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Optimization of the procedure for the determination of polycyclic aromatic hydrocarbons and their derivatives in fish tissue: Estimation of measurements uncertainty

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Abstract
Three alternative procedures were employed for the 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: (1) Soxhlet extraction, (2) batch extraction enhanced by sonication, and (3) saponification of the sample followed by re-extraction of analytes into hexane. Soxhlet extraction using hexane-acetone (1:1, v/v) was the most efficient extraction technique, with analyte recoveries in the range 70–108%. Within optimization of the 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 the most appropriate for purifying of crude extracts before 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 by employing two alternative approaches. Both provided similar results: the expanded uncertainties obtained for individual PAHs by the 'top–down' approach were in the range 9–53%, their values resulting from application of the 'bottom–up' approach 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 last decade, many studies concerned with the monitoring of PAH inputs (these may result from industry incineration, transport, uncontrolled spills, surface runoff and/or atmospheric deposition), their fluxes and fate, particularly in an aquatic environment, have been initiated (Hellou et al. 1997; Varanasi 1989; Baoliang et al. 2004; Van der Oost et al. 2003; Grimalt et al. 2004; Koh et al. 2004; Reeves et al. 2004; Ross and Oros 2004; Hellou et al. 2005; Shi et al. 2005). Note that the list of 16 US EPA PAHs is different from that established recently by European Union (2005).

Exposure of habitant biota to PAHs is often assessed through the 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 the analytical procedure is needed to generate reliable data. Within the optimization of the analytical process, the availability of reference materials with certified concentrations of target analytes incurred in the 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

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monitoring studies concerned with the occurrence of PAHs in various biotic matrices, are not commercially available.

Similar to the analysis of other organic pollutants, extraction of PAHs from the respective matrix represents a critical step in their determination scheme. Various techniques have been applied for the isolation of these substances from biotic and/or abiotic samples. Particularly Soxhlet extraction (Jaouen-Madoulet et al. 2000; Frankenius and Asklund 2001), batch extraction enhanced by sonication (Wenzel et al. 1997, 1998) and alkaline saponification followed by re-extraction (Chen and Lin 1997) represent common methods in routine laboratories. The use of supercritical fluid extraction (SFE) has also been described in some studies (De Castro et al. 1994; Taylor 1996; Heemken et al. 1997; Librando et al. 2004). However, its routine use for the processing of biotic samples is limited, probably due to a strong dependence of optimal parameters setting on sample composition (fat, moisture), which is the cause of a rather low robustness of SFE-based procedures. At present, a growing interest in alternative extraction techniques such as pressurized liquid extraction (PLE) (Lopez-Avila et al. 1994; Dean 1996; Heemken et al. 1997; Saim Nor'ashikin et al. 1997; Schantz et al. 1997; Wenzel et al. 1998) and microwave-assisted extraction (MAE) (Lopez-Avila et al. 1994; Leray et al. 1995; Budzinski et al. 1996; Latellier et al. 1996; Saim et al. 1997) can be noticed. Organic solvents such as methanol, acetone (Franzaring et al. 1992; Knulst et al. 1995), chloroform (Cejpek et al. 1995; Tomaniová et al. 1998, 2000), dichloromethane (Marvin et al. 1999; Howsam et al. 2000; Gratz et al. 2000), hexane (Weiss et al. 2000) and cyclohexane (Brouwer et al. 1994), or mixtures of them, e.g. hexane-acetone (Lopez-Avila et al. 1995; Chee et al. 1996; Budzinski et al. 1999; Shu et al. 2000), dichloromethane-acetone (Fischer et al. 1997; Popp et al. 1997; Tao et al. 2002), hexane-acetone-toluene (Berset et al. 1999) and hexane-dichloromethane (Hubert et al. 2001) are typically used for performance of the extraction step.

When analysing complex samples such as plants, biota or foodstuffs, many other matrix components are unavoidably co-isolated together with target analytes. Oils, waxes, essential oils and natural pigments such as carotenoids and chlorophylls are the most typical matrix components occurring in extracts obtained from plant samples. In animal tissues, lipids represent the major class of co-extracts. Effective removing of these substances, which can adversely affect identification and/or quantification of PAHs, is the crucial prerequisite for obtaining credible results. However, not only good separation of target analytes from co-extracts, but also the overall throughput of the clean-up step (which is largely dependent on the possibility of its automatization) is an important criterion to consider when choosing an optimal procedure. At present, gel permeation chromatography (GPC) employing fractionation according to differences in the molecular size (or more exactly effective molecular volume) of impurities and target contaminants together with adsorption chromatography based on differences in polarity/chemical structure of sample components, represent nowadays dominating purification techniques applied in the processing of primary extracts in PAHs analysis. The GPC procedure may employ several types of gels for the purification of primary extracts, e.g. styrene divinylbenzene copolymer gels Bio-Beads S-X3 (Czuczwa and Alford-Stevens 1989; Kaupp and Sklorz 1996), S-X12 (Fernández et al. 1988), XAD-2 (Codina et al. 1994), PL gel (Schantz et al. 1997) and Phenogel (Nyman et al. 1993). The elution of sample components is usually carried out by different elution solvents, e.g. toluene (Kaupp and Sklorz 1996), dichloromethane (Schantz et al. 1997), a mixture of dichloromethane-cyclohexane (Kaupp and Sklorz 1996) and ethyl acetate-cyclohexane (Czuczwa and Alford-Stevens 1989). As regards adsorption chromatography, the use of the following sorbent/eluent combinations was reported in the literature: (1) silica with cyclohexane (Karl and Leinemann 1996; Popp et al. 1997; Tao et al. 2002), hexane (Chee et al. 1996; Falcón et al. 1996; Berset et al. 1999), pentane (Budzinski et al. 1999), (2) florisil with hexane (Wenzel et al. 1997), dichloromethane (Wang et al. 1999) and their mixtures (Chen and Lin 1997), and (3) alumina with dichloromethane (Marvin et al. 1999) and mixtures of pentane-dichloromethane (Jaouen-Madoulet et al. 2000).

