J Fluoresc (2014) 24:549–556
DOI 10.1007/s10895-013-1324-5

ORIGINAL PAPER

Novel Comparative Synchronous Spectrofluorimetric Study of Benzo(a)pyrene Using Beta-Cyclodextrin and Calix(8)arene as Fluorescence Enhancers

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Received: 15 August 2013 / Accepted: 7 November 2013 / Published online: 19 November 2013

Abstract Successfully benzo(a)pyrene could be quantitified in environmental samples by a novel synchronous spectrofluorimetric techniques at a constant wavelength difference $\Delta\lambda=120$ nm, using beta-cyclodextrin 'β-CD' and calix(8)arene as fluorescence enhancers, where a linear calibration curve was obtained in a concentration range of 900–14,400 pg mL^{-1} and 18–360 pg mL^{-1} and the detection limit of 380.00 pg mL^{-1} and 12.08 pg mL^{-1} (which is well below the maximum contaminant concentration for benzo(a)pyrene set by the Environmental Protection Agency 'EPA') using both enhancers, respectively. The method can be easily adopted for determination of benzo(a)pyrene in aqueous media including tap water, river water and complex water samples. The recoveries obtained were 85.13–113.36 % with RSD<4 %. The proposed method was validated according to International Conference of Harmonization (ICH) guide lines and successfully applied to determine benzo(a)pyrene in pure form and in water samples including contaminated environmental water samples. All the results obtained were compared with those of a published method, where no significant difference was observed.

Keywords Benzo(a)pyrene · Beta-cyclodextrin · Calix(8)arene · Synchronous spectrofluorimetry · Fluorescence enhancement

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are environmental pollutants which are known for their teratogenic, carcinogenic and mutagenic potential [1, 2], consequently their monitoring is of great importance.

Benzo(a)pyrene (Fig. 1a) is one of the members of polycyclic aromatic hydrocarbons, consisting of five fused benzene rings and it is one of the most potent carcinogens [3]. It is one of the 16 PAHs listed by the EPA as priority pollutants [4], also it is selected as an indicator for the presence of other PAHs in different matrices, such as water, air and food [5–7].

Benzo(a)pyrene, as other PAHs, is generated from incomplete combustion of different organic materials as in case of motor vehicle traffic and forest fires [8, 9], thus it can reach humans through either ingestion of food, contaminated air or water [5].

The EPA has set a maximum admissible concentration for benzo(a)pyrene of 10 ng L^{-1} in water intended for human consumption [10] and a maximum contaminant level (MCL) of 200 ng L^{-1} [11].

In order to determine benzo(a)pyrene at such low levels, usually pre-concentration steps are required prior to the analysis. Different pre-concentration approaches have been used including liquid-liquid extraction, solid-phase extraction, solid-phase microextraction, stir-bar sorptive extraction or membrane extraction [12].

Methods reported for analysis of benzo(a)pyrene in literature were HPLC with UV–VIS, fluorimetric and amperometric detection, GC-MS and GC-FID [13–22]. Most of these methods require pre-concentration steps which render them tedious and time-consuming, so presenting a disadvantage in case of routine environmental monitoring.

