, the skin and bones were removed. Homogenised sample (3 kg of fish fillets) was
20 16 stored at - 20 °C.
21 17

22 18 23 19 *Chemicals and materials*

24 20
25 21 Chloroform and acetone (analytical reagent grade, Lachema Brno, Czech Republic) were
26 22 redistilled in glass before use. Acetonitrile (gradient grade, for chromatography, Merck,
27 23 Germany), hexane (for organic trace analysis, Merck, Germany), dichloromethane (for gas
28 24 chromatography, Merck, Germany), methanol (gradient grade, for chromatography, Merck
29 25 Germany) cyclohexane (for gas chromatography, Merck, Germany) and ethyl acetate (for
30 26 pesticide residue analysis, Scharlau, Spain) were used as supplied. Deionised water was
31 27 obtained from Milli-Q water purification system (MILLIPORE, USA). Anhydrous sodium
32 28 sulphate (Penta Praha, Czech Republic) was dried at 500 °C for 5 hours and then stored in a
33 29 tightly capped glass bottle. Potassium hydroxide (analytical reagent grade, Penta Praha, Czech
34 30 Republic) was used for saponification.

35 31 The standard mixture 1647d of 16 priority PAHs - naphthalene (Naph), acenaphthene
36 32 (Ace), fluorene (Flu), phenanthrene (Phe), anthracene (Ant), fluoranthene (Flt), pyrene (Pyr),
37 33 benz[*a*]anthracene (B[*a*]A), chrysene (Chr), benzo[*b*]fluoranthene (B[*b*]F),
38 34 benzo[*k*]fluoranthene (B[*k*]F), benzo[*a*]pyrene (B[*a*]P), dibenz[*a,h*]anthracene (DB[*ah*]A),
39 35 benzo[*g,h,i*]perylene (B[*ghi*]P) and indeno[*1,2,3-cd*]pyrene (I[*cd*]P) dissolved in acetonitrile
40 36 was supplied by National Institute of Standards and Technology (NIST, USA). Standards of
41 37 individual PAHs derivatives - 1-methylnaphthalene (1-MeNaph), dibenzothiophene (DBT), 2-
42 38 methylanthracene (2-MeAnt), 1-methylpyrene (1-MePyr), 5-methylchrysene (5-MeChr),
43 39 benzo[*b*]naphtho[2,1-*d*]thiophene (B[*b*]N[*d*]T), benzo[*e*]pyrene and (B[*e*]P) dissolved in
44 40 acetonitrile were supplied by Dr. Ehrenstorfer (Germany). Purity of individual standards was
45 41 not less than 95 %. Working standard solutions were prepared in acetonitrile and stored at 4
46 42 °C.
47 43

48 44 Before the use, all glassware was washed with detergent, rinsed with distilled water and
49 45 acetone and then heated.
50 46

51 47 52 48 *Equipment*

53 49 A laboratory blender (WARING blender, 38BL-40, Waring Commercial, USA) was used for
54 50 homogenization of samples, an ultrasonic bath Sonorex RK 510 (Bandeline, Germany) and
55 51 Soxhlet extractor with cellulose extraction thimbles (Whatman, U.K) were used for sample
56 52 extraction.
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1 An automated GPC (gel permeation chromatography) system consisting of 305 MASTER
2 pump, fraction collector, automatic regulator of loop 231 XLI, microcomputer (software 731
3 PC via RS232C), dilutor 401C (GILSON, France) and stainless steel columns (i) 500 x 8 mm
4 i.d. packed with gel Bio-Beads S-X3, 200 – 400 mesh ~ 37-75 µm (Bio-Rad Laboratories,
5 USA) and (ii) 500 x 7.5 mm i.d. packed with PL gel, particles size 10 µm, pores size 50 Å
6 (Polymer Laboratories, U.K.) and (iii) two columns connected in series packed with
7 Envirogel, with dimensions 150 x 19 mm and 300 x 19 mm i.d., particles size 15 µm, pores
8 size 100 Å (Waters, USA) were used for clean-up of extracts.