As regards the determinative step, both gas chromatography coupled with mass spectrometry (GC/MS) and reversed-phase high-performance liquid chromatography employing fluorimetric detector (HPLC/FLD) are used for quantification (De Boer and Law 2003). Nowadays, several dedicated HPLC as well as GC columns are available on the market for separation of complex PAH mixtures.

Since no measurement is entirely free of random and/or systemic errors, uncertainties in the results unavoidably exist. According to the Eurachem Guide (Ellison et al. 2000), uncertainty is defined as a 'parameter, associated with the result of a measurement, that characterises the dispersion of the values that could reasonably be attributed to the measurand'. In practice, information on uncertainty is needed in a test report whenever (1) it is relevant to the validity or application of the test results, (2) a client so requires it for a particular purpose, and (3) the uncertainty may affect compliance to a

specification limit. Especially the last requirement for uncertainty declaration is important in PAH analysis, the results of which are dedicated to the decision-making process. Supposing, for example, that hygienic limits established for toxic chemicals occurring in specified (environmental/food) matrices are to be controlled, then the uncertainty value has an obvious implication for the interpretation of the analytical results (compliance – non compliance). Detailed analysis of uncertainty sources can also provide identification of critical points in an analytical flowchart. Identification of the uncertainty source is an important stimulus for further improvements of method performance and, consequently, for achieving better quality generated data.

Uncertainty can be expressed as a standard uncertainty. The relationship between the combined standard uncertainty $u_c(y)$ of y and the uncertainty of the independent parameters $x_1, \ldots, x_n$ on which it depends is characterized by:

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

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

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

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

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

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

Alternative concepts aimed at a practical and understandable way of measurement uncertainty calculations based mainly on already existing quality control and validation data, namely the use of certified reference materials (CRM), participation in interlaboratory comparisons (proficiency testing) and recovery tests are described in the *Handbook for Calculation of Measurement Uncertainty in Environmental Laboratories* (Magnusson et al. 2003).

The presented study concerning PAHs analysis was focused on the following tasks: (1) a critical assessment of extraction efficiencies of most conceivable isolation techniques represented by Soxhlet extraction, batch extraction enhanced by sonication and saponification of the sample followed by re-extraction; (2) to find a suitable experimental configuration of GPC for the purifying of crude extracts within determination of PAHs and some of their derivatives in fish samples; and (3) an estimation of uncertainties of optimized methods frequently employed in food analysis (using 'bottom–up' and 'top–down' approaches) with respect to their practical use.

Materials and methods

Experimental materials

The sample of smoked fish (mackerel) used for optimization of the analytical procedure was obtained from the common market of the Czech Republic. Before homogenization in a blender, the skin and bones were removed. A homogenized sample (3 kg fish fillets) was stored at -20°C.

Chemicals and materials

Chloroform and acetone (analytical reagent grade, Lachema Brno, Brno, Czech Republic) were redistilled in glass before use. Acetonitrile (gradient grade, for chromatography), hexane (for organic trace analysis), dichloromethane (for gas chromatography), methanol (gradient grade, for chromatography) cyclohexane (for gas chromatography; all Merck, Darmstadt, Germany) and ethyl acetate (for pesticide residue analysis; Scharlau, Spain, Barcelona) were used as supplied. Deionized water was obtained from Milli-Q water purification system (Millipore, Bedford, MA, USA). Anhydrous sodium sulphate (Penta Praha, Praha, Czech Republic) was dried at 500°C for 5 h, then stored in a tightly capped

glass bottle. Potassium hydroxide (analytical reagent grade; Penta Praha, Praha, Czech Republic) was used for saponification.

The standard mixture 1647d of 16 priority PAHs: naphthalene (Naph), acenaphthene (Ace), fluorene (Fln), phenanthrene (Phe), anthracene (Ant), fluoranthene (Flt), pyrene (Pyr), benz[*a*]anthracene (B[*a*]A), chrysene (Chr), benzo[*b*]fluoranthene (B[*b*]F), benzo[*k*]fluoranthene (B[*k*]F), benzo[*a*]-pyrene (B[*a*]P), dibenz[*a*,*h*]anthracene (DB[*ah*]A), benzo[*g*,*h*,*i*]perylene (B[*ghi*]P) and indeno[1,2,3-*cd*]pyrene (I[*cd*]P) dissolved in acetonitrile was supplied by the National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA). Standards of individual PAHs derivatives: 1-methyl-naphthalene (1-MeNaph), dibenzothiophene (DBT), 2-methylanthracene (2-MeAnt), 1-methyl-pyrene (1-MePyr), 5-methylchrysene (5-MeChr), benzo[*b*]naphtho[2,1-*d*]thiophene (B[*b*]N[*d*]T), benzo[*e*]pyrene and (B[*e*]P) dissolved in acetonitrile were supplied by Dr Ehrenstorfer (Ausburg, Germany). The purity of individual standards was not less than 95%. Working standard solutions were prepared in acetonitrile and stored at 4°C.

