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# Determination of Benzo(a)pyrene in Cigarettes Stubs by High Performance Liquid Chromatography

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In present studies, a standard method for the quantitative determination of benzo(a)pyrene in cigarette stub has been developed. The analysis procedure involves a solid phase extraction after extracted with sonicator in cyclohexane. The determination with sensitive detection was attained by reversed-phase high-performance liquid chromatography with fluorescence detector. The detection limits of benzo(a)pyrene was 0.01 ng $mL^{-1}$ at a signal/noise ratio of 3. The contents of benzo(a)pyrene in the stubs of several flue-cured cigarettes were 1-3 ng $cig^{-1}$.

**Key Words: Cigarette stub, Benzo(a)pyrene, Solid phase extraction, HPLC.**

## INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous compounds. PAHs are one of the most contaminating groups of pollutants in the environment because of their mutagenic sources[1]. Benzo(a)pyrene (BaP), the most representative one in PAHs, is found as a result of incomplete combustion of organic materials. BaP has been identified in petroleum and coal shale[2], as well as in food either through environment contamination or as a result of cooking process. It has also reported to be presented in cigarette mainstream smoke[3-5].

It is known that the filter of cigarette could intercept plenty of harmful materials of mainstream smoke. In addition, smoker exposure to BaP occurs mainly by cigarette mainstream smoke and cigarette stub. Accurate quantification of BaP in cigarette stubs will be helpful to the research of problem of smoking and health.

The objective of this investigation was to develop a cleanup and detection method suitable for the quantification of BaP in cigarette stub. Furthermore, we studied the factors affecting the separation of BaP in cigarette stub such as conditioning of SPE cartridge, flow-rate of the sample

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loaded on the cartridge. The effects of step wash stub extracts with acid; alkali and strip extracted with acetonitrile were also presented in this paper. Reversed-phase high-performance liquid chromatography with fluorescence detector (HPLC-FLD) as the final analysis allowed for high resolution and sensitivity. The method of analysis introduced has been applied to determine BaP in the stubs of several flue-cured cigarettes.

## EXPERIMENTAL

Benzo(a)pyrene (BaP, 48.3 μg $mL^{-1}$ in methanol) were purchased from Research Center of National Standard Materials (Beijing,China), which was then diluted to 483 ng $mL^{-1}$ with acetonitrile and the solutions was stored at 0-4ºC, in the dark. Cyclohexane, methanol and acetonitrile (HPLC grade) were supplied by Tedia Co. (USA). Distilled water received from Pure Water Ltd., University of Science and Technology of China (USTC) and the all the solution were filtered through a 0.45 μm pore-size membrane filter prior to their injection into HPLC.

An Agilent 1100 series high performance liquid chromatography equipped with a G1312A binpump, a G1313A auto-injector and a G1321A fluorescence detector. Data were acquired and processed with an Agilent 1100 Chemstation (Agilent Co., USA). Reversed-phase liquid chromatographic separation of BaP was performed on a Inertsil ODS-3 C18 column (GL Sciences Inc. Co., Japan; 4.6 mm i.d.× 250 mm, 5 μm particle size). The fluorescence detector has a 10 nm spectral brandwidth and it allows both the excitation (λex) and emission (λem) wavelength to be changed during the run to optimize the sensitivity of BaP. The sample was collected on a single-pore smoking machine (Borgwaldt Co., German). The SPE column was packed with Bond Elut Jr. Si (Dikma Co., USA).

**Sample collection:** Before cigarettes smoking, cigarettes were conditioned for at least 48 h at 21 ± 1ºC and a relative humidity of 60 ± 2 %. After selected by weight, the cigarettes were smoked under standard conditions of 35 mL puff of 2 s duration on a single-pore smoking machine. Then took 40 stubs of smoked, stripped off the wrapping paper carefully, cut the stubs into fragments, collected all the fragments in another 200 mL Erlenmeyer flask and immersed them in 60.0 mL of cyclohexane.

**Pretreatment of sample:** The sample was extracted for 40 min in an ultrasonic bath, then laid aside statically for several min. Took out 40.0 mL of solution of stubs sample, washed it with 50 mL per time of 5 % sodium hydroxide for 3 times first, then with the same volume of 5 % hydrochloric acid for 3 times and finally cleaned the sample with distilled water to neutralization.

