Journal of Chromatography A, 1218 (2011) 5628–5637

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of primary aromatic amines in the mainstream waterpipe smoke using liquid chromatography–electrospray ionization tandem mass spectrometry

Jens Schubert*, Oliver Kappenstein, Andreas Luch, Thomas G. Schulz

German Federal Institute for Risk Assessment (BfR), Department of Product Safety, Thielallee 88-92, 14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 24 March 2011
Received in revised form 16 June 2011
Accepted 16 June 2011
Available online 26 June 2011

Keywords:
Primary aromatic amines
LC–MS/MS
Waterpipe
Mainstream smoke

ABSTRACT

In recent years waterpipe smoking has spread worldwide and emerged as global health issue. Yet only little is known on the composition of waterpipe smoke. Here, we present a study on the identification and quantification of primary aromatic amines (PAAs) in this complex environmental matrix. Smoking of the waterpipe was conducted with a smoking machine and particulate matter was collected on glass fiber pads. We developed a fast, simple and specific liquid chromatography–electrospray ionization tandem mass spectrometry (LC–MS/MS) approach to simultaneously detect 31 different PAAs in this matrix. The detection limits comprised a range of 0.45–4.50 ng per smoking session, represented by 2-aminobiphenyl and 3,4,5-trichloroaniline, respectively. Intra- and inter-day precision were determined and proved excellent. We detected 31.3 ± 2.2 ng aniline and 28.0 ± 1.6 ng 4,4′-oxydianiline in the smoke of one waterpipe session. The water in the bowl exerted a small but considerable filter effect on PAAs. The method worked-out showed excellent sensitivity and specificity and is thus highly suited for the determination of PAAs in mainstream waterpipe smoke.

1. Introduction

Recent studies on the use of the waterpipe, also called shisha, hookah, or argileh, show that it has become extremely popular worldwide, especially for young people. In a study performed in England among university students nearly 40% had tried a waterpipe at least once [1]. Among Turkish university students this rate was even 45% [2]. In a survey in Germany 38% of adolescents aged 12–17 stated to have experienced waterpipe smoking at some point [3]. These data clearly demonstrate that, with regard to tobacco consumption, waterpipe smoking follows right next to cigarette smoking. Although research is now under way for no less than 40 years in the field of waterpipe smoking [4], compared to cigarette smoking there is still insufficient knowledge on the health hazards related to waterpipe smoking. However, in recent years Saliba and coworkers demonstrated the presence of significant amounts of harmful substances such as polycyclic aromatic hydrocarbons (PAHs) and aldehydes in the waterpipe smoke [5,6].

During the combustion of a cigarette, a variety of different reactions occur, including pyrolysis, distillation and sublimation [7]. Due to these processes, carcinogenic primary aromatic amines (PAAs) may be formed among other products [8]. Combustion processes also occur during smoking of the waterpipe, for instance, in the burning charcoal. It seems thus conceivable that PAAs are also present in the waterpipe smoke. In the case of cigarettes a number of investigations have been released that addressed the contents of PAAs in both mainstream and sidestream smoke [8–11]. So, among others the carcinogenic amines 2-naphthylamine (2-ANP) and 4-aminobiphenyl (4-ABP) have been detected in cigarette smoke [10]. These two substances are without any doubt associated with the formation of bladder cancer in humans [12]. To the best of our knowledge no research on PAAs is as yet published for the waterpipe smoke. We therefore developed a specific and sensitive method for the detection of 31 PAAs in this complex matrix.

Hoffmann and Masuda reported in 1969 for the first time on the determination of 1- and 2-ANP by applying a gas chromatography electron capture detector (GC-ECD) technique [8,13]. In the following four decades a variety of methods for the analysis of PAAs in cigarette smoke were developed [8–11,13–20]. Most of these methods employed GC–MS instrumentation for the detection of PAAs in tobacco smoke. Moreover, various extraction techniques were utilized such as simultaneous distillation & extraction (SDE) [17] or solid phase extraction (SPE) [10]. In 2007, Kataoka et al. [21] used GC coupled to a nitrogen-phosphorus detector (GC-NPD) to determine 20 different PAAs and in a recent study Saha et al. [11] used an LC–MS/MS technique to analyze six carcinogenic PAAs in mainstream cigarette smoke. For the determination of PAAs in other matrices such as aqueous food simulants usually LC–MS/MS serves as technique of choice [22–25].

Previously published methods clearly suffer from extensive sample preparation which was requiring scavenging of PAAs out of the smoke followed by extraction, additional clean-up steps

* Corresponding author. Tel.: +49 30 18412 3963; fax: +49 30 18412 4928.
E-mail address: Jens.Schubert@bfr.bund.de (J. Schubert).

