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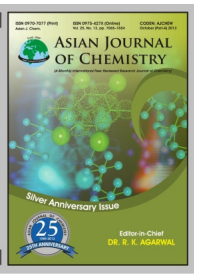

Influence of Steryl Esters on the Delivery of Polycyclic Aromatic Hydrocarbons in Tobacco Mainstream Smoke

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Steryl esters, stigmasteryl oleate, cholesteryl oleate and β-sitosteryl oleate were synthesized and used as an additive in the cigarettes for studying the influence on the delivery of polycyclic aromatic hydrocarbons by GC-MS. The results showed that the delivery of polycyclic aromatic hydrocarbons in tobacco mainstream smoke was closely related to the addition levels of those steryl esters, indicated that the steryl esters are possible the most potential precursors of polycyclic aromatic hydrocarbons in tobacco mainstream smoke.

Key Words: Steryl ester, Polycyclic aromatic hydrocarbons, GC-MS.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs), presenting in automobile exhaust[1] and tobacco smoke[2], have been proved to be widespread environmental pollutants[3-4]. Polycyclic aromatic hydrocarbons are potential carcinogenic effects. Numerous reports have focused on the detection and quantification of PAHs, in order to get a better understanding of the biological activity and toxicity of PAHs.

Phytosterols, acting as the important structural components of plant membranes[5], are the important precursors of those tumorigenic PAHs[6-8]. Stigmasterol, cholesterol, β-sitosterol and campesterol have been shown to be the characteristic sterols in tobacco[9]. They exist in tobacco as either free sterols (FSs) or conjugates which include steryl esters (SEs), steryl glycosides (SGs) and acylated steryl glycosides (ASGs)[10-11]. Accordingly, studies have shown that phytosterols had a significant influence on the delivery of PAHs in tobacco smoke. Rodgman *et al.*[12] proved that adding phytosterols into tobacco could improve the delivery of PAHs in mainstream smoke. Two times of addition amount caused the delivery of PAHs increased by 13 %, while by 18 % when adding three times. Liu *et al.*[13] showed that there were significantly positive correlations between the quantity of free phytosterols and the delivery of PAHs. In particularly, the delivery of four, five, six membered ring PAHs were significantly influenced by stigmasterol.

Because of that the tetracyclic cyclopenta[*a*]phenanthrene ring structures in phytosterols tend to form PAHs during pyrolysis. Some groups used pyrolytic expriments to demonstrate that phytosterols were the important precursors of PAHs in tobacco smoke. Johnston and Plimmer[14] reported that pyrolysis of stigmasterol under 750 °C could produce benzo[a]pyrene. Schepartz *et al.*[15] showed that approximately 61 % benzo[a]pyrene in tobacco smoke was produced through the pyrolysis of hexane-extractable fraction of phytosterols. Meanwhile, study by Britt[16,17] showed that PAHs and their monomethylated derivatives were formed in the absence of bimolecular reactions and the yields of PAHs were dependent on the structures of the steroids.

In tobacco, only 15-25 % phytosterols exist in free form and the majority of phytosterols exist as conjugates[18]. In plants, most sterols were esterified with long chain fatty acids, mainly unsaturated C_{18}, saturated C_{16} and C_{20}-C_{32} fatty acids[6]. The report of Schlotzhauer and Chorthy[6] indicated the steryl esters may be the most potential PAH precursors. Britt[17] also reported that the yields of PAHs produced by cholesteryl esters per mole was 1.1 to 1.4-fold larger than the free cholesterol. Meanwhile, Saiz-Jimenez[19] had also reported that aliphatic chains lead to the formation of alkylbenzenes, indenes or naphthalenes by cyclization and aromatization reactions. No matter the tetracyclic cyclopenta[a]phenanthrene ring structures or the long aliphatic chains in steryl esters could form PAHs, so steryl esters in tobacco tend to have a significant influence on the delivery of PAHs.

In our study, stigmasterol, cholesterol and β-sitosterol were esterified by C_{18} fatty acid and the esterification products were added in the cigarettes using the external addtion method to investigate the influence of steryl esters on the delivery of PAHs

in tobacco mainsteam smoke, in order to get a better understanding of the PAHs precursors in tobacco.