9 A Vacuum evaporator (Büchi Rotavapor R-114 a Waterbath B-480, Switzerland) was
10 used for concentration of extracts.

11 A high performance liquid chromatographic system (HPLC) Hewlett-Packard 1100 Series
12 composed of quaternary pump system with degasser, autosampler, column thermostat,
13 fluorescence detector (FLD) (Hewlett Packard, USA) and a LiChroCART 250-4 (250 × 4 mm
14 i.d.) column and guard column 4-4 (4 × 4 mm i.d.) both with the sorbent LiChrospher PAHs®
15 (Merck, Germany), was used for PAH analysis.

16 Analytical procedures

17 Procedure flow chart is shown in Figure 1.

18 [Insert Figure 1 about here]

19 *Soxhlet extraction.* Twenty grams of homogenised sample mixed with 60 g of anhydrous
20 sodium sulphate in a grinding mortar were placed into the extraction cellulose thimble,
21 covered with glass wool and inserted into the Soxhlet extractor. Thimbles were pre-extracted
22 for 2 hours with an extraction solvent to obtain lower PAHs procedure blank. Extraction was
23 carried out with 170 ml of extraction solvent (hexane-acetone, 1:1, v/v) for 6 hours (10
24 cycles/hour). The Soxhlet apparatus was covered with an aluminium foil to avoid access of
25 daylight (to prevent the risk of photodegradation). The extraction solvent was then carefully
26 evaporated by rotatory vacuum evaporator at 40°C just to dryness and quantitatively
27 transferred into a 10-ml volumetric flask by chloroform.

28 *Batch extraction enhanced by sonication (“Sonication”).* Twenty grams of homogenised
29 sample dried with 60 g of anhydrous sodium sulphate in a grinding mortar were transferred
30 into an Erlenmeyer flask with 100 ml of extraction solvent (chloroform; hexane-acetone, 1:1,
31 v/v and hexane-dichloromethane, 1:1, v/v were tested). The flask was covered with an
32 aluminium foil to avoid access of daylight (to prevent the risk of photodegradation) and
33 placed for 20 min into an ultrasonic bath. The extract was then filtered through a layer of
34 anhydrous sodium sulphate. Extraction was repeated twice with 50 and 30 ml of extraction
35 solvent, in either case for 20 min. Combined filtrates were evaporated by rotatory vacuum
36 evaporator at 40 °C just to dryness and quantitatively transferred into a 10-ml volumetric flask
37 by chloroform.

38 *Saponification.* Ten grams of homogenised sample were transferred into a flask with 50 ml of
39 2 M methanolic solution of potassium hydroxide and boiled under reflux for 2 hours.
40 Apparatus was covered with an aluminium foil to avoid access of daylight (to prevent the risk
41 of photo degradation). The content of flask was filtered to a separatory funnel and then
42 extracted by intensive shaking for 5 min with 50 ml of hexane. The extraction was repeated
43 with separated water layer under the same conditions. Combined hexane layers were filtered
44 through a layer of anhydrous sodium sulphate, carefully evaporated by rotatory vacuum

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3 1 evaporator at 40 °C just to dryness and quantitatively transferred into a 5-ml volumetric flask
4 2 by chloroform.
5 3

6 4 *Clean-up.* Experimental set-ups of all gel / mobile phase combinations used for optimisation
7 5 of crude extract purification are summarised in Table I.
8 6

9 7 [Insert Table I about here]
10 8

11 9 Within optimisation of isolation step, the clean-up procedure carried out by gel
12 10 permeation chromatography (GPC) employing gel Bio-Beads S-X3 and mobile phase
13 11 chloroform was used. Flow rate of the mobile phase was 0.6 ml/min and injection volume 1
14 12 ml. After discarding the first 15.5 ml of eluate, the next 14.5 ml were collected. The purified
15 13 extracts were subsequently subjected to concentration by rotatory vacuum evaporator at 40°C
16 14 just to dryness. The residue after evaporation was immediately dissolved in 0.5 ml of
17 15 acetonitrile and the solution was transferred into a 2-ml amber vial.
18 16

