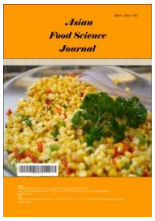

Asian Food Science Journal

14(1): 1-10, 2020; Article no.AFSJ.53375
ISSN: 2581-7752

Evaluation of Effective and Safe Extraction Method for Analysis of Polycyclic Aromatic Hydrocarbons in Kolanuts from Côte d'Ivoire

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Authors' contributions

This work was carried out in collaboration among all authors. Author KKR designed the study, wrote the protocol, fitted the data and wrote the first draft of the manuscript. Author NYB checked the first draft of the manuscript and achieved the submitted manuscript. Authors BGH, AAY and CA performed the statistical analysis, managed the literature and assisted the experiments implementation. Author BGH expertized the results interpretations. All authors managed the literature, read and approved the submitted manuscript.

Article Information

DOI: 10.9734/AFSJ/2020/v14i130118
Editor(s):
(1) Dr. Kresimir Mastanjevic, Associate Professor, Faculty of Food Technology, University in Osijek, Franje Kuhaca, Croatia.
Reviewers:
(1) Esraa Ashraf Ahmed ElHawary, Cairo University, Egypt.
(2) Imtiaz Ahmad, University of Peshawar, Pakistan.
Complete Peer review History: http://www.sdiarticle4.com/review-history/53375

Original Research Article

Received 22 October 2019
Accepted 27 December 2019
Published 14 January 2020

ABSTRACT

Aims: The current study targets the achievement of a reliable process for the determination of PAH contents in kola nuts for better appreciation of the risks incurred from the consumption of such food products.
Study Design: Kolanuts collected from two big storage centers were analyzed after the validation of the proposed analytical method.

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Place and Duration of Study: Central Laboratory for Food Hygiene and Agro-Industry, LANADA in Abidjan, Côte d'Ivoire, running 2018.
Methodology: Two references were used for the validation of the analytical method, namely the French standard NF V 03-110 and the ISO directive ISO/DIS/15753 applicable in Liquid Chromatography. The PAH contents of some samples collected from different stores were then determined.
Results: From the data, a significant regression chart was recorded for the PAH detection graphs. All the analysis exhibited good linearity with significant correlation coefficients (R^2> 0.99). The relative standard deviations of the repeatability and reproducibility assays are below 3%, whereas standard additions of PAH are fully recovered, with percentages close to 100%.
Conclusion: Using this analytical method, kola nuts contamination by PAH have been determined with satisfactory.This analytical method could help in ensuring effective sanitary control at different critical points of kola nut distribution channel for promoting a good management of the toxicity concerns in such products.

Keywords: PAH; Cola nitida; method validation; HPLC.

1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) represent a class of relatively stable organic contaminants with at least two confined aromatic rings consisting essentially of carbon and hydrogen atoms [1,2]. These are important environmental pollutants that are formed and released during incomplete combustion or pyrolysis of organic matter, during industrial processes and other human activities [3,4]. Indeed, the formation of PAH goes through a process of carbonization, where the initial matrix undergoes a chemical transformation and a rearrangement to give a more condensed polycyclic aromatic structure [5].

PAHs can be of natural or anthropogenic origin [2,6]. From these origins arise environmental and professional exposure [7]. Food can be contaminated through the environment by PAHs from air, soil, water, automobile exhaust, and during industrial or domestic cooking or cooking [8,9]. Thus, PAHs have been detected in certain foods such as vegetable oils, seafood, meat, tea, coffee, rice, tomato, potato, fruit and infant foods [10,11,6]. According to Walker [12], PAHs show a high toxicity towards living organisms including carcinogenic, teratogenic and mutagenic properties. Also, many epidemiological studies have shown that PAHs are responsible for cancers of the lungs, skin and bladder, stunting in living organisms [13,14,15].

This proven toxicity is a real public health problem for many governments and a hindrance to the export of some agricultural products that are widely prized by Western industries such as kola nuts. Indeed, kola is the first agricultural product of trade between the countries of the Community of West African States (CEDEAO). Also, Côte d'Ivoire is the largest producer and exporter of kola nuts in the world with an annual export earnings reaching 130 billion FCFA [16,17].

Kola nuts have an increasing interest for industries, mainly because of their richness in bioactive and functional compounds such as polyphenols, caffeine and theobromine [18,19]. They constitute an important raw material in the formulation of pharmaceutical, food, cosmetic and textile products [20,21,19].

