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Rapid and simultaneous analysis of ten aromatic amines in mainstream cigarette smoke by liquid chromatography/electrospray ionization tandem mass spectrometry under ISO and "Health Canada intensive" machine smoking regimens

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ABSTRACT

Ten primary aromatic amines (AAs) in mainstream cigarette smoke under both ISO and "Health Canada intensive" machine smoking regimens were determined in this work, which were suspected to be carcinogenic compounds. The measured AAs included aniline, ortho-toluidine, meta-toluidine, para-toluidine, 1-naphthylamine, 2-naphthylamine, 3-aminobiphenyl, 4-aminobiphenyl, meta-phenylenediamine and meta-anisidine. For rapidly and sensitively analyzing these AAs, a liquid chromatography-electrospray ionization tandem mass spectrometric (LC–MS/MS) method coupled with solid phase extraction (SPE) was developed. The particulate phase of mainstream cigarette smoke was collected on a Cambridge filter pads, while the gas phase was trapped by 25 mL 5% HCl solution. Then, the pad was extracted in an ultrasonic bath with the impinger HCl solution. After being neutralized with NaOH, the extract was purified with a HLB solid phase extraction column, and then was analyzed with LC–MS/MS using isotope-labeled internal standard. The overall sample pretreatment and analysis time was less than 1.5 h. The limits of detection for all targets ranged from 0.05 ng cig^{-1} to 0.96 ng cig^{-1} with the recoveries in the range of 75.0–131.8%. And the intra-day and inter-day precisions were less than 10% and 16%, respectively. Under HCI machine smoking regimen, the AAs yields in mainstream cigarette smoke were much higher and the average increases were greater than 100% compared with those under ISO smoking condition.

1. Introduction

Primary aromatic amines (AAs) such as aniline and its substituted analogs are important and widely used industrial intermediates in the manufacture of carbamate and urethane pesticides, dyestuffs, cosmetics (hair dyes), and medicines [1–3]. In the case of cigarettes AAs have been tested in a number of investigations in both mainstream (MS) and sidestream (SS) smoke [4,5]. Many AAs are toxic compounds and/or suspected human carcinogens. It has been known that occupational exposure to 2- naphthylamine (2-NA), 4-aminobiphenyl (4-ABP) and o-toluidine (o-TOL) was causally associated with the occurrence of human bladder cancer [6–10]. In this work, we studied the determination of 10 AAs (Table 1) in MS smoke, containing aniline (ANL), o-, m-, p-TOL, 1-naphthylamine (1-NA), 2-NA, 3-aminobiphenyl (3-ABP), 4-ABP, m-phenylenediamine (PDA) and m-anisidine (ASD). Most of them are classified as human carcinogen by the International Agency for Research on Cancer (IARC) [11]. 2-NA and 4-ABP were involved in Hoffmann list of tobacco carcinogens [12]. Moreover, 1-NA, 2-NA, 3-ABP and 4-ABP were contained in the test list of toxic components in cigarette smoke propounded by Health Canada [13].

Up to now, various methods have been developed for the determination of AAs, such as gas chromatography electron-capture detection (GC-ECD) technique [4,14], GC-mass spectrometry (GC–MS) [5,15–17], high performance liquid chromatography-ultraviolet-visible (HPLC-UV–vis) [18,19], LC–MS/MS [20–23], and so on. Hoffmann and Masuda [4,14] reported in 1969 for the first time on the determination of aromatic amines in cigarette smoke by applying a GC-ECD technique. However, GC–MS is the most popular method for the determination of AAs in cigarette smoke. For example, in 1992, Pieraccini et al. [15] introduced a GC–MS technique for determination of 17 AAs in cigarette smoke in two Italian commercial brand cigarettes. Due to their high polarity and thermal instability, for GC–MS method AAs were derivatized with pentafluoropropionyl [15,16], trifluoroacetic anhydride [17] and heptafluorobutyric anhydride [5], and so on. On the other hand,

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Table 1
The IARC group of ten AAs.

AAs	IARC group[a]
o-TOL, 2-NA, 4-ABP	1
ANL, o-,m-, p-PDA, 1-NA, p-ASD	3
3-ABP, m-, p-TOL, m-ASD	–
o-ASD	2B

[a] IARC classification groups: 1=carcinogenic to humans; 2B=possibly carcinogenic to humans; 3=not classifiable as to its carcinogenicity to humans.

as the ultra-low yields of some carcinogenic AAs in cigarette smoke, multi-step liquid–liquid extraction and preconcentration were involved in the pretreatment procedure for GC–MS method. Therefore, the pretreatment procedure for GC–MS method is tedious and time-consuming. LC–MS/MS has been shown to be efficient technique for the determination of polar compounds, such as AAs [20–23] and heterocyclic aromatic amines [24]. By using LC–MS/MS, the derivatization procedures could be avoided. Furthermore, LC–MS/MS generally has a higher selectivity and sensitivity. In the recent study, Saha et. al [20] used LC–MS/MS technique to analyze 6 AAs in cigarette smoke. However, liquid–liquid extraction and preconcentration process were adopted in their research, which made the pretreatment procedure tedious. Furthermore, only 4-ABP was determined, while 3-ABP was not mentioned in their study. Moreover, Schubert et. al [21] reported a LC–MS/MS method for the analysis of AAs in the mainstream waterpipe smoke. In their research, 3- and 4-ABP could not be separated at all, and the two isomers were analyzed together. It has been known that AAs isomers show different biological activity, e.g., of the two isomers of aminobiphenyl (3 and 4-ABP), only 4-ABP is a carcinogen [11]. Therefore, the simultaneous separation and determination of AAs and related isomers are very important for the evaluation of carcinogenic risk for cigarette smoke.

