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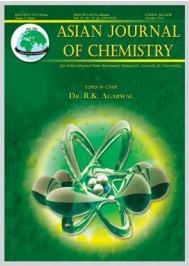

Determination of 14 Polycyclic Aromatic Hydrocarbons in Mainstream Smoke from Flue-Cured Cigarettes by GC-MS Using a New Internal Standard

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A method for the determination of 14 polycyclic aromatic hydrocarbons in cigarette mainstream smoke using 1,2-*bis*(9-anthryl)ethane as an internal standard has been developed and was applied to the analysis the Chinese cigarettes. The analytical method involves an ultrasonic extraction of Cambridge filters with cyclohexane followed by a solid-phase extraction on a Si cartridge for cleanup. After being concentrated, the samples were directly injected for gas chromatography-mass spectrometry detection. The mainstream smoke from the flue-cured cigarettes had individual polycyclic aromatic hydrocarbons ranging from benzo[k]fluoranthene at levels below 3.0 ng cig^{-1} to phenanthrene at levels of around 160 ng cig^{-1}. The polycyclic aromatic hydrocarbon levels varied among the different cigarette brands, with the amount of the total 14 polycyclic aromatic hydrocarbons ranging from 0.67 μg cig^{-1} to 0.80 μg cig^{-1}. Moreover, we compared levels of polycyclic aromatic hydrocarbons in mainstream smoke with phytosterols in tobacco of cigarettes. The results indicated that the free phytosterols were highly correlated with total polycyclic aromatic hydrocarbons ($r = 0.952$, $P < 0.001$) and carcinogenic polycyclic aromatic hydrocarbons ($r = 0.977$, $P < 0.001$).

Key Words: Cigarette mainstream smoke, Polycyclic aromatic hydrocarbons, Phytosterols 1,2-*bis*(9-anthryl)ethane.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous organic pollutants that are released into the environment during the incomplete combustion of organic materials such as tobacco. Due to their recalcitrance and suspected carcinogenicity[1], 16 polycyclic aromatic hydrocarbons were identified as priority pollutants by EPA. Among the 16 polycyclic aromatic hydrocarbons, benzo[a]pyrene has drawn the most attention because of its ability to induce lung tumors and injures central nerves at a certain degree[2].

Currently, cigarette smoking is associated with about 90 % of lung cancer cases and it accounts for about 30 % of all cancer cases in developed countries. In developing countries, the promotion of this habit resulted in a huge increase in smoking-associated disease and death[3]. Along with the intensively studies on the cigarette smoking, the cigarette smoke ranks as a major risk pollutant associated with negative health effects. Cigarette smoke is an ever changing and extremely complex mixture of chemicals. Over 4800 chemical constituents are generated in cigarette smoke, many of which are very harmful compounds such as the 16 priority polycyclic aromatic hydrocarbons. Due to the potential carcinogenicity of many polycyclic aromatic hydrocarbons, one of the important factors to reduce the risk of tobacco harm and develop less harmful cigarettes is removal or reduction of their levels in cigarette smoke. Therefore, the detection of polycyclic aromatic hydrocarbon levels in mainstream smoke from cigarettes are of considerable interest in tobacco analysis.

For the determination of polycyclic aromatic hydrocarbons in the cigarettes smoke, the most frequently analytical methods including gas chromatography-mass spectrometry (GC-MS)[4-9], high-performance liquid chromatography have been proposed in the literature[10-12]. After solid-phase extraction cleanup, the mostly used quantification methods are GC-MS and HPLC coupled with fluorescence detection. Due to the possibility of the application of internal standard, GC-MS provides a more accurate quantification in cigarette smoke, especially in the studies quantified more than 10 polycyclic aromatic hydrocarbons. Most studies reported in the literature by GC-MS were focused on the blend cigarettes, while little information of polycyclic aromatic hydrocarbon levels is available for flue-cured cigarettes from China.

The aim of the present study is to establish and evaluate an accurate and effective analytical method for the determination of polycyclic aromatic hydrocarbons in cigarette mainstream smoke. To meet these requirements, we developed a

gas chromatography/mass spectrometry detection using 1,2-*bis*(9-anthryl)ethane as an internal standard. The proposed method was successfully applied to the determination levels of 14 polycyclic aromatic hydrocarbons in mainstream smoke from flue-cured cigarettes. Furthermore, we comparatively analyzed the levels of total polycyclic aromatic hydrocarbons and the total phytosterols and indicated the effects of different existed form of phytosterols on the emission of polycyclic aromatic hydrocarbons in mainstream smoke.