The SPE cartridges were conditioned to humid enough with 10-20 mL of methanol, followed by 10-20 mL of cyclohexane until the cartridges

became transparent. The cartridge was not permitted to run dry during the whole conditioning procedure. 15 mL of solution from the treated sample was loaded onto the SPE column. Solution passed through the column with a flow of 1 mL $min^{-1}$ under pressure. Elution was performed with 3 portions of 5 mL cyclohexane. For the special characters of the packing in SPE, the cartridge didn't absorb the BaP fraction but retain components of the sample matrix, which would otherwise disturbed the analysis. All the effluent and the eluant should be collected to ensure all the BaP was recovered. Transferred the isolated BaP fraction to a 100 mL funnel, extracted with 10 mL of acetonitrile for 5 times and took the acetonitrile solution of BaP in a 100 mL flask.

After all extractions were completed, transferred acetonitrile solution to a round-bottom flask and evaporated solvent to dryness by rotary evaporation under 50-60ºC, vacuum condition. The residue was then dissolved in 1 mL of acetonitrile and filtered sample with a 0.45 μm membrane to remove particulate matter before transfer to a 3 mL of sample bottle with a syringe for HPLC analysis.

**Chromatographic method:** An aliquot (6 μL) of the acetonitrile solution was injected into the HPLC system and eluted with acetonitrile-water (65:35, v/v) at a constant flow-rate of 1.5 mL $min^{-1}$. To quantify the BaP, the fluorescence detector was set at excitation wavelength 382 nm and emission wavelength 417 nm.

## RESULTS AND DISCUSSION

**Amount of sample processed:** The filter of cigarette could intercept plenty of total particulate matters (TPM) of mainstream smoke. The number of compounds in TPM may be exceed the column capacity when the sample was loaded on the SPE cartridge and affected the separation of HPLC. Furthermore, the removal of interferences should increase the consumption of toxic organic solvent and increase the analytical time. The results show that 40 cigarettes stubs can satisfy the requirement of experiment.

**Optimum time of ultrasonic extraction:** The silicon-magnesium absorbent as the reference material was prepared for optimum time of ultrasonic extraction[27]. The experiment shows that the extraction efficiency gradually increases before 40 min and BaP reached the maximum extraction yield in 40 min (Fig. 1). The absorption affinity between the silicon-magnesium absorbent and BaP is stronger than that between the stub and BaP, so 40 min extraction time can ensure that most of the BaP is extracted from the stubs. Further experiments were performed selecting this time.

**Optimization of SPE:** The conditioning step is important for the SPE procedures. The first step of conditioning was to wet the SPE cartridge

with methanol. Wetting of the cartridge can open up the groups on the sorbent surface and thus, increases the surface area available for interaction with the analyte. It can also remove some impurities that might interfere with the analysis, especially the low concentration materials imported during the process of housing, frit and packing of the cartridge. The second step was to wash the sorbent bed with a solvent to prepare the suitable surface for the adsorption of analyte. The second solvent will have weaker or equal eluting strength than that of sample solution. Since, the sample solution of BaP was prepared in cyclohexane, the choice of secondary solvent was cyclohexane.