EXPERIMENTAL

Tobacco samples were supplied by technology center of China Tobacco Chuanyu Industrial Corporation. All samples were conditioned at 22 °C and 60 % relative humidity for at least 48 h. The PAH standards, acenaphthylene (ACL), acenaphthene (AC), fluorene (FL), phenanthrene (PHE), anthracene (AN), fluoranthene (FA), pyrene (PY), benz(*a*)anthracene (BaA), chrysene (CHR), benzo[*b*]fluoranthene (BbF), benzo[k]fluoranthene (BkF), benzo[*a*]pyrene (BaP), indeno[1,2,3-c,*d*]pyrene (IcdP), dibenz[a,h]anthracene (DahA), benzo[*g*,*h*,*i*]perylene (BghiP) and their isotopically labeled analogues were all purchased from Sigma-Aldrich (97.0 %). Stigmasterol, cholesterol and β-sitosterol were purchased from Sigma-Aldrich (95.0 %). All organic solvents were HPLC grade. Oleic acid, dicyclohexylcarbodiimide (DCC) and 4-dimethylamino pyridine (DMAP) were reagent grade and used as purchased without further purification. The yields of PAHs in mainstream smoke was analyzed by HP6890GC/5973MSD (U.S. Perkinelmer Company).

Synthesis of steryl esters: The esters of stigmasterol, cholesterol and β-sitosterol were prepared as reported[20]: the mixture of sterol (5 mmol), oleic acid (5.6 mmol), DCC (5.6 mmol) and DMAP (1.2 mmol) were dissolved in dichloromethane (100 mL), stirred for 72 h at room temperature. The excess of DCC was removed in vaccum and the resulting residue was purified by silica gel column chromatography (ethyl acetate: petroleum ether, 5:1) to give the steryl esters. IR spectra: ν 1732 cm^{-1} for C=O, 1195 cm^{-1} for C-O-C. The synthetic routes were shown in Fig. 1.

Sample pretreatment: According to the results of previous report[21] (Table-1), a suitable content of steryl esters (0.5, 1, 2, 4 and 8 times of quantity) were dissolved into dichloromethane, then added into cigarettes evenly by using a microinjector. In order to improve the accuracy of the experimental results, three samples for each experiment trail were made.

TABLE-1
CONTENTS OF THE THREE STERYL ESTERS IN CIGARETTES

	Steryl esters		
Content (μg/cig)	Stigmasteryl ester	Cholesteryl ester	β-sitosteryl ester
	137.34	60.25	198.71

Smoke collection: Mainstream smoke TPM generated under ISO smoking conditions (60 s puff interval, 2 s puff duration and 35 mL puff volume) was collected on individual CFPs using a RM20/CS smoking machine.

Sample preparation: The samples were prepared by published method[22]. After smoking, the TPM collected on each CFP was extracted using 60 mL cyclohexane with 1 mL internal standard solution. The mixture was placed on the ultrasonic generator and shaken 40 min. Then 15 mL of cyclohexane extracts were loaded on a solid phase extraction cartridge and washed with 50 mL of cyclohexane. The eluent was evaporated to approximately 0.5 mL and analyzed by GC/MS, each sample determined three times.

GC/MS analysis: Separation was performed on a DB-5MS column (30 m × 0.25 mm I.D. × 0.25 μm film thickness), the splitless injector was set to 270 °C, a constant flow of 1.2 mL/min of helium carrier gas was maintained through the column and injection volume was 1 μL. The temperature program was: the oven was heated with the initial temperature of 50 °C and held for 1 min, after ramping up to 150 °C at 25 °C min^{-1}, then raised to 280 °C at 4 °C min^{-1} and held for 10 min, then raised to 300 °C at 25 °C min^{-1} and maintained for another