19 17 *Identification and quantification.* The HPLC/FLD determination was carried out under the
20 18 following conditions: gradient elution (A – acetonitrile, B – water; 0 min – 55 % A, 40 min –
21 19 100 % A, 42 min – 100 % A), mobile phase flow rate 1 ml min⁻¹, injection volume 20 µl,
22 20 column temperature 35°C, FLD settings are shown in Table II.
23 21

24 22 An example of PAHs determination in standard solution and fish sample by HPLC/FLD
25 23 is demonstrated in Figure 2.
26 24

27 25 [Insert Table II about here]
28 26

29 27 [Insert Figure 2 about here]
30 28

31 29 *Recovery experiments.* Because of unavailability of reference fish tissue with certified content
32 30 of PAHs, alternative strategy represented by *spiking* the matrix by target analytes was used
33 31 for trueness check. It should be emphasised that *spiking* may affect the recovery information
34 32 generated this way due to the potential difference between extractability of naturally incurred
35 33 and in solvent spiked target analytes (in the latter case extraction of analytes is more
36 34 straightforward). In our experiments samples spiked at four different levels (50, 100, 150 and
37 35 200 % of 0-spike level i.e. natural PAHs content) were analysed; the measured and theoretical
38 36 values were compared by linear regression. Recovery was obtained as a slope of this curve
39 37 multiplied by 100. The incubation time of the spike (in acetone solution) in a sample was 16
40 38 hours. For the purpose of uncertainty calculation, four repeated measurements of level spiked
41 39 at 150% of natural PAHs content (0-spike level) were done.
42 40

43 41 **Results and discussion**

44 42 Regardless of a large number of existing methods dedicated for analysis of PAHs and their
45 43 derivatives in various matrices (see Introduction), only few of published studies attempted to
46 44 compare and critically assess conceivable strategies of sample handling that is to be carried
47 45 out prior to the final determinative step. In following paragraphs the most common alternative
48 46 approaches to conduct isolation and purification procedures preceding HPLC/FLD
49 47 identification/quantification are documented; the choice of optimal strategy is discussed. In
50 48 addition, all relevant considerations on the basis of which the estimation of uncertainties of
51 49 measurement is made are described in detail.
52 50

1 *Optimisation of clean-up step*

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In the first part of our study the attention was paid to the optimisation of crude extracts clean-up. Based on our previous experience with purification of lipid-rich extracts obtained from animal tissues when analysing lipophilic pollutants in biota, GPC was identified as the method of choice.

Three different elution systems employing two types of polystyrene divinylbenzene copolymer gels (PSDVB), one “soft” gel - Bio-Beads S-X3 and two “rigid” gels - PL gel and Envirogel were tested (experimental configurations and procedure codes are listed in Table III). The latter type of gel (routinely used in our laboratory for clean-up of pesticide samples) offers some practical advantages since it is resistant to higher operating pressures and its fluctuation, hence the column lifetime is higher. Moreover, the overall ruggedness of clean-up step when employing rigid gel columns is improved. Another advantage of using rigid gels for GPC fractionation is a possibility to operate a column at different elution solvents without a risk of gel volume changes (swelling after switching between two miscible eluents).

Attention was also paid to the selection of optimal GPC mobile phase. In our earlier studies chloroform was used as an elution solvent in clean-up step employing BioBeads SX-3 column (Cejpek et al. 1995, Tomaniová et al. 2000, Tomaniová et al. 1998) Considering the work place hazard as well as ecological aspects posed by this solvent ethyl acetate-cyclohexane (1:1, v/v) solvent mixture, which is commonly used for purification of extracts in pesticide residue analysis was tested as an alternative for its replacement. For evaluation of performance of clean-up procedure, several parameters were considered: (i) efficiency of PAHs separation from co-extracts (purity of PAHs fraction), (ii) column capacity for co-extracts (sample equivalent that can be safely loaded into GPC system), (iii) the total solvent volume needed to accomplish GPC clean-up and (iv) time needed for complete elution of PAHs fraction.

