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Validation of a Method for the Quantification of Polycyclic Aromatic Hydrocarbons in Fish

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Abstract

This study aimed to meet the need for a reliable method for the quantification of polycyclic aromatic hydrocarbons in fish. To this end, the standard NFV03-110 was used to validate internally a method for the determination of six polycyclic aromatic hydrocarbons (PAHs): benzo(a)pyrene (BaP), benzo(b)fluoranthene (BbF), benzo(g,h,i)perylene (BghiP), benzo(k)fluoranthene (BkF), fluoranthene (F), indeno(1,2,3-c,d)pyrene (IcdP) in samples of fresh and smoked fish. This study was conducted in accordance with the specific requirements of the standard ISO/DIS/15753 applicable to the High Performance Liquid Chromatography. Pearson coefficients for the six PAHs ranged from 0.998 to 0.999. The limits of detection were between 0.04 µg/kg for benzo(g,h,i)perylene and 0.15 µg/kg for benzo(b)fluoranthene and benzo(k)fluoranthene. The limits of quantification varied between 0.1 µg/kg for benzo(g,h,i)perylene and 0.15 µg/kg for benzo(b)fluoranthene and benzo(k)fluoranthene. The coefficients of variation for the repeatability and reproducibility tests were less than 4% for standards at 2.5 µg/L and 5 µg/L. The recovery percentages for the tests of adequacy were close to 100%. Moreover, the compliance test revealed no significant differences between PAHs concentrations and the certified values. As a result, the method used to determine PAHs concentrations is valid. This method was then used to quantify PAHs concentrations in samples of fresh and smoked fish intended for human consumption. The concentrations measured varied from one molecule to another and according to the physiological status of the fish.

Keywords: PAHs, fresh and smoked fish, validation test, HPLC

Introduction

Polycyclic aromatic hydrocarbons are toxic substances that are formed in foods during preserving treatments such as traditional smoking [3][4][20][21]. In Côte d'Ivoire, smoked fish account for over 80% of the total consumption of fish [1][5]. According to data from the literature, any form of cooking at high temperature promotes the development of lipophilic, carcinogenic, mutagenic and teratogenic substances [16][19][20]. To protect the consumer, the European Commission took, in 2006, a stringent regulation laying down the maximum residue levels of benzo(a)pyrene at 5 µg/kg and 2 µg/kg respectively in the flesh of smoked and fresh fish [6]. However, in 2007, following health checks, Ivorian Customs found benzo(a)pyrene in batches of smoked fish from countries of West Africa at concentrations (7-17 µg/kg) exceeding the limit values [6]. These results have led to the reinforcement of the check on smoked fish exporters and coerced the Ivorian authorities into taking up measures to reduce the chemical contamination of fish products. A prerequisite for achieving this goal definitely relies upon the validation of the method used for the determination of polycyclic aromatic hydrocarbons.

Material and Methods

Biological Material

A reference sample of grapeseed oil (1-1544-0018-2009), from BIPEA, was used. It contained benzo(a)pyrene (15.7±2.4 µg/kg), benzo(b)fluoranthene (18.3±3.2 µg/kg), benzo(g,h,i)perylene (12.3±2.8 µg/kg), benzo(k)fluoranthene (7.7±0.6 µg /kg), fluoranthene(97.7±7.0 µg/kg), indeno(1,2,3-c,d)pyrene (11.8±1.8 µg/kg). Some samples of fresh and smoked fish, collected in the second half of 2010, by official inspection services of the Department of Animal Production and Fish Resources (MIPARH) were also used. The species considered in this study were: Pandora (*Pagellus erythrinus*),

Roncador (*Sarpa Salpa*), Carp (*Cyprinus carpio*), Mackerel (*Scomber scombrus*), Bonito (*Sarda spp*) and Pike (*Esox lucius*).

