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A comparison of the extraction procedures and quantification methods for the chromatographic determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish

O. Viegas[a,b], P. Novo[a], O. Pinho[a,b], I.M.P.L.V.O. Ferreira[a,*]

[a] REQUIMTE, Laboratório de Bromatologia e Hidrologia, Departamento de Ciências Químicas, Faculdade de Farmácia da Universidade do Porto, Portugal
[b] Faculdade de Ciências da Nutrição e Alimentação da Universidade do Porto, Portugal

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ABSTRACT

A method for analysis of 15 PAHs in charcoal-grilled meat/fish was established by high performance liquid chromatography and fluorescence detection. Gradient elution was performed with methanol/water/ethyl acetate. Maxima excitation and emission wavelengths were selected for each PAH. Retention times were very stable with coefficients of variation below 0.24% within analytical day and below 0.60% across analytical days. Two different methods of cleanup and pre-concentration steps were compared. Solvent extraction assisted by sonication carried out with *n*-hexane on 2 g of lyophilized meat or 1 g of lyophilized fish allowed to obtain high sensitivity, reproducibility and better extraction efficiency. Limits of quantification (LOQs, $s/n = 10$) were lower than 0.01 ng/g of meat wet weight and lower than 0.02 ng/g of fish wet weight for all PAHs (except for Na, Fl and IP that were lower than 0.1 ng/g). Two different quantification methods were compared. Standard addition method compensated PAHs losses due to incomplete extraction and it is recommended for analyses of grilled meat and fish samples that usually contain very low amounts of the eight high molecular weight PAHs (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA).

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are primary formed during incomplete burning (pyrolysis) of carbon-containing materials, such as oil, wood, garbage or coal. Environmental contamination and food processing are the main reasons for the presence of PAHs in foodstuff [1,2]. In mammalian cells PAHs undergo metabolic activation to diol epoxides that bind covalently to cellular macromolecules, including DNA, thereby causing errors in DNA replication and mutations that start the carcinogenic process [3].

The US Environmental Protection Agency (US-EPA) proposed to use a selection of 16 PAHs which are frequently found in environmental monitoring samples, namely, naphthalene (Na), acenaphthene (Ac), acenaphthylene (Ace), fluorene (F), anthracene (A), phenanthrene (Pa), fluoranthene (Fl), pyrene (P), benzo[a]anthracene (BaA), chrysene (Ch), benzo[b]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[a]pyrene (BaP), dibenzo[a,h]anthracene (DhA), benzo[g,h,i]perylene (BgP), indeno[1,2,3-cd]pyrene (IP) [4]. In 2005 EU recommended monitorization of 15 priority PAHs including eight high molecular weight from the US-EPA list (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA). BaP was chosen as a marker of the occurrence and carcinogenic potency of the entire class of carcinogenic and genotoxic PAHs [5]. EFSA CONTAM Panel (2008) concluded that BaP is not a suitable indicator for the occurrence of PAHs in food and that eight high molecular weight PAHs (PAH8) are the most suitable indicators of PAHs in food [6]. Recently, Commission Regulation (EU) n° 836/2011 of 19 August 2011 established analytical performance criteria for BaP, BaA, BbF and Ch in relevant food matrices [836/2011] [7].

The consumption of smoked meat or fish has been associated to high incidence of stomach cancer in some population that consume such products with high frequency [8]. The amount of PAHs in smoked meat and fish have been extensively reported [9–11]. However, information concerning grilled foods is scarce.

When food, particularly, meat and fish, are cooked over an open flame, PAHs are formed [12,13]. If the meat/fish is in direct contact with flame, pyrolysis of the fats from the meat/fish generates PAHs that can become deposited on its surface. Even if not in direct contact, fat dripping on to the flame or hot coals generates these compounds that are carried back on the surface of the meat/fish [13]. The presence of PAHs in charcoal grilled meat/fish should be a matter of concern and alert to consumers, because even if present in low levels, the intake of this type of food can be quite frequent and

* Corresponding author. Tel.: +351 222078926; fax: +351 222003977.
E-mail address: isabel.ferreira@ff.up.pt (I.M.P.L.V.O. Ferreira).

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representing a high portion (~120 g or more per meal). However, PAHs extraction and quantification in charcoal grilled meat and fish is difficult because they occur in food at ppb or lower levels and many other organic components such as proteins, lipids and compounds similar to PAHs that also result from thermal processing, such as heterocyclic aromatic amines that can be co-extracted from the matrix [14]. Additionally, meat and fish contain plenty of lipids, which have similar polarity to PAHs, the variable fat content influences extraction yield, and appropriate conditions for each type of food should be selected.