EXPERIMENTAL

Polycyclic aromatic hydrocarbons used for calibrating standard solutions were obtained from Sigma Chemical (St. Louis, MO, USA). Campesterol (98 %), stigmasterol (95 %) and β-sitosterol (95 %) were purchased from Sigma Chemical (St. Louis, MO, USA). 1,2-*Bis*(9-anthryl)ethane as an internal standard was synthesized as reported previously[13]. Dichloromethane, methanol and cyclohexane were HPLC grade from Tedia (USA). Water was obtained by purifying demineralized water using a Milli-Q system from Millipore (Belford, MA, USA). BondElut Si cartridges (500 mg, 3 mL) were purchased from Varian (Palo Alto, CA, USA). Other chemicals and reagents were obtained from Shanghai No. 3 Chemical Reagents Company (China).

The 92 mm diameter Cambridge filters and cigarette samples were supplied by Technology Center of Qingdao Cigarette Factory, China.

Preparation of standard solutions: A standard mixture containing all 14 polycyclic aromatic hydrocarbons (acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, benz[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, indeno(1,2,3-cd)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)-perylene) was prepared in cyclohexane. Stock solutions (20 μg mL^{-1}) were prepared by dilution of this standard with cyclohexane; this solution was further diluted with the same solvent when necessary. Standard polycyclic aromatic hydrocarbon solutions were stored at -20 ºC in glass vials wrapped in aluminum foil to avoid possible photodegradation of some polycyclic aromatic hydrocarbons. The stock solution of 1,2-*bis*(9-anthryl)ethane was prepared in dichloromethane. The spike level of the 1,2-*bis*(9-anthryl)ethane in the Cambridge filters was 1 μg mL^{-1}.

Smoke collection: Six brands of cigarettes (all of them from Qingdao cigarette factory) were analyzed in the study. The cigarettes were stored for at least 24 h maintained at 22 ºC and 60 % relative humidity and were then selected by weight (± 20 mg of average weight of 200 cigarettes) and by draw resistance (± 7 % of average draw resistance of 50 cigarettes selected by weight). The cigarettes were smoked to a butt length of 23 mm or to the length of the filter overwrap plus 3 mm, whichever was longer. The cigarette smoking conditions were one puff per minute; puff duration 2 s; puff volume 35 mL.

Cigarettes were smoked with a Phipps and Bird 20-port smoking machine. After smoking, the filters containing the cigarette smoke of twenty cigarettes were collected for extraction.

Preparation of the cigarette sample: The collected filters were transferred to 100 mL flasks and spiked with 100 μL of 1,2-*bis*(9-anthryl)ethane solution in cyclohexane (1 μg mL^{-1}). After addition of 60 mL of cyclohexane, the filters were extracted in an ultrasonic bath for 40 min. The Si cartridge was preconditioned twice with 5 mL methanol and 5 mL cyclohexane. After being washed, 10 mL of the extraction fraction were eluted with 20 mL cyclohexane. The cyclohexane extracts were evaporated under nitrogen to approximately 1 mL and a 1 μL aliquot was used for GC-MS analysis.

Gas chromatography and mass spectrometry: Quantification was performed on a J & W Scientific (Folsom, CA) DB-5MS fused-silica capillary column (30 m × 0.25 mm × 0.25 μm), protected by a J & W Scientific guard column (DB-1, 3 m × 0.32 mm × 0.5 μm). A constant flow of 1.5 mL min^{-1} of helium carrier gas was maintained through the column. The following temperature program was: 50 ºC held for 1 min, 25 ºC min^{-1} to 150 ºC, 4 ºC min^{-1} to 280 ºC (hold for 15 min), 5 ºC min^{-1} to 290 ºC (hold for 15 min). The splitless injector was set to 270 ºC.

The detection of the different compounds was performed in the scan mode in the mass range m/z from 50 to 650. The ion source temperature was 200 ºC and the GC-MS-interface was set 270 ºC. The ionizing energy was 70 eV. Quantitative analysis was performed in SIM mode by selected ion monitoring mode (SIM).