Reagents

Standard individual solutions of polycyclic aromatic hydrocarbons certified by the Environmental Protection Agency (EPA) were used: Fluoranthene F (1 mg/mL), Benzo(b)fluoranthene (0.2 mg/mL), Benzo(k)fluoranthene (0.2 mg/mL), Benzo(a)pyrene (0.01 mg/mL), Benzo(g,h,i)perylene (0.02 mg/mL) and indeno(1,2,3-cd)pyrene (0.04 mg/mL). Different solvents such as acetonitrile HPLC grade (Scharlau Chemie SA), acetone HPLC grade (Scharlau Chemie SA), methanol HPLC grade (Scharlau Chemie SA), N-hexane HPLC grade (Carlo Erba), toluene grade HPLC (Labosi), dichloromethane HPLC grade (Carlo Erba), deionized water HPLC grade (Panreac Quimica SA) and hydrochloric acid 37% (Carlo Erba) were also used.

Apparatus

The determination of PAHs was performed with the help of a High Performance Liquid Chromatograph (Prominence Dual brand from HGE) equipped with a detector Shimadzu UV-Visible (UV-Vis Prominence SPD 20 A), a pump (LC 20AD Prominence Chromatograph), an automatic injector (Autosampler Prominence SIL 20AC) and a column (Column Prominence Owen CTO 20A) type Prevail C18 (15 x 4.6 mm x 5 microns). A Soxhlet equipment (Sibat 34/45) with 6 positions was also used.

The determination of PAHs was performed under the operating conditions listed in Table 1 (room temperature, column temperature, gradient, flow rate, wavelength).

Table 1: HPLC operating conditions

Room temperature	26°C
Cartridge temperature	40°C
Binary gradient	Solvent A: water Solevent B: acetonitrile
Flow rate	1mL/minute
Wavelength	254 nm

Method of Validation

The method validation was conducted using the method of the French Association for Standardization (NFV03-110/1998) [7]. This procedure includes the study of the linearity of the calibration range, the determination of the limits of detection and quantification, the calculation of the coefficient of variation for the tests of repeatability and reproducibility, and the calculation of the percentage recovery for testing accuracy. A reference sample of grapeseed oil was used to compare the PAHs concentrations obtained with the certified values.

Test for Linearity

The linearity was tested between 0 and 10 µg/L using 5 points calibration (0 µg/L, 2.5 µg/L, 5 µg/L, 7.5 µg/L and 10 µg/L) for each of the 6 PAHs. Five separate tests were performed for each compound.

Limits of Detection and Quantification

The limits of detection (LD) and quantification (LQ) were calculated from the blank and 10 separate trials were analyzed by HPLC.

$$\text{Limit of Detection (LD)} = mb + 3\sigma$$

$$\text{Limit of Quantification (LQ)} = mb + 10\sigma$$

(mb= Average concentration with the blank; σ = Standard deviation of blank values)

Tests of Repeatability and Reproducibility

To test the repeatability, 10 trials of extract from a reference sample of grapeseed oil were analyzed by HPLC. For reproducibility, five separate test samples from a reference sample of grapeseed oil were analyzed by HPLC at intervals of several days.

Test of Accuracy

Ten separate trials from reference samples were analyzed to assess the recovery rate by the method used for the determination of the 6 PAHs.

Method for the Determination of Fat

Fats were extracted by Soxhlet method [8]. The fat content was calculated using the formula:

$$\% \text{ Fat} = (\text{M2}-\text{M1}) \times 100 / \text{M}$$

M = mass of test sample

M1 = mass of flask + mass of hexane

M2 = mass of flask after extraction and drying

Method for the Extraction of PAHs

Extraction and analysis of PAHs were performed according to ISO 15753/2004 [9]. A sample of 2.5 g of fish flesh or reference sample is introduced into a centrifuge tube and then 10 mL of acetonitrile/acetone (V/V, 60/40) are added. The whole is homogenized by vortexing 30 seconds and 5 minutes in ultrasonic bath before being centrifuged for 5 minutes at 4000 rpm. The upper phase was removed and transferred into a conical tube and the solvent is evaporated using a rotary evaporator at 35°C. The extraction was repeated twice with 10 mL of acetonitrile/acetone. The extract is then purified on the cartridges of bonded phase C18 (Waters Sep Pack). To do this, 2 mL of acetonitrile/acetone are introduced into a conical tube containing the sample. The whole was vortexed 15 seconds and centrifuged for 30 seconds. The upper phase was transferred into a tube and the operation was repeated twice. The supernatants were transferred onto a C18 cartridge previously conditioned with 12 mL of methanol and 12 mL of acetonitrile. The elution was performed with 5 mL of acetonitrile/acetone at atmospheric pressure. Then the eluate is concentrated to 50 mg using a rotary evaporator at 35°C. The purified extract is recovered in 1 mL of hexane. The tube is crimped and stored at -18°C before analysis.