The most common methods for the isolation of PAHs from foods involve saponification of lipids by methanolic KOH solution followed by extraction procedures to isolate the PAHs-containing fraction. Different procedures are described for PAHs extraction, namely, liquid–liquid, Soxhlet or sonication extraction [14–16], solid-phase extraction (SPE) [13,15,17–19], solid-phase microextraction [20–22], supercritical-fluid extraction [23] and microwave-assisted extraction [24,25]. Owing to the complexity of the matrices these methods, in general, present inconstant recoveries and in some cases interfering peaks in the chromatograms.

High performance liquid chromatography with fluorescence or UV diode array detection has often been used for quantitative determination of PAHs [13–19,23–29]. The eluent usually used for chromatographic separation is acetonitrile–water under isocratic conditions [15] or gradient elution [24,25]. Water–methanol–acetonitrile [16] is also described for PAHs separation by high performance liquid chromatography/fluorescence detection (HPLC/FLD). Alternative elution solvents can be used to make PAHs analysis less expensive, namely methanol–water, however, in general poor peak resolution is obtained [14].

External calibration curve method is currently the most popular in the quantitative determination of PAHs in various food matrices [12–30]. However, analyte losses can occur in the course of sample preparation due to incomplete extraction, and cause underestimated results. At the same time, overestimated results can also be obtained (matrix effect) if the chromatographic separation of PAHs and co-extractive substances is inadequate. The use of the external calibration curve method for the quantitative evaluation of PAHs does not take into account the effect of systematic errors due to either sample preparation or chromatographic separation and detection. The standard addition method has not found use in the determination of PAHs in food matrices. This method implies the use of the analyte as an internal standard, the number of sample preparation operations increase, but the effect of systematic errors decreases, thus it can be a good choice when low levels of analyte are quantified.

The aim of this study was to compare two extraction procedures and two quantification methods for the accurate determination of polycyclic aromatic hydrocarbons in charcoal grilled meat and fish by HPLC/FLD using an acetonitrile free eluent. Fifteen EPA-priority PAHs were selected as they include the eight PAHs previously indicated as the most suitable indicators of the presence of carcinogenic and genotoxic PAHs in foodstuffs [6]. Two different extraction and clean-up approaches were adapted from methods described in the literature [15,24]. The first approach was based on saponification, followed by extraction on diatomaceous earth and use of tandem solid-phase extraction with propylsulphonic acid (PRS) and silica (SiO_2) SPE columns as stationary phase [15]. The second approach was based on extraction of PAHs from lyophilized meat/fish with *n*-hexane and clean-up with SiO_2 cartridges [24]. The extraction procedure that presented best sensitivity, precision and accuracy was used for comparison between quantification by external calibration curve method and standard addition method.

2. Materials and methods

2.1. Reagents and standards

All the solvents used were of HPLC grade (Sigma, St Louis, MO, USA). Water was purified with a Milli-Q System (Millipore, Bedford, MA, USA). The standard PAHs mixture in 1 ml of acetonitrile (Supelco, Bellefone, PA, USA) consisted of: 10 μg/ml of Na, Ac, Ace, F, A, Pa, Fl, P, BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA (47940-U, Supelco, Bellefonte, PA, USA). The glassware, mostly amber, was carefully washed and rinsed with distilled solvent (acetone and hexane) before use.

2.2. Apparatus

An ultrasonic bath (FungiLab SA, Barcelona, Spain) was used to carry out the extraction step. Separation and quantification of PAHs was performed by HPLC/FLD. The chromatographic analysis was carried out in an analytical HPLC unit (Jasco, Japan) equipped with one Jasco PU-1580 HPLC pump, a MD 910 and a type 7125 Rheodyne injector equipped with a 20 μL loop. The column was a C18 reversed phase: Supelcosil™ LC-PAH (25 cm length; 4.6 mm internal diameter; 5 μm particle size) (Supelco, Bellefonte, PA, USA), thermostated at 32.0 ± 0.2 °C. The Borwin PDA Controller Software (JMBS Developments, Le Fontanil, France) was also used. The mobile phase was as follows: solvent A: 75% methanol (in water); solvent B: 100% methanol, solvent C: 100% ethyl acetate with a flow rate 1 ml/min. The linear gradient program was: 0–18 min, 0–80% B in A; 18–19 min, 80–100% B in A; 19–20 min, 100–90% B in C; 20–28.5 min, 90–82% B in C; 28.5–37.5 min, 82–80% B in C; 37.5–40 min, 80–100% B in C, 40–45 min 100–0% B in A, rinsing and re-equilibration of column to the initial conditions. Excitation/emission wavelengths selected were 276/330 nm for Na, Ac and F; 250/336 nm for Pa; 250/402 nm for A; 270/460 nm for Fl; 270/390 nm for P, BaA and Ch; 260/430 nm for BbF; 290/410 nm for BkF, BaP, DhA, and BgP; 290/470 nm for IP.

