

Optimization of the Benzo(A)Pyrene Determination Procedure in Cachaça

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Neste trabalho foi otimizada uma metodologia de rotina para quantificar e confirmar a presença de benzo(a)pireno (BaP) em cachaça. Foram avaliados três procedimentos de extração; um, utilizando extração em fase sólida (EFS) tanto para extração como para purificação e dois, empregando extração líquido-líquido, seguida de limpeza por cromatografia em coluna com sílica gel. O BaP foi quantificado por cromatografia líquida de alta eficiência (CLAE), com detecção por fluorescência e confirmação por cromatografia gasosa com detector de massas. Os limites de detecção e quantificação obtidos foram, respectivamente, 0,03 e 0,10 $\mu\text{g L}^{-1}$. Os resultados de recuperação, em 3 níveis de concentração (0,1; 1,0 e 3,0 $\mu\text{g L}^{-1}$) situaram-se na faixa de 82,9 a 97,0%, com coeficientes de variação de 0,5 a 4,9%. Estes resultados são similares aos relatados em trabalhos anteriores. Os 3 procedimentos de extração foram considerados apropriados para quantificar BaP em cachaça, entretanto a EFS revelou ser o método mais barato, rápido e com menor consumo de solvente. As concentrações de BaP variaram de não detectado ($\text{LOD} < 0,03 \mu\text{g L}^{-1}$) a 0,49 $\mu\text{g L}^{-1}$ em vinte amostras de cachaça analisadas.

The present study evaluated and optimized a routine method to quantify and confirm BaP in Brazilian sugar cane spirit (cachaça). Three extraction procedures were tested, one using SPE for extraction and clean-up, while two others were liquid-liquid partition extraction followed by silica gel chromatography clean-up. BaP was quantified by high performance liquid chromatography with fluorescence detection and confirmed by gas chromatography-mass spectrometry. HPLC detection and quantification limits were 0.03 and 0.10 $\mu\text{g L}^{-1}$, respectively. The recovery results ranged from 82.9 to 97.0% with coefficients of variation (CV) ranging from 0.5 to 4.9% at concentration levels of 0.1, 1.0 and 3.0 $\mu\text{g L}^{-1}$, which are in accordance with those described by other authors. The three procedures were appropriated to quantify BaP in cachaça, although SPE extraction is the cheapest, fastest and the least solvent-consuming. The levels of BaP contamination on twenty cachaça samples ranged from no detected ($\text{LOD} < 0.03 \mu\text{g L}^{-1}$) to 0.49 $\mu\text{g L}^{-1}$.

Keywords: benzo(a)pyrene, polycyclic aromatic hydrocarbons, cachaça, sugar cane spirit, HPLC

Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental and food contaminants.^{1,2} In the last years, they have attracted considerable interest, because some of them are highly carcinogenic in laboratory animals and have been implicated in breast, lung, and colon cancers in humans. These chemicals commonly enter human body through inhalation of cigarette smoke or polluted air, dermal contact or ingestion of contaminated food and beverages. Among these two pathways, dietary intake of PAHs constitutes an important source of exposure in humans.³

Benzo(a)pyrene (BaP) is the most studied PAH and it usually is generated together with others PAHs.⁴ Scientific Committee on Food⁵ concluded that BaP could be used as a marker for the occurrence and effect of carcinogenic PAH in beverages and food.⁶

The control of BaP in food and beverages chain is required due to potential hazard to human health. The European Union has recently established a maximum level of BaP in different foodstuff, such as, oils and fats, smoked meat, crustaceans, bivalve mollusks, and infants and baby foods.⁷ During the last decade, many studies concerning BaP monitoring in food and water have been developed.⁸⁻¹² However, only a few studies about BaP contamination of alcoholic beverages were published.