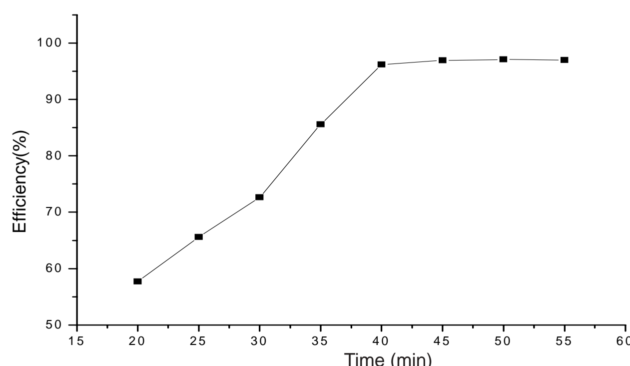



Fig. 1. Ultrasonic extraction curve

The SPE treatment can purify the extracts of the stub and can protect the chromatographic column in subsequent analysis. It is the primary step of pretreatment of samples during the determination of BaP. Various flow-rates of sample loading that range from 1 to 100 mL $min^{-1}$ depending on the sample processed have been reported. As the normal-phase column, it does not adsorb the BaP fraction, but retain constituents of the sample matrix, which would otherwise contaminate the analytical column, disturb the separation of HPLC. At higher flow-rate, non-equilibrium process between adsorbent and polar compounds can lead to lower retention volumes of the analytes. This may result in a difficult separation between interference and BaP. It can be seen from the HPLC chromatograms (Fig. 2) of samples cleaned-up with SPE treatment at different velocity that interference peaks in the analysis of BaP is reduced. The sample volume in our experiment is relatively small and sample preparation is not a problem, 1 mL $min^{-1}$ of flow rate of sample loading can be suggested to achieve a better separation.

**Effect of wash:** The stubs contain a great deal of acidic and alkaline compounds and other hydrophilic compounds such as phenols, organic acid, nitrogenous compounds, which can dissolve in water. So some undesired constituents can be removed by washing with alkaline and acid. During the

treatment of sample, some analysts washed the sample with 5 % hydrochloric acid, 5 % sodium hydroxide solution or sulfuric acid, saturated sodium bicarbonate, sodium hydroxide solution as washing solution. The comparison between washed and unwashed sample was shown in Fig. 3. The chromatogram indicates that wash can clean up a great deal of polar compounds. The number of interfering material peaks is reduced and the interfering peaks area is obviously decreased, but the BaP peak area has no apparent change. It can also be proved by the phenomenon that the colour of sample changed from brown to light yellow. So wash is not redundant for the purification of sample and it can improve the efficiency of isolation.

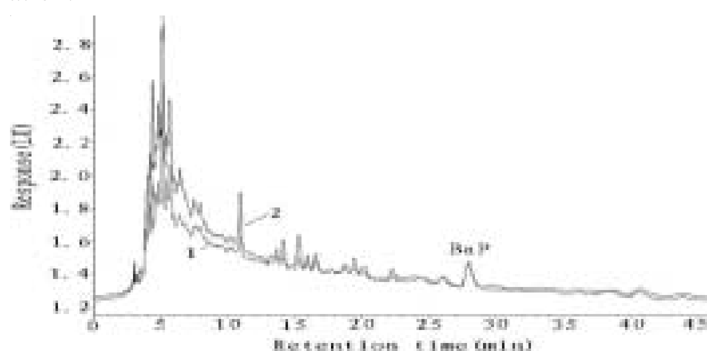



Fig. 2. The effect of eluant velocity on the separation of sample 1) 1.0 mL $min^{-1}$ 2) 1.5 mL $min^{-1}$

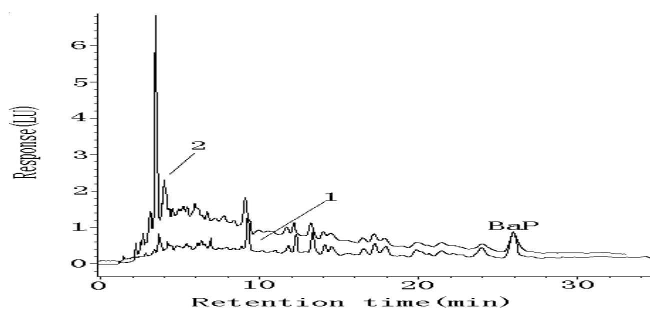



Fig. 3. The chromatographic comparison between sample washed and unwashed 1) sample washed 2) sample unwashed

**Effect of strip extraction with acetonitrile:** Solvents with low polarity are disadvantageous for reversed-phase HPLC separation. If BaP is determined directly from the eluate, the nonpolarities of analyte would deposit on the reversed column through Van der Waals force and considerable band broadening will hinder the separation and reliable quantitative analysis. But when sample was stripextracted with acetonitrile (ACN), the nonpolar compounds that would damage the reversed column were reserved in the cyclohexane phase. The sample was cleaned up, which would be

helpful to reduce the interference peaks in the analysis of BaP, protect the chromatographic column, prolong the lifespan and maintain the precision, reproducibility and stability of determination in subsequent HPLC analysis. Fig. 4 shows the HPLC chromatograms of sample cleaned up with and without stripextraction. It indicates that the polar peaks have no great changes than before, but the nonpolar peaks can be removed obviously.