RESULTS AND DISCUSSION

Selection and identification of the internal standard: Polycyclic aromatic hydrocarbons (PAHs) generally mean the class of hydrocarbon compounds whose molecular structure includes two or more fused benzene rings[14]. Just as its name implied, polycyclic aromatic hydrocarbons comprise several compound classes: bi-aryls, alkylated aromatics, aromatic-substituted aliphatic hydrocarbons. In this study, 1,2-*bis*(9-anthryl)ethane was selected as an internal standard for the GC-MS determination of polycyclic aromatic hydrocarbons in cigarette mainstream smoke due to the similarity of their structure. Compared with the labelled internal standards, 1,2-*bis*(9-anthryl)ethane can be easily synthesized and with no radioactivity.

The compound whose purity confirmed by GC-MS was identified and characterized as follows. Mass spectrum (GCT-MS, 70ev) (Fig. 1.), m/z 382 (M^+, 0.22), 192 (1.07), 191 (100), 190 (0.28), 189 (1.10), 165 (0.14); Elemental analysis (Vario EL III Universal Chnos Elemental Analyzer), calculated: C, 94.20; H, 5.80. Found: C, 94.23, H, 5.82; ^{1}H NMR (Bruker Avance Av 400 MHz, $CDCl_3$), δ 4.07(s, 4, CH_2), 8.39 (d, 4, H_1), 7.50 (m, 8, $H_{2,3}$), 8.03 (d, 4, H_4), 8.41 (s, 2,H_{10}).

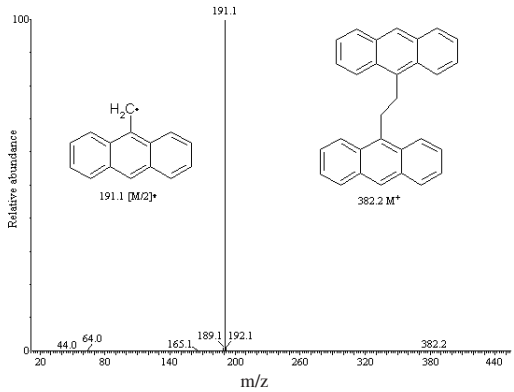


Fig. 1. Mass spectrum and chemical structure of 1,2-*bis*(9-anthryl)ethane

Validation of the method

Polycyclic aromatic hydrocarbon SIM group, linearity and limit of detection: The total ion chromatograph of all 14 polycyclic aromatic hydrocarbons showed sensitivity and resolution (Fig. 2). The total ion chromatographs from cigarette smoke extracts was much more complicated due to the background chemical noise, but the selected ion chromatograms demonstrated good separation for 14 polycyclic aromatic hydrocarbons (Fig. 3). Analytical parameters including retention times and ion masses for the 14 polycyclic aromatic hydrocarbons measured were selected (Table-1). During the analysis of the sample extract, the 14 polycyclic aromatic hydrocarbons were divided into nine SIM groups (Table-1) to improve sensitivity. Calibration curves were constructed for each polycyclic aromatic hydrocarbon with detection levels ranging from 2 ng mL^{-1} to 2 µg mL^{-1}. The correlation coefficient and the limit of detection (LOD) for each polycyclic aromatic hydrocarbon were given. The LOD was determined according to standard practice as the concentration that provided a signal to a noise ratio of 3[15]. The calculated LODs of polycyclic aromatic hydrocarbons ranged from 0.35 ng mL^{-1} of benzo[k]fluoranthene to 3.54 ng mL^{-1} of dibenz(a,h)anthracene (Table-1).

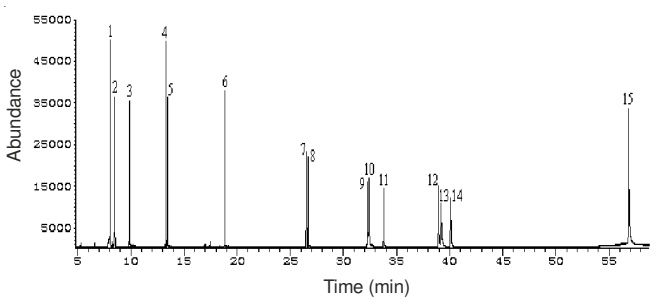
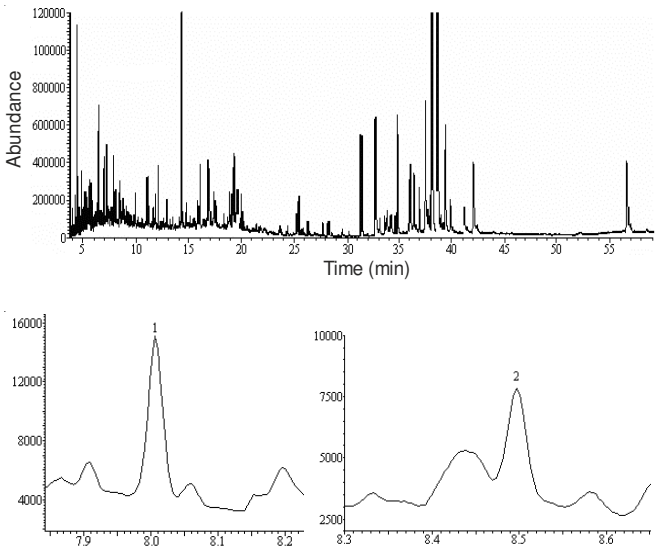