Besides fish oil also rape seeds oil representing plant lipids was used to characterize the elution curves of these matrix constituents. The maximum load of lipids used for comparison of tested systems i.e. amount injected onto GPC column was 300 mg. The first set of experiments employing GPC-BB-C system revealed rape seed oil as less resolved from PAHs elution band (compared to fish oil and/or triolein). Representing the candidate for the worst separation scenario, rape seed oil was employed as model lipid for construction of elution profiles in the other GPC systems. Differences in elution profiles of rape seed oil and fish oil might be attributed to different types of triacylglycerols (contrary to plant matrices high amounts of long chain polyene fatty acids with 20 – 24 carbon atoms are present in fish oil) and other natural lipophilic compounds such as waxes, vitamins, sterols...) and/or triacylglycerols breakdown products possibly occurring in these materials. In GPC-BB-C system elution of pure triolein was also measured. Elution curve of this pure chemical was fairly narrower as compared to fish oil, the elution band of which was rather tailing, probably due to the presence of some lower molecular weight components (see above).

Based on results summarised in Table III, the columns packed with gel Bio-Beads S-X3 and/or PL gel operated with ethyl acetate-cyclohexane (1:1, v/v) as a mobile phase were identified as the most promising.

Similar trends in separation efficiencies of PAHs and co-extracts were recognised in the both soft gel systems. In GPC-BB-EC 8% of lipids weight loaded onto column occurred in PAHs fraction and in the system GPC-BB-C 6% of lipids weight loaded onto column occurred in PAHs fraction. However, it should be noted that the elution volume of PAHs fraction in ethyl acetate-cyclohexane (1:1, v/v) was higher - about by 30% than in chloroform. No differences in elution profiles between fish oil and rape seeds oil were observed anymore

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3 1 in acetate-cyclohexane (1:1, v/v) mobile phase. Diverse separation capabilities of tested GPC
4 2 systems are documented.

5 3 The column packed with rigid PL gel operated in chloroform was found fairly unsuitable
6 4 for purification purpose due to the severe overlap of PAHs and lipids fraction (about 30% of
7 5 injected lipids co-eluted with PAHs). On the other hand a complete separation of PAHs and
8 6 oil was achieved on PL gel column when using ethyl acetate-cyclohexane (1:1, v/v) as a
9 7 mobile phase. Unfortunately elution volume of PAHs fraction was more than twice higher as
10 8 compared to GPC-P-C. It should be also noted that in both GPC systems employing ethyl
11 9 acetate-cyclohexane (1:1, v/v) as a mobile phase (GPC-B-EC and GPC-P-EC), even
12 10 separation of PAH groups occurred. Complete separation of 3- and 5- ring, 3- and 6-ring and
13 11 also 4- and 6-ring PAHs on the PL gel column was obtained. Contrary to size exclusion
14 12 theory, the elution volume of PAHs increased with increasing amount of aromatic rings. The
15 13 reason of this reversed elution order (“small” PAHs eluted before those with higher molecular
16 14 weight) might be caused by strong unspecific interactions of analytes with gel structure, e.g. π
17 15 - π interactions. No adsorption phenomena occurred for lipids; their elution order follows the
18 16 principles of size exclusion chromatography only.

19 17 Regarding other tested systems consisting from column packed with rigid gel Envirogel
20 18 and mobile phase chloroform, under conditions listed in Table I complete separation from co-
21 19 extracts was obtained, however, PAHs were eluted at volume as large as 75 – 115 ml (i.e. 40
22 20 ml), which is three times higher compared to GPC-BB-C. On this account (good separation in
23 21 small solvent volume i.e. in a shorter time) the latter system was used throughout all
24 22 following experiments. On the other hand, it should be also noted, that higher amount of
25 23 sample is possible to load on this clean-up column and thus limits of detection/quantitation of
26 24 procedure could be set lower.