Before use, all glassware was washed with detergent, rinsed with distilled water and acetone, and then heated.

Equipment

A laboratory blender (Waring blender, 38BL-40, Waring Commercial, New Hartford, CT, USA) was used for the homogenization of samples; an ultrasonic bath Sonorex RK 510 (Bandeline, Berlin, Germany) and a Soxhlet extractor with cellulose extraction thimbles (Whatman, Maidstone, UK) were used for sample extractions.

An automated GPC (gel permeation chromatography) system consisting of 305 MASTER pump, fraction collector, automatic regulator of loop 231 XLI, microcomputer (software 731 PC via RS232C), dilutor 401C (GILSON, Paris, France) and stainless steel columns (1) 500 × 8 mm i.d. packed with gel Bio-Beads S-X3, 200–400 mesh about 37–75 μm (Bio-Rad Laboratories, USA) and (2) 500 × 7.5 mm i.d. packed with PL gel, particles size 10 μm, pores size 50 Å (Polymer Laboratories, Shropshire, UK) and (3) two columns connected in series packed with Envirogel, with dimensions 150 × 19 and 300 × 19 mm i.d., particles size 15 μm, pores size 100 Å (Waters, Milford, MA, USA) were used for the clean-up of extracts.

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

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

Analytical procedures

The procedure flow chart is shown in Figure 1.

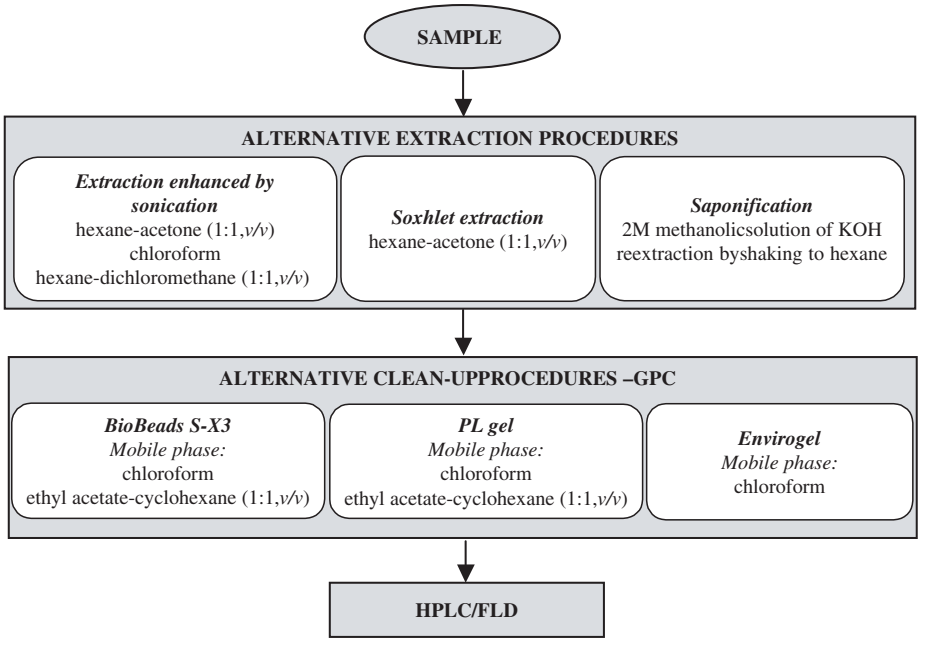

Figure 1. Procedure flow chart.

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

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

Saponification. Homogenized sample (10 g) was transferred into a flask with 50 ml 2 M methanolic solution of potassium hydroxide and boiled under reflux for 2 h. The apparatus was covered with aluminium foil to avoid access of daylight (to prevent the risk of photo degradation). The content of the flask was filtered to a separatory funnel and then extracted by intensive shaking for 5 min with 50 ml hexane. The extraction was repeated with separated water layer under the same conditions. Combined hexane layers were filtered through a layer of anhydrous sodium sulphate, carefully evaporated by rotatory vacuum evaporator at 40°C just to dryness and quantitatively transferred into a 5-ml volumetric flask by chloroform.

Clean-up. Experimental set-ups of all gel/mobile phase combinations used for optimization of crude extract purification are summarized in Table I.

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

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

An example of PAH determination in standard solution and a fish sample by HPLC/FLD is shown in Figure 2.

Recovery experiments. Because of the unavailability of reference fish tissue with a certified content of PAHs, an alternative strategy represented by spiking the matrix by target analytes was used for a trueness check. It should be emphasized that spiking may affect the recovery information generated this way due to the potential difference between the extractability of naturally incurred and in solvent spiked target analytes (in the latter case the extraction of analytes is more straightforward). In our

Table I. Characterization of GPC systems used for PAHs—co-extract separation.

GEL	Column dimensions (mm)	Particle size/pore size	Mobile phase	Flow rate of mobile phase (ml min^{-1})[a]	Code of procedure
Bio-Beads S-X3	500 × 8	200–400 mesh (about 37–75 µm)	chloroform	0.6	GPC-BB-C
			EtAc-CyH (1:1, v/v)*	0.6	GPC-BB-EC
PL gel	500 × 7.5	10 µm/50 Å	chloroform	0.6	GPC-P-C
			EtAc-CyH (1:1, v/v)*		GPC-P-EC
Envirogel	450 × 19 (150 × 19 and 300 × 19)	15 µm/100 Å	chloroform	3.0	GPC-E-C

*Ethyl acetate-cyclohexane (1:1, v/v).
[a]The same flow rate was used for similar column dimension (GPC-BB and GPC-P set-up).