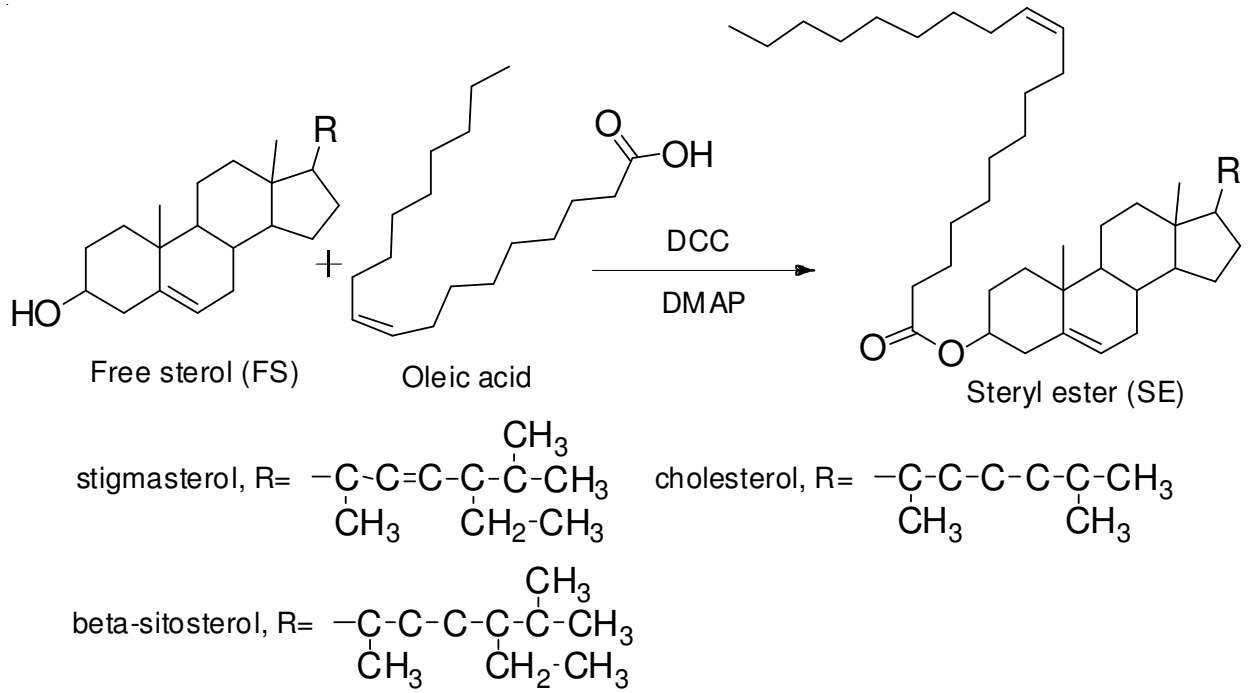

Fig. 1. The synthetic routes of steryl esters

5 min, giving a total run time of 53.3 min. The mass spectrometer was operated in the electron impact mode (EI), the ion source temperature was 200 °C and the GC-MS-interface set to 280 °C. The analysis was performed by selected ion monitoring (SIM). Polycyclic aromatic hydrocarbons yields were calculated using a calibration curve obtained from the analysis of standard solutions, the response of PAHs to its concentration showed a good linear relationship with linear correlation coefficients higher than 0.999.

RESULTS AND DISCUSSION

In this study, we utilized an external addtion method to evaluate the influence of steryl esters on the delivery of PAHs in mainstream smoke and the results were summarized in (Tables 2-4). Based on the ring numbers of PAHs, the 15 types PAHs investigated in our experiment were divided into four groups, including three-membered ring PAHs (ACL, AC, FL, PHE and AN), four-membered ring PAHs (FA, PY, BaA and CHR), five-membered ring PAHs (BbF, BkF, BaP and DahA) and six-membered ring PAHs (BghiP and IcdP). Among them, PAHs with ring numbers ranging from 4-6 are carcinogenic, whereas 2-3 ring PAHs are either toxic or carcinogenic[22]. In order to maintain the accuracy and reproducibility of the experiment, the yields of PAHs were all average of three experiments. The increment was calculated as following: Increment (%) = 100 × (B-A)/A, B was the highest yield of PAH after adding steryl esters into cigarettes, while A was the yield of PAH without adding any steryl esters.

According to the results, the PAHs yields were enhanced significantly after adding steryl esters into cigarettes and the highest yield could be obtained by two times of adding amount. As shown in Table-2, adding stigmasteryl oleate obviously increased the delivery of PAHs in tobacco mainstream smoke, especially for four-six-membered ring PAHs, BaA (15.41 %), BkF (13.46 %), BaP (11.54 %), DBahA (16.49 %) and BghiP (26.47 %). Only a relatively small increment of 9.11 % and 8.57 % was observed for three-membered ring PAHs, AC and AN, respectively, while the amount of ACL, FL and PHE were influenced slightly. Cholesteryl oleate performed similarly (Table-3) to stigmasteryl oleate. Its addition also caused a notable increase in four-six-membered ring PAHs delivery, PY (15.51 %), BaA (22.32 %), CHR (16.26 %), BbF (18.40 %), BkF (9.71 %), BaP (9.55 %), DahA (23.91 %) and BghiP (19.79 %) and likewise a small increase in three-membered ring PAHs such as AN (9.51 %). By contrast, β-sitosteryl oleate (Table-4) had a significant influence on the delivery of three-membered ring PAHs in tobacco mainstream smoke, while only a little influence on the yields of four-six-membered ring PAHs, except PY (29.58 %).