27 25 Considering the throughput of sample and purification efficiency, currently used
28 26 automated system GPC-BB-C (gel Bio-Beads S-X3 and mobile phase chloroform), was found
29 27 as the most effective for routine processing of crude PAHs extracts. In this system, PAHs
30 28 derivatives and PAHs sulphur analogues are eluted in the same fraction as the un-substituted
31 29 PAHs. Although the volume of chloroform used for clean-up step is very low compared to for
32 30 example extraction step, the current trend is to replace chlorinated solvents completely due to
33 31 their toxicity and ecological reasons. Therefore the system GPC-BB-EC (gel Bio-Beads S-X3
34 32 and mobile phase ethyl acetate-cyclohexane, 1:1, v/v) could be recommended as an
35 33 alternative. It should be noted that the total time needed for a sample is about 50% longer than
36 34 in system GPC-BB-C.

37 35
38 36
39 37 [Insert Table III about here]

40 38 41 39 *Optimisation of extraction step*

42 40
43 41 In general, the use of certified reference material (CRM) provides the best way to assess the
44 42 trueness of respective procedure. Unfortunately, at present no certified reference material
45 43 relevant to matrices involved in our study is commercially available. For this reason the
46 44 optimisation of extraction step was performed with fish tissue containing naturally incurred
47 45 PAHs. The evaluation of individual experiments efficiency was carried out on the relative
48 46 basis taking the highest result as the reference concentration (100%). However, it should be
49 47 noted that the evaluation of all experiments was somehow difficult because of low levels of
50 48 target analytes naturally present in the testing fish sample. The levels of some analytes were
51 49 close to the limits of quantification, which ranged, depending on the individual PAH, from
52 50 0.03 to 0.12 $\mu\text{g kg}^{-1}$.

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3 1 Procedure blank samples were handled together with extracts of tested material in the same
4 2 way as real sample. The values of PAHs determined in blanks were subtracted from obtained
5 3 results to compensate contamination from the external environment.
6 4

7 4
8 5 *Choice of a suitable extraction solvent.* To identify the most efficient extraction solvent,
9 6 experiments employing sonication for PAHs isolation were carried out. Chloroform, hexane-
10 7 acetone (1:1, v/v) and hexane-dichloromethane (1:1, v/v) were used for this purpose. Relative
11 8 efficiencies obtained within individual extraction experiments are summarised in Table IV.
12 9 For majority of all target analytes an extraction mixture hexane-acetone (1:1, v/v) was found
13 10 as the most efficient isolation solvent, which is in the agreement with studies already
14 11 mentioned (Shu et al. 2000, Budzinski et al. 1999, Chee et al. 1996, Lopez-Avila et al. 1995).
15 12 For all other validation experiments only this solvent mixture was used.
16 13

17 13
18 14 [Insert Table IV about here]
19 15

20 15
21 16 *Choice of a suitable extraction technique.* Within this experiment, Soxhlet extraction,
22 17 sonication and saponification, i.e. techniques, which are commonly used for the isolation of
23 18 PAHs from lipids and protein-rich matrices, were tested. The relative efficiencies of
24 19 individual extraction methods are summarised in Table V. Based on the previous experiment
25 20 hexane-acetone (1:1, v/v) was chosen as an extraction solvent for sonication and Soxhlet
26 21 extraction. The analyte content obtained by Soxhlet extraction was set as 100 %. In overall,
27 22 generated data showed - with a few exceptions, practical equivalency of all the tested isolation
28 23 techniques in terms of 2- and 3-ring PAHs (Naph, Ace, Flt, DBT, Phe, Ant, 2-MeAnt)
29 24 extraction efficiency. For the 4, 5 and 6- ring PAHs the best recoveries were achieved by
30 25 Soxhlet extraction.
31 26