Table II. FLD settings for PAHs determination.

PAHs	Time window (min)	λ excitation (nm)	λ emission (nm)
Naph, 1-MeNaph	0–1.0	217	338
Ace, Fln	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

experiments, samples spiked at four different levels (50, 100, 150 and 200% of zero-spike level, i.e. natural PAHs content) were analysed; the measured and theoretical values were compared by linear regression. Recovery was obtained as a slope of this curve multiplied by 100. The incubation time of the spike (in acetone solution) in a sample was 16 h. For the purpose of uncertainty calculation, four repeated measurements of level spiked at 150% of natural PAHs content (zero-spike level) were performed.

Results and discussion

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

Optimization of the clean-up step

In the first part of our study, attention was paid to the optimization of the clean-up of crude extracts. Based on our previous experience with the purification of lipid-rich extracts obtained from animal tissues when analysing liphophilic 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 (for the experimental configurations and procedure codes, see Table I). The latter type of gel (routinely used in our laboratory for the clean-up of pesticide samples) offers some practical advantages since it is resistant to higher operating pressures and their fluctuation, hence the column lifetime is higher. Moreover, the overall ruggedness of the clean-up step when employing rigid gel columns is improved. Another advantage of using rigid gels for GPC fractionation is the 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 a clean-up step employing BioBeads SX-3 column (Cejpek et al. 1995; Tomaniová et al. 1998, 2000). Considering the workplace hazard as well as ecological aspects posed by this solvent ethyl acetate-cyclohexane (1:1, v/v) solvent mixture, which is commonly used for the purification of extracts in pesticide residue analysis, was tested as an alternative for its replacement. For the evaluation of performance of the clean-up procedure, several parameters were considered: (1) the efficiency of PAH separation from co-extracts (purity of PAH fraction), (2) column capacity for co-extracts (sample equivalent that can be safely loaded into the GPC system), (3) the total solvent volume needed to accomplish GPC clean-up and (4) the time needed for complete elution of the PAH fraction.

Besides fish oil, rape seeds oil representing plant lipids were also used to characterize the elution curves of these matrix constituents. The maximum load of lipids used for comparison of tested systems, i.e. the amount injected onto GPC column, was 300 mg. The first set of experiments employing the GPC-BB-C system revealed rape seed oil as less resolved from the PAH elution band (compared with fish oil and/or triolein). Representing the candidate for the worst separation scenario, rape seed oil was employed as a model lipid for the 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 present in fish oil) and other natural liphophilic compounds such as waxes, vitamins, sterols, etc.) and/or triacylglycerol breakdown products possibly occurring in these materials. In the GPC-BB-C system, elution of pure triolein was also measured. The elution curve of this pure chemical was narrower as compared with fish oil,