In the present wok, a simple, sensitive and practical method for the determination of 10 AAs in MS smoke under both ISO and "Health Canada intensive" machine smoking regimens with LC–MS/MS technique based on multiple reaction monitoring (MRM) was developed. At the same time, a simple and efficient solid phase extraction (SPE) technique was used to purify samples. Moreover, through the optimization of liquid phase chromatography conditions, five groups of AAs isomers containing 1-, 2-NA, 3-, 4-ABP, o-, m-, p-TOL, o-, m-, p-PDA, o-, m-, p-ASD were separated in this work. The proposed method was suited to significantly reduce the sample preparation time, simultaneously separate and determine AAs isomers.

2. Experimental

2.1. Reagents and cigarettes

The AAs (analytical standard) were obtained from Sigma-Aldrich Chemie GmbH (Munich, Germany). ANL-d5, 1-NA-d7 and 4-ABP-d9 were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). ANL-d5 was used as the internal standard for ANL, o-, m-, p-TOL, m-PDA and m-ASD. And 1-NA-d7 was used for 1-NA and 2-NA, while 4-ABP-d9 was for 3-ABP and 4-ABP. Acetonitrile and methanol were HPLC grade and purchased from Dikama Corporation. Hydrochloric acid used here was of analytical-reagent grade. All other reagents were analytical standard. Water was purified with a Milli-Q system (Millipore, Bedford, MA, USA). Standard stock solutions were prepared in methanol with a concentration of 1.0 mg mL^{-1} each and stored in −18 °C for up to 6 months. Only m-PDA was kept refrigerated no longer than one month. Calibration solutions were prepared with methanol. A working solution containing m-PDA was prepared separately every week.

Two reference cigarettes and five commercial cigarettes with different tar level were selected as test sample. Kentucky reference cigarette 3R4F was purchased from the Tobacco and Health Research Institute at the University of Kentucky, while CM6 was obtained from Cooperation Centre for Scientific Research Relative to Tobacco (CORESTA). Five commercial cigarettes were collected from China market, with packet labeled tar ranged from 5.0 to 12.0 mg cig^{-1}.

2.2. Smoke generation

Before smoking, the cigarettes were conditioned at (22 ± 1) °C and (60 ± 2)% humidity for 48 h. MS smoke for the analysis of AAs was generated on a linear smoking machine (SM450 Cerulean, UK) under two kinds of smoking regimens. One is FTC/ISO (i.e. US Federal Trade Commission/the International Organization for Standardization) smoking regimen, i.e. 35 cm^3 puff volume, 2-s puff duration, 1-min puff interval. The other is the Health Canada intensive smoking regimen (HCI), i.e. 55 cm^3 puff volume, 2-s puff duration, 30-s puff interval, 100% ventilation block.

2.3. Sample preparation

The particulate phase (PP) of cigarette smoke was collected on a Cambridge filter pad, while the gas vapor phase (GP) was trapped with one impinger containing 25 mL 5% HCl solution. Five cigarettes were smoked. After smoking, the filter pad was extracted with 5% HCl

Table 2
The specific parameters of AAs for MRM analysis.

Analytes	Retention time (min)	Precursor ion (*m/z*)	Production ion (*m/z*)	Dwell time (ms)	CE (eV)	DP (eV)	EP (eV)	CXP (eV)
ANL	3.14	94.0	77.0[a] 51.0[b]	50	26	100	10	12
ANL-d5	3.10	99.1	82.0[a] 54.0[b]	50	30	80	10	12
p-TOL	4.03	108.0	91.0[a] 65.0[b]	50	24	60	10	12
o-TOL	4.36	108.0	91.0[a] 65.0[b]	50	24	60	10	12
m-TOL	4.49	108.0	91.0[a] 65.0[b]	50	24	60	10	12
1-NA	14.00	144.0	127.0[a] 77.0[b]	50	30	150	10	12
2-NA	12.11	144.0	127.0[a] 77.0[b]	50	30	100	10	12
1-NA-d7	13.37	151.0	132.0[a] 81.0[b]	50	30	100	10	12
3-ABP	16.24	170.0	153.0[a] 127.0[b]	50	30	100	10	12
4-ABP	16.55	170.0	153.0[a] 127.0[b]	50	30	100	10	12
4-ABP-d9	15.90	179.0	160.0[a] 134.0[b]	50	30	100	10	12
m-PDA	2.70	109.0	92.0[a] 65.0[b]	50	25	70	10	12
o-PDA	3.01	109.0	92.0[a] 65.0[b]	50	25	70	10	12
p-PDA	1.94	109.0	92.0[a] 65.0[b]	50	25	70	10	12
m-ASD	4.70	124.1	92.1[a] 108.9[b]	50	30	90	10	12
o-ASD	4.14	124.1	92.1[a] 108.9[b]	50	30	90	10	12
p-ASD	3.37	124.1	92.1[a] 108.9[b]	50	30	90	10	12