However, 90% of the production of fresh cola is consumed daily by the population and this, in many sociocultural rituals such as weddings, baptisms, expressions of friendship, funerals and rituals of sacrifice [22,20]. Thus, after the harvesting of the nuts, a large quantity of the production undergoes a more or less long road which leaves from the producer to the big stockage centers while passing by various warehouses [17,23]. This situation could lead to an exposure of populations from here and elsewhere to the serious problems of toxicity of several substances in the environment such as PAHs. Among these molecules, Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(a)anthracene, Chrysene, Fluoranthene, Benzo(k)fluoranthene, Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene and Indeno (1,2,3, c, d) pyrene are commonly analyzed in the different components of the environment because of their presence in water and food and their carcinogenic properties [24]. Considering the health risks and taking into account the consumer protection, there is a need to set up the systematic control of PAH levels in

foodstuffs in order to guarantee the safety of the noted molecules. This control involves a fast, reliable and significant method. The objective of this study is the establishment and the validation of the quantification method of PAH in the kola nuts from Côte d'Ivoire.

2. MATERIALS AND METHODS

2.1 Biological Material

A sample of cereal references (1-1844-0031) composed of polycyclic aromatic hydrocarbon concentrations obtained from the BIPEA network was used: Benzo(a)pyrene (15.7 ± 0.86 µg/L), Benzo(a)anthracene (11.3 ± 0.56 µg/L), Benzo(b)fluoranthene (18.3 ± 0.91 µg/L), Chrysene (12.8 ± 0.65 µg/L), Fluoranthene (97.7 ± 3.81 µg/L), Benzo(k)fluoranthene (7.7 ±0.23 µg/L), Dibenzo(a,h)anthracene (14.4 ± 0.66 µg/L), Benzo(g,h,i)perylene (12.3 µg/L) and Indeno(1,2,3,c,d)pyrene (11.8 ± 0.47 µg/L).

Cola nitida Vent. (Schott & Endl.) samples collected from two big storage centers of Anyama (kola nut city in Côte d'Ivoire) served as an experimental matrix. Kolanuts was authenticated by a botanist in the laboratory of Botany, Training and Research Unit of Biosciences, Felix HOUPHOUËT-BOIGNY University where a voucher specimen was documented.

2.2 Reagents

Standard individual solutions of polycyclic aromatic hydrocarbons certified by the Environmental Protection Agency (EPA) were used: Benzo(a)pyrene (0.01 g/l), Benzo(b)fluoranthene (0.2 g/l), Benzo(a)anthracene (1 g/l), Chrysene (1 g/l), Fluoranthene (1 g/l), Benzo(k)fluoranthene (0.2 g/l), Dibenzo(a,h)anthracene (1 g/l), Benzo(g,h,i)perylene (0.02 g/l) and Indeno(1,2,3,c,d)pyrene (0.04 g/l). Different solvents and chemical reagents used in this study were HPLC grade: acetonitrile (Scharlau Chemie S.A), N-hexane (Carlo Erba), toluene (Labosi) and deionized water (Panreac Quimica SA).

2.3 Apparatus

An Adept brand High Performance Liquid Chromatograph (HPLC) equipped with an ultraviolet (UV) / visible CE 4200 (CECIL) detector was used for the identification and quantification of PAHs. This device was equipped with a CE 4104 (CECIL) pump, an Optimas automatic injector (SPARK), a CE 4040 (CECIL) degasser, an HPLC column (Prevail C18 column, 150 mm x 4.6 mm - particles 5 µm). The column should be maintained at a constant temperature of 40°C by an oven (CECIL) CE 4600. The system controller (CECIL) CE 4900 has an automatic follow-up analysis.

A METTLER TOLEDO AB 104-S scale was used to weigh the samples. BUCHI R-215 rotary evaporator with vacuum Comtroler V-850, vaccum Pump v-700; Heating Bath: B-491 was used to concentrate the extracts of the samples. The operating conditions of the HPLC assay for PAHs are presented in Table 1.

2.4 Validation of Analytical Method

The method of validation was conducted by using the method of the French Association for Standardization (NFV03-110/1998), respecting the specific requirements of ISO/DIS/15753 applicable in High Performance Liquid Chromatography [25,26]. 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 cereal was used to compare the PAHs concentrations obtained with the certified values.

2.4.1 Test for linearity

The linearity was tested between 0 and 1 mg/L using 6 points calibration (0 µg/L, 100 µg/L, 250 µg/L, 500 µg/L, 750 µg/L and 1 mg/L) for each of the 9 PAHs. Five separate tests were performed for each compound.