Fig. 2. Typical chromatogram obtained by GC-MS from the 14 polycyclic aromatic hydrocarbon standard (2.0 µg mL^{-1}) and the internal standard (1.0 µg mL^{-1}). Peak identities: 1 = acenaphthylene, 2 = acenaphthene, 3 = fluorene, 4 = phenanthrene, 5 = anthracene, 6 = fluoranthene, 7 = benz[a]anthracene, 8 = chrysene, 9 = benzo[b] fluoranthene, 10 = benzo[k]fluoranthene, 11 = benzo[a]pyrene, 12 = indeno(1,2,3-cd)pyrene, 13 = dibenz(a,h)anthracene, 14 = benzo-(g,h,i) perylene, 15 = 1,2-*bis*(9-anthryl)ethane.



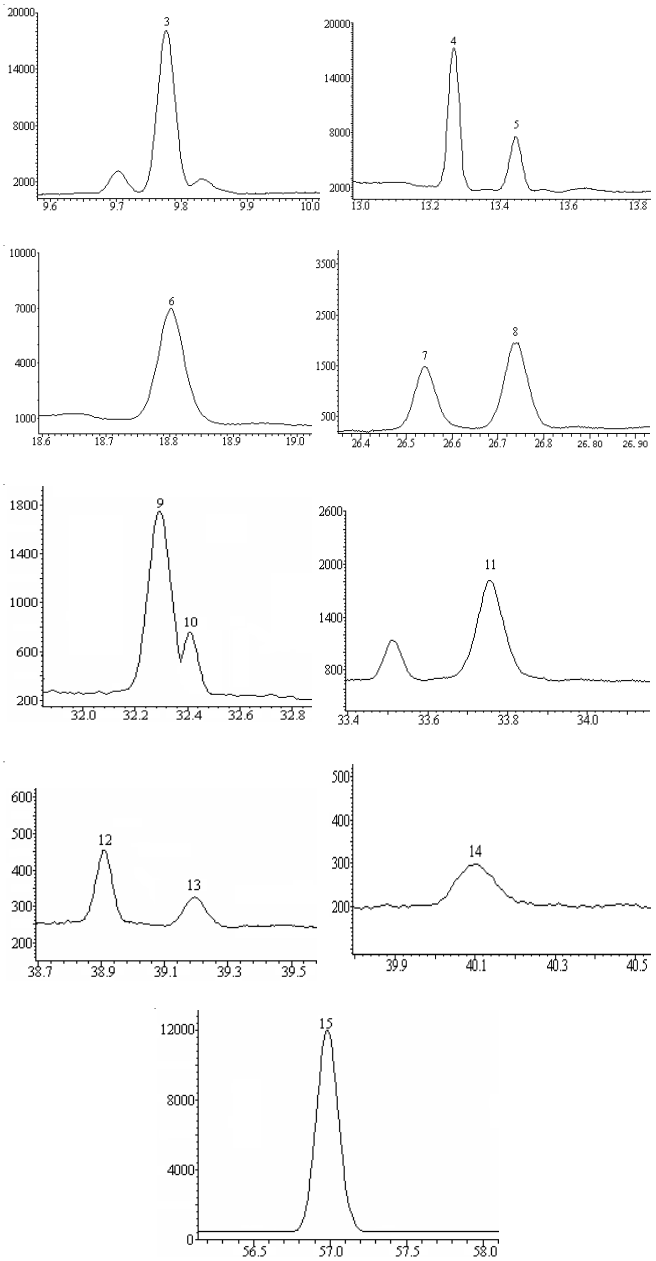


Fig. 3. Total ion chromatogram and selected reconstructed ion chromatograms of 14 polycyclic aromatic hydrocarbons in mainstream smoke from a cigarette sample. Peak identities were as for Fig. 2

Method accuracy (recovery) and precision: Under controlled conditions 14 polycyclic aromatic hydrocarbons in mainstream smoke from one sample were analyzed five times. The results are presented in Table-2. The current method demonstrated good precision with relative standard deviation below 10 % for all target polycyclic aromatic hydrocarbons.