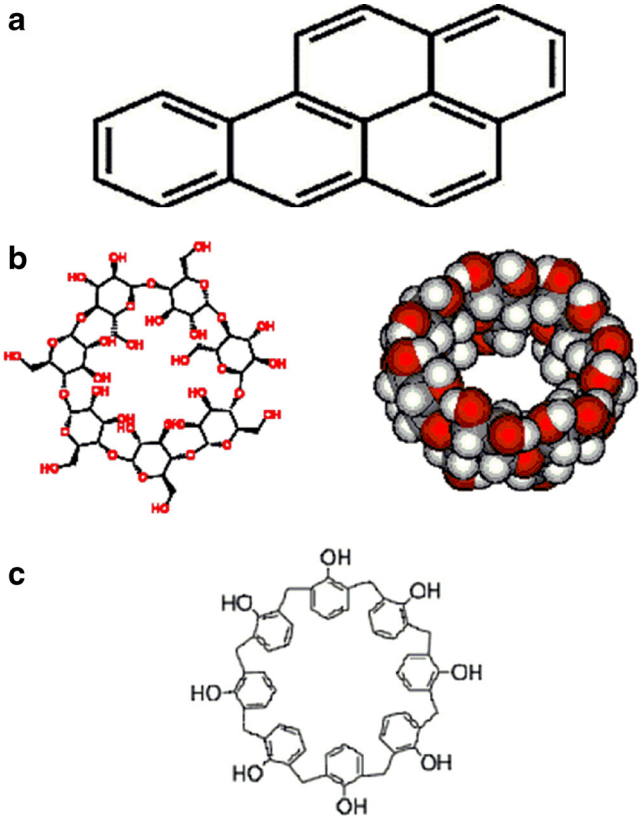

Fig. 1 **a** Structure of benzo(a)pyrene, **b** Chemical structure and three dimensional model of β-CD, **c** Chemical structure of calix(8)arene

Therefore, we intended to develop a highly sensitive and simple method for the determination of benzo(a)pyrene in different aqueous samples reaching low detection limits and consequently the pre-concentration step will not be required.

Fluorescence spectroscopy is a technique of high sensitivity, as usually it determines analytes in ng mL^{-1} range, thus it is well suited for environmental monitoring, especially, in case of polycyclic aromatic hydrocarbons which have high native fluorescence [23].

But, there are some drawbacks in conventional fluorescence spectroscopy, including the need for adjustment of excitation and emission wavelengths, which could be a problem for new substances when no excitation or emission wavelength is known. In addition to that the emission spectra of many substances tend to be broad and not symmetric [23].

Synchronous fluorescence spectrometric technique provides an improvement of the fluorescence spectroscopy by simplification of fluorescence spectra and its well known band-narrowing effect [24]. Furthermore, there is no need to handle two different spectra (exCitation and emission). Instead, only one synchronous spectrum is obtained by adjusting a suitable wavelength difference $\Delta\lambda$, where a good peak shape can be obtained in most of the cases [25].

Synchronous spectrofluorimetry has been mainly used for resolution of mixtures which were not easily resolved by conventional spectrofluorimetry [26–28].

Calix[n]arenes (CAs) and cyclodextrins (CDs) are among common macrocyclic hosts or cavitands which are water-soluble and posses a hydrophobic cavity compared to water, so they can interact with different organic molecules to form inclusion complexes in their hydrophobic cavity, modifying the physicochemical properties of the guest such as increasing the fluorescence emission [29, 30].

CAs are basket-shaped metacyclophanes synthesized by condensation of p-alkyl-substituted phenols and formaldehyde in the presence of alkalis. Furthermore, they can be functionalized by substitution of (−R) by groups such as HSO_3^-, to increase their water solubility [30, 31].

Cyclodextrins are cyclic oligosaccharides consisting of α-d-(+)-glucopyranose units linked by α-(1,4′)-glycosidic bonds. According to the number of units 6, 7 and 8 we obtain α-CD, β-CD (Fig. 1b) and γ-CD, respectively [32].

They have the shape of a truncated cone with non-polar cavity and can be modified by chemical and enzymatic treatments to obtain for example hydroxypropyl-β-cyclodextrin which shows different properties from the native β-CD such as higher water solubility [32].

Although both hosts have a hydrophobic nanocavity with similar dimensions, their features, structural properties and nature of the driving interactions for the complexes formation differ significantly [29].

In the present paper, a novel comparison study could be successfully adopted for benzo(a)pyrene determination, using a simple synchronous spectrofluorimetric technique by utilizing calix(8)arene (Fig. 1c) and β-CD as fluorescence enhancers, where a more sensitive and selective analysis procedure for benzo(a)pyrene could be obtained.

The delivered novel technique could be applied for determination of benzo(a)pyrene in tap water, river water and complex water samples.

Experimental

Chemicals and Reagents

Benzo(a)pyrene was obtained from Riedel-dehaen, Sigma-Aldrich, Germany and certified as analytical standard for environmental analysis and certified to contain >96 %.

Calix(8)arene (technical≥90 %) and beta-cyclodextrin (≥ 97 %) were obtained from Riedel-dehaen, Sigma-Aldrich, Germany. Methanol and dimethyl formamide (DMF) were obtained from ADWIC, Egypt.

All chemical and reagents used through this work are of spectrofluorimetric analytical grade.

Bi-distilled water is used throughout the whole work and is indicated by the word “water”.