32 26
33 27 Since the Soxhlet extraction was recognised as the most suitable, in following
34 28 experiments the bias of extraction step was determined only for this approach. As shown in
35 29 Table V, recoveries for all analytes ranged within 70 – 108%. The lowest recoveries were
36 30 found for the most volatile PAHs i.e. Naph and 1-MeNaph.
37 31

38 31
39 32 [Insert Table V about here]
40 33

41 33 *Estimation of uncertainty*

42 34

43 35 The uncertainty of PAHs measurement (12 of 16 PAHs defined by US EPA as priority
44 36 pollutants) was quantified using experimental data generated by optimised analytical
45 37 procedure consisting of following steps (i) Soxhlet extraction employing hexane-acetone (1:1,
46 38 v/v), (ii) GPC clean-up employing gel Bio-Beads S-X3 and mobile phase chloroform and (iii)
47 39 HPLC/FLD for identification and quantitation of target analytes. Two alternative approaches
48 40 were applied in estimating the overall uncertainty.
49 41

50 41
51 42 *The “Top-down” approach.* Considering that only a small number of the possible sources of
52 43 uncertainty make a significant contribution to the total uncertainty, components that are more
53 44 than 3-4 times smaller than the largest component can usually be ignored. Based on ISO
54 45 21748:2004 (ISO 2004), only the most relevant uncertainty contributions i.e. recovery of
55 46 analytical procedure, repeatability of analysis and purity of calibration standards are sufficient
56 47 to be involved in “top-down” calculation of the total uncertainty. Generally, “top-down”
57 48 represents simpler, although not so extensive approach for estimation of uncertainty as
58 49 compared to the “bottom-up” approach.
60 49

In our study the uncertainty of recovery was estimated on the basis of experiments described in paragraph 'Recovery experiments' and calculated by formula for the rectangular distribution:

$$u_{REC} = \frac{0.5 \times (1 - REC)}{\sqrt{3}} \quad (3)$$

Contribution of repeatability was expressed as a relative standard deviation (RSD, %) of the overall procedure (n=6):

$$u_{RSD} = RSD \quad (4)$$

Calculation of uncertainty of standard solution purity was based on a manufacture certificate information and calculated as

$$u_{STD} = \frac{u_p}{\sqrt{3}} \quad (5)$$

where u_p is a standard uncertainty (%) of standard solution purity declared in the manufacture certificate.

The total uncertainty was obtained by combination of the individual standard uncertainties using Equation (1). Results obtained by "top-down" approach are summarised in Table VI. As can be seen, the contribution of uncertainty of standard solution purity is not significant thus could be ignored.

[Insert Table VI about here]

The "Bottom-up" approach. The comprehensive "bottom-up" approach (Ellison et al. 2000) in the first phase requires identification and quantification of the individual relevant sources of uncertainty and in the next phase combination of the individual uncertainty estimates follows (see Equation (1)). Although this approach is rather complicated and laborious, it enables to distinguish, which sources of uncertainty are the most important and which are insignificant and can be neglected.

The process of the total uncertainty calculation is shown in Figure 3, where the analytical procedure is divided into specific blocks representing individual steps.

Generally, uncertainty contributions can be classified into two groups. The first one includes random and the second one systematic components of uncertainty. In the later case, the uncertainty contribution is assessed by considering a recovery and also information from other sources, including manufacture's specifications, calibration certificates or general knowledge about the behaviour and properties of relevant materials or instruments, etc. Both random and systematic uncertainty components of most individual steps cannot be determined directly as it is not possible to measure them in a separate way without considering contributions of the subsequent sample handling steps and the determinative HPLC/FLD procedure (see Figure 3). To determine the uncertainty (u), which can be assigned to each individual step, subtraction of variances (σ^2) of the following steps from the variance calculated for this particular step must be performed. In this text the symbol u_{1-8} is used for uncertainty associated with only one individual procedure step and symbol σ_{1-8} is applied for variance involving also contributions of the subsequent steps. Subscripts 1-8 correspond to codes (numbers) of the procedure steps as demonstrated in the Figure 3.