2.4.2 Limits of detection and quantification

The limits of detection (LOD) and quantification (LOQ) were calculated from the analysis of 10 separate assays of blank matrices. These parameters were measured by using the following formulas:

$$LOD = Mx + 3S$$

$$LOQ = Mx + 10S$$

With: LOD: Limits of detection, LOQ: Limits of quantification, Mx: Average from 10 assays of blank matrices, S: Standard deviation of blank values.

Table 1. HPLC operating conditions

Precolumn	Security guard, 20 mm x 4.6 mm
Column	Prevail C18, 150 mm x 4.6 mm
Detector, wavelength	ultra-violet (UV),λ = 255 nm
Binary gradient	Acetonitrile and Water
Injected volume (µL)	20 µl
Flow (mL/mn)	2.5
Column temperature (°C)	40
Rinse solvent	Acetonitrile /Water(50/50)
Analysis time (min)	6

2.4.3 Tests of repeatability and reproducibility

The repeatability of the analysis was probed into 10 assays of reference sample. As far as the reproducibility is concerned, 5 separate assays were achieved with the reference sample in a period of several days intervals.

2.4.4 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 9 PAHs.

2.5 Evaluation of the PAH Contents in Kolanuts

2.5.1 Extraction procedure

In a 50 ml conical tube containing 10 g of kola ground material was added 30 ml of hexane. The whole was homogenized with the magnetic stirrer for 1 hour, then in an ultrasonic bath for 5 minutes, before being centrifuged for 5 minutes at 4000 rpm. The upper phase was removed by using a pasteur pipette and transferred to a calibrated conical tube. The solvent was evaporated by using a rotary evaporator at 35°C (Buchi R-215). This extraction was repeated twice and extracts obtained were combined.

2.5.2 Purification

Purification was performed using C18 (Waters Sep-Pak® Vac) cartridges. The extract obtained was recovered with 2 ml of cyclohexane and transferred to a cartridge previously conditioned with 15 ml of cyclohexane. Each cartridge was placed on top of a previously calibrated conical tube. After recovery of the extract in the tube, 2 ml of cyclohexane was added thereto. The whole was vortexed for 15 seconds and then filtered through a cartridge. The tube was rinsed with 2 x 2 ml of cyclohexane and transferred to the cartridge. Elution was done with 4 ml of cyclohexane at atmospheric pressure. The eluate was concentrated by using a rotary evaporator at 35°C (Buchi R-215) until a volume V = 1 ml, to which was added 0.5 ml of toluene. A new evaporation was carried out until a volume of 50 µl of extract was obtained. The final volume of the extract was supplemented to 2 mL with ACN.

2.6 Statistical Analysis

The statistical analysis was performed using SPSS (version 20.0). Statistical significance was set at p = .05. The average concentrations of the 9 PAHs were calculated with their respective standard deviation. The coefficients of variation were obtained to express the repeatability and reproducibility and the squared Pearson correlation coefficient (R^2) was calculated to assess linearity. The recovery percentage 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 kola nuts contamination. The test of conformity at 5% risk between the reference method and our method was used to compare the concentrations obtained with the certified values of reference sample.

3. RESULTS

3.1 Validation Parameters for PAH Quantification

The validation data deal with the values of linearity, repeatability, reproducibility, soundness, and limits of detection and quantification involved in the PAHs determination.

Fig. 1 shows the chromatogram of the assay of 9 PAHs with a concentration of 1 mg/L by using the proposed method. We noted that the method used allows good separation and identification of the compounds analyzed from their different

retention times (Table 2). It can be concluded that the method used allows good separation and identification of the compounds analyzed from their different retention times (Rt).

All PAH exhibited good linearity other the evaluated range with significant correlation coefficient ($R^2 \geq 0.99$).

Table 3 displays the statistical validity of the linearity over the full calibration range according to the statistical Fisher rule. Indeed, the F1 values calculated for regression (ranged between 3526.79 to 46386.84) are higher than the critical Fischer value (8.10). On the other hand, the F2 values calculated for the error trend (ranged between 0.16 to 2.64) are lower than the critical Fisher value (4.94). The regression model and the calibration domain reflect a statistical acceptability of the method used.