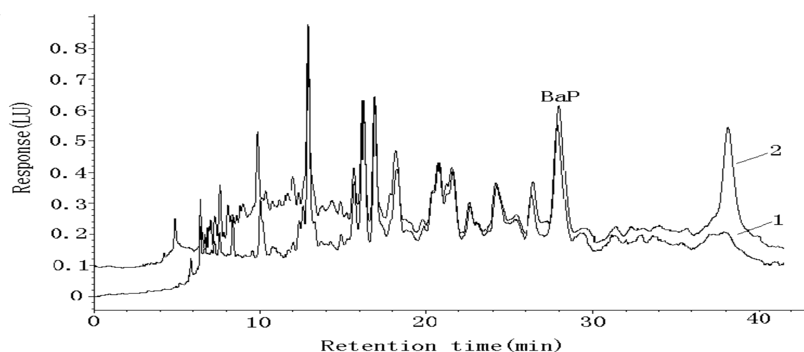
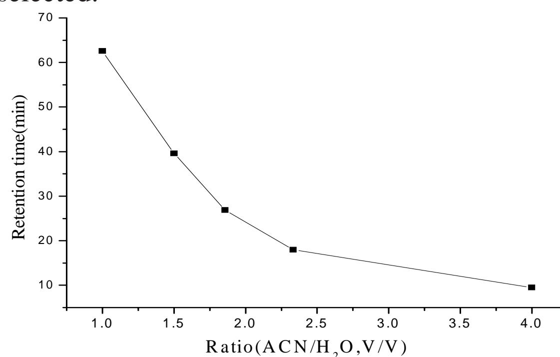



Fig. 4. The chromatographic comparison between the sample stripextracted and unstripextracted

**Optimization for the chromatographic conditions:** Fluorescence spectra of BaP in acetonitrile (ACN) were studied for the detection of BaP. The 382 nm excitation and 417 nm emission wavelengths are the optimum settings for sensitivity. In order to get the best separation at the least cost of time and toxic solvent (ACN) consumption, the influence of velocity and ratio of mobile phase (ACN: $H_2O$) on the separation were evaluated. With the decrease of selected velocity, the retention time of BaP is increased, but the BaP still can get good separation. But with the increment of the mobile phase polarity, the retention time is shortened obviously (Fig. 5) and the resolution of BaP with other interferences decreases. Comparing the separation effect and analytical time consumed, the chromatographic condition of flow = 1.5 mL $min^{-1}$, ACN/$H_2O$ = 65/35, column temperature = 40ºC was selected.




ig. 5. The relation between mobile ratio and retention time

**Confirmation of BaP:** The identity of the analyte was confirmed through the retention time and the increment of chromatographic peak. This technique has been widely used for the quantification of analyte with HPLC. The chromatogram of standard BaP and sample was shown in Figs. 6 and 7, respectively.

**Precision:** Table-1 shows a chromatographic relative standard deviation (RSD) of 1.48 % for a standard solution equivalent to BaP content of 14.48 ng cig$^{-1}$. The overall precision of the method when applied to 6 different smokes of the same sample had also been listed in the Table-1.