The principles of uncertainty calculation of individual procedure steps are described in the following paragraphs. Uncertainties of the individual analytical steps (u_{1-8}), which were involved in calculation of the combined uncertainty, are summarised in Table VII.

The variance (σ_1^2) of *weighing of sample (%)* was calculated based on the principle given by Equation (1):

$$\sigma_1^2 = u_{RSD1}^2 + u_w^2 \quad (6)$$

where u_{RSD1} is repeatability of weighing calculated using Equation (4) and u_w uncertainty of the balance calibration, calculated according to the Equation (7):

$$u_w = \frac{a}{m \times \sqrt{3}} \times 100 \quad (7)$$

where a is a weighting tolerance declared in the calibration certificate of the balance ($\pm 0.33 \times 10^{-3}$ g) and m is the weight of the sample (20 g).

Uncertainties of *extraction*, *evaporation (RVO 1, RVO 2)* and *clean-up (GPC)* steps are composed of two main components – random error (expressed as repeatability) and systematic error (represented by recovery). To estimate the uncertainty of recovery of each particular step Equation (3) was used. The repeatability was obtained as a relative standard deviation of repeated measurements ($n=6$), see Equation (4). The variances of these steps were obtained using Equation (1)

$$\sigma_i^2 = u_{REC,i}^2 + u_{RSD,i}^2 \quad (8)$$

where i represents procedure steps numbered 2, 3, 5, 6; σ_2^2 represents variance of extraction, σ_3^2 and σ_4^2 represent variances of evaporation steps and σ_6^2 represents variance of GPC clean-up.

For the calculation of the uncertainty of the “*make-up to 10 ml*” procedure, the repeatability (u_{RSD4}), uncertainty of volumetric glass calibration (u_{VOL}) and the influence of temperature-related solvent volume expansion (u_{ex}) were taken into account. Repeatability was estimated as a relative standard deviation of 10 results obtained by making-up of 10 ml volumetric flask to the mark with chloroform and subsequent weighing (Equation (4)).

Uncertainty of volumetric flask volume was calculated as

$$u_{VOL} = \frac{a}{\sqrt{3}} \quad (9)$$

where a is a tolerance provided by manufacturer ($\pm a$), i.e. 0.25 % for 10 ml flask.

The uncertainty of the solvent volume expansion was calculated as

$$u_{ex} = \frac{\Delta V}{V} \times 100 \quad (10)$$

where ΔV (ml) is a volume expansion of 10 ml of solvent calculated for temperature range from 20 to 24 °C and V is volume of the solvent (10 ml).

The variance (σ_4^2) was calculated using Equation (1):

$$\sigma_4^2 = u_{RSD4}^2 + u_{VOL}^2 + u_{ex}^2 \quad (11)$$

The variance of the “*transferring into acetonitrile*” (σ_7^2) was calculated as the uncertainty of syringe used for volume measurement:

$$\sigma_7^2 = u_s^2 + u_1^2 \quad (12)$$

where u_s is standard deviation of ten repeated weighing of relevant volume and $u_1 = \sigma_1$ is combined uncertainty of weighing (see Equation 7 and Figure 3).

The main contributions, which form the uncertainty associated with *HPLC* analysis are following:

- (i) the uncertainty of standard solution concentration
- (ii) uncertainty of volume measurement when using pipette for the transfer of sample within the dilution process (calculation of this uncertainty comprises repeatability, calibration of pipette and the influence of temperature on acetonitrile volume expansion)

- 1
2
3 1 (iii) making-up of this solution (see (ii)) to 10 and 25 ml (calculation of uncertainty
4 2 comprises of repeatability, calibration of volumetric flask and influence of
5 3 temperature on acetonitrile volume expansion; data for selected standard solution
6 4 at level 3-83 ng ml⁻¹ were used)
7 5 (iv) repeatability of 20 µl injection which comprises also fluctuation of detector
8 6 response

9 7 For the calculation of uncertainty of these components the same approach as was shown in the
10 8 previous text was applied, i.e.:

11 9 *ad (i):* for the calculation the Equation (5) was used

12 10 *ad (ii) and (iii):* for the calculation the Equations (4, 9, 10, 11) were used

13 11 *ad (iv):* for the calculation the Equation (4) was used.