Based on our experiment results, it can be seen that steryl esters, especially stigmasteryl oleate and cholesteryl oleate, had a significant influence on the delivery of PAHs in tobacco mainstream smoke and the yields of four-six-membered ring PAHs were enhanced obviously. Compared to free sterols, there is a long aliphatic chain in the steryl esters, in order to make clear whether the aliphtic chain or the parent steroid backbone contributes to the PAHs formation, free sterols were added into cigarettes for comparison, using an external addtion experiment as reported[13] and the results were listed in Table-5. Stigmasterol which had been demonstrated an important precursors of PAHs in tobacco smoke by a lot of groups[12-14], increased the PAHs yields obviously. After adding stigmasteryl oleate into cigarettes, the enhancement was quite similar with stigmasterol. However under the same reaction conditions, the cholesteryl oleate and β-sitosteryl oleate exhibited much larger enhancement than the corresponding free sterols. Cholesteryl oleate increased the yields of four-six-membered ring PAHs more significantly than the cholesterol. In contrast, β-sitosteryl oleate produced more three-four-membered ring PAHs than β-sitosterol. Those results indicated that aliphatic carbon chain of the steryl ester could increase the PAHs delivery in tobacco mainstream smoke and in some cases, the increment was significant.

Saiz-Jimenez[23] reported that aliphatic carbon chains in humic acid could help to the produce alkylbenzenes, indenes naphthalenes, *etc*. through cyclization and aromatization reactions. All of these products, which can decompose into small reactive molecules and/or free radicals during the high

TABLE-2
INFLUENCE OF STIGMASTERYL OLEATE ON THE DELIVERY OF PAHS IN MAINSTREAM SMOKE

Stigmasteryl oleate (ug/cig)	PAHs (ng/cig)						Increment (%)
	0	68.67	137.34	274.68	549.36	1098.72	
ACL	212.78	213.02	216.72	221.12	222.37	216.47	4.51
AC	66.09	71.55	69.31	71.82	72.11	70.27	9.11
FL	227.97	232.98	232.51	236.77	233.37	228.25	3.86
PHE	269.05	280.06	274.85	285.11	273.21	272.85	5.97
AN	76.54	83.00	80.90	83.10	77.33	77.38	8.57
FA	77.47	79.50	80.86	82.40	81.05	79.03	6.36
PY	65.26	67.46	68.36	70.01	66.50	66.14	7.28
BaA	15.12	16.23	16.50	17.45	15.89	17.11	15.41
CHR	24.45	25.64	26.11	26.67	26.54	24.09	9.08
BbF	14.77	14.96	15.24	15.74	15.10	14.94	6.57
BkF	3.12	3.17	3.45	3.54	3.47	3.30	13.46
BaP	12.83	13.80	13.99	14.31	13.75	13.56	11.54
DBahA	10.25	10.25	10.81	11.94	10.81	10.59	16.49
BghiP	2.04	2.44	2.55	2.58	2.41	2.34	26.47
IcdP	6.18	6.34	6.37	6.38	6.37	6.27	3.24

TABLE-3
INFLUENCE OF CHOLESTERYL OLEATE ON THE DELIVERY OF PAHs IN MAINSTREAM SMOKE

Cholestery loleate (ug/cig)	PAHs (ng/cig)						Increment (%)
	0	30.13	60.25	120.50	241.00	482.00	
ACL	213.43	220.49	218.96	218.90	216.26	214.85	3.31
AC	70.14	70.50	71.81	73.85	72.15	70.04	5.29
FL	226.77	232.73	234.39	238.14	235.82	233.55	5.01
PHE	265.63	272.77	275.90	282.14	279.74	275.35	6.22
AN	75.60	78.26	79.31	82.79	79.19	75.90	9.51
FA	78.67	80.74	82.00	84.05	82.20	82.10	6.84
PY	64.81	67.99	69.67	74.86	71.24	70.36	15.51
BaA	15.23	15.96	16.54	18.63	17.17	16.00	22.32
CHR	25.52	26.54	28.24	29.67	28.28	27.14	16.26
BbF	14.29	15.24	15.54	16.92	16.21	15.32	18.40
BkF	3.40	3.36	3.58	3.73	3.50	3.42	9.71
BaP	13.20	14.00	14.04	14.46	14.22	13.65	9.55
DBahA	10.79	11.85	12.08	13.37	11.85	11.49	23.91
BghiP	1.92	2.27	2.12	2.30	2.23	2.27	19.79
IcdP	6.13	6.22	6.42	6.48	6.24	6.30	5.71