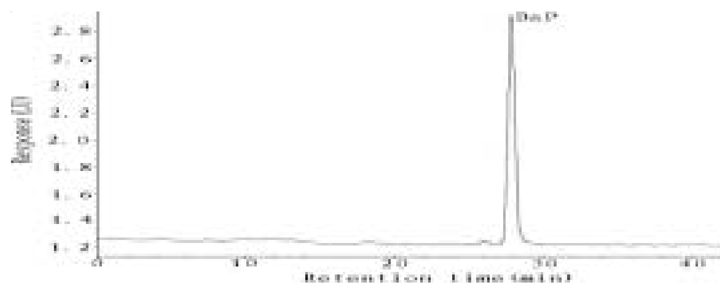



Fig. 6. Chromatogram of standard BaP

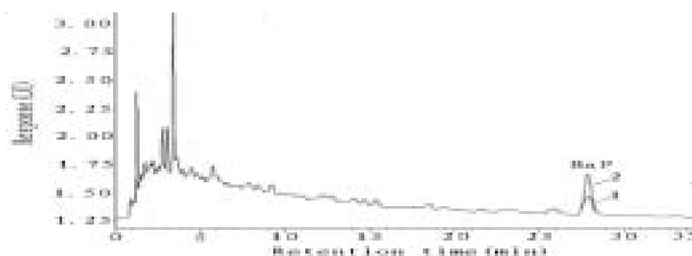



Fig. 7. Chromatogram of BaP in cigarette stubs 1) stubs 2) Standard BaP added

TABLE-1
THE PRECISION OF INSTRUMENT AND METHOD

| | Content of BaP (ng cig$^{-1}$) | | | | | | Average (ng cig$^{-1}$) | RSD (%) |
|---|---|---|---|---|---|---|---|---|
| Instrument | 14.30 | 14.45 | 14.28 | 14.62 | 14.84 | 14.39 | 14.48 | 1.48 |
| Method | 1.95 | 2.07 | 2.2 | 1.94 | 2.14 | 1.04 | 2.04 | 1.09 |

**Recovery:** To evaluate the recovery of BaP in stubs, a series standard BaP solution were spiked into the sampled collected, determined the total amount of BaP. Results in Table-2 shows the recovery is 76.15-81.78 % for cigarette stubs. The loss of BaP should be caused in the procedure of ultra-sonic extraction, wash, transfer, acetonitrile extraction, concentration and dilution.

**Linearity:** The calibration curves for quantification were based on the analysis of solutions (in acetonitrile) containing 2.01. 8.05, 24.15, 48.3, 96.6, 120.8 and 161 ng $mL^{-1}$ of BaP, three injections of each standard solution were made and the calibration curves were obtained by plotting the peak-area *vs.* analyte concentration. It shows a good linearity of BaP because the regression equation is $C = 0.5911A + 0.2588$, with the correlation coefficient of 0.9999. The detection limit calculated with a signal to noise ratio of three (IUPAC criterion) was 0.01 ng $mL^{-1}$.

TABLE-2
RECOVERY FOR BaP FROM STUBS OF CIGARETTES

| Amount added* (ng $cig^{-1}$) | Amount founded* (ng $cig^{-1}$) | Amount recovered (ng $cig^{-1}$) | Recovery (%) | Average (%) |
|---|---|---|---|---|
| 3.02 | 4.34 | 2.30 | 76.15 | - |
| 6.04 | 6.96 | 4.92 | 81.46 | 81.78 |
| 9.06 | 9.99 | 7.95 | 87.74 | - |

*On five reduplicate measurements each

**Application:** To illustrate its utility, this method was used to measure BaP in stubs from flue-cured cigarettes. Table-3 shows that the content of BaP in stubs is 1-3 ng $cig^{-1}$, which is about 7-20 % of BaP in main stream smoke of cigarettes and the BaP in blank filter had not been detected.

TABLE-3
CONTENTS OF BaP IN CIGARETTES STUBS

| Sample | Content (ng $cig^{-1}$) | Sample | Content (ng $cig^{-1}$) | Sample | Content (ng $cig^{-1}$) |
|---|---|---|---|---|---|
| 1 | 1.69 | 5 | 2.51 | 9 | 1.59 |
| 2 | 1.15 | 6 | 1.75 | 10 | 2.35 |
| 3 | 1.94 | 7 | 2.16 | 11 | 2.42 |
| 4 | 2.04 | 8 | 2.35 | 12 | 1.85 |

## Conclusion

Normal phase SPE column was used for the isolation of BaP in cigarette stubs. Sample obtained was analyzed with HPLC coupled with a fluorescence detector. The method is linear over a wide concentration range. It can not only be used on the determination of BaP in cigarette stubs, but also on the determination of BaP in cigarette mainstream smoke. The amount of sample processed, SPE flow-rate, wash and strip extraction step are essential for decreasing the interfering materials, improving the separation of BaP.

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