To study recovery, one smoke sample solution was divided to four parts; one had no standard solution added, the other three were mixed with standard solution in the approximate volume ratios 2:1, 1:1 and 1:2. After thorough mixing these solutions were injected to the GC-MS. The recoveries exceed 80 % for most polycyclic aromatic hydrocarbons except dibenz(a,h)anthracene. The low recovery of dibenz-(a,h)anthracene may be due to the comparatively higher standard deviation.

TABLE-1
ANALYTICAL PARAMETERS AND SIM GROUP OF 14 PAHs

PAH (SIM[a] group)	Ion masses (m/z)	Retention time (min)	Correlation coefficient	LOD[b] (ng mL^{-1})
Acenaphthylene (1)	152	8.06	0.9934	1.61
Acenaphthene (2)	154	8.50	0.9992	0.57
Fluorene (3)	166	9.87	0.9956	0.82
Phenanthrene (4)	178	13.26	0.9948	0.69
Anthracene (4)	178	13.44	0.9991	2.15
Fluoranthene (5)	202	18.82	0.9945	1.73
Benz[a]anthracene (5)	202	26.54	0.9984	0.74
Chrysene (6)	228	26.74	0.9993	0.91
Benzo[b]fluoranthene (7)	252	32.27	0.9991	1.43
Benzo[k]fluoranthene (7)	252	32.40	0.9987	0.35
Benzo[a]pyrene (7)	252	33.75	0.9976	0.87
Indeno(1,2,3-c,d)pyrene (8)	276	38.92	0.9943	2.78
Dibenz(a,h)anthracene (9)	278	39.25	0.9959	3.54
Benzo(g,h,i)perylene (8)	276	40.10	0.9947	2.19
1,2-*bis*(9-anthryl)ethane	191	56.90		

[a]Selected ion monitoring for GC-MS; [b] S/N=3

TABLE-2
SUMMARY OF THE PRECISION AND ACCURACY OF THE METHOD (*n*=5)

PAHs	Mean accuracy (%)	Precision (%)
Acenaphthylene	88.1	5.9
Acenaphthene	85.4	2.1
Fluorene	89.7	3.4
Phenanthrene	104.3	2.7
Anthracene	92.9	1.9
Fluoranthene	94.5	4.8
Benz[a]anthracene	101.8	2.3
Chrysene	90.6	3.2
Benzo[b]fluoranthene	92.7	4.7
Benzo[k]fluoranthene	104.3	5.4
Benzo[a]pyrene	90.1	4.4
Indeno(1,2,3-c,d)pyrene	103.6	5.7
Dibenz(a,h)anthracene	75.2	9.8
Benzo(g,h,i)perylene	94.7	4.1

Application of the method: Six cigarette samples from China were analyzed. The results obtained (Table-3) revealed that different quantities of polycyclic aromatic hydrocarbon compounds were obtained from different varieties of cigarette. This may be related to tobacco leaf quality, art and craft of production and materials added to the cigarettes. The cigarettes had common characteristics: acenaphthylene, fluorene and phenanthrene were the most abundant polycyclic aromatic hydrocarbon compounds. And these three polycyclic aromatic hydrocarbon compounds were all three ring polycyclic aromatic hydrocarbon compounds. Although levels of the four ring and five ring polycyclic aromatic hydrocarbon compounds were lower, they possessed higher carcinogenic potential.