2.3. Samples

The meat samples used in this study were obtained from the *Longissimus dorsi* muscle of middle-aged bovine carcasses. The meat was obtained from a major butchery in Porto, Portugal. The beef samples six steaks with 1.5 cm of thick, and weighing about 253 g (±49.4 g) were chilled for 24 h in a cooling room (5 ± 1 °C). Following the chilling process, all trimmable fat and connective tissue (epimysium) were removed from the *Longissimus dorsi* muscle.

Samples of fresh salmon were obtained in a fish market in the same city. Three fillets of salmon with 2 cm of thick and weighting 236.6 (±50.65 g) were prepared.

For preparation of charcoal barbecued meat and fish, a bed of charcoal was prepared and ignited using an appropriate device of 35 cm width, 52 cm length, and 15 cm height. When all flames had subsided, the bed was leveled by raking. The meat and fish were then barbecued over charcoal samples close to the heat source (10–12 cm). The meat and fish were turned once during grilling at half the total cooking time. No salt or oil was applied to meat and fish before or after grilling. Temperatures were measured by using a digital thermocouple (0560 9260, Testo 926, Lenzkirch, Germany) with a surface probe (0603 1992, Testo 926, Lenzkirch, Germany). The temperature near to the charcoal was 280–300 °C. To obtain "well-done" doneness, the grilling time was 9 min for beef, and 15 min for salmon. After cooking, the three samples of each kind of muscle food were mixed all together in a grinder, obtaining a representative and homogeneous amount of sample.

2.4. Sample pre-treatment, extraction and clean-up

Two different extraction approaches were adapted from methods described in the literature. The first approach (extraction method 1) for extraction and clean up procedures was based on the method described by Janoszka, et al. [15] for identification and quantitative analysis of five PAHs in meat. Meat samples were extracted by saponification, followed by extraction on diatomaceous earth and use of SPE columns filled with PRS. The eluted PAHs fraction was cleaned by SPE SiO_2 column, packed manually. Briefly, each meat/fish sample (5 g) was homogenized for 1 min shaking with a vortex shaker (Vortex mixer, EU-plug, VWR International, Darmstadt, Germany), in 25 ml of cold NaOH solution (1 M). Each sample was mixed with Extrelut refill material, diatomaceous earth (17 g) and the mixture obtained was placed in a 20 ml Extrelut column (Extrelut®, Merck, Darmstadt, Germany). Then the column was connected to a 500 mg PRS SPE column (Bond Elut PRS, 500 mg, 3 ml from Agilent Technologies, USA), where the PAHs fraction was retained, and eluted with a 95:5 (v/v) dichloromethane/toluene (60 ml). The dichloromethane extract was evaporated to dryness with a rotatory evaporator (Rotavapor Büchi RE-111, coupled with a water bath Büchi 461, BÜCHI, Flawil, Switzerland) at 40 °C and the residue was dissolved in *n*-hexane (1 ml), the residue was then placed on the top of the column which was manually packed with deactivated silica gel (10 g) and preconditioned by using *n*-hexane (25 ml). The column was eluted with 60:40 (v/v) *n*-hexane/dichloromethane (60 ml) to collect the PAHs fraction. The solvent was evaporated by rotatory evaporator and the PAHs residue was dissolved in acetonitrile (100 μl) and injected into the HPLC/FLD.

The second approach (extraction method 2) for extraction and clean up procedures was based on the method of Moret and Conte [18], applied for quantification of 15 PAHs in vegetable oils [18] and smoked meat [24]. A representative amount of meat sample was freeze-dried with a freeze dryer (Cryodos-90, from Telstar®, Terrassa, Spain) and reduced to a fine powder with a knife mill (Grindomix GM 200, Retsch, Hann, Germany). The lyophilization step has the advantage to eliminate water facilitating extraction of PAHs with *n*-hexane (24). Briefly, 2 g of meat lyophilized were weighed into a flask, added of 20 ml of *n*-hexane and sonicated for 1 h at room temperature. After this period the sample was filtered on paper, the solvent was evaporated in a round flask and taken near to the dryness with a rotatory evaporator at room temperature. The *n*-hexane residue was quantitatively transferred with 3 ml of *n*-hexane into a 5 g silica cartridge (Mega BE-Si, 5 g, 20 ml, from Agilent Technologies, USA) (previously washed with 20 ml of dichloromethane, dried completely by means of vacuum, and conditioned with 20 ml of *n*-hexane), and eluted through the column with 17 ml of a mixture of *n*-hexane/dichloromethane (70:30) (v/v). The first 8 ml of eluate were discharged and the following fraction, containing the PAHs was collected in a vial. The flow rate was adjusted at about 1 drop per second.

The collected fraction was evaporated to dryness under nitrogen stream at room temperature, in order to minimize volatile losses. The residue was dissolved in 100 μl of acetonitrile and injected into the HPLC/FLD.

2.5. Analytical performances

The validation of HPLC/FLD method for quantification of PAHs in barbequed meat and fish was accomplished by testing the linearity, the detection limit, the precision (repeatability and reproducibility) and the accuracy.