Statistical Analysis

The statistical analysis was performed using SPSS 12. Statistical significance was set at 0.05. The average concentrations of the six PAHs were calculated with their respective standard deviation, then the coefficients of variation were obtained to express the repeatability and reproducibility. The squared Pearson correlation coefficient (R²) was calculated to assess linearity. Test of Fisher-Snedecor (risk 1%) was used to validate the calibration range. The percentage recovery was calculated to express the efficiency of extraction. Average concentrations and range of variation in concentrations of PAHs were used to describe the level of contamination of samples of fresh and smoked fish. The test of conformity at 1% risk between the reference method and our method was used to compare the concentrations obtained with the certified values of reference sample.

Results

Results of the Validation

Figure 1: Chromatogram of the determination of the 6 PAHs at 5 µg/L

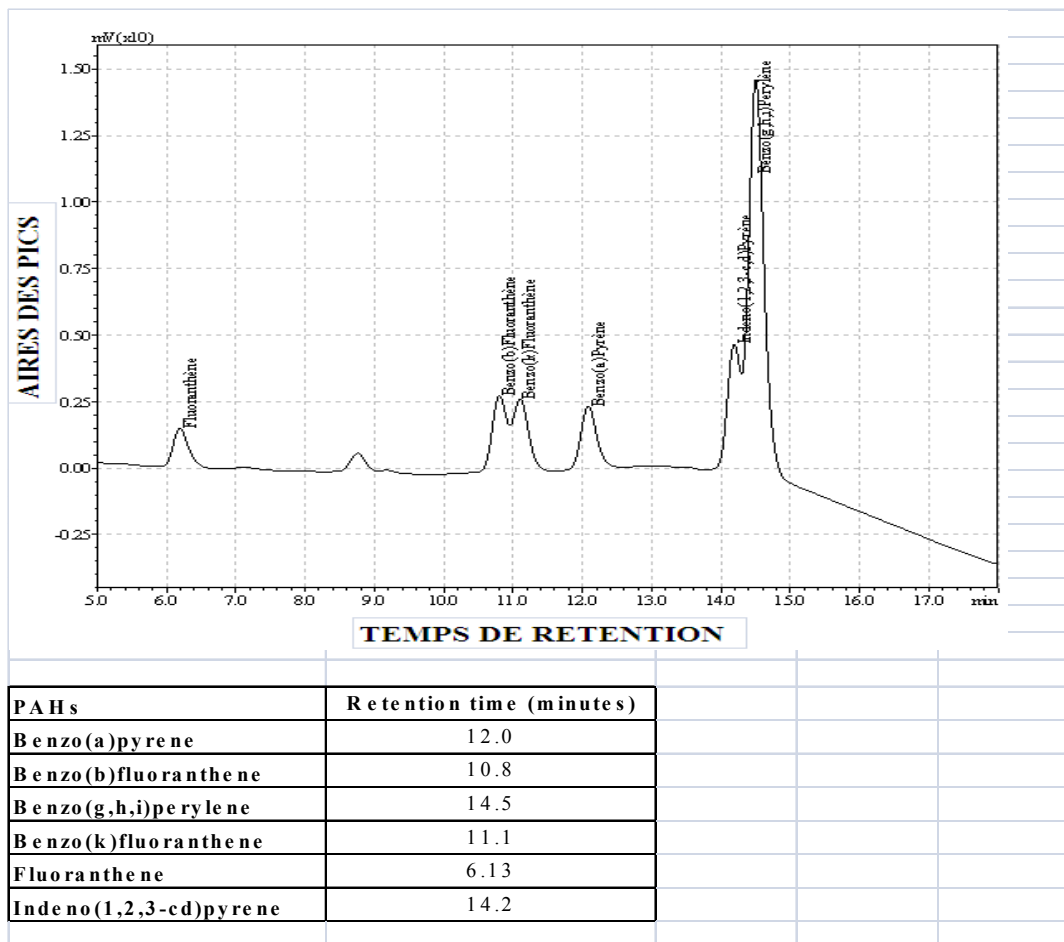


Figure 1 shows the chromatogram of the assay of the 6 PAHs at a concentration of 5 µg/L. The determination coefficients obtained for the study of the linearity of PAHs are between 0.9987 for BghiP and 0.9999 for BkF (Table 2). The linearity of the calibration range was validated by Fisher-Snedecor test at 1% risk (Table 3). The detection limits of the different compounds varied between 0.04 µg/kg for BghiP and 0.15 µg/kg for BkF and BbF. As to the limits of quantification, they were between 0.06 µg/kg and 0.28 µg/kg (Table 4). The coefficients of variation calculated for the tests of repeatability ranged from 0.80 to 1.63% for the standard solution at 2.5 µg/L and between 0.40 to 1.45% for the standard solution at 5 µg/L (Table 5). For the test of reproducibility, coefficients of variation ranged from 1.77 to 2.99% for the standard solution at 2.5 µg/L and from 1.93 to 2.79% for the standard solution 5 µg/L (Table 5). The results of these tests clearly reflect the accuracy of the method of determination of PAHs and the precision of the chromatographic technique. Regarding the accuracy