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BaP is extracted using different techniques prior to clean-up and purification. Normally, after complete dissolution or alkaline digestion of food samples, BaP is extracted from sample by a selective solvent.^{4,6} Extracted samples are usually purified from interfering substances by adsorption column chromatography.¹³ C-18 cartridges have been used for both extraction and clean-up of water and beverage samples.¹⁴⁻¹⁶

A common analytical procedure used in the determination of BaP in food is high performance liquid chromatography (HPLC) with fluorescence detection,^{4,15,17} sometimes combining gas-chromatography (GC-MS) for identifying BaP compounds.¹⁷

Brazilian sugar cane spirit (cachaça) is a typical Brazilian distilled alcoholic beverage obtained from distillation of fermented sugar cane juice. Its annual production is estimated at around 1.4 billion liters,¹⁸ 1% of which are exported,¹⁹ being the third spirit most consumed in the world.¹⁷ The BaP contamination of cachaça may occur from raw material, because sugar cane is burnt during the harvesting season. In addition, graxes and oils from the equipments used during the industrial process¹⁶ and maturing also may be responsible for BaP contamination of cachaça.^{20,21}

Considering both high production and consumption of cachaça in Brazil, the objective of the present study was to propose a reliable routine method for determination of BaP in cachaças.

Experimental

Chemicals

All chemicals employed were analytical grade, except acetonitrile, cyclohexane, methanol and isopropanol, which were HPLC grade, purchased from Merck (Darmstadt, Germany). BaP standard with 97% purity was purchased from Sigma (ST Louis, USA). The Accubond SPE C-18 (500 mg, 6 mL) columns were purchased from Agilent (Palo Alto, USA). All solutions were prepared with deionized water (resistivity > 18 M Ω cm⁻¹, Toraypure, Shimadzu, Japan).

Standard solution

Stock standard solution was prepared by BaP dissolution in acetonitrile. Aliquots were taken to prepare analytical solutions in acetonitrile ranging from 0.05 to 30 μ g L⁻¹ to calibrate HPLC fluorescence detector. This stock solution was also used to spike the samples.

Samples

A total of 20 samples of cachaça produced in 4 different Brazilian states were purchased in supermarkets, and cachaça stores in the City of São Paulo, State of São Paulo, Brazil, during the period of March to July of 2006. All samples were analyzed in triplicate.

Methods

Extraction

Three different extraction procedures were tested to quantify BaP in cachaça, one using SPE and the others liquid-liquid partition as extraction step. In all cases, both identification and quantification were done by HPLC system with fluorescence detection. The confirmation of BaP was carried out using a GC-MS equipment.

SPE extraction

Extraction and clean-up procedures were conducted according to Bettin and Franco¹⁶ with some modifications. SPE cartridge was conditioned with 10 mL methanol before sample injection. An analytical aliquot of 10.0 mL of sample was transferred to the cartridge. The vacuum was adjusted to elute the sample at about 1 drops s⁻¹. After the entire sample had been eluted, the column was rinsed with 1 mL isopropanol, and completely drained. BaP was eluted with 10 mL cyclohexane. The eluate was dried under N₂ at 40 °C. The residue was dissolved in 2.0 mL acetonitrile, and filtered through a 0.45 μ m membrane filter before LC analysis.

Partition extraction with cyclohexane

This procedure was an adaptation of the methods described by Toussaint and Walker,²² and Kleinjans *et al.*²³

50 mL of cachaça were transferred to separating funnel, and 7 g of NaCl were added. This mixture was extracted with three portions of 30 mL of cyclohexane. The combined extract was filtered through filter paper containing 2 g Na₂SO₄. The solution was reduced to 4 mL under N₂ at 40 °C. The extract was cleaned-up through a silica-gel column as described in "Silica-gel clean-up".

Partition extraction with dimethylformamide/water

This methodology was first described by Grimmer and Böhne¹³ and has been utilized with some modifications in many studies of BaP or PAHs determination in foods.

20 mL of cachaça were transferred to separating funnel, and 7 g NaCl were added. This mixture was extracted with 120 mL (portions of 50, 35 and 35 mL) of cyclohexane. The cyclohexane combined portions were extracted with 100 mL

(portions of 50, 25 and 25 mL) dimethylformamide-water (9:1, v/v). The extraction solution was diluted with 50 mL of 2% sodium sulfate aqueous solution and re-extracted with 120 mL (portions of 50, 35 and 35 mL) of cyclohexane. The extracts were filtered through filter paper containing 2 g of anhydrous sodium sulfate. The solution was reduced to 4 mL under N_2 at 40 °C. The extract was cleaned-up by silica-gel column as described following.