Instruments

Fluorescence was measured on Kontron SFM25 (BIO-TEK Kontron, Switzerland) spectrofluorimeter, equipped with a 150 W Xenon lamp and a photomultiplier detector.

The spectrofluorimeter was controlled by computer, using SFM25 software, the photomultiplier tube voltage was adjusted at 450 V and the optimum scan speed of 500 nm s^{-1} was used.

All measurements took place in a standard 10 mm path length quartz cell, where the excitation and emission monochromators were scanned simultaneously with a constant difference $\Delta\lambda$=120 nm and a response time of 8 s.

The slit width of both monochromators was 5 nm and the synchronous spectra were recorded in an excitation scale.

Membrane filter (PTFE, 0.45 μm pore size) were used for filtration of environmental samples.

Standard Solutions

Standard Solutions of Benzo(a)pyrene

Stock standard solution of benzo(a)pyrene, having a concentration of 720 ng mL^{-1} was prepared in methanol and then further diluted with the same solvent giving a solution having a concentration of 36 ng mL^{-1} to be used as a working standard solution.

Solutions of Beta-Cyclodextrin and Calix(8)arene

Stock standard solutions of beta-cyclodextrin and calix(8)arene, having concentrations of 400 and 460 μg mL^{-1}, respectively, were freshly prepared in DMF and used as working standard solutions.

Procedure

General Procedure

Aliquots of benzo(a)pyrene standard working solution were mixed with 0.8 mL beta-cyclodextrin and 0.3 mL calix(8)arene separately in two series of 10 mL volumetric flasks and then diluted with water to obtain a concentration range of 900–14,400 pg mL^{-1} and 18–360 pg mL^{-1}, respectively.

The above mentioned procedure was adopted, using synchronous spectra where a constant wavelength difference $\Delta\lambda$=120 nm was used.

In all cases, the blank spectra were drawn and subtracted by the software from the corresponding spectra and the measurements were done at 280 nm in case of beta-cyclodextrin and at 276 nm in case of calix(8)arene as the vertical distance from the peak to the baseline.

The benzo(a)pyrene sample concentrations were determined by constructing calibration curves and adopting regression equations.

Sample Collection

Tap water was used directly from tap and river water, collected from Nile River in Cairo at a place near Tahrir Square in amber glass containers, was used after filtration using Membrane filters (PTFE, 0.45 μm pore size).

Analysis of Spiked Tap and River Water

Tap and river water were spiked with different amounts of benzo(a)pyrene and the mentioned procedure under "General Procedure" was utilized.

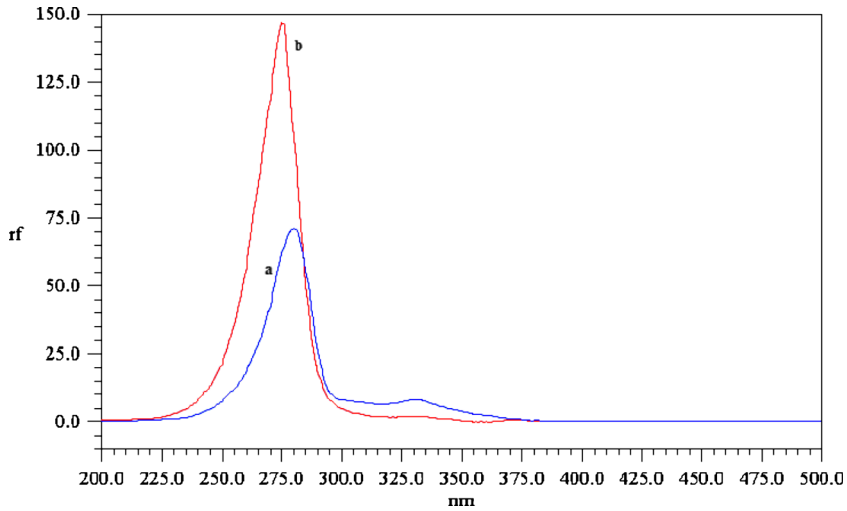

Fig. 2 Comparison of the fluorescence intensity enhancement of 3.6 ng mL^{-1} benzo(a)pyrene produced by (*a*) 32 μg mL^{-1} β-CD and (*b*) 13.8 μg mL^{-1} calix(8)arene