Comparative analysis of total polycyclic aromatic hydrocarbons and phytosterols: Polycyclic aromatic hydrocarbons was always contributed by complex molecules such as solanesol, phytosterols, aliphatic hydrocarbons, sugars, amino acids, nicotine, lipids and many other tobacco components[16]. It was thought that phytosterols might be potential precursors to polycyclic aromatic hydrocarbons in many studies[17,18]. Phytosterols existed in tobacco as free sterol (FS) and conjugates including steryl esters (SE), steryl glycosides (SG). While data on phytosterols and total polycyclic aromatic hydrocarbons in the flue-cured cigarettes are lacking in the literature, we compared levels of total polycyclic aromatic hydrocarbons in mainstream smoke with phytosterols in tobacco of cigarettes from China. The method for the determination of phytosterols by GC-FID was used as reported previously[19].

TABLE-3
PAH LEVELS (ng cig^{-1}) OF DIFFERENT CIGARETTE SAMPLES

PAHs	H1	H2	H3	Y1	Y2	T1
Acenaphthylene	166.3	160.8	141.5	135.7	126.3	124.3
Acenaphthene	25.9	24.5	24.3	27.2	25.1	26.2
Fluorene	143.5	134.3	135.7	130.8	130.5	133.6
Phenanthrene	164.7	161.7	161.6	159.6	149.7	157.8
Anthracene	87.2	86.6	83.8	79.5	78.2	80.1
Fluoranthene	70.6	64.1	66.2	63.4	69.4	59.4
Benz[a]anthracene*	45.1	42.5	34.6	30.4	29.8	25.6
Chrysene*	48.6	46.3	37.8	35.2	34.8	28.6
Benzo[b]fluoranthene*	10.2	10.4	9.3	8.7	7.6	8.1
Benzo[k]fluoranthene*	2.1	2.3	1.8	1.5	1.7	0.9
Benzo[a]pyrene*	12.7	12.4	12.4	10.9	9.8	9.3
Indeno(1,2,3-c,d)pyrene	11.6	11.0	9.8	10.3	9.5	8.9
Dibenz(a,h)anthracene*	12.1	10.6	9.4	8.7	7.3	6.5
Benzo(g,h,i)perylene	8.0	7.9	7.1	6.5	6.8	7.0

*Sum of six carcinogenic PAH

The result obtained (Table-4) showed that the free phytosterols were highly correlated with total polycyclic aromatic hydrocarbons ($r = 0.952$, $P < 0.001$) and carcinogenic polycyclic aromatic hydrocarbons ($r = 0.969$, $P < 0.001$). The total phytosterols were also correlated with total polycyclic aromatic hydrocarbons ($r = 0.863$, $P < 0.05$) and carcinogenic polycyclic aromatic hydrocarbons ($r = 0.890$, $P < 0.05$). We demonstrated stigmasterol was correlated with benzo[a]pyrene that was in accordance with the previous study[18]. But the conjugated phytosterols had no significant correlation with polycyclic aromatic hydrocarbons, this might be caused by the addition of tobacco flavouring which lead to the hydrolysis of the conjugated phytosterols[20].

TABLE-4
COMPARATIVE ANALYSIS OF PHYTOSTEROLS AND PAHs

Brand	Phytosterols in cigarette (μg g^{-1})					PAHs in smoke (ng g^{-1})		
	Total	FS	SE	SG	Stigmasterol	Total	Carcinogenic	B(a)P
	2660	1091	720.7	848.2	692.8	808.6	130.8	12.7
	2516	1048	690.5	777.3	625.9	775.4	124.5	12.4
	2494	1006	687.3	801.2	590.4	735.3	105.3	12.4
	2404	954.5	676.5	773.0	500.2	708.4	95.4	10.9
	2386	917.1	647.1	822.2	462.1	686.5	91.0	9.8
	2209	860.3	569.9	778.9	408.6	676.3	79.0	9.3

Conclusion

The GC-MS method presented here enables simultaneous analysis of 14 polycyclic aromatic hydrocarbon compounds. Under the standardized machine smoking conditions and the pretreatment process, the polycyclic aromatic hydrocarbons were successfully separated and determined by the proposed method using a new internal standard. We applied the method to the analysis of six cigarette samples from China. The precision was good and detection limits were low, confirming the utility of the method for determination of polycyclic aromatic hydrocarbon compounds in cigarette smoke.

Through the comparatively analysis of polycyclic aromatic hydrocarbons and phytosterols, we find the correlations between polycyclic aromatic hydrocarbons and phytosterols in the cigarette samples. Therefore, monitoring the phytosterols in tobacco of cigarette might help indicating the polycyclic aromatic hydrocarbon levels in mainstream smoke, especially in improvement the safety of cigarettes.

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