Silica-gel clean-up

5 grams of silica gel for adsorption chromatography (with a content of 15% of water) were packed into a glass column. The BaP extract was transferred to the column and eluted with 100 mL of cyclohexane. The eluate was dried under N_2 at 40 °C and the residue was dissolved in 2.0 mL of acetonitrile. This solution was filtered through 0.45 μ m membrane filter before LC analysis.

HPLC chromatographic conditions

The determination of BaP was carried out in a Shimadzu (Kyoto, Japan) high performance liquid chromatography (HPLC) system equipped with a pumping system (LC-10AD), a degasser (DGU-14A), a fluorescence detector (RF-10A XL), a 20 μ L injector (Rheodyne LP), and a computer software (Class LC10). The BaP was separated with a reversed-phase C-18 column (Varian, Lake Forest, USA) (250 x 4.6mm, 5 μ m) protected with a guard column C-18 (Varian, Lake Forest, USA) (2.0 x 4.6 mm, 5 μ m) using isocratic elution. The mobile phase was a mixture of acetonitrile:water (70:30, v/v), and the flow-rate was 1.0 mL min^{-1} .¹⁷ The BaP was detected by fluorescence detector at 295 nm excitation wavelength and 405 nm emission wavelength.

Quantification

Peak areas of the sample chromatograms were correlated with the concentrations according to the calibration curve. The linearity was determined in the range of 0.05-30 μ g L^{-1} using ten BaP standard solutions with equidistant concentrations. Standard solution and sample volumes of 20 μ L were injected in triplicate.

GC-MS chromatographic conditions

The confirmation of BaP was carried out in a Shimadzu (Kyoto, Japan) chromatograph system equipped with gas chromatograph (GC 17A), mass detector (QP 5000), and computer software (Class 5000). The BaP was separated with DB5-MS column (J&W Scientific, Folson, USA)

(5% phenyl-95% methylpolysiloxane, 30 m x 0.254 mm, 0.25 μ m film thickness) using helium as carrier gas at 0.9 mL min^{-1} . The GC temperature programming rates were as follows: initial column oven temperature, 120 °C, then programmed to rise to 250 °C at 5 °C min^{-1} and thereafter to 280 °C at 4 °C min^{-1} . Detector temperature was 285 °C, and injector temperature 250 °C. Injection volume was 1 μ L using splitless by 1 min, after that, the split valve was opened (split ratio: 1/10). Mass spectra were obtained in SIM mode, monitoring selected fragments at 126 and 252 m/z, and the ionization voltage was 70 eV.

Validation

Detection limit was calculated by spiking blank cachaça samples at levels of the BaP equivalent to three times the standard deviation of noise on analysis ($n = 5$), while quantification limit was calculated from BaP concentrations that provided signals equal to ten times of noise on analysis ($n = 5$). The spiked samples were prepared in five replicates independently using a blank cachaça sample.

Recovery was assessed by performing tests on cachaça blank samples. Independent aliquots were fortified with 0.1, 1.0, and 3.0 μ g L^{-1} . At each level, the analyses were performed at least in independent triplicates. Recoveries were determined by back-calculation.

Repeatability of the method was evaluated through the coefficients of variation (CV%) associated with measurements of BaP ($n = 5$) in one choiced cachaça sample with low and natural contamination using three different extraction methods.

Method application

Twenty different cachaça brands obtained in the market in the City of São Paulo, State of São Paulo, Brazil, were evaluated using SPE extraction, HPLC quantification and confirmation of BaP identity by GC-MS. These samples were from four different states of Brazil, nine cachaça samples from Minas Gerais, nine from São Paulo, one from Ceará, and one from Pernambuco.

Results and Discussion

In order to define HPLC chromatographic conditions, the analytical mobile phase composition was investigated considering different proportions of acetonitrile and water using a fortified cachaça sample. The percentages of acetonitrile ranged from 65 to 80% at a constant flow-rate of 1.0 mL min^{-1} . The 70% acetonitrile composition was the best proportion to evaluate optimal peak sharpness and efficient

separation from others extraneous substances (Figure 1). After chromatographic conditions standardization, different analytical excitation and emission wavelengths were tested to detect BaP. The best results were obtained using 295 nm excitation wavelength and 405 nm emission wavelength.