The linearity of the method was checked through the calibration curves, which were calculated for each PAH and obtained by linear regression of the peak area versus concentration of each PAH in the injected solution. The detection limit values (LODs) were based on a signal-to-noise ratio of 3:1, and the quantification limits (LOQs) were established as the amount of analyte that produces a signal-to-noise of 10:1.

One standard solution containing the mixture of PAHs was analyzed daily, repeating the analysis over three days. The repeatability was calculated as the RSD of peak areas and retention times across days. The reproducibility was studied by running three consecutive replications of the same mixture and calculating the RSD for peak areas and retention times.

The precision of the two extraction approaches was evaluated by estimating the standard deviation of three different extractions of the same meat sample. Recovery studies were carried out to determine the accuracy of the two extraction procedures. The extraction procedure that presented best precision and accuracy was further improved to guarantee reliable quantification of PAHs even if present in trace amounts.

The identities of the compounds were established by comparing the retention times of the peaks with those obtained from a standard mixture of PAHs and from spiked samples analyzed under the same conditions. Quantification of PAHs in meat and fish samples was performed by external calibration curve method and by standard addition method (using two fortified levels 20 and 40 ng of PAHs for fish samples and 10–20 ng/g for meat samples and unspiked samples).

2.6. Statistics

The averages of triplicate analysis were calculated for each PAH. The results were statistically analyzed by analysis of variance. Differences (*t*-test) were considered significant for $p < 0.05$. Statistical analyses were all performed with SPSS for Windows version 18 (SPSS Inc, Chicago, IL).

3. Results and discussion

3.1. Validation of HPLC/FLD method for PAHs separation

The mobile phase containing three solvents: 75% methanol (in water), 100% methanol, and 100% ethyl acetate was selected as an alternative elution, less expensive than acetonitrile for PHAs elution. The addition of ethyl acetate improved resolution between DhA and BgP. The gradient conditions described in Section 2.2 allowed resolving correctly all target compounds as shown in Fig. 1.

Excitation and emission wavelengths were selected from literature in order to choose the most appropriate excitation and emission wavelengths for each PAH under study, creating a new program to obtain higher sensitivity [19,24,27].

To evaluate the analytical performance of the HPLC-FLD method, calibration curve parameters, limits of detection, limits of quantification, repeatability or run-to-run precision and reproducibility or day-to-day precision were determined. Results are summarized in Table 1. Calibration curves were constructed by injecting in duplicate 7 diluted standard solutions (in the range 0.2–500 ng/ml) and plotting the mean peak area against PAHs standard concentration. For all peaks, there was a tight relationship between the amounts of each PAH and the detector response as indicated by *r* values that exceeded 0.99. Limits of detection ranged between 0.07 and 0.47 ng/ml and limits of quantification ranged between 0.22 and 1.44 ng/ml. Results indicate that the precision (repeatability and reproducibility) was good and comply with the requirements of the criteria for the chromatographic separation found in the European guidelines (Commission Regulation (EU) No 836/2011) [7]. The RSD values for retention times (RT) were below 0.24% within analytical day (repeatability) and below 0.60% across

Table 1
Parameters of regression equations for calibration curves, limit of detection (LOD), limit of quantification (LOQ), repeatability and reproducibility for PAHs under study.

PAHs	Slope[a] (area count/ng)	Intercept[a] (area count)	Regression coefficient	LOD (ng/ml)	LOQ (ng/ml)	Repeatability ($n=3$)		Reproducibility ($n=6$)	
						RT (RSD%)	Area (RSD%)	RT (RSD%)	Area (RSD%)
Na	412.8	2162	0.9985	0.22	0.73	0.24	0.75	1.24	5.54
Ac	1119	5997	0.9982	0.07	0.22	0.15	0.60	1.42	4.88
F	1176	6249	0.9984	0.13	0.37	0.15	0.95	1.42	8.05
Pa	1376	3170	0.999	0.13	0.37	0.13	0.57	1.33	4.07
A	7324	45493	0.9977	0.07	0.22	0.12	0.50	0.36	6.98
Fl	845.1	5245	0.9977	0.46	1.43	0.12	0.43	1.07	5.37
P	3229	17908	0.998	0.07	0.22	0.12	0.39	1.99	8.17
BaA	4556	12762	0.997	0.07	0.22	0.07	0.33	0.44	5.15
Ch	2284	6235	0.997	0.12	0.37	0.06	0.34	0.24	5.10
BbF	2159	1989	0.998	0.07	0.22	0.08	0.34	0.62	7.76
BkF	13352	21301	0.997	0.07	0.22	0.09	0.34	0.59	6.35
BaP	7298	-23575	0.996	0.07	0.22	0.10	0.35	3.36	6.91
DhA	2593	730	0.998	0.07	0.22	0.12	0.34	3.79	7.75
BgP	2029	1876	0.997	0.07	0.22	0.11	0.30	3.44	8.15
IP	400.4	-25.7	0.997	0.47	1.44	0.14	0.29	2.16	4.49

[a] Seven points were considered for the regression. Each point represents the average of two injections of each standard solution.

analytical days (reproducibility). Values of RSD for peak areas were below 3.79% within day and below 8.17% across days. All RSD values were similar to those reported in literature for within- and between-days variation [15].