The lowest concentration that can be quantified (LOQ) with acceptable accuracy and precision were 0.04 µg/L, 0.07 µg/L, 0.09 µg/L, 0.13 µg/L, 0.17 µg/L, 0.18 µg/L, 0.22 µg/L, 0.45 µg/L and 0.88 µg/L for Dibenzo(a,h)Anthracene, Benzo(k)Fluoranthene, Benzo(a)pyrene, Indeno(1,2,3,c,d)Pyrene, Benzo(b)fluoranthene, Chrysene, Benzo(g,h,i)Perylene, Benzo(a)anthracene and Fluoranthene, respectively (Table 2). Furthermore, the limit of detection (LOD) ranged from 0.02 µg/L to 0.55 µg/L corresponding to Dibenzo(a,h)Anthracene and Fluoranthene, respectively.

For the standard solution at 10 µg/L, the coefficients of variation calculated for the repeatability tests were between 0.89% and 2.28% for Benzo (a) anthracene and Benzo (k) fluoranthene, respectively. While those calculated for the reproducibility tests were between 1.4% for Benzo(a)pyrene and 2.67% for Benzo(k)fluoranthene (Table 4). The results of these tests reflect the accuracy of the method of PAHs determination and the precision of the chromatographic analysis used. Regarding the accuracy of the method, the recovery rates and the results of the test of compliance with the certified values were performed. The recovery rates obtained were between 96.1% and 102.31% corresponding to Benzo(k)fluoranthène and Benzo(b)fluoranthène, respectively (Table 5).

Table 2. Retention time and calibration data obtained using matrix matched standards for the selected PAHs

PAHs	Retention time	Regression equation[a]	R²	LOD (µg/L)	LOQ (µg/L)
Benzo(a)pyrene (B[a]P)	2.35	y = 21294.6x	0.9954	0.04	0.09
Benzo(a)anthracene (B[a]A)	1.10	y = 19277.4x	0.9954	0.27	0.45
Benzo(b)fluoranthene (B[b]F)	1.47	y = 20948.1x	0.9929	0.07	0.17
Chrysene (CHR)	1.21	y = 13022.7x	0.9966	0.10	0.18
Fluoranthene (FLA)	0.49	y = 48258.3x	0.9985	0.55	0.88
Benzo(k)Fluoranthene (B[k]F)	2.07	y = 9155.8x	0.9921	0.03	0.07
Dibenzo(a,h)Anthracene (D[ah]A)	3.03	y =8018.72x	0.9944	0.02	0.04
Benzo(g,h,i)Perylene (B[ghi]P)	3.43	y = 20564.6x	0.9915	0.14	0.22
Indeno(1,2,3,c,d)Pyrene (IcdP)	4.01	y = 9691.72x	0.9951	0.09	0.13

a: y – absorbance; x – concentration (µg/L)

Table 3. Linearity traits deriving from the experimental domain calibration

PAH	F1	Critical F-value	Regression model	F2	Critical F-value	Calibration range
B[a]P	3526.79		Validated	1.27		Validated
B[a]A	3773.38		Validated	1.34		Validated
B[b]F	5023.01		Validated	1.16		Validated
CHR	5985.75		Validated	1.57		Validated
FLA	46386.84	8.10	Validated	0.16	4.94	Validated
B[k]F	11597.01		Validated	1.78		Validated
D[ah]A	6561.04		Validated	2.64		Validated
B[ghi]P	3608.03		Validated	1.39		Validated
IcdP	5372.60		Validated	2.39		Validated

F1: F-value for the regression trend; F2: F-value for the statistical error trend

Table 4. Repeatability, reproducibility and Recovery test of the standard solution

PAHs	Repeatability[a] (n= 10)		Reproducibility [a] (n = 5)	
	Measured Value*	CV** (%)	Measured Value*	CV**(%)
B[a]P	9.98 ± 0.20	2.24	9.99 ± 0.14	1.4
B[a]A	10.01 ± 0.09	0.89	10.07 ± 0.22	2.18
B[b]F	10.07 ± 0.21	2.08	10.02 ± 0.16	1.59
CHR	10.01 ± 0.14	1.39	10.11 ± 0.25	2.47
FLA	10.02 ± 0.1	0.99	10.05 ± 0.16	1.59
B[k]F	10.08 ± 0.23	2.28	10.08 ± 0.27	2.67
D[ah]A	10.07 ± 0.19	1.88	10.21 ± 0.27	2.64
B[ghi]P	9.99 ± 0.13	1.30	10.03 ± 0.23	2.29
IcdP	9.97 ± 0.16	1.60	9.98 ± 0.16	1.60