[a] The qualitative ion pair.
[b] Quantitative ion pair.

solution in the impinger and an aliquot of internal standard solution was added (i.e. ANL-d5, 1-NA-d7 and 4-ABP-d9 were 1200 ng, 60 ng and 12 ng, respectively) in an ultrasonic bath for 30 min with temperature maintained at below 40 °C. 20 mL of the extraction solution was transferred accurately into another Erlenmeyer flask and was neutralized with 10 M NaOH solution (about 4 mL) to pH 13. Solid phase extraction (SPE) was used for the sample purification with a Waters Oasis HLB (200 mg, 6 mL) column preconditioned with 10 mL methanol. The total basified extraction solution was passed through the cartridge at a flow rate of 1–1.5 mL/min. After washing with 10 mL water, the target compounds were eluted with 10 mL methanol for the analysis of LC–MS/MS.

2.4. Instrumental conditions

The chromatographic separation was achieved using an Agilent 1200 HPLC (USA) coupled with an AB SCIEX Triple QUAD 5500 mass spectrometer (AB Sciex Instruments, Germany). Chromatography was performed on a Waters Symmetry Shield™ RP18 column (150 mm × 2.1 mm i.d., 3.5 μm) at 30 °C with the sample injection volume of 10 μL. The mobile phase solvents A was water with 0.1% formic acid, while B was acetonitrile with 0.1% formic acid. HPLC separation was achieved running a gradient under following conditions: 0–3 min: 100% A, 3.1–16 min: 75% A, 16.1–20 min: 0% A, 20.5–25 min: 100% A; the flow rate: 0–16 min: 200 μL/min, 16.1–25 min: 400 μL/min.

Mass detection conditions were as follows: ionization mode, positive ESI; ion spray voltage, 5500 V; ion source temperature, 500 °C; curtain gas, nitrogen; setting: 30 psi; ion source gas 1 (GS1), setting: 70 psi; ion source gas 2 (GS2), setting: 70 psi. Compound-dependent parameters were optimized by flow injection analysis. For each analyte, the two most intense ion transition was used under scheduled multiple reaction monitoring (MRM) mode. The parameters of precursor ion, qualitative and quantitative ion pair, dwell time, collision energy (CE), declustering potential (DP), entrance potential (EP), collision cell exit potential (CXP), as well as retention time were summarized in Table 2.