3.2. Improvement of extraction procedure for meat/fish

The precision (RSD%) and recoveries (%) data obtained for charcoal-grilled meat samples were determined by two different extraction procedures (extraction method 1 and extraction method 2). The recoveries were calculated (in triplicate) by comparing the difference between spiked and unspiked meat samples with two levels of PAHs added (Table 2). The RSD% of triplicate extractions was evaluated. As can be seen in Table 2, the recoveries for meat samples ranged from 15.37 to 145% and from 16.1 to 82.12%, respectively for extraction method 1 and 2. Several authors also describe great variation on recovery percentages for analyses of PAHs in thermally treated meat [14,15,23,27]. Pan and Cao describe recoveries ranging between 68.5 and 102.8% [14] for the same PAHs using saponification followed by solid phase extraction, Purcaro et al. [25] reported recoveries that ranged between 9.7 and 102.5% for the analyses of the same PAHs using microwave-assisted extraction.

Method 1 uses 5 g of wet meat samples and involves several steps of extraction and clean up, finally, the residue is dissolved in 100 μl of acetonitrile, whereas method 2 uses 2 g of lyophilized meat sample (corresponding to 4 g of wet sample used in method 1) and few purification steps, only extraction with *n*-hexane and purification with silica column, finally, the residue is dissolved in 100 μl of acetonitrile. Method 2 with the referred modifications

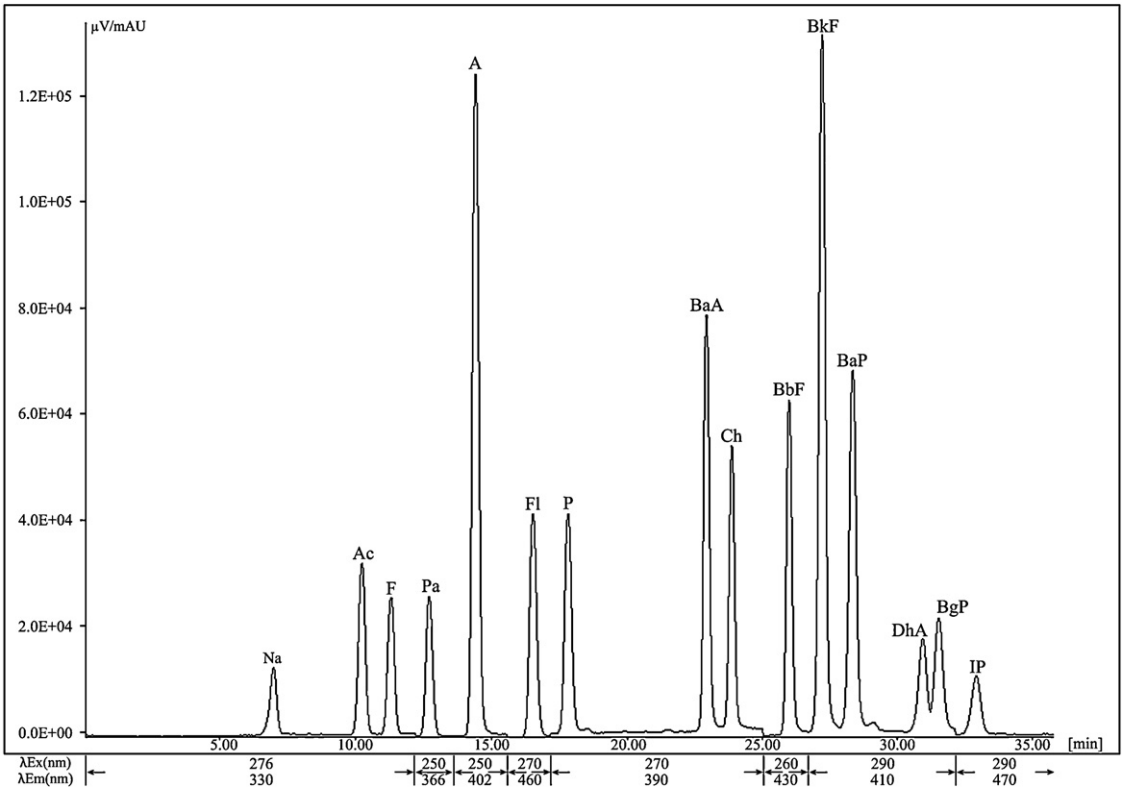

Fig. 1. HPLC chromatogram of standard solution (500 ng/ml). For conditions, see text.

Table 2
Recovery and precision of PAHs, obtained for charcoal-grilled meat samples, determined by two different extraction procedures.