of the method, the percentage recovery and the results of the test of compliance with the certified values were performed. The recovery percentages obtained were between 100.4±7.20% for Benzo(a)pyrene and 108±7.60% for Indeno(1,2,3-cd)pyrene. Comparing the values obtained with those certified through compliance testing of the accuracy to risk 1% revealed no significant difference (Table 6).

Table 2: Equations of calibration and coefficients of determination of PAHs

PAHs	Calibration equations	Pearson coefficients (R2)
Benzo(a)pyrene	$y = 0.047456x$	0.9997
Benzo(b)fluoranthene	$y = 0.043288x$	0.9987
Benzo(g,h,i)perylene	$y = 0.28492x$	0.9998
Benzo(k)fluoranthene	$y = 0.039504x$	0.9999
Fluoranthene	$y = 0.033624x$	0.9997
Indeno(1,2,3-cd)pyrene	$y = 0.05344x$	0.9997

PAHs Concentrations in Fresh and Smoked Fish Analyzed

Figure 2 shows the change in fat content and average concentrations of PAHs found in fresh and smoked fish of different species. The fat content is between 3.19% and 41.5%. PAH concentrations are highest in *Sphraena sp*, *Sarda sp* and *Scomber sp* and are respectively 68.8 µg/kg, 95.3 µg/kg, 94.6 µg/kg (Table 7). These fresh fish had BaP concentrations above 2 mg/kg. These concentrations were respectively 6.91 µg/kg, 31.3 µg/kg and 15.3 µg/kg. Concerning smoked fish, PAH concentrations are higher than those of fresh fish (Table 7 and 8).

Table 3: Results of linearity tests

PAHs	F1	Critical value	Regression model	Fnl	Critical value	Calibration range
Benzo(a)pyrene	126578	8.10	Validated	4.82	4.94	Validated
Benzo(b)fluoranthene	132362		Validated	3.38		Validated
Benzo(g,h,i)perylene	17941		Validated	1.21		Validated
Benzo(k)fluoranthene	320840		Validated	1.26		Validated
Fluoranthene	85753		Validated	1.84		Validated
Indeno(1,2,3-cd)pyrene	112683		Validated	3.80		Validated

F1 values calculated for the regression with the threshold 1% are higher than the value of Fisher equal to 8,10; the model of regression is acceptable. With the threshold 1%, the Fnl values calculated for the error of model is lower or equal to the value of Fisher equalizes to 4,94, the field of calibration is then validated.