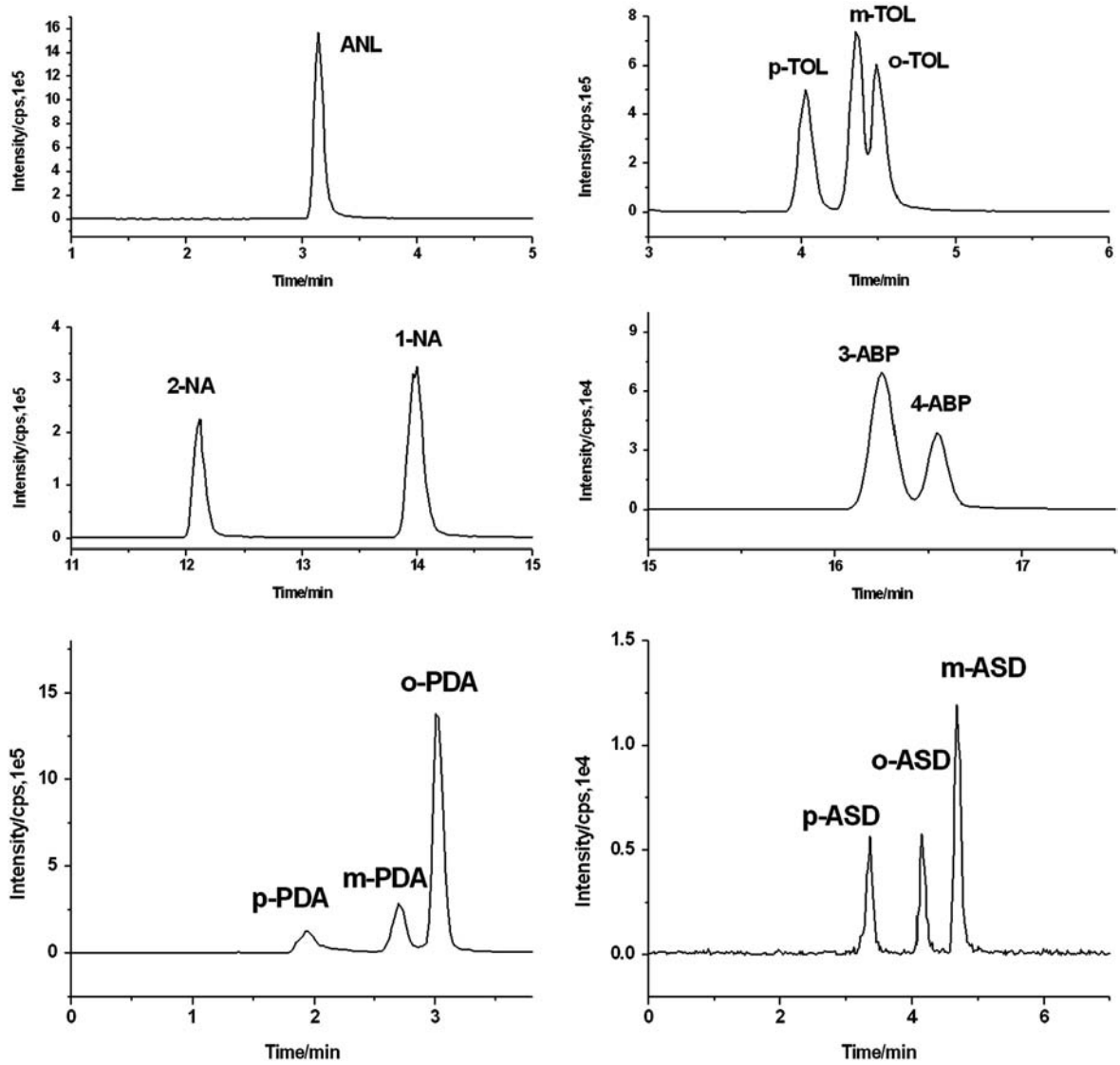

Fig. 1. LC–MS/MS chromatograms of AAs standard solution. The selected ions and concentrations of AAs as following: ANL: 94.0/77.0, 36.0 ng/mL; o-TOL: 108.0/91.0, 10.9 ng/mL; m-TOL: 108.0/91.0, 13.8 ng/mL; p-TOL: 108.0/91.0, 11.5 ng/mL; o-PDA: 109.0/92.0, 59.0 ng/mL; m-PDA: 109.0/92.0, 50.0 ng/mL; p-PDA: 109.0/92.0, 40.0 ng/mL; o-ASD: 124.1/92.1, 0.58 ng/mL; m-ASD: 124.1/92.1, 0.52 ng/mL; p-ASD: 124.1/92.1, 0.46 ng/mL; 1-NA: 144.0/127.0, 5.0 ng/mL; 2-NA: 144.0/127.0, 2.5 ng/mL; 3-ABP: 170.0/153.0, 1.0 ng/mL; 4-ABP: 170.0/153.0, 0.5 ng/mL. The mobile phase A: water containing 0.1% formic acid, B: acetonitrile containing 0.1% formic acid. HPLC gradient conditions: 0–3 min: 100% A, 3.1–16 min: 75% A, 16.1–20 min: 0% A, 20.5–25 min: 100% A; the flow rate: 0–16 min: 200 μL/min, 16.1–25 min: 400 μL/min.

3. Results and discussion

3.1. The optimization of HPLC conditions

The isomers of AAs show similar chemical structure and property, especially 3-ABP and 4-ABP. It has been reported that 3- and 4-ABP could not be separated at all [21]. Thus, we tested the following set of HPLC columns with similar column efficiency: Waters Symmetry Shield™ RP18 (150 mm × 2.1 mm i.d., 3.5 μm), Agilent ZORBAX Eclipse Extend C18 (5 μm, 2.1 × 150 mm), Dikma Spursil™ C18 (3 μm, 2.1 × 150 mm), Waters XBridge™ C18 (5 μm, 2.1 × 150 mm). The isomers of o, m, p-TOL, 3, 4-ABP and o, m, p-ASD could not be separated on the first three columns. Best separation was achieved on Waters Symmetry Shield™ RP18 column, and the chromatograms of a standard mixture of AAs obtained are shown in Fig. 1. Baseline separation of 1-, 2-NA, 3-, 4-ABP, o-, m-, p- PDA and o-, m-, p-ASD isomers were achieved. The chromatographic resolutions of 1- and 2-NA, 3- and 4-ABP, o- and m-PDA, m- and p- PDA, o- and m-ASD, p- and o-ASD, m- and p-TOL are 7.71, 1.25, 1.39, 2.78, 2.40, 3.59, 1.79, respectively, which indicate their good separation. However, there are some overlaps between m-TOL and o-TOL with the resolution of 0.62, these two peaks were integrated in manual mode.

For separation of AAs isomers different mobile phases and additives were tested. With the proportion of organic phase increasing, AAs eluted faster. As the composition of mobile phases was 75% water and 25% acetonitrile, both with 0.1% formic acid, respectively, the baseline separation of 3-ABP and 4-ABP was achieved. Addition of formic acid to mobile phases could help the ionization of AAs and the enhancement of signal intensity. However, the addition of ammonium acetate resulted in the loss of signal intensity. Although there was no significant temperature effect on the separation, an oven temperature of 30 °C was used in this work.