PAHs	Extraction method 1						Extraction method 2					
	Initial content (ng/g)[a]	RSD (%)	Addition (ng/g)[a]	Measured content (ng/g)[a]	RSD (%)	Recovery (%)	Initial content (ng/g)[a]	RSD (%)	Addition (ng/g)[b]	Measured content (ng/g)[a]	RSD (%)	Recovery (%)
Na	1.91	30.79	8	3.23	58.22	16.5	2.90	26.76	10	3.58	57.06	16.12
			16	5.01	36.52	19.37			20	5.64	76.21	16.30
Ac	8.67	19.29	8	10.82	14.65	26.87	7.44	16.25	10	9.99	13.64	49.08
			16	14.56	15.32	35.7			20	15.18	12.02	74.63
F	5.28	2.71	8	8.97	10.91	46.2	5.94	1.77	10	8.89	7.95	56.81
			16	13.42	5.29	50.87			20	14.03	3.19	77.86
Pa	37.92	12.05	8	49.3	4.12	142	31.50	10.25	10	39.44	3.11	79.40
			16	61.21	10.39	145			20	47.41	8.34	79.55
A	0.91	9.89	8	4.99	11.51	51.00	2,13	10.02	10	5.68	6.21	68.42
			16	9.54	12.41	53.93			20	9,89	9.49	74.77
Fl	1.31	3.55	8	6.4	6.22	51.12	4.06	12.15	10	7.83	4.01	72.67
			16	10.0	8.99	54.31			20	12.58	7.22	82.12
P	15.43	16.09	8	22.9	5.27	93.37	18.24	6.69	10	25.39	4.38	71.50
			16	31.5	10.35	100.4			20	33.98	5.70	78.71
BaA	n.q.	–	8	3.99	11.23	49.87	0.51	14.64	10	4.01	5.98	67.66
			16	7.54	8.54	47.13			20	8.32	7.96	75.32
Ch	n.q.	–	8	3.41	10.23	42.62	1.04	13.78	10	4.77	5.56	72.00
			16	8.43	17.72	52.68			20	8.84	7.42	75.19
BbF	n.q.	–	8	4.46	12.91	55.75	0.37	12.74	10	3.48	4.97	59.98
			16	8.1	12.96	50.62			20	7.28	8.96	66.61
BkF	0.16	5.39	8	3.71	12.12	44.4	0.16	9.49	10	6.44	6.38	62.79
			16	9.4	14.72	57.75			20	13.49	8.72	66.67
BaP	2.64	7.20	8	5.28	1.69	33.2	0.84	9.28	10	6.84	6.69	59.60
			16	10.6	11.12	49.7			20	14.06	9.11	66.12
DhA	n.q.	–	8	3.14	8.56	39.2	0.21	15.19	10	6.56	10.93	63.50
			16	7.88	15.22	49.25			20	13.94	19.99	68.62
BgP	n.q.	–	8	3.51	6.38	43.87	0.52	5.05	10	6.53	6.38	60.08
			16	8.02	12.39	50.12			20	14.01	11.19	67.49
IP	n.q.	–	8	7.1	5.99	88.7	0.75	17.28	10	6.88	5.49	61.30
			16	13.5	11.27	84.37			20	12.96	7.47	61.05

[a] Analyte concentration expressed as ng/g of wet weight.
[b] Analyte concentration expressed as ng/g of dry weight (1 g of dry weight corresponds to approximately 2 g of wet weight, thus, the added amount corresponds to 5 and 10 ng/g of wet weight).

proved to be more sensible since all PAHs under study were quantified in the charcoal-grilled meat sample, only 8 analytes were quantified with method 1, additionally, in general, higher RSD and lower recoveries were obtained with method 1. Thus, the method 2 with modifications was selected for further studies and its application extended for quantification of PAHs in the analysis of fatty fish.

Several experiments were performed to optimize meat extraction conditions using method 2, for this purpose the *n*-hexane residue was quantitatively transferred with 3 ml of *n*-hexane into a silica cartridge (previously washed with 20 ml of dichloromethane, dried completely by means of vacuum, and conditioned with 20 ml of *n*-hexane), and eluted through the column with 17 ml of a mixture of *n*-hexane/dichloromethane (70:30). Analyses of PAHs, in fortified meat samples, were performed in the eluate collected in fractions of 2 ml. These fractions of 2 ml were evaporated to dryness under nitrogen stream. The residues were dissolved in 100 μl of acetonitrile and injected into the HPLC/FLD to investigate the presence of PAHs. No PAHs were detected on the first 4 fractions of 2 ml of eluate, corresponding to the first 8 ml that are discharged. As expected the following 4 fractions of 2 ml contained PAHs, the same occurred in the next 2 ml of eluate, but no PAHs were detected in the next 2 ml of eluate, indicating that the most appropriate volume of eluate to guarantee that all PAHs were recovered was 10 ml after discharge of the first 8 ml. Limits of detection (LODs, $s/n = 3$) using 2 g of meat sample were lower than 0.003 ng/g wet weight for all PAHs except for Na, Fl, and IP (that were lower than 0.01 ng/g). Limits of quantification (LOQs, $s/n = 10$) using 2 g of meat sample were lower than 0.01 ng/g wet weight for all PAHs except for Na, Fl, and IP (that were lower than 0.04 ng/g).