Two different HPLC analytical columns were evaluated, an Inertsil ODS-P column (GL Science) (150 × 4.6 mm, 5 μm) and a C-18 column (Varian, Lake Forest, USA) (250 × 4.6 mm, 5 μm). The latter column was chosen because Inertsil column showed both high affinity to analyte resulting a retention time of 64 minutes and poor resolution of BaP peak (resolution < 1.5).

Fluorescence detector was linear for BaP from 0.05-30 μg L⁻¹. Data least-square regression analysis for ten calibrators resulted in excellent straight-line fits over the examined concentration curve described by equation $A = 331713 \text{ BaP} - 625,95$ ($r^2 = 0.9995$, where A is the peak area and BaP the benzo(a)pyrene concentrations). Detection (LOD) and quantification (LOQ) limits were 0.03 and 0.10 μg L⁻¹, respectively. These results are

considered satisfactory for BaP determination²⁴ and they are in accordance with values reported by others authors for BaP analysis in food and beverages.^{8,10,15-18,25}

Extraction procedures were evaluated to maximize recovery and decrease both time and solvent consumption. Three different extraction procedures were tested, one using only SPE cartridge for extraction and clean-up steps, and two others, solvent partition to extraction and silica gel column to clean-up. The recovery results from three extraction procedures were summarized in Table 1. The precision was expressed as CV%, and the accuracy was determined using fortified blank cachaça at 0.1, 1.0 and 3.0 μg L⁻¹ and expressed as the percent deviation between amount found and amount added at three concentration levels.

The recovery values summarized in Table 1 have good similarity, ranging from 82.9 to 97.0% with CV% from 0.5 to 4.9% at concentration levels of 0.1, 1.0 and 3.0 μg L⁻¹. However, the best results were obtained from SPE extraction, ranging from 91.7 to 96.3% and CV from 1.9 to 4.9%.

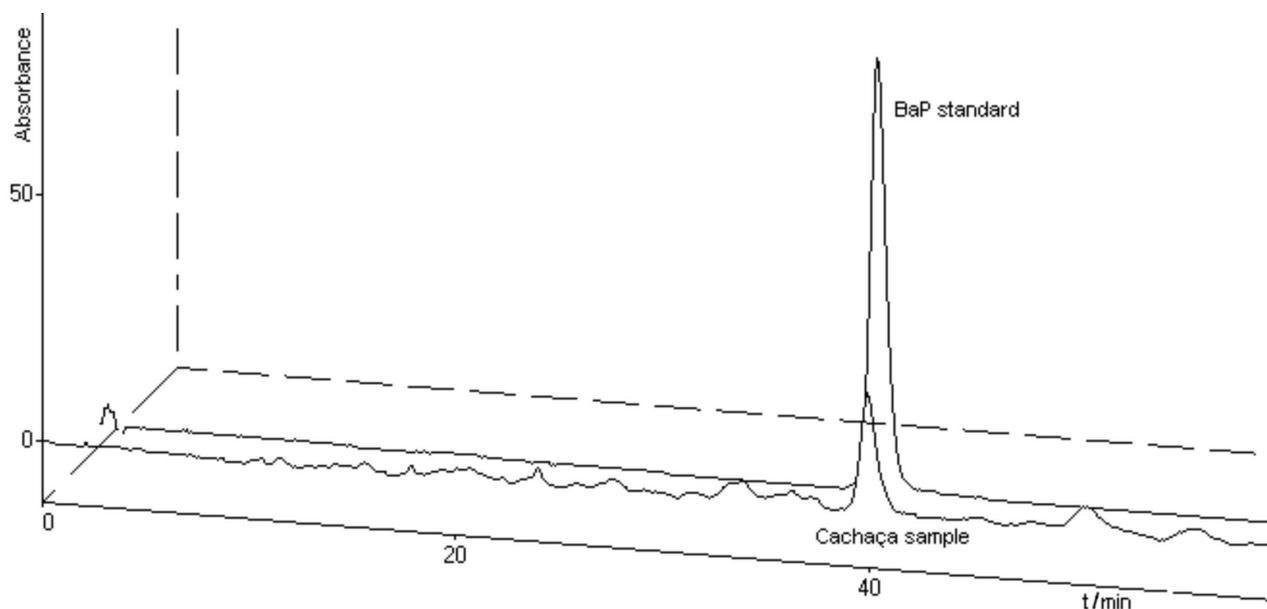


Figure 1. Chromatograms obtained by HPLC with fluorescence detection for BaP standard and cachaça sample solutions.