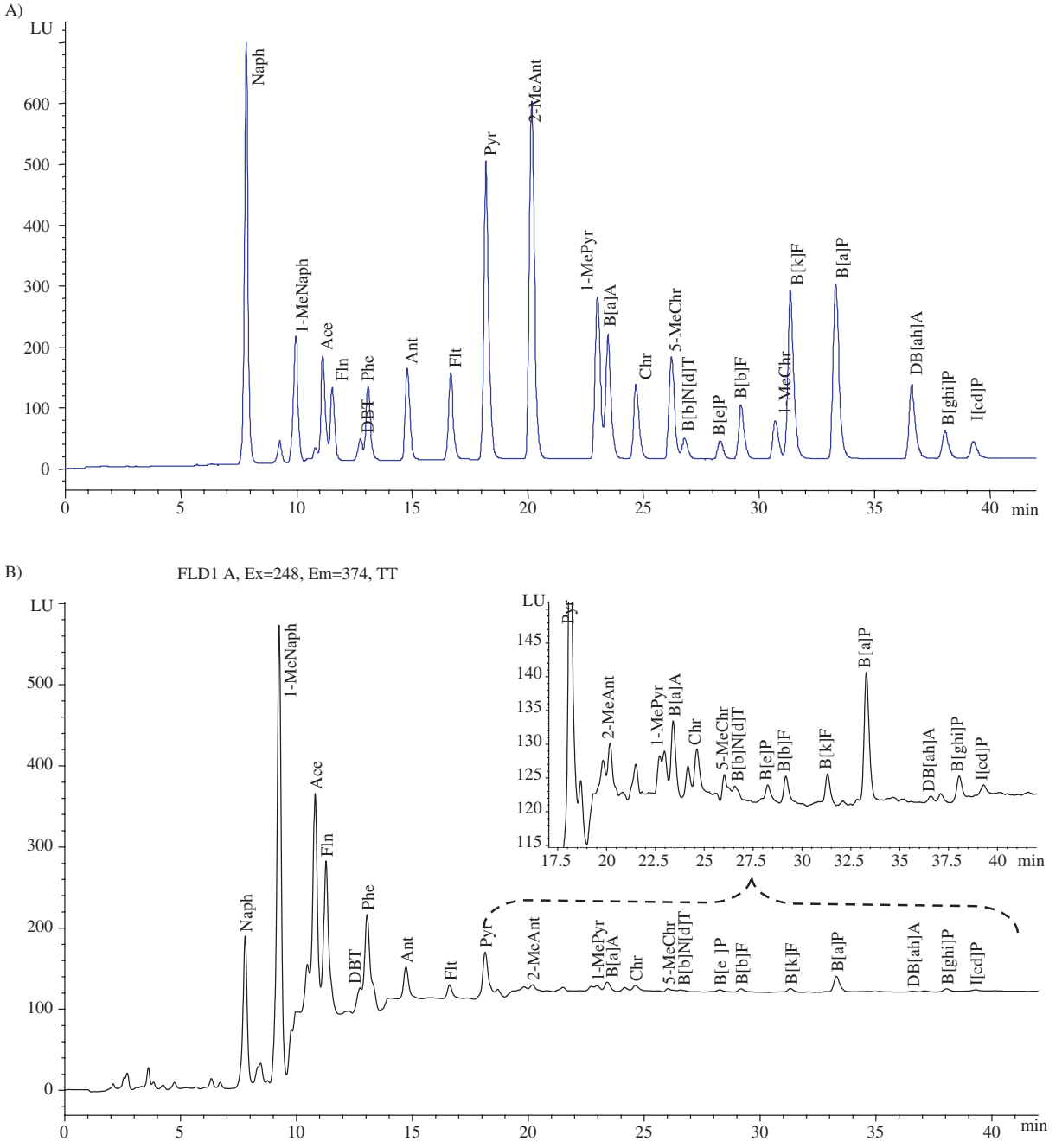

Figure 2. HPLD/FLD analysis: (A) standard mixture (PAHs from 3 to 83 ng ml^{-1}) and (B) purified fish extract (80 mg of matrix-equivalent corresponding to 20 µl injected).

which elution band was rather tailing, probably due to the presence of some lower molecular weight components (see above).

Based on the results summarized 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 recognized in both soft gel systems. In GPC-BB-EC, 8% of lipids' weight loaded onto the column occurred in the PAH fraction, and in system GPC-BB-C, 6% of lipids' weight loaded onto the column occurred in the PAH fraction. However, note that the elution volume of the PAH fraction in ethyl acetate-cyclohexane (1:1, v/v) was higher (by about 30%) than in chloroform. No differences in elution profiles between fish oil and rape seeds oil were observed in the acetate-cyclohexane (1:1, v/v) mobile phase. The diverse separation capabilities of tested GPC systems are documented.

The column packed with rigid PL gel operated in chloroform was found to be fairly unsuitable for purification purposes due to the severe overlap of

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

Code of procedure	PAH fraction (ml)	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 the percentage of the lipid fraction from the total lipids loaded on the column that is co-eluted with the PAH fraction.

PAHs and the lipids' fraction (about 30% of injected lipids co-eluted with PAHs). On the other hand, a complete separation of PAHs and oil was achieved on a PL gel column when using ethyl acetate-cyclohexane (1:1, v/v) as a mobile phase. Unfortunately, the elution volume of the PAH fraction was more than twice higher as compared with GPC-P-C. Note that in both GPC systems employing ethyl acetate-cyclohexane (1:1, v/v) as a mobile phase (GPC-B-EC and GPC-P-EC), even separation of the PAH groups occurred. Complete separation of 3- and 5-ring, 3- and 6-ring, and 4- and 6-ring PAHs on the PL gel column was obtained. Contrary to size exclusion theory, the elution volume of PAHs increased with an increasing amount of aromatic rings. The reason of this reversed elution order ('small' PAHs eluted before those with higher molecular weight) might be caused by strong unspecific interactions of analytes with gel structure, e.g. π–π interactions. No adsorption phenomena occurred for lipids; their elution order follows the principles of size exclusion chromatography only.

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

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

Optimization of extraction step

In general, the use of CRM provides the best way to assess the trueness of the respective procedures. Unfortunately at present no CRM relevant to matrices involved in our study is commercially available. For this reason, the optimization of the extraction step was performed with fish tissue containing naturally incurred PAHs. The evaluation of individual experiments' efficiencies was carried out on a relative basis taking the highest result as the reference concentration (100%). However, note that the evaluation of all experiments was somehow difficult because of low levels of target analytes naturally present in the testing fish sample. The levels of some analytes were close to the limits of quantification, which ranged, depending on the individual PAH, from 0.03 to 0.12 $\mu g\,kg^{-1}$.

Procedure blank samples were handled together with extracts of tested material in the same way as a real sample. The PAHs determined in blanks were subtracted from the obtained results to compensate for contamination from the external environment.

Choice of a suitable extraction solvent. To identify the most efficient extraction solvent, experiments employing sonication for PAH isolation were carried out. Chloroform, hexane-acetone (1:1, v/v) and hexane-dichloromethane (1:1, v/v) were used for this purpose. Relative efficiencies obtained within individual extraction experiments are summarized in Table IV. For the majority of all target analytes, an extraction mixture hexane-acetone (1:1, v/v) was the most efficient isolation solvent, which is in the agreement with the literature (Lopez-Avila et al.

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

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

*Hexane-acetone (1:1, v/v) was set as 100%.
[a]Content of PAHs was calculated on a fresh weight basis (the moisture of the sample was 62.5%).

1995; Chee et al. 1996; Budzinski et al. 1999; Shu et al. 2000). For all other validation experiments only this solvent mixture was used.