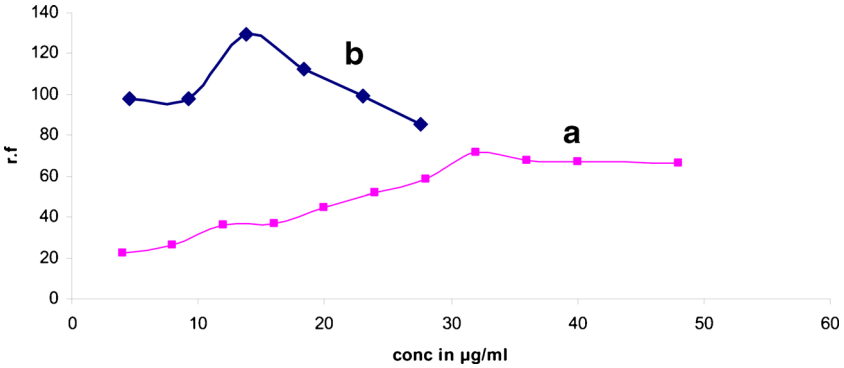

Fig. 3 **a** Effect of concentration of the cavidant β-CD on the fluorescence intensity of 3.6 ng mL^{-1} benzo(a)pyrene, **b** Effect of concentration of the cavidant calix(8)arene on the fluorescence intensity of 3.6 ng mL^{-1} benzo(a)pyrene

Analysis of Contaminated Environmental Water Samples

Water samples from sources which are likely to be contaminated with benzo(a)pyrene were collected from "shisha" (water pipe) and analyzed by the mentioned procedure under "General Procedure", where the water sample was analyzed by utilizing 0.5 mL and 0.1 mL of the sample in case of beta-cyclodextrin and calix(8)arene, respectively.

Results and Discussion

Effect of Different Cavidants and Solvents

Benzo(a)pyrene as a member of polycyclic aromatic hydrocarbons has a high native fluorescence, but this native fluorescence is not sufficient to determine benzo(a)pyrene at very low concentrations such as the maximum admissible concentration for benzo(a)pyrene set by the EPA.

Thus, in order to achieve a higher sensitivity, two different cavidants, namely beta-cyclodextrin and calix(8)arene were used and tested for enhancement of benzo(a)pyrene fluorescence intensity (Fig. 2). At the same time, the same utilized concentrations of benzo(a)pyrene were prepared without any cavidant, in order to determine the enhancement ratio of each one in water.

The enhancement ratio is defined as the ratio between the fluorescence intensity obtained from the solution containing enhancer and the fluorescence intensity obtained without enhancer. The different enhancement ratios were 4.74 and 8.58 for beta-cyclodextrin and calix(8)arene, respectively.

These results show that, beta-cyclodextrin and calix(8)arene are good candidates for the enhancement of benzo(a)pyrene fluorescence; consequently, they were used for further studies.

In order to investigate the effect of solvent on the obtained host-guest inclusion fluorescent-complex, methanol and DMF were used as alternative solvents instead of water, where the fluorescence enhancement ratio was tested, but no real fluorescence enhancement could be observed, thus, water was selected as the best solvent leading to high fluorescence enhancement.

This could be due to the high polarity of water leading to a great difference between the external environment (solvent)