Fig. 2 depicts the chromatograms of AAs in MS smoke sample. Applying the present method, the ANL, o-TOL, m-TOL, p-TOL, 1-NA, 2-NA, 3-ABP, 4-ABP, m-PDA, m-ASD were detected in mainstream cigarette smoke, the resolution and sensitivity for target AAs are good enough for the quantitative analysis. However, p-PDA, o-PDA, o-ASD and p-ASD were not detected in mainstream cigarette smoke due to the yields lower than the detection limit or the interference with some impurities.

3.2. The trapping efficiency

In order to investigate the trapping efficiency for AAs in cigarette smoke, two impingers each containing 25 mL 5% HCl solution were connected in series after the Cambridge filter pads. The particulate phase was collected with Cambridge filter pads. The two impingers were used for the collection of the gas vapor phase (i.e. GP1 and GP2). The filter pads were extracted with 25 mL 5% HCl solution and cleaned up according to the procedure mentioned in Section 2.3. The solution in each of impingers was cleaned up with the same procedure. Then the final 3 extracts were analyzed using LC–MS/MS, respectively. The yields of AAs in filter pads and two impingers solutions were determined for calculating trapping efficiency, which was calculated according to that the yield of AAs in filter pads or one of impingers divided by the total yields of AAs in filter

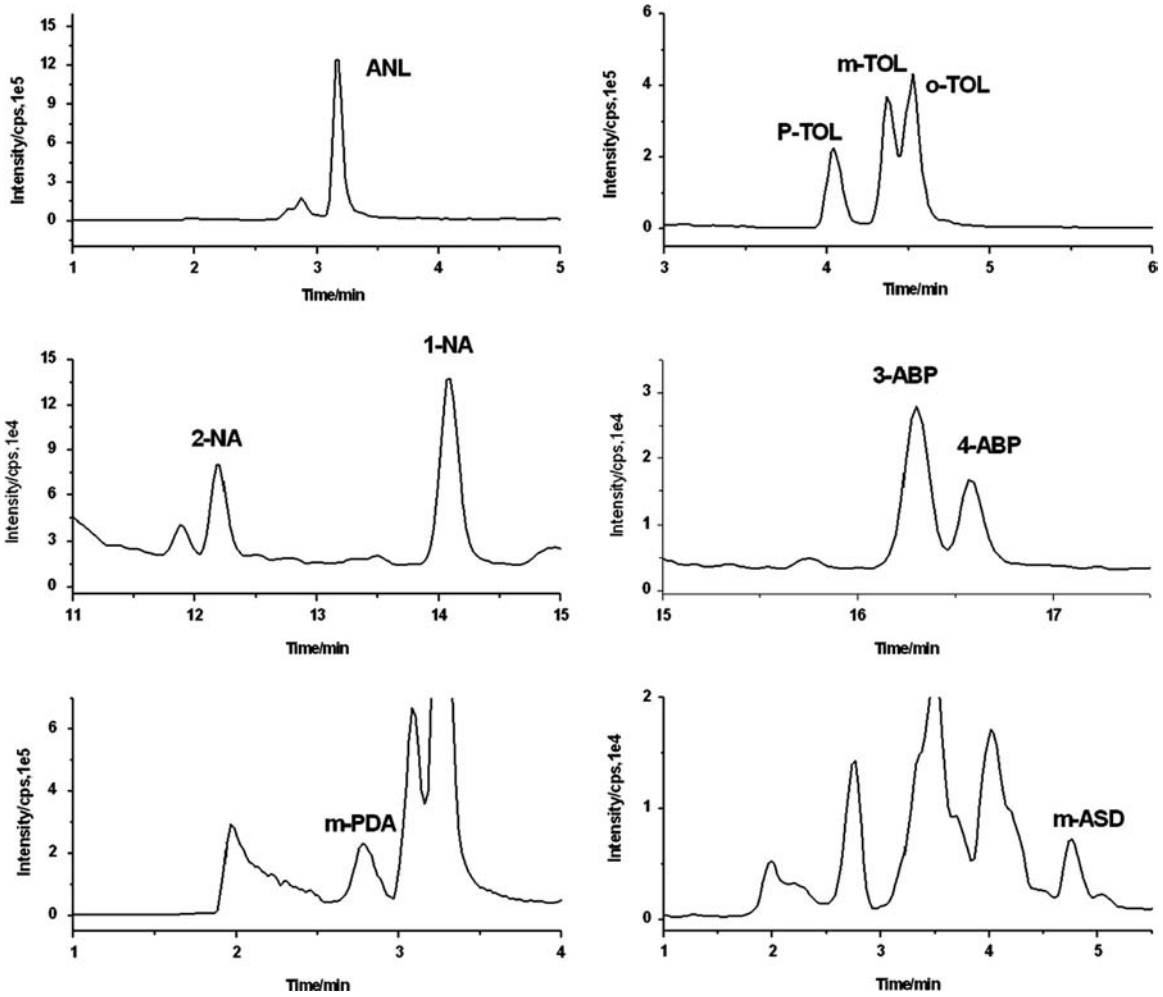

Fig. 2. LC–MS/MS chromatograms of AAs in mainstream smoke sample. The selected ions and HPLC conditions the same as Fig. 1.

pads and two impingers. The trapping efficiency was evaluated under both ISO and HCI smoking conditions, which was shown in Table 3. The trapping efficiency of ten AAs in filter pads was all over 85%, while it was all below 5% in the second impinger. Moreover, the trapping efficiency for only p-TOL was over 5% in the first impinger under ISO smoking regimen. It was shown that ten AAs were mainly distributed in particulate phase of mainstream cigarette smoke under two smoking conditions, and the total trapping efficiency of filter pad and the first impinger for ten AAs was all over 95%. Therefore, Cambridge filter pads coupled with one impinger containing HCl solution were employed for the collection of AAs in mainstream smoke under two smoking conditions in this work.