A similar procedure was performed in fortified salmon samples, however, for this purpose only 1 g of lyophilized salmon was used, since 2 g of sample contained very high level of fat and exceeded the capacity of silica column of retaining fat [18,31]. No PAHs were detected on the first 4 fractions of 2 ml of eluate, corresponding to the first 8 ml that should be discharged. The following 6 fractions of 2 ml contained PAHs, but no PAHs were detected in the next 2 ml of eluate, indicating that the most appropriate volume of eluate to guarantee that all PAHs were recovered was 12 ml. Limits of detection using 1 g of salmon sample were lower than 0.006 ng/g wet weight for all PAHs except for Na, Fl, and IP (that were lower than 0.02 ng/g). Limits of quantification using 1 g of fish sample were lower than 0.02 ng/g wet weight for all PAHs except for Na, Fl and IP (lower than 0.1 ng/g). The LODs and LOQs values obtained for meat and fish samples were lower than those referred by Commission Regulation (EU) No 836/2011 (LOD < 0.3 μg/kg; LOQ < 0.9 μg/kg) [7].

3.3. Analysis of PAHs by standard addition method meat/fish

Two methods of quantitative analysis were comparatively evaluated using meat and fish samples: external calibration curve method and standard addition method using two fortified levels (20 and 40 ng/g of PAHs for fish samples and 10–20 ng/g for meat samples). Typical chromatograms obtained for an unspiked and two levels spiked meat sample are shown in Fig. 2. Table 3 summarizes the results obtained. The PAHs concentrations obtained by the two quantification methods are significantly different ($p < 0.05$ running a *t*-test, except for BgP and DhA in salmon samples). Ratio between the concentration determined by the different methods of

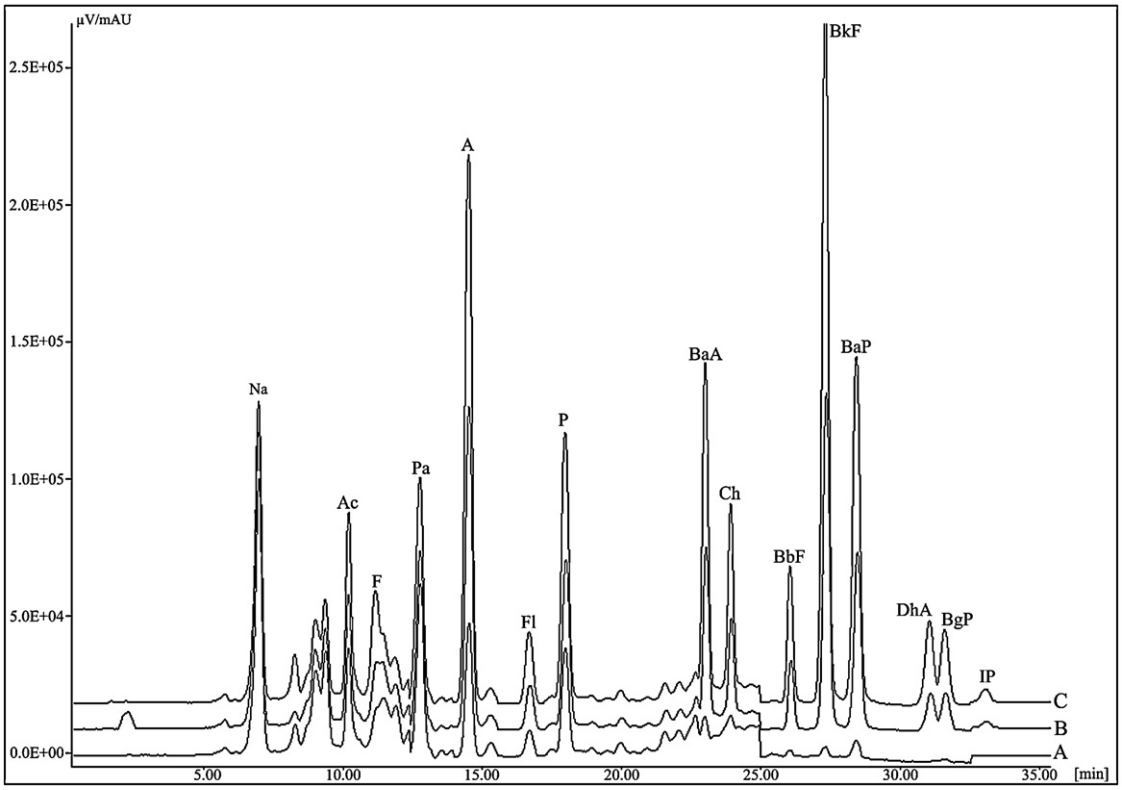

Fig. 2. Typical chromatograms obtained for an unspiked (A) and two levels (10–20 ng/g) spiked meat sample (B and C, respectively). For conditions, see text.