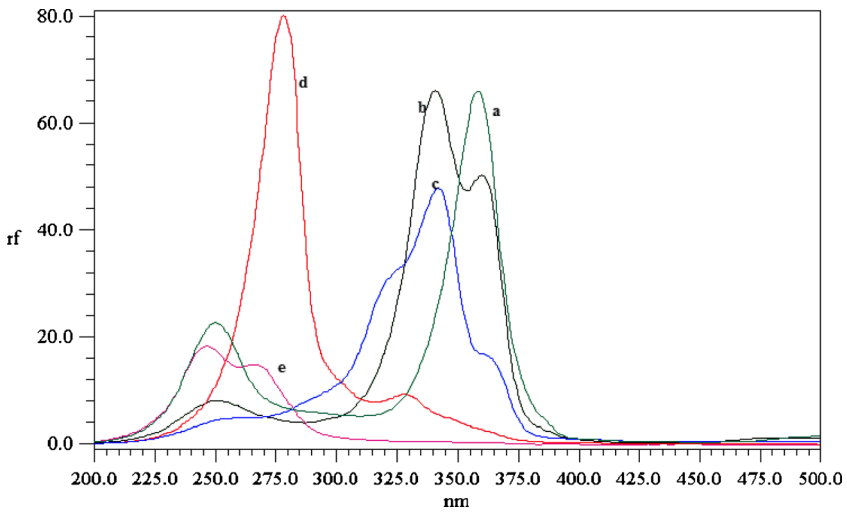

Fig. 4 Synchronous spectra of 36 ng mL^{-1} benzo(a)pyrene in methanol at different Δλ obtaining the best spectrum at Δλ=120 nm (*a*) Δλ=40 nm, (*b*) Δλ=60 nm, (*c*) Δλ=80 nm, (*d*) Δλ=120 nm, (*e*) Δλ=180 nm

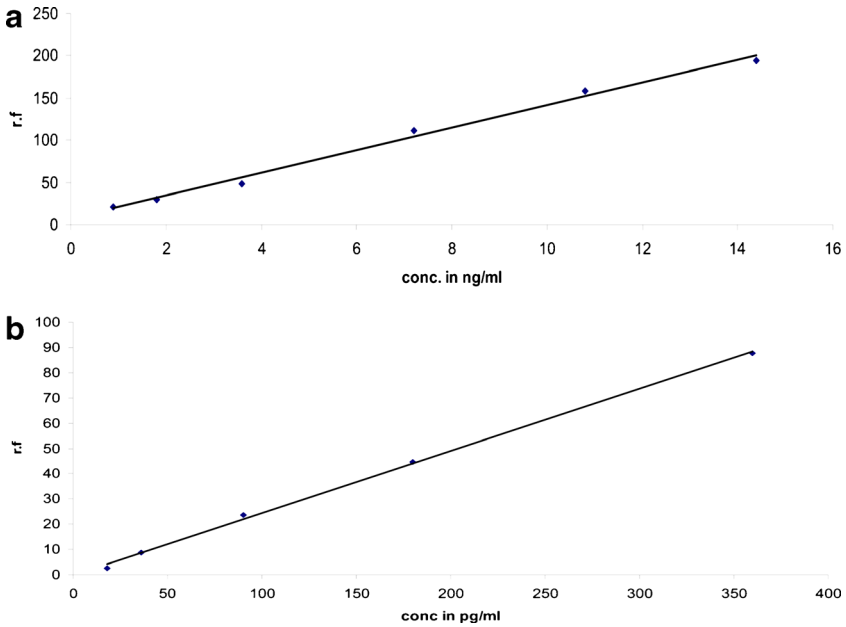

Fig. 5 Calibration curve for benzo(a)pyrene using **a** 32 μg mL^{-1} β-CD and **b** 13.8 μg mL^{-1} calix(8)arene

and the internal nanocavity of the cavidant. Thus, this increases the tendency of the non-polar and highly hydrophobic solute benzo(a)pyrene to enter the nanocavity of the cavidant to be protected from the polar environment.

But in case of DMF and even methanol, this is not the case as benzo(a)pyrene has a greater solubility in these solvents than in water.

The increase in fluorescence intensity in micellar medium is generally attributed to the formation of inclusion complex, where the analyte is located in the nanocavity of the cavidant, so it is protected from any fluorescence quenchers. This new microenvironment permits the stabilization of the excited singlet states [33].

The fluorescence enhancement obtained in case of calix(8)arene is much greater than that obtained in case of beta-cyclodextrin. The predicted reason for this great enhancement in case of calix(8)arene is that the cavity of calix(8)arene consists of eight benzene rings, allowing greater interaction with the aromatic system of benzo(a)pyrene through π-π interactions, consequently extending the aromatic system leading to a highly fluorescent complex.

The effect of pH on fluorescence intensity was not performed, since the studied compound has no ionizable groups.

Effect of Cavidant Concentration

The effect of cavidant concentration was also investigated to determine the optimum concentration for fluorescence enhancement (Fig. 3a and b).

The results obtained illustrate that, increasing the beta-cyclodextrin concentration is accompanied by increasing the fluorescence intensity, where the highest fluorescence intensity was obtained at 32 μg mL^{-1}, since at concentrations higher than 32 μg mL^{-1}, the fluorescence intensity begin to decline again. While, in case of calix(8)arene the optimum concentration for fluorescence enhancement was 13.8 μg mL^{-1}.