Table 4: Limits of detection and quantification

PAHs	Detection limit	Quantification limit
Benzo(a)pyrene	0.09	0.19
Benzo(b)fluoranthene	0.15	0.28
Benzo(g,h,i)perylene	0.04	0.06
Benzo(k)fluoranthene	0.15	0.28
Fluoranthene	0.13	0.24
Indeno(1,2,3-cd)pyrene	0.13	0.19

Concentration in µg/kg

Table 5: Results of the tests of repeatability and reproducibility

PAHs	REPEATABILITY			
	Standard solution at 2.5 µg/L		Standard solution at 5 µg/L	
	Value measured	CV	Value measured	CV
Benzo(a)pyrene	2.53±0.05	1.94	5.06±0.09	1.79
Benzo(b)fluoranthene	2.51±0.04	1.38	5.03±0.07	1.40
Benzo(g,h,i)perylene	2.52±0.03	1.22	5.04±0.06	1.22

Table 5: Results of the tests of repeatability and reproducibility - continued

Benzo(k)fluoranthene	2.52±0.02	0.80	5.05±0.04	0.76
Fluoranthene	2.51±0.03	1.28	5.04±0.03	0.57
Indeno(1,2,3-cd)pyrene	2.51±0.05	2.14	5.03±0.09	1.79
PAHs	REPRODUCIBILITY			
	Standard solution at 2.5 µg/L		Standard solution at 5 µg/L	
	Value measured	CV	Value measured	CV
Benzo(a)pyrene	2.53±0.06	2.35	5.18±0.16	3.03
Benzo(b)fluoranthene	2.56±0.07	2.90	5.14±0.16	3.12
Benzo(g,h,i)perylene	2.52±0.04	1.46	5.06±0.14	2.81
Benzo(k)fluoranthene	2.54±0.05	1.78	5.05±0.07	1.44
Fluoranthene	2.52±0.06	2.21	5.03±0.18	3.48
Indeno(1,2,3-cd)pyrene	2.56±0.08	2.96	5.07±0.14	2.84

Values measured in µg/kg, coefficient of variation - CV – in %

Table 6: Results of the study of the accuracy

PAHs	Certified value	Value measured	Percentage recovery	 X-Z
	(X)	(Z)	(%)	
Benzo(a)pyrene	15.7±2.4	15.8±0.2	100.4±7.20	0.07
Benzo(b)fluoranthene	18.3±3.2	18.9±0.4	103.8±3.70	0.63
Benzo(g,h,i)perylene	12.3±2.8	12.8±0.1	103.4±11.6	0.46
Benzo(k)fluoranthene	7.70±0.60	8.0±0.2	104.3±29.2	0.33
Fluoranthene	97.7±7.0	100.5±1.0	102.9±14.9	2.84
Indeno(1,2,3-cd)pyrene	11.8±1.8	12.8±0.1	108.5±7.60	1.00
Mean difference (M d)				0.8883
Standard deviation (Sd)				1.0053
W (M d/Sd)				0.8837

Concentration in µg/kg

When W is lower or equal to 3, the values found by the alternative method are similar to the values certified with a risk of error of 1%