*[a]:Standard solution at 10 µg/L; *: µg.L^{-1}; **: Coefficient of variation*

Table 5. PAH recovery rate of the standard solution

PAHs	Certified values (µg/L)	Measured content (µg/L)	Recovery ±RSD (%)
B[a]P	15.7 ± 0.86	15.3 ± 0.7	97.45± 4.60
B[a]A	11.3 ± 0.56	10.9 ± 0.52	96.46 ± 4.77
B[b]F	18.3 ± 0.91	18.8 ± 0.65	102.73 ± 3.45
CHR	12.8 ± 0.65	13.0 ± 0.5	101.56 ± 3.84
FLA	97.7 ± 3.81	98.1 ± 4.3	100.41 ± 4.32
B[k]F	7.7 ±0.23	7.4 ± 0.37	96.1 ± 3.5
D[ah]A	14.4 ± 0.66	14.1 ± 0.6	97.92 ± 5.87
B[ghi]P	12.3 ± 0.59	11.9 ± 0.57	96.75 ± 5.51
IcdP	11.8 ± 0.47	11.6 ± 0.53	98.30 ± 5.2

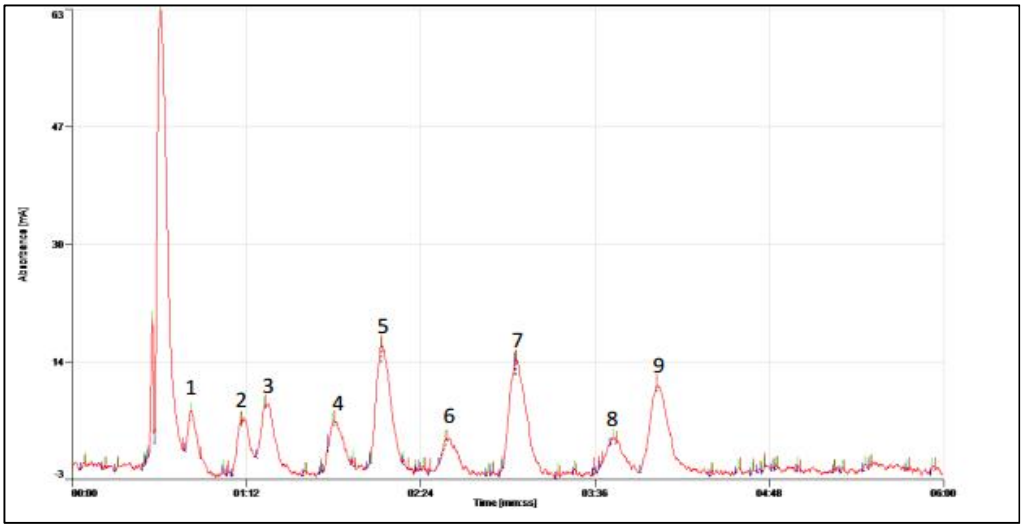

Fig. 1. Chromatogram of the determination of 9 PAHs at 1 mg/L

Fluoranthene (1), Benzo(a)anthracene (2), Chrysene (3), Benzo(b)fluoranthene (4), Benzo(k)fluoranthene (5), Benzo(a)pyrene (6), Dibenzo(a,h)anthracene (7), Benzo(g,h,i)perylene (8) and Indeno(1,2,3,c,d)pyrene (9)

3.2 Application of the Proposed Method: PAHs Concentration in Kolanuts

Fig. 2 shows the variation of PAH levels in kola samples collected. Using the analytical method proposed, kola nuts contamination by PAH have been determined with satisfactory. The results indicate that only Benzo(a)pyrene concentrations are below the LOQ for all samples analyzed. The concentrations of PAH vary from 0.51 µg/kg to 2.15 µg/kg for Chrysene and Indeno(1,2,3,c,d)Pyrene, respectively.