Table 3
Comparison of PAHs concentrations in meat and fish samples determined by external calibration curve method and by standard addition method (triplicate analyses were performed using the extraction method 2).

PAHs	Beef samples						Salmon samples					
	External calibration curve method (A)		Standard addition method (B)		*t*-test *p* value[b]	Ratio between the conc. determined by different methods of quantitative analyses (B)/(A)	External calibration curve method (A)		Standard addition method (B)		*t*-test *p* value[b]	Ratio between the conc. determined by different methods of quantitative analyses (B)/(A)
	Mean conc. (ng/g)[a]	±Standard deviation	Mean conc. (ng/g) [a]	±Standard deviation			Mean conc. (ng/g)[a]	±Standard deviation	Mean conc. (ng/g) [a]	±Standard deviation		
Na	3.75	0.73	5.81	0.62	0.009	1.54	24.76	1.34	36.06	1.98	0.000	1.45
Ac	6.83	0.53	10.94	1.28	0.001	1.60	4.59	0.60	5.06	0.40	0.000	1.10
F	6.09	0.13	7.25	0.08	0.001	1.19	7.04	0.04	9.37	0.83	0.002	1.33
Pa	15.76	0.50	27.09	0.76	0.000	1.71	28.42	0.17	57.01	1.34	0.001	2.00
A	2.02	0.10	3.14	0.11	0.000	1.56	3.81	0.03	6.49	0.90	0.000	1.70
Fl	3.80	0.23	5.39	0.36	0.002	1.41	11.47	0.71	14.95	1.20	0.000	1.30
P	4.18	0.08	5.98	0.57	0.000	1.43	9.05	0.64	13.65	1.70	0.022	1.51
BaA	0.41	0.08	0.68	0.04	0.018	1.64	2.69	0.22	3.98	0.37	0.041	1.48
Ch	0.86	0.15	1.37	0.04	0.002	1.59	4.02	0.19	7.04	0.20	0.012	1.75
BbF	0.32	0.04	0.48	0.06	0.040	1.51	2.14	0.03	3.72	0.37	0.031	1.74
BkF	0.07	0.01	0.14	0.05	0.037	2.02	0.36	0.01	0.80	0.09	0.000	2.21
BaP	0.39	0.04	0.60	0.05	0.007	1.55	1.66	0.07	2.85	0.06	0.001	1.70
DhA	0.11	0.01	0.22	0.02	0.002	2.12	0.59	0.10	0.59	0.04	0.538	1.00
BgP	0.25	0.02	0.43	0.10	0.075	1.71	0.97	0.19	1.47	0.26	0.426	1.51
IP	0.33	0.05	0.50	0.08	0.016	1.51	1.27	0.02	2.06	0.23	0.008	1.63

[a] Mean concentration of triplicate analyses expressed as ng/g of wet weight.
[b] *t*-test, $p < 0.05$ indicate significant differences.

quantitative analyses: standard addition method (B)/external calibration curve method (A) ranged between 1.19–2.12 and 1.00–2.21, respectively for beef and salmon samples, indicating that standard addition method compensates PAHs losses at different stages of sample preparation, and decreases the contribution of systematic errors and improves the accuracy of the results [32]. Therefore it can be recommended for analyses of PAHs in grilled meat and fish samples that contain variable amounts of PAHs. The eight high molecular weight PAHs (BaA, Ch, BbF, BkF, BaP, IP, BgP, DhA) present at traces levels could be quantified.

4. Conclusions

This work describes optimization of a methodology for determination of 15 PAHs (including the 8 high molecular weight EU

priority PAHs) in charcoal grilled foods (extraction, HPLC conditions and quantification method).

The analytical strategy consisted in extraction using sonication followed by purification on SPE, based on an extraction procedure for oils (rich in fat) and smoked foods (with high levels of PAHs per gram) with appropriate modifications for the matrix under study. Standard addition method was used to measure at trace levels these analytes with high sensitivity and specificity. Application on meat and fish samples permitted to prove its suitability and to collect data on PAHs contamination profile in this type of foods. The LODs and LOQs values obtained for meat and fish samples were lower than those referred by new European Legislation.

Time consumption and, consequently, the cost of routine analyses with the use of standard addition method increased as compared with the external calibration curve method. This is due to the replications of sample preparation and chromatographic analyses. However, the proposed HPLC method is less expensive than others that require acetonitrile as the eluent. Additionally, the extraction procedure is not very laborious and uses only one silica cartridge per extraction (three for each analysis).

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