Table 7: Concentrations of PAHs found in fish analyzed

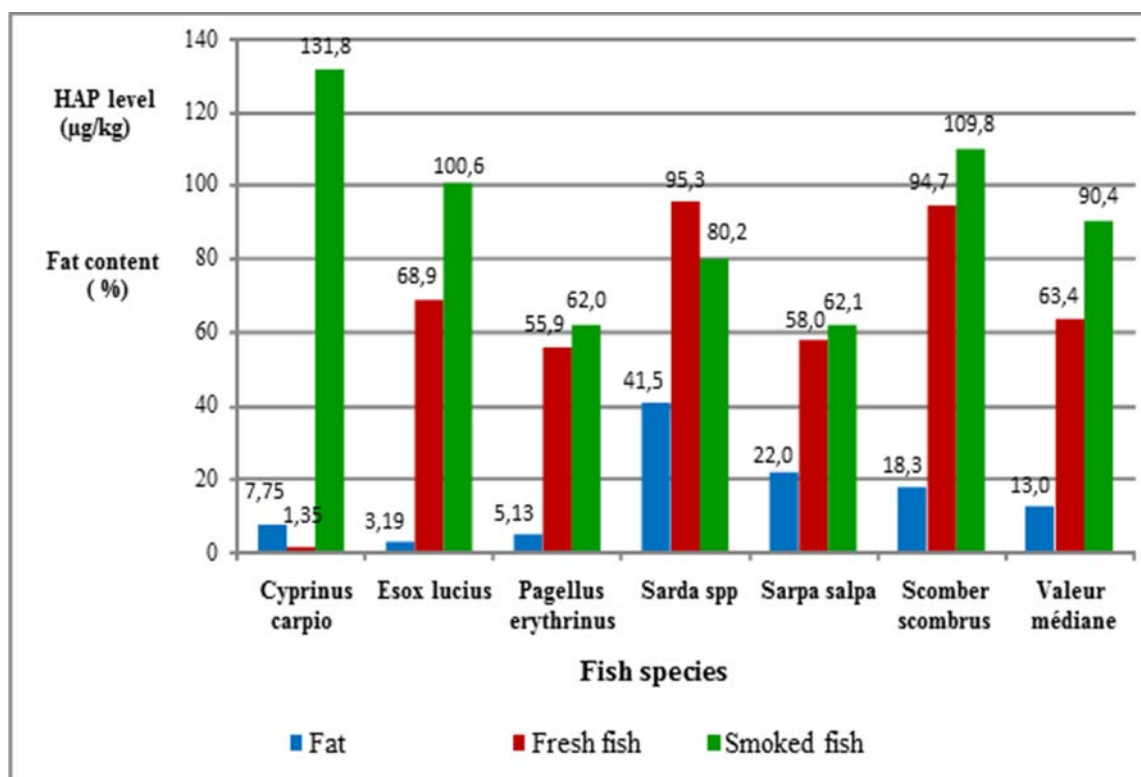
FISH		POLYCYCLIC AROMATIC HYDROCARBONS					
STATUS	Species	BaP	BbF	BkF	BghiF	F	IcdP
FRESH	<i>Cyprinus carpio</i>	0.48	0.11	0.17	0.01	0.57	0.01
	<i>Esox lucius</i>	6.91	0.07	0.07	3.17	47.0	11.7
	<i>Pagellus erythrinus</i>	0.14	0.61	0.67	0.01	54.4	0.01
	<i>Sarda spp</i>	31.4	3.99	5.21	1.26	48.8	4.72
	<i>Sarpa salpa</i>	0.46	1.84	1.78	0.03	53.9	0.01
	<i>Scomber scombrus</i>	15.4	0.21	0.24	0.55	76.2	2.08
SMOKED	<i>Cyprinus carpio</i>	16.9	46.5	9.30	2.26	48.8	8.03
	<i>Esox lucius</i>	56.5	3.84	8.93	1.16	25.6	4.52
	<i>Pagellus erythrinus</i>	36.7	3.49	2.93	1.99	13.1	3.84
	<i>Sarda spp</i>	55.4	3.20	1.22	1.34	13.6	5.40
	<i>Sarpa salpa</i>	18.0	2.61	2.54	0.35	37.0	1.54
	<i>Scomber scombrus</i>	78.7	9.93	1.93	2.10	8.25	8.89

Concentration in µg/kg

Table 8: Median concentrations of PAHs found in fish analyzed

PAHs	FRESH FISH		SMOKED FISH	
	Median value	[Min-Max]	Median value	[Min-Max]
Benzo(a)pyrene	3.70	0.46-31.4	46.0	16.9-78.7
Benzo(b)fluoranthene	0.41	0.07-3.99	3.67	2.61-46.5
Benzo(g,h,i)perylene	0.46	0.07-5.21	2.74	1.22-9.30
Benzo(k)fluoranthene	0.29	0.01-3.17	1.67	0.35-2.26
Fluoranthene	51.3	0.57-76.2	16.6	8.25-48.7
Indeno(1,2,3-cd)pyrene	1.05	0.01-11.6	4.96	1.54-8.89