The decrease in fluorescence at high micelle concentration may be due to the decrease in incident light of excitation that

Table 1 Validation parameters of the proposed spectrofluorimetric method for benzo(a)pyrene using the two different cavidants

Parameters	In presence of beta-cyclodextrin	In presence of calix(8)arene
Range	0.9–14.4 ng mL^{-1}	18–360 pg mL^{-1}
Slope	13.44	0.2458
Intercept	7.4	−0.2234
S_b	0.59	0.00079
S_a	4.81	0.03711
LOD	0.38 ng mL^{-1}	12.08 pg mL^{-1}
LOQ	0.96 ng mL^{-1}	18.31 pg mL^{-1}
Correlation coefficient (r)	0.9961	0.9993
Accuracy (mean ± SD)	101.03±2.47	98.97±2.30
RSD %	2.45	2.33
%Er	1.00	1.04

LOD limit of detection; *LOQ* limit of quantification; S_a standard deviation of the intercept; S_b standard deviation of the slope, *%RSD* relative standard deviation (%RSD = SD × 100/X where SD is the standard deviation and X is the mean recovery), *%Er* percent error (%Er=RSD/√n where n is the number of values)

Table 2 Repeatability (intra-assay precision)

Beta-cyclodextrin as fluorescence enhancer					Calix(8)arene as fluorescence enhancer				
Amount added (ng mL^{-1})	Amount found[a] (ng mL^{-1})	Recovery %	RSD %	Er %	Amount added (pg mL^{-1})	Amount found[a] (pg mL^{-1})	Recovery %	RSD %	Er %
0.9	0.91	101.38	2.86	1.65	90	92.45	102.72	0.44	0.25
1.8	1.79	99.18	2.73	1.58	180	182.36	101.31	0.22	0.13
3.6	3.59	99.59	0.75	0.43	360	345.36	95.94	0.96	0.55

[a] Each result is the average of three separate experiments

reaches the benzo(a)pyrene molecules, as it could be partially dispersed and absorbed by the crowded cavidant molecules.

Effect of Temperature

A study of the effect of temperature on the fluorescence intensity of benzo(a)pyrene, using the different cavidants shows a decrease in fluorescence intensity with increasing temperature, therefore, all measurements were done at room temperature.

The decrease in fluorescence in case of both cavidants could be explained by weakening the interactions between the hydrophobic analyte benzo(a)pyrene and the hydrophobic cavity of the cavidant, due to increased kinetic energy transferred to the molecules at higher temperature.

Adjustment of the Instrumental Parameters for Synchronous Scanning

The use of synchronous spectrofluorimetry enhances minor spectral features and allows more reliable identification of chemical species [24]. Also, synchronous spectrofluorimetry has been used to determine single compounds rather than mixtures [34] in order to decrease matrix interference and to obtain better peak shape and resolution which is important in environmental analysis.

Since, the adjustment of instrumental parameters is very important to obtain a symmetric spectrum shape with a narrow bandwidth, different $\Delta\lambda$ have been tested at 10 nm intervals and each time the synchronous spectrum has been plotted.

The change in $\Delta\lambda$ leads to a considerable change in the spectrum shape and the best synchronous spectrum was obtained for $\Delta\lambda$=120 nm (Fig. 4). Consequently, this $\Delta\lambda$ was selected for performing the synchronous scans of benzo(a)pyrene.

Also, in order to obtain the best shape and response, other instrumental parameters were adjusted, including the voltage of the photomultiplier, the scan speed and the response time, where (450 V), (500 nm min^{-1}) and (8 s) were chosen.

The synchronous spectra were plotted and the response was recorded at a wavelength of 280 nm and 276 nm in case of beta-cyclodextrin and calix(8)arene, respectively, as the vertical distance from the peak to the baseline. This signal is proportional to benzo(a)pyrene concentration and it was utilized for construction of the calibration graph.

Analytical Characteristics

By applying the previously mentioned conditions, the calibration graph was plotted, obtaining regression equations [$y=13.444x+7.4$] and [$y=0.2458x-0.2234$] for both cavidants, respectively, (Fig. 5 and Table 1).