Table 1. Recovery values for three different extraction procedures of BaP from cachaça samples

Procedure	Recovery					
	0.1 ppb		1.0 ppb		3.0 ppb	
	Mean / (%)	CV / (%)	Mean / (%)	CV / (%)	Mean / (%)	CV / (%)
Partition with cyclohexane	93.3	1.4	92.3	0.5	82.9	1.3
Partition with dimethyl-formamide	90.8	0.7	97.0	1.2	87.9	1.9
SPE	96.3	2.4	91.7	4.9	94.0	1.9

CV = coefficients of variation.

The recovery values are satisfactory for determinations at these levels ($\mu\text{g L}^{-1}$)^{24,26} and they were in accordance with those reported by others authors.^{8,15,18,25} All peaks obtained in the recovery experiments were analyzed by GC-MS. This technique showed itself sensible and reliable to confirm the BaP peak identity.

Tfouni *et al.*¹⁷ analyzing cachaça samples have found BaP recovery value of 74.5% and CV of 11.5%. In another work related to quantify PAH in cachaça, the authors had reported BaP recovery of 99.4% and CV of 4.2%.¹⁶ Garcia-Falcon and Simal-Gandara²⁰ had obtained BaP recovery result of 94% and CV of 9% for white rum and recovery result of 100% and CV of 9% for red wine.

The repeatability was also evaluated for three extraction procedures and the results were summarized in Table 2. In this study, BaP concentration was determined in the same sample of cachaça in quintuplicate. All extraction procedures presented good repeatability.

Table 2. Repeatability values for three different extraction procedures of BaP from cachaça samples

Procedure	Repeatability		
	Mean, n = 5 / (ppb)	sd / (ppb)	CV / (%)
Partition with cyclohexane	0.149	0.004	2.7
Partition with dimethyl-formamide	0.183	0.004	2.2
SPE	0.177	0.003	1.7

sd = standard deviation, CV = coefficients of variation.

Based on the results showed in Tables 1 and 2, the three extraction procedures showed precision, accuracy and sensitivity to quantify BaP in cachaças. During both liquid-liquid extraction procedures, the formation of emulsion was observed, resulting on low recovery values as described by Toussaint and Walker.²² In order to avoid emulsion, a portion of sodium chloride was added in the sample at the beginning of the extraction step, resulting on better recovery results. The solvent partition extraction steps using N,N-dimethylformamide were similar to procedure described by Tfouni *et al.*,¹⁷ which is an adaptation from the original method proposed by Grimmer and Böhnke.¹³ However, the use of only 50 mL of 2% sodium sulphate aqueous solution instead of 100 mL of 1% sodium sulphate aqueous solution as proposed by Tfouni *et al.*¹⁷ produced higher recovery values.

A modification of the procedure previously described by Bettin and Franco¹⁶ was used in SPE extraction. In order to define the best experimental conditions to elute BaP from the cartridge, different solvent volumes of isopropanol and acetonitrile or cyclohexane were tested. Analyzing

the recovery values, it was concluded that the use of isopropanol is important to remove residual water present in the cartridge, increasing recoveries. The best results were obtained using 1 mL of isopropanol to rinse the cartridge before the BaP elution with 10 mL of cyclohexane.

Considering solvent and time consumption and costs related to each extraction method, SPE extraction drastically reduced analysis time, volume of solvents and sample manipulations, and it was also suitable for routine analysis. This technique combines both clean-up and extraction. It was cheapest, and the least toxic among of the three techniques tested.