14 12 The combined uncertainty σ_3 was calculated using Equation (1).

15 13
16 14 [Insert Figure 3 about here]
17 15

18 16 Based on a detailed analysis of uncertainty budget, extraction step and both evaporation
19 17 steps (RVO 1 and RVO 2) followed by GPC clean-up were identified as the most critical
20 18 phases of the analytical procedure (see Figure 4; Table 8). The examples of recognized
21 19 contributions of individual procedure steps to the uncertainty of various PAHs classes
22 20 measurement are shown in Figure 4 (representatives of 3-ring - Phe; 4-ring - Chr; 5-ring -
23 21 B[b]F and 6-ring - B[ghi]P PAHs were selected for comparison). It can be seen that with the
24 22 increasing molecular weight of analytes (what is accompanied by volatility decrease) the
25 23 contribution of the evaporation step to the total uncertainty declines, while the contribution of
26 24 uncertainty associated with the extraction step raises.

27 25
28 26 [Insert Figure 4 about here]
29 27

30 28 More detailed analysis of individual contributions to total uncertainty was carried out for
31 29 *HPLC/FLD* determinative step (see Figure 5). The repeatability of injection together with
32 30 uncertainty of standard dilution was identified as the most significant contributions to total
33 31 uncertainty of this step.

34 32
35 33 [Insert Figure 5 about here]
36 34

37 35 Comparing the total uncertainties obtained by both approaches (see Tables VII and VIII)
38 36 it is evident that there is no significant difference between the values of combined
39 37 uncertainties calculated by either the “*bottom-up*” or “*top-down*” strategy. The former
40 38 approach provided expanded uncertainty for individual PAHs measurements within the range
41 39 of 12 - 43%, the latter one from 20 to 42%. Based on these results it can be concluded that in
42 40 practice for the assessment of analytical result uncertainty simpler “*top-down*” approach is
43 41 possible to apply.

44 42 Reduction of uncertainties of isolation and, possibly, purification steps, could be
45 43 accomplished e.g. by utilization of automated extraction device (e.g. pressurised liquid
46 44 extraction, PLE). Relative standard deviations and also recoveries thus could be improved. To
47 45 minimize losses of more volatile analytes during vacuum evaporation and to improve
48 46 repeatability of this step, a keeper, e.g. 1-hexanol and 1-octanol (Matthiessen 1997) might be
49 47 used. Based on our experience, addition of 20% mixture of 1,2-propandiol in 2-propanol to
50 48 the sample solution prior to its evaporation is also a feasible option. Since uncertainties of the
51 49 most critical procedure steps are usually given by used instrumentation and by operator
52 50 (human factor), further improvement is difficult to control.

[Insert Table VII about here]

Conclusions

In this study an extraction efficiency of three routinely used extraction techniques were critically assessed. The Soxhlet extraction with hexane-acetone (1:1, v/v) was identified as the most efficient procedure for isolation of PAHs (including their methyl derivatives and sulphur analogues from fish samples as compared to sonication and saponification. To demonstrate purification efficiency of several conceivable GPC systems, the elution profiles of the main co-extracts and PAHs were constructed. As the most convenient, clean-up procedure employing column filled by gel BioBeads S-X3 and mobile phase chloroform was chosen. For most of target PAHs comparable results are obtained by optimised analytical procedure regardless the estimation of uncertainty is carried out using either "bottom-up" or "top-down" approaches. While the "bottom-up" approach is based on the construction of an "uncertainty budget" with estimates of each source of error, a simplified "top-down" approach uses experimental determination of the accuracy of a measurement method by calculating measures of repeatability, reproducibility and intermediate precision. In our study the application of "top-down" approach was shown to be more convenient option, which can be used in the case that data from method validation studies or proficiency testing are available.

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