TABLE-4
INFLUENCE OF β-SITOSTERYL OLEATE ON THE DELIVERY OF PAHs IN THE MAINSTREAM SMOKE

β-sitosteryl oleate (ug/cig)	PAHs (ng/cig)						Increment (%)
	0	99.34	198.71	397.42	794.84	1589.68	
ACL	212.67	221.42	220.85	232.02	217.61	222.75	9.10
AC	68.41	75.88	79.49	85.36	69.50	70.59	24.78
FL	229.38	242.36	252.55	263.82	246.78	230.54	15.01
PHE	260.67	267.09	273.89	280.97	263.96	266.78	7.79
AN	76.62	79.47	79.74	87.84	80.98	80.17	14.64
FA	72.63	74.95	75.24	77.31	74.43	74.40	6.44
PY	66.57	73.67	86.26	80.43	69.44	69.82	29.58
BaA	15.43	16.08	16.25	16.69	16.17	16.24	8.17
CHR	23.18	24.04	24.61	25.45	24.85	25.06	9.79
BbF	13.13	13.47	13.14	13.29	13.24	13.41	2.59
BkF	3.68	3.85	3.77	3.83	3.71	3.89	5.71
BaP	12.80	12.83	13.36	13.76	13.41	13.51	7.50
DBahA	10.38	11.31	11.24	11.10	11.17	10.63	8.96
BghiP	1.91	1.97	1.97	2.01	1.96	1.95	5.24
IcdP	6.23	6.25	6.26	6.28	6.29	6.32	1.44

TABLE-5
COMPARISON OF THE INFLUENCE BETWEEN FREE STEROLS AND STERYL ESTERS[a]

Sterol	Increment (%)					
	A	A-oleate	B	B-oleate	C	C-oleate
ACL	5.78	4.51	9.23	3.31	7.91	9.10
AC	8.02	9.11	21.64	5.29	-0.38	24.78
FL	7.76	3.86	7.19	5.01	5.17	15.01
PHE	6.66	5.97	3.28	6.22	2.46	7.79
AN	10.22	8.57	2.48	9.51	8.92	14.64
FA	3.42	6.36	0.55	6.84	1.44	6.44
PY	8.18	7.28	4.8	15.51	5.06	29.58
BaA	11.69	15.41	5.08	22.30	3.26	8.17
CHR	12.56	9.08	6.65	16.27	4.39	9.79
BbF	4.04	6.57	-0.01	18.40	4.27	2.59
BkF	11.69	13.46	8.77	9.71	5.97	5.71
BaP	15.22	11.54	0.95	9.55	2.96	9.38
DBahA	7.5	16.49	13.54	23.91	2.13	8.96
BghiP	8.38	26.47	1.51	19.79	2.18	5.24
IcdP	8.15	3.24	5.28	5.71	12.5	1.44

[a]A: Stigmasterol, B: cholesterol, C: β-sitosterol

temperature smoking processes, followed by recombination reactions, can possible form larger PAHs during tobacco smoke[7,24-26]. Accordingly, it can be tentativly concluded that, in our experiment, the aliphatic carbon chain in the cholesteryl oleate and β-sitosteryl oleate was also involed in the process of PAHs formation. This perhaps accounts for the increment

of PAHs yields in tobacco mainstream smoke, caused by steryl esters compared to corresponding free sterols.

Conclusion

In this study, three steryl esters were synthesized from corresponding sterols and oleic acid. The somking experiments showed that their addtion into the cigarettes could influence the delivery of PAHs. Stigmasteryl oleate and cholesteryl oleate increased the delivery of four-six-membered ring PAHs significantly, while β-sitosteryl oleate increased the production of three-membered ring PAHs. The results indicated that steryl esters were possibly the most responsible for PAHs delivery in tobacco mainstream smoke.

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