SPE extraction was applied in the quantification of twenty cachaça samples purchased in the market in São Paulo City, State of São Paulo, Brazil. The sampling was formed by cachaça brands from sugar cane burnt and not burnt. The levels of BaP contamination ranged from no detected (LOD < 0.03 $\mu\text{g L}^{-1}$) to 0.49 $\mu\text{g L}^{-1}$. Table 3 summarizes the concentrations of BaP determined in different brands of cachaça and their respective producer state.

Table 3. Levels of BaP in cachaça samples from different Brazilian States

Total samples	State sample	Concentration range, n=3 / (ppb)
09	São Paulo	< LOD-0.49
09	Minas Gerais	< LOD-0.12
01	Ceará	0.19
01	Pernambuco	< LOD

LOD = 0.03 ppb.

The identity of all BaP peaks was confirmed by GC/MS method, monitoring selected fragments at 126 and 252 m z^{-1} . Brazilian regulations have not established BaP limit for alcoholic beverages; however, considering European regulation for foods, that establishes a BaP maximum level ranged from 2.0 $\mu\text{g L}^{-1}$ to 10.0 $\mu\text{g L}^{-1}$ depending of the kind of the food,⁷ all analyzed cachaças presented BaP contents above these limits.

The results shown in Table 3 are in accordance with those described by others authors,¹⁶⁻¹⁸ and the variation of the BaP levels could be related to different processing or differences on the type of sugar cane, burnt or not burnt. Bettin and Franco¹⁶ analyzed 28 cachaça samples and have found BaP levels ranged from LOD (0.001 $\mu\text{g L}^{-1}$) to 1.55 $\mu\text{g L}^{-1}$, and they observed a highest PAH contamination in cachaça produced from burnt sugar cane. Tfouni *et al.*¹⁷ analyzed 25 brands of cachaça and have reported BaP levels ranged from LOD (0.011 $\mu\text{g L}^{-1}$) to 0.36 $\mu\text{g L}^{-1}$. In another study, 131 cachaça samples from burned and non-burned

sugar crops were analyzed and BaP levels ranged from LOD ($< 0.001 \mu\text{g L}^{-1}$) to $0.045 \mu\text{g L}^{-1}$.¹⁵

Studies conducted in Brazil have shown that sugar cane burnt is an important source of PAHs emission and could be responsible for contamination of sugar cane by-products.^{15,16} Other possible sources of BaP contamination are the contact of the product with coal tar that coats the reservoirs utilized to store the cachaça during the old step and the sugar added in some cachaça brands.¹⁶

The control of BaP in food and beverages has been required due to its mutagenic and carcinogenic potential in vertebrates. During the last decade, many studies concerning monitoring of PAH and BaP in food have been developed. However, only a few studies about BaP or PAH contamination of alcoholic beverages were published.^{15-17,20} Food contamination studies by BaP are included in the JECFA (Joint FAO/WHO Expert Committee on Food Additives) priority list.²⁷ In addition, European Commission has just published the Regulation (EC) N. 208/2005, establishing maximum level of BaP in some foods.⁷

Considering both expressive production and high national consumption of Brazilian cane sugar spirit (cachaça), and the use of burnt sugar cane as raw material to produce this alcoholic beverage, the control of BaP contamination is very important to guarantee the safety and quality of this product. Consequently, a reliable routine method to determine BaP in cachaça samples is important for aiming the quality control.

Conclusions

The resolution obtained by HPLC isocratic elution using SPE C18 extraction and clean-up is a reliable method to determine BaP in cachaça samples. HPLC detection and quantification limits were 0.03 and $0.10 \mu\text{g L}^{-1}$, respectively. The recovery results ranged from 82.9 to 97.0% with CV% ranging from 0.5 to 4.9% at concentration levels of 0.1, 1.0 and $3.0 \mu\text{g L}^{-1}$. The levels of BaP contamination on twenty cachaça samples ranged from no detected (LOD $< 0.03 \mu\text{g L}^{-1}$) to $0.49 \mu\text{g L}^{-1}$. The analytical method presents appropriated accuracy, precision and sensitivity levels, for the quality control of this product and all the results found in the present work are in accordance with those described by others authors.

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