Choice of a suitable extraction technique. Within this experiment, Soxhlet extraction, sonication and saponification, i.e. techniques commonly used for the isolation of PAHs from lipids and protein-rich matrices, were tested. The relative efficiencies of individual extraction methods are summarized in Table V. Based on the previous experiment, hexane-acetone (1:1, v/v) was chosen as an extraction solvent for sonication and Soxhlet extraction. The analyte content obtained by Soxhlet extraction was set as 100%. Overall, generated data showed — with a few exceptions — practical equivalency of all the tested isolation techniques in terms of 2- and 3-ring PAHs (Naph, Ace, Fln, DBT, Phe, Ant, 2-MeAnt) extraction efficiency. For the 4-, 5- and 6-ring PAHs, the best recoveries were achieved by Soxhlet extraction.

Since the Soxhlet extraction was recognized as the most suitable, in the following experiments the bias of the extraction step was determined only for this approach. As shown in Table V, recoveries for all analytes ranged within 70–108%. The lowest recoveries were found for the most volatile PAHs, i.e. Naph and 1-MeNaph.

Estimation of uncertainty

The uncertainty of PAHs measurement (12 of 16 PAHs defined by the US EPA as priority pollutants) was quantified using experimental data generated by an optimized analytical procedure consisting of following steps: (1) Soxhlet extraction employing hexane-acetone (1:1, v/v), (2) GPC clean-up employing gel Bio-Beads S-X3 and mobile phase chloroform, and (3) HPLC/FLD for the identification and quantitation of target analytes. Two alternative approaches were applied in estimating the overall uncertainty.

'Top–down' approach. Considering that only a small number of the possible sources of uncertainty make a significant contribution to the total uncertainty, components that are more than three to four times smaller than the largest component can usually be ignored. Based on ISO 21748:2004 (2004a), only the most relevant uncertainty contributions, i.e. recovery of the analytical procedure, the repeatability of analysis and the purity of calibration standards are sufficient to be involved in the 'top–down' calculation of the total uncertainty. Generally, 'top–down' represents a simpler, although not so extensive, approach for the estimation of uncertainty as compared with the 'bottom–up' approach.

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

*Soxhlet extraction was set as 100%.
[a]Content of PAHs was calculated on a fresh weight basis (the moisture of sample was 62.5%).

In the present study, the uncertainty of recovery was estimated based on experiments described above and calculated by an equation for the rectangular distribution:

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

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

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

The calculation of uncertainty of standard solution purity was based on a manufacture certificate information:

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

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

Total uncertainty was obtained by combination of the individual standard uncertainties using equation (1). Results obtained by the 'top–down' approach are summarized in Table VI. The contribution of uncertainty of standard solution purity is not significant and thus could be ignored.

Table VI. Uncertainties of the optimized analytical procedure obtained by a 'top–down' approach (%).

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

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

'Bottom–up' approach. The comprehensive 'bottom–up' approach (Ellison et al. 2000) in the first phase requires the identification and quantification of the individual relevant sources of uncertainty and in the next phase combination of the individual