3.3. Optimization of SPE

After the neutralization of extracting solution with NaOH, AAs was transferred from their hydrochloride form into molecular state. However, the salt-containing solution could not be analyzed by LC–MS/MS. And some ultra-trace AAs could not be detected due to the complicated matrix of cigarette smoke. Thus, solid-phase extraction (SPE) technique was employed to purify sample. Several solid-phase extraction cartridges with different retention characteristics were tested, such as Agilent Bond Elut C18 (200 mg, 3 mL), Varian Bond Elut JR-Si (500 mg, 100/PK), Waters Oasis HLB (200 mg, 6 mL), and so on. However, ten AAs could not been retained in C18 and Si SPE cartridges completely. Only HLB cartridge could make AAs retained and was chosen for sample purification.

A simulated sample composed of a certain AAs standard aqueous solution was prepared for the study of the retention and elution characteristics of AAs on HLB cartridge. The effluent solution during SPE sampling and clean procedure were collected and analyzed by LC–MS/MS, respectively. And no AAs were detected, which revealed that AAs were retained on HLB cartridge completely. Furthermore, the elution volume was optimized. Each 5 mL methanol was used to elute continuously and the elution solutions were analyzed separately. The contents of AAs were all below 5% of their total amounts in the third 5-mL elution volume. Thus, 10 mL methanol was the optimum elution volume.

3.4. Recovery and precision

A spiked recovery method was applied in 3R4F cigarette smoke extract. The standard AAs solution was spiked in three different concentration levels. And the medium spiked concentration was selected to nearby concentration of the yield of AAs in cigarette smoke. Three replicate measurements were performed at each concentration level of each AA. Recovery tests were carried out with standard solution spiked both before extraction (Recovery I) and in the final extracts (Recovery II). The recovery was calculated as $(C_b-C_a)/C_s$, where C_a and C_b were the determined concentration before and after adding standard solution respectively; C_s was the spiked concentration. The results (Table 4) revealed that recoveries I and recoveries II of ten AAs were in the range of 75.0%–131.8% and 84.3%–124.8%, respectively. There was no significant difference between the two kinds of recoveries. It indicated that the matrix effect was not serious for this method though isotopes were not used as internal standard for p-,m-,o-TOL, m-PDA and m-ASD. Intra-day and inter-day precision were determined using 3R4F cigarette. For intra-day precision the cigarette was analyzed 6 times on the same day, which was less than 10%. For inter-day precision the cigarette was analyzed on 5 different days, which was less than 16%.

Table 3
The trapping efficiency of AAs in MS smoke under ISO and HCI regimens.

Analyte	ISO			HCI		
	PP(%)	GP1(%)	GP2(%)	PP(%)	GP1(%)	GP2(%)
ANL	97.0	3.0	0.0	95.4	4.6	0.0
p-TOL	87.5	9.1	3.4	100.0	0.0	0.0
m-TOL	98.0	1.9	0.1	97.0	2.2	0.8
o-TOL	99.0	1.0	0	98.2	1.7	0.1
1-NA	100.0	0.0	0.0	100.0	0.0	0.0
2-NA	100.0	0.0	0.0	100.0	0.0	0.0
3-ABP	100.0	0.0	0.0	100.0	0.0	0.0
4-ABP	100.0	0.0	0.0	100.0	0.0	0.0
m-PDA	100.0	0.0	0.0	100.0	0.0	0.0
m-ASD	100.0	0.0	0.0	100.0	0.0	0.0

3.5. Calibration curves, LODs and LOQs

The concentrations of ANL-d5, 1-NA-d7, 4-ABP-d9 for each working solutions were 96 ng mL^{-1}, 4.8 ng mL^{-1}, 0.96 ng mL^{-1}, respectively. The 6-point calibration curves were carried out by the internal standard method. Linear range, linear equation and linear correlation coefficient (R) were shown in Table 4. The calibration curves showed excellent linearity in the linear concentration range with R between 0.9972 and 1.0000. The calibration range could be extended to higher and lower amounts, but this was not routinely done since this working calibration range was sufficiently wide to measure smoke AAs from current cigarette samples. For the study of limit of detection (LOD) and limit of quantification (LOQ), the working solution of lowest calibration standard was added in the blank filter pads extract used as matrix and the extract was determined in ten replicates. LOD and LOQ were obtained from three and ten times of the standard deviation of these determinations [20] after being calibrated with recovery I

Table 4
Intra-day, inter-day precision, and recoveries of ten AAs.