Concentration in $\mu\text{g}/\text{kg}$

Figure 2: Fat content and PAH levels according to the physiological status of fish

Discussion

The study demonstrated the reliability of the method for the determination of the six PAHs considered (BaP, BBF, BghiP, BkF, F and IcdP) in fishery products and prioritized by the French Agency for Food Safety [14]. Indeed, the test of linearity demonstrated the normality of the distribution at the limits of the interval of the calibration range [0 to 10 $\mu\text{g}/\text{L}$]. The Fisher-Snedecor test helped to highlight the linearity of the models (Table 3). Furthermore, the determination coefficients (R^2) obtained during this study are all close to 1 and meet the criterion of the limit of linearity (Table 2). The limits of detection and quantification are on average equal to those advocated in the standard

ISO/DIS-15753-2004 [9] that is 0.2 µg/kg for most compounds. The method therefore presents a good level of quantification for the determination of these six (6) PAHs. Whatever the standard used, the variation coefficients obtained during tests of repeatability are less than 2%. These results account for the stability and reliability of the HPLC chromatograph. The tests of reproducibility corroborate the results of repeatability because the coefficients of variation are less than 3%. The recovery percentages and concentrations of PAHs obtained in testing the accuracy with the reference sample shows the accuracy of the results. Moreover, no significant differences were obtained with the compliance test. The results of the study on the validity of the method for the determination of PAHs are consistent with values showing the acceptability of an analytical technique as stated by experts of the Joint FAO / IAEA [10] and documented in the collection on standard methods of American Public Health Association [11]. It is important to note that the analysis time of our alternative method (20 minutes) is shorter than that prescribed by ISO 15753 (45 minutes) that is a time savings of at least twenty minutes per sample. According to Bories (1980) [17], lightening a methodology is interesting in terms of sanitation because of the gradual adoption in different countries of regulations laying down permissible concentrations of some PAHs in food products and this requires that simplified and less costly methods be available and can be implemented by laboratories not having a high degree of specialization. The dosage of the six PAHs revealed the presence of total PAHs concentrations ranging from 1.34 to 95.32 µg/kg and from 61.97 to 131.7 µg/kg of flesh respectively for fresh and smoked fish. The highest concentrations are found in samples of smoked fish: for example, the concentrations of BaP were 43.7±24.3 µg/kg and 9.12 ± 12.4 µg/kg respectively in smoked and fresh samples. The presence of high concentrations of PAHs in smoked foodstuffs has long been known [16]. Preservative, flavoring and coloring functions are some reasons why fish are smoked. However, smoke also carries PAHs [12][13][14]. In their study on the smoking of fish in West Africa for local and export markets, Rivier *et al.* [2] demonstrated the importance of smoking in the contamination of fish by PAHs. Furthermore, our study also reveals that the concentrations of BaP are higher in samples of fat and smoked fish (Figure 2). In 1995, Knockaert [15] showed the importance of fat in the pyrosynthesis of PAHs. The concentrations obtained were all below the limit values (2 µg/kg in the flesh of fresh fish and 5 µg/kg in the flesh of smoked fish) recommended by the European Union regulation 1881/2006 [6].

Conclusion

This study substantiated the acceptability of the method proposed for the determination of benzo(a)pyrene (BaP), benzo(b)fluoranthene (BBF), benzo(g,h,i)perylene (BghiP), benzo(k)fluoranthene (BkF), fluoranthene(F), indeno(1,2,3-c,d)pyrene (IcdP) in fish. Validation is essential for accurate health risks assessment. Quantifying polycyclic aromatic hydrocarbons not only in fish but more widely in foodstuffs is of utmost importance for guaranteeing safer foods for local and foreign markets as well as ensuring a sustainable management of the toxicity of these chemicals.

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