The limit of detection (LOD) was determined according to ICH Q2(R1) recommendations [35], by analyzing an appropriate number of samples near the detection limit and calculating the standard deviation of y-intercepts of the obtained

Table 3 Intermediate precision (inter-day assay)

Beta-cyclodextrin as fluorescence enhancer					Calix(8)arene as fluorescence enhancer				
Amount added (ng mL^{-1})	Amount found[a] (ng mL^{-1})	Recovery %	RSD %	Er %	Amount added (pg mL^{-1})	Amount found[a] (pg mL^{-1})	Recovery %	RSD %	Er %
0.9	0.91	101.66	2.86	1.65	90	95.29	105.88	1.86	1.07
1.8	1.81	100.42	2.73	1.58	180	182.36	98.30	2.86	1.65
3.6	3.51	97.59	0.75	0.43	360	345.36	94.54	1.18	0.68

[a] Each result is the average of three separate experiments

Table 4 Statistical comparison between the proposed method and a published method

Values	Beta-cyclodextrin as fluorescence enhancer		Calix(8)arene as fluorescence enhancer	
	Proposed method	Published method	Proposed method	Published method
Mean	101.03	100.10	98.97	100.10
Standard deviation	2.47	1.27	2.30	1.27
Variance	6.10	2.02	5.30	2.02
N	6	5	5	5
F	3.02 (6.26)[a]		2.62 (6.39)[a]	
Student's *t* test	0.748 (2.262)[a]		0.933 (2.306)[a]	

[a] Figures in parenthesis are the corresponding theoretical values for F and t at the 95 % confidence level

regression lines (σ) followed by using the equation LOD=3.3 σ/S, where S is the slope of the calibration curve.

The results of inter-day and intra-day precision of the method were obtained by using [0.9, 1.8 and 3.6 ng mL^{-1}] and [90, 180 and 360 pg mL^{-1}] of benzo(a)pyrene in case of beta-cyclodextrin and calix(8)arene, respectively. The precision is acceptable due to the low values of SD and RSD%.

Also, the low values of Er% indicate a good inter-day and intra-day accuracy when using the proposed method, (Tables 2 and 3).

The results of the proposed method for determination of benzo(a)pyrene were compared with those of the published method [26] and the statistical comparison between the results was performed using the student's *t*-test and F ratio at 95 % confidence level, as shown in (Table 4).

The proposed method was found to be accurate and precise, since there was no significant difference between both, the proposed and the published methods.

Applications

The proposed method was successfully applied for rapid determination of benzo(a)pyrene in tap water and river water (Nile water), by analyzing spiked water samples, as shown in Table 5.

It was also successfully applied for analysis of a contaminated environmental water sample (shisha water) as shown in (Table 5).

Conclusion

The results obtained from the present study show that cavidants, especially calix(8)arene, are able to enhance the fluorescence of benzo(a)pyrene and thus other polycyclic aromatic hydrocarbons in water in such a way that they can be determined at very low concentrations as suggested by the EPA.

Thus, the present work presents determination of benzo(a)pyrene in aqueous samples by a novel simple, sensitive and time saving method for routine environmental analysis of benzo(a)pyrene with good sensitivity and precision without the need of a pre-concentration step.

At the same time this paper highlights the use of calix(n)arenes as successful cavidants for enhancement of

Table 5 Results obtained from the analysis of real and polluted water samples

Sample	Using β-CD as fluorescence enhancer				Using calix(8)arene as fluorescence enhancer			
	Added (ng mL^{-1})	Found[a] (ng mL^{-1})	Relative Recovery %	RSD % (n=3)	Added (pg mL^{-1})	Found[a] (pg mL^{-1})	Relative recovery %	RSD % (n=3)
Tap water	0	ND	–	–	0	ND	–	–
	0.90	0.77	85.13	0.56	90.00	92.72	103.02	1.16
	1.80	1.86	103.17	0.93	180.00	181.41	100.78	1.76
	3.60	4.08	113.36	0.68	288.00	280.27	97.32	0.72
Nile water	0	ND	–	–	0	ND	–	–
	1.00	1.07	107.61	0.26	90.00	77.80	86.44	1.85
	1.80	2.14	112.75	1.60	180.00	163.64	90.91	1.39
	3.60	3.97	107.42	0.61	288.00	251.93	87.47	0.61
Shisha water (water pipe)	0	78.85	–	–	0	6941.99	–	–

[a] Each result is the average of three separate experiments

fluorescence intensity of highly conjugated aromatic systems such as polycyclic aromatic hydrocarbons.

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