Analytes	Intra-day precision (RSD, $n=6$)	Inter-day precision (RSD, $n=5$)	Spiked concenration	Recovery I[a] ($\pm$SD, $n=3$,%)	Recovery II[b] ($\pm$SD, $n=3$,%)
ANL	5.37%	8.99%	Low	109.5 ± 3.5	107.2 ± 3.3
			Mid	128 ± 4.0	123.6 ± 5.2
			High	128.1 ± 5.6	120.9 ± 8.1
p-TOL	8.28%	9.77%	Low	110 ± 7.0	94.2 ± 6.0
			Mid	123.5 ± 5.3	109.6 ± 4.0
			High	120.9 ± 5.4	89.6 ± 4.5
m-TOL	7.67%	15.4%	Low	115 ± 6.1	108.9 ± 5.4
			Mid	131.8 ± 3.8	103.1 ± 4.2
			High	131.3 ± 4.8	124.8 ± 5.9
o-TOL	6.85%	13.6%	Low	108 ± 6.2	110.1 ± 4.9
			Mid	125.2 ± 5.0	105.6 ± 4.1
			High	123.6 ± 4.9	120 ± 2.0
1- NA	8.89%	12.1%	Low	101.8 ± 3.8	98.5 ± 3.5
			Mid	110.1 ± 2.1	100.4 ± 1.9
			High	113.6 ± 4.1	108.9 ± 6.5
2- NA	9.46%	13.2%	Low	92.9 ± 4.5	92 ± 3.6
			Mid	80.6 ± 5.5	89.6 ± 4.5
			High	75 ± 5.0	87.3 ± 3.1
3-ABP	3.23%	8.95%	Low	80.6 ± 4.6	89.4 ± 2.9
			Mid	86.1 ± 4.4	84.3 ± 4.5
			High	88.5 ± 3.6	95.9 ± 3.8
4-ABP	6.2%	12.9%	Low	81.1 ± 5.4	91.1 ± 3.2
			Mid	86.5 ± 4.8	90.9 ± 3.6
			High	89.4 ± 4.9	91.8 ± 2.7
m-PDA	2.54%	13.8%	Low	113.9 ± 4.8	112.8 ± 4.7
			Mid	109.2 ± 6.2	99.8 ± 4.7
			High	108.9 ± 5.7	101 ± 3.6
m-ASD	8.24%	15.4%	Low	102.5 ± 2.2	109.9 ± 4.8
			Mid	124.9 ± 4.9	103.1 ± 3.7
			High	114.1 ± 4.4	105.7 ± 5.2

[a] Recovery with standard solution spiked before extraction.
[b] Recovery with standard solution spiked in the final extracts.

(Table 4). As shown in Table 5, LOD and LOQ of ten AAs ranged from 0.05 to 0.96 ng cig^{-1} and 0.18 to 3.20 ng cig^{-1}, respectively.

3.6. Sample determination

Two reference cigarettes (Kentucky Reference Cigarette 3R4F, CORESTA Monitor Cigarette CM6) were analyzed by the developed method. Ten AAs in mainstream cigarette smoke were determined under both ISO and HCI smoking conditions. And the results were shown in Table 6.

The 3R4F cigarette is a new reference cigarette to replace the depleting supply of the 2R4F cigarette, but there are no available AAs yields literature results for the 3R4F cigarettes. So, the yields of AAs in mainstream smoke of 3R4F were compared with the literature values of 2R4F. According to the literatures [16,20], under ISO smoking conditions, the yields of ANL in 2R4F mainstream smoke ranged from 251.60 to 258.73 ng cig^{-1}, 1-NA from 15.06 to 15.97 ng cig^{-1}, 2-NA from 8.6 to10.65 ng cig^{-1}, 4-ABP from 1.60 to 1.92 ng cig^{-1}, and o-TOL from 42.42 to 56.10 ng cig^{-1}. Considering the minor differences in cigarette design between 2R4F and 3R4F, the determined values in the present work were close to those of the literature values.

Applying this developed method, five commercial cigarettes (labeled as 1# to 5#) from China market were determined (Table 6). The packet labeled tar levels of these cigarettes are in the range of 5.0–12.0 mg cig^{-1}, and sample 1, 2, 3 are Virginia type cigarettes, sample 4, 5 are American blended type cigarettes.

Under ISO machine smoking regimen, the most abundance aromatic amines in mainstream smoke of commercial cigarettes is m-PDA, yields ranged from 171 ng cig^{-1} to 419 ng cig^{-1} with the average of 278 ng cig^{-1}. For the most carcinogenic aromatic amines, the yields of 2-NA were between 1.85 ng cig^{-1} and 5.96 ng cig^{-1} with the average of 3.34 ng cig^{-1}; the yields of 4-ABP were between 0.30 ng cig^{-1} and 0.72 ng cig^{-1} with the average of 0.46 ng cig^{-1}; the yields of o-TOL were between 36.4 ng cig^{-1} and 93.1 ng cig^{-1} with the average of 56.2 ng cig^{-1}.