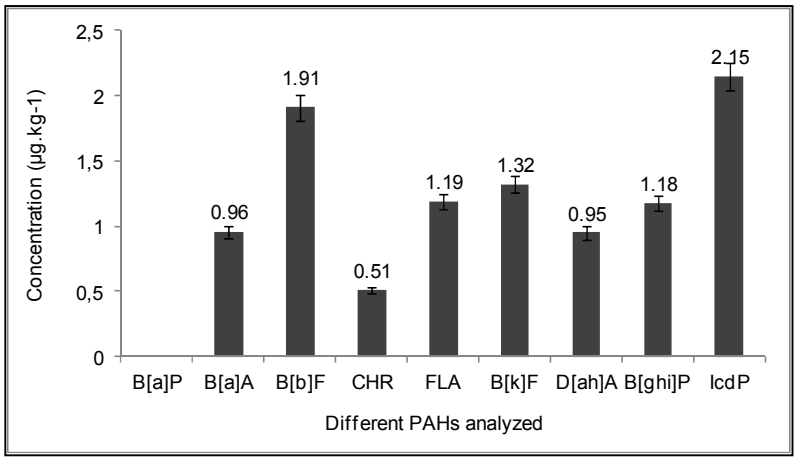

Fig. 2. PAHs content in kolanut sample

4. DISCUSSION

The study has demonstrated the reliability of the method for the determination of nine carcinogenic PAHs (B [a] P, B [a] A, B [b] F, CHR, FLA, B [k] F, D [ah] A, B [ghi] P and IdcP) in kolanut samples [24].

Indeed, the linearity test highlights the normality of the distribution across the range of the calibration range (0 to 1 mg/L). In addition, the Pearson coefficients (R^2) obtained during this study are all close to 1 and meet the criterion of linearity.

The detection and quantification limits are on average close to those recommended by the ISO/DIS-15753-2004 standard, ie 0.2 µg/kg for most PAHs studied [26]. These results are in agreement with those of Ake et al. [27] on PAHs identified and analyzed in fish consumed in Côte d'Ivoire. Thus, the method has a high sensitivity for the nine PAHs analyzed. With respect to fidelity (repeatability and reproducibility), the coefficients of variation are all less than 3%. Values ranged from 0.89% to 2.28% for repeatability and from 1.4% to 2.67% for reproducibility. Thus, the results of the reproducibility tests corroborate those of the repeatability. This indicates the possibility of obtaining good results from the operating conditions set. In addition, it appears that by testing the precision with the reference sample, the calculated recovery percentages vary between 96.1% and 102.73%. The different indices obtained highlight the stability and the fidelity of the High Performance Liquid Chromatography (HPLC) as well as its precision.

The determination of the PAH concentrations of the certified sample shows the accuracy of the results of the assay of the different samples and the reliability of the method of extraction of the compounds because no significant difference was obtained with the conformity test. The results of the study of the validity of the method for determining PAHs are in accordance with the values demonstrating the acceptability of an analytical technique as affirmed by the experts of the Joint FAO/IAEA Committee [28] and documented in the American Association of Public Health's standard methods book [29].

This method also has the advantage of reducing the analysis time of a sample (6 minutes) compared to those proposed by the ISO 15753 standard (45 minutes) and Ake et al. [27] (20 minutes) for six (6) PAHs analyzed.

Analysis of the kolanuts samples collected revealed the presence of PAHs. This more or less significant presence is due to a post-harvest contamination by human or during kolanuts transport and storage [30]. Since the nuts after maturation and harvest are contained in the pods and coated with a thick protective membrane [19]. According to some authors, the low water solubility and the relatively high octanol/water partition coefficient (Kow) of PAHs give them a high adsorption potential on airborne particles in water and food [31,32]. Thus, the concentrations of PAHs in kola nuts can be attributed to the high atmospheric PAH pollution. In fact, the daily passage of gasoline engines or diesel engines of the various trucks in transit, combined with soot and smoke of all origins, from the exhaust gases of the combustion engines to the smoke of

cigarette and the incineration of the agricultural waste, wood combustion, coal or garbage could justify this PAH content [33,4,34]. Otherwise, Anyama, gateway to Abidjan, which concentrates most of the national manufacturing productive park with a high population density (20.8% of the population) and an intense daily road traffic is subject to more pollution than rural zone. In addition, the main compounds released by the exhaust gases of combustion engines, in particular those of the vehicles, are benzo(a)pyrene, benzo(k)fluoranthene, benzo(b)fluoranthene, benzo(g, h i)perylene and benzo(a)anthracene [35]. As for fluoranthene, pyrene and chrysene, they are emitted by domestic heating [36].

5. CONCLUSION

The study showed the acceptability of the method of determination of Benzo(a)pyrene, Benzo(b)fluoranthene, Benzo(a)anthracene, Chrysene, Fluoranthene, Benzo(k)fluoranthene, Dibenzo(a, h)anthracene, Benzo(g, h, i)perylene and Indeno(1,2,3, c, d)pyrene in kola nuts. The tests made it possible to highlight the linearity of the calibration lines in the chosen concentration ranges, the reproducibility and repeatability of the method used. This method has considerably reduced sample analysis time and could ensure effective health control not only of kola but other foodstuffs in order to ensure sustainable management and control of the toxicity of these food samples for the consumer.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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