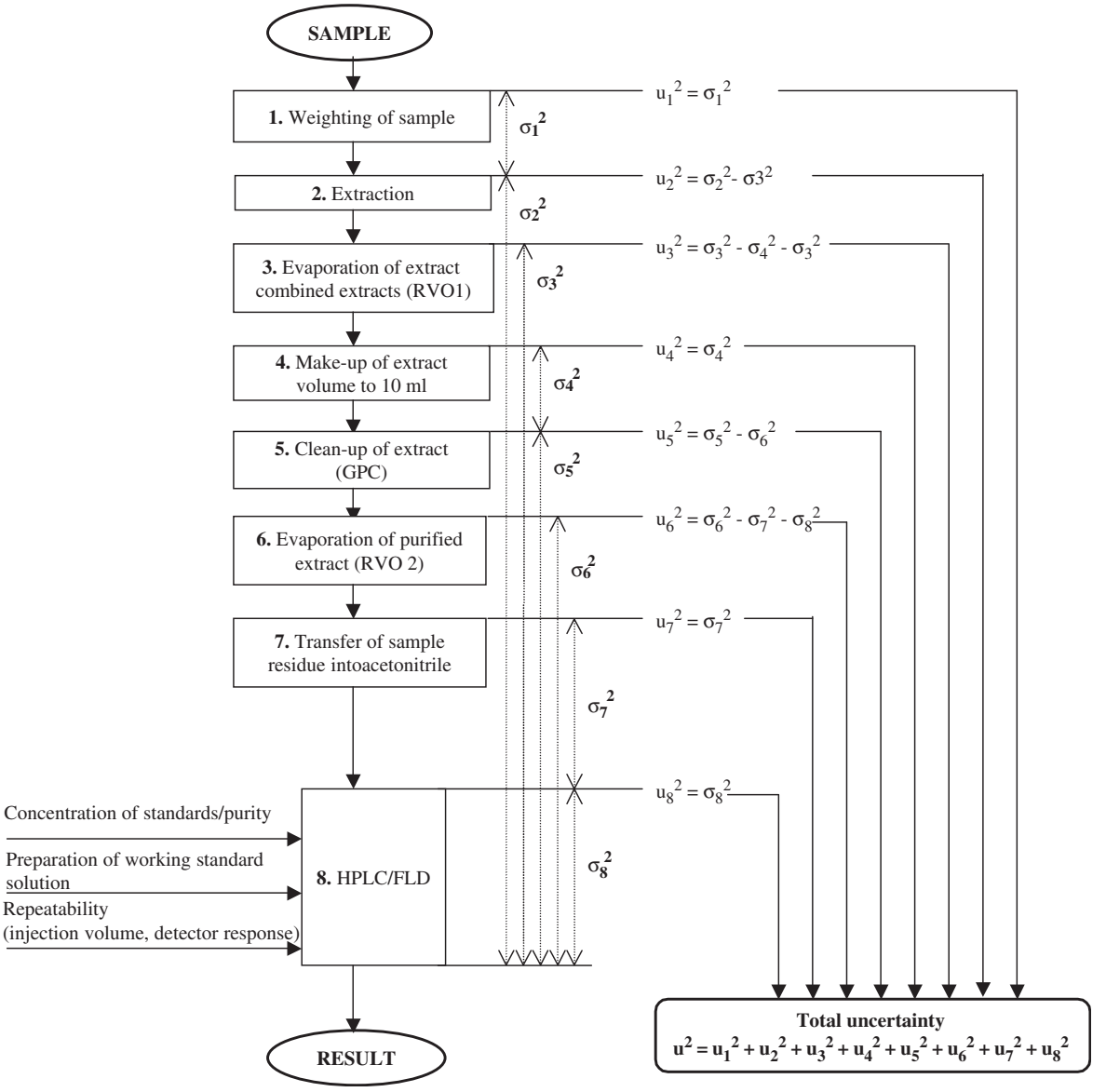

Figure 3. Flow chart of the total uncertainty calculation.

uncertainty estimates follows (equation 1). Although this approach is rather complicated and laborious, it enables one 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 includes random and the second systematic components of uncertainty. In the later case, the uncertainty contribution is assessed by considering a recovery and also information from other sources, including the manufacturer'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 separately without considering contributions of the subsequent sample-handling steps and the determinative HPLC/FLD procedure (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. Here, u_{1-8} is used for uncertainty associated with only one individual procedure step and σ_{1-8} is applied for variance also involving contributions of the subsequent steps. Subscripts 1–8

correspond to codes (numbers) of the procedure steps, as shown in Figure 3.

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

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

$$\sigma_1^2 = u_{\mathrm{RSD1}}^2 + u_w^2 \tag{6}$$

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

$$u_w = \frac{a}{m \times \sqrt{3}} \times 100 \tag{7}$$

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 ($n = 6$) (equation 4). The variances of these steps were obtained using equation (1):

$$\sigma_i^2 = u_{\mathrm{REC},i}^2 + u_{\mathrm{RSD},i}^2 \tag{8}$$

where i is procedure steps 2–6; σ_2^2 is the variance of extraction, σ_3^2 and σ_4^2 are variances of evaporation steps, and σ_6^2 is the 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 ten results obtained by the making-up of a 10-ml volumetric flask to the mark with chloroform and subsequent weighing (equation 4).

The uncertainty of volumetric flask volume was calculated as:

$$u_{\mathrm{VOL}} = \frac{a}{\sqrt{3}} \tag{9}$$

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

The uncertainty of the solvent volume expansion was calculated as:

$$u_{\mathrm{ex}} = \frac{\Delta V}{V} \times 100 \tag{10}$$

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

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

$$\sigma_4^2 = u_{\mathrm{RSD4}}^2 + u_{\mathrm{VOL}}^2 + u_{\mathrm{ex}}^2 \tag{11}$$

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

$$\sigma_7^2 = u_s^2 + u_1^2 \tag{12}$$

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

The main contributions that form the uncertainty associated with HPLC analysis are the following:

(1) Uncertainty of the standard solution concentration.
(2) Uncertainty of volume measurement when using a pipette for the transfer of the sample within the dilution process (calculation of this uncertainty comprises repeatability, calibration of the pipette and the influence of temperature on acetonitrile volume expansion).
(3) Making-up of this solution (see above) to 10 and 25 ml (the calculation of uncertainty comprises repeatability, the calibration of the volumetric flask and the influence of temperature on acetonitrile volume expansion; data for a selected standard solution at level 3–83 ng ml^{-1} were used).
(4) Repeatability of a 20-µl injection that also comprises fluctuation of the detector response.

For the calculation of uncertainty of these components the same approach was used as shown above, i.e.:

- *ad* (1): for the calculation equation (5) was used.
- *ad* (2) and (3): for the calculation equations (4, 9–11) were used.
- *ad* (4): for the calculation the equation (4) was used.

The combined uncertainty σ_8 was calculated using equation (1).

Based on a detailed analysis of uncertainty budget, extraction step and both evaporation steps (RVO 1 and RVO 2) followed by GPC clean-up were identified as the most critical phases of the analytical procedure (Figure 5; Table VIII). The examples of recognized contributions of individual

Table VII. Uncertainties (%) of the optimized analytical procedure obtained by a '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 the 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
	u_1	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
	u_2	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
	u_3	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 $22\pm2^{\circ}$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
	u_4	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
	u_5	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
	u_6	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
	u_7	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
	u_8	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

$u_{1–8}$, total uncertainties of individual steps of the procedure (Figure 4).
$u_{8a–c}$, total uncertainties of individual operations related to HPLC/FLD.
u, total uncertainty of the procedure, calculated according to equation (1).
U, expanded uncertainty of the procedure (see equation 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).