Because the ISO regimen constitutes a set of puffing parameters that systematically underestimate smoking behavior in humans [25], it is widely acknowledged to be inappropriate for purposes of setting regulatory restrictions. “Health Canada intensive” machine smoking regimen is an alternative machine smoking regimens because of its more intensive puffing parameters and the increased filter ventilation blocking.

The results indicated that the AAs yields of cigarette smoke are great differences between HCI and ISO smoking regimens, the ISO smoking regimen significantly underestimates human exposure for the AAs. Under HCI machine smoking regimen, the AAs yields in mainstream cigarette smoke were much higher and the average increases were greater than 100% compared with those under ISO smoking condition. The average increases of ten AAs under HC condition compared with ISO conditions were as follows, 163% for ANL, 119% for p-TOL, 150% for m-TOL, 133% for o-TOL, 125% for 1-NA, 95% for 2-NA, 146% for 3-ABP, 141% for 4-ABP, 127% for m-PDA and 140% for m-ASD.

Table 5
Calibration curves, LODs and LOQs of ten AAs.

Analytes	Linear range (ng mL^{-1})	Linear equation	R	LOD (ng cig^{-1})	LOQ (ng cig^{-1})
ANL[a]	7.23–361.5	$y=0.014x+0.067$	0.9972	0.52	1.72
p-TOL[a]	2.29–114.7	$y=0.013x-0.0063$	0.9999	0.41	1.35
m-TOL[a]	2.18–109	$y=0.022x+0.0053$	0.9998	0.83	2.75
o-TOL[a]	2.76–138	$y=0.021x+0.0060$	0.9998	0.96	3.20
1- NA[b]	1–50	$y=0.453x+0.22$	0.9996	0.11	0.37
2- NA[b]	0.5–25	$y=0.462x+0.076$	0.9999	0.11	0.36
3-ABP[c]	0.2–10	$y=1.22x-0.058$	0.9993	0.15	0.51
4-ABP[c]	0.1–5	$y=1.18x-0.0052$	0.9998	0.06	0.19
m-PDA[a]	10–500	$y=0.0023x+0.033$	0.9979	0.50	1.66
m-ASD[a]	0.103–5.15	$y=0.0102x+0.00092$	1.0000	0.05	0.18

[a] ANL-d5 as internal standard.
[b] 1-NA-d7 as internal standard.
[c] 4-ABP-d9 as internal standard.

4. Conclusions

A LC–MS/MS method coupled with solid phase extraction technique was developed for the simultaneous determination of ten AAs in mainstream cigarette smoke under both ISO and “Health Canada intensive” machine smoking regimens. The whole sample pretreatment showed high efficiency, which takes just 1.5 h. The separation of five isomers, etc. o, m, p-TOL, 1, 2-NA, 3, 4-ABP, o, m, p-PDA and o, m, p-ASD was achieved through the optimization of HPLC conditions in this work. The developed method showed lower

Table 6
AAs yields in MS smoke of cigarette samples under ISO and HCI regimens.

Samples	Smoking Ćconditions	ANL Ć (ng cig^{-1})	p-TOL Ć (ng cig^{-1})	m-TOL Ć (ng cig^{-1})	o-TOL Ć (ng cig^{-1})	1-NA Ć(ng cig^{-1})	2-NA Ć (ng cig^{-1})	3-ABP Ć (ng cig^{-1})	4-ABP Ć (ng cig^{-1})	m-PDA Ć (ng cig^{-1})	m-ASD Ć (ng cig^{-1})
3R4F	ISO	232	48.8	33.1	47.9	13.6	9.38	1.58	0.72	325	1.36
	HCI	668	124.4	115.0	161.9	31.0	24.0	4.55	2.05	875	3.67
CM6	ISO	491	97.0	115.0	181.3	22.8	11.6	1.63	0.64	709	2.65
	HCI	838	103.1	138.1	228.8	42.5	20.5	3.57	1.24	838	3.22
1#	ISO	263	45.3	49.0	93.1	9.4	3.63	1.19	0.45	419	1.43
	HCI	583	85.3	112.5	185.9	18.9	7.22	2.85	1.11	784	2.72
2#	ISO	129	20.2	18.2	36.4	5.7	2.14	0.83	0.33	171	0.56
	HCI	332	39.4	42.3	77.2	11.2	4.41	2.16	0.93	356	1.56
3#	ISO	203	27.5	34.3	60.1	5.7	1.85	0.80	0.30	266	1.06
	HCI	463	59.8	63.0	145.0	12.8	2.98	1.97	0.62	531	2.94
4#	ISO	245	42.9	35.8	52.9	11.3	5.96	1.72	0.72	299	1.05
	HCI	688	99.7	126.6	144.7	24.9	10.5	3.76	1.56	953	2.65
5#	ISO	193	29.6	26.3	38.4	6.7	3.13	1.04	0.50	238	0.61
	HCI	653	77.2	64.3	102.5	19.4	7.50	2.97	1.33	535	1.43

detection limit, better recovery and an excellent reproducibility. The ISO smoking regimen significantly underestimates human exposure for the AAs. Under HCI machine smoking regimen, the AAs yields in mainstream cigarette smoke were much higher and the average increases were greater than 100% compared with those under ISO smoking condition.

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