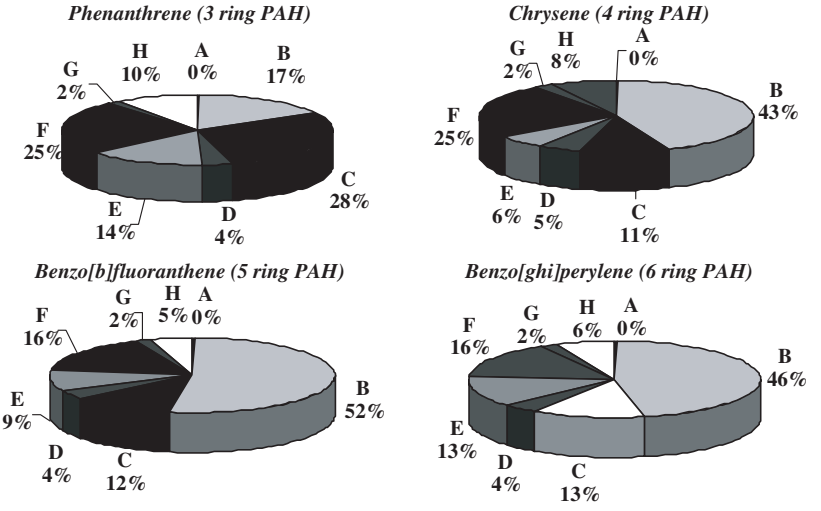

Figure 4. Contributions of partial uncertainties of the individual steps of procedure to the total uncertainty: A, weighting of the sample; B, extraction; C, RVO 1; D, make-up to 10 ml; E, GPC; F, RVO 2; G, transferring into acetonitrile; H, HPLC.

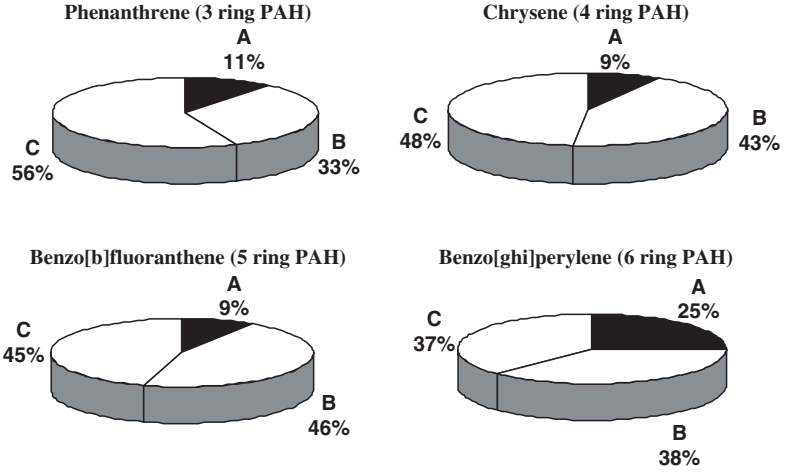

Figure 5. Contributions of partial uncertainties to the total uncertainty of the HPLC/FLD step: A, concentration of standard solution; B, diluting of working standard solution; C, repeatability.

procedure steps to the uncertainty of various PAH classes measurement are shown in Figure 4 (representatives of 3-ring — Phe; 4-ring — Chr; 5-ring — B[*b*]F; and 6-ring — B[*ghi*]P PAHs were selected for comparison). With the increasing molecular weight of analytes (what is accompanied by a decrease in volatility), the contribution of the evaporation step to the total uncertainty declines, while the contribution of uncertainty associated with the extraction step raises.

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

By comparing the total uncertainties obtained by both approaches (Tables VI and VII), it is evident that there is no significant difference between the combined uncertainties calculated by either the 'bottom–up' or 'top–down' strategies. The former approach provided expanded uncertainty for individual PAH measurements within the range 12–43%, the latter from 20 to 42%. Based on these results, it can be concluded that in practice for the assessment of the analytical result uncertainty, a simpler 'top–down' approach is possible.

Reduction of uncertainties of isolation and, possibly, of purification steps could be accomplished, for example, by use of an automated extraction device (e.g. pressurized liquid extraction). Relative standard deviations and recoveries thus could be improved. To minimize losses of more volatile

analytes during vacuum evaporation and to improve the repeatability of this step, a keeper, e.g. 1-hexanol and 1-octanol (Matthiessen 1997), might be used. Based on our experience, addition of a 20% mixture of 1,2-propandiol in 2-propanol to the sample solution before its evaporation is also a feasible option. Since uncertainties of the most critical procedure steps are usually given by the used instrumentation and by the operator (the human factor), further improvement is difficult to control.

Conclusions

In this study, an extraction efficiency of three routinely used extraction techniques was critically assessed. The Soxhlet extraction with hexane–acetone (1:1, v/v) was identified as the most efficient procedure for the isolation of PAHs (including their methyl derivatives and sulphur analogues from fish samples as compared with sonication and saponification. To demonstrate the 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 a column filled by gel BioBeads S-X3 and mobile phase, chloroform was chosen. Estimation of uncertainty calculated either by 'bottom–up' or 'top–down' approach provided comparable results for most of target PAHs. While the 'bottom–up' approach is based on the construction of an 'uncertainty budged' 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 the present study, the application of a 'top–down' approach was shown to be the more convenient option, which can be used in the case that data from method validation studies or proficiency testing are available. However 'bottom–up' approach is still recommended by EURACHEM as a method of the first choice. Moreover evaluation of individual source could be used for identification of critical steps in the analytical procedure.

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