0021-9673/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.chroma.2011.06.072

and derivatization procedures. Here we report on an improved method offering the advantage of determination of PAAs directly from the extraction solution. For separation and detection we used LC–MS/MS that enables high sample throughput and an analyte-specific detection based on multiple reaction monitoring (MRM). The method introduced is suited to significantly reduce the sample preparation time to a minimum and to provide highest accuracy and reproducibility with limits of detection in the lower nanogram range.

2. Materials and methods

2.1. Reagents and materials

Analytical grade standards of 33 different PAAs, as listed in Table 1, were purchased from Sigma–Aldrich Chemie GmbH (Munich, Germany) and Dr. Ehrenstorfer GmbH (Augsburg, Germany). Internal Standards were obtained from Dr. Ehrenstorfer except for aniline-d_5 which was purchased from Sigma–Aldrich. All other analytical chemicals (LC–MS grade) were also purchased from Sigma–Aldrich. Standard stock solutions were prepared in analytical grade methanol with a concentration of 1 mg/ml each and stored in brown 20 ml headspace vials sealed with a PTFE screw cap in a refrigerator for up to 6 months. Only *para*-phenylenediamine (*p*-PDA) was kept refrigerated no longer than 1 month. By diluting of the stock solutions with methanol, working solutions of 8–11 PAAs were freshly prepared with 5.0 μg/ml each every 2 weeks. A working solution containing *m*- and *p*-PDA was prepared separately every week. A spiking solution of the four internal standards was prepared in methanol with 12.5 μg/ml each.

Waterpipe tobacco was purchased from Nakhla Tobacco (Two Apples flavour, Nakhla Tobacco, Egypt). Quick lighting charcoal (Ø 40 mm) was obtained from Three Kings (The Netherlands) and 92 mm glass fiber filter pads were purchased from Borgwaldt KC (Hamburg, Germany). Perforated aluminum foil (Ø 15.5 cm, 25 holes) was obtained from Falu (Ballingen, Germany).

2.2. Automated smoking conditions

Waterpipe smoking was performed by connecting a Borgwaldt Shisha Smoker machine to a standard laboratory waterpipe (Borgwaldt KC) with a plastic hose (see Fig. 1). Each smoking session consisted of 171 puffs of 530 ml each and 2.6 s duration every 20 s. These parameters were obtained from a field study that looked into the smoking behavior of waterpipe users in the Lebanon [26]. We decided to use these smoking parameters since detailed informations for European waterpipe smokers are not available and also for better comparability with published data. The bowl was filled with 750 g distilled water and the stem was placed 30 mm underneath the water surface. Ten grams of waterpipe tobacco was loaded into the head of the pipe and covered with perforated aluminum foil in a way that the tobacco did not touch the aluminum foil. A single quick lighting charcoal disk was lit and placed after 60 s atop the perforated foil to start the smoking session. The total particulate matter (TPM) was collected by aspirating the smoke of an entire session through a 92 mm glass fiber filter pad. TPM was determined gravimetrically by weighing the filter holder (including filter pad) before and after smoking. To avoid overloading the filter pads were always changed after puff #105. Method blanks were performed by smoking the waterpipe without charcoal and tobacco.

2.3. Sample preparation

Smoking of the waterpipe and sample preparation were performed at the same day. For extraction of TPM the 92 mm filter pads were transferred to a 500 ml Erlenmeyer flask covered with aluminum foil, spiked with 100 μl of the internal standard solution and 50 ml methanol was added subsequently. The filter pads were then agitated for 1 h on an HS 250 basic shaker. The samples were filtered directly into autosampler vials through a 0.45 μm PTFE syringe filter and analyzed by LC–MS/MS (see Fig. 1).

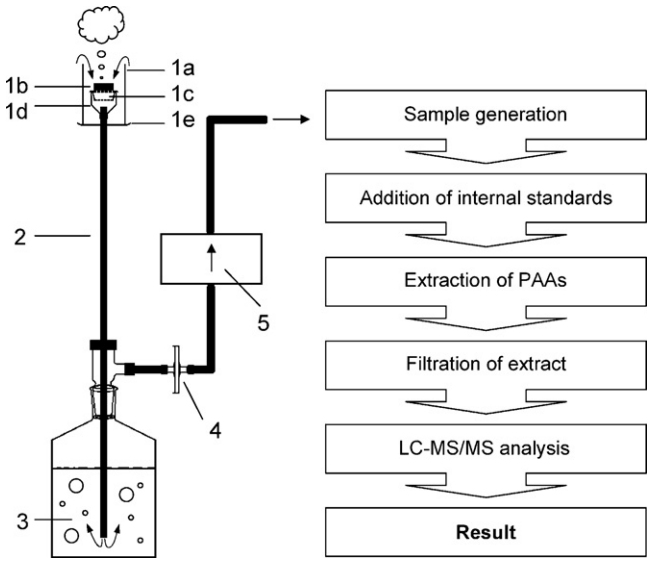

Fig. 1. Experimental set-up: wind cover (1a); charcoal (1b); tobacco (1c); head (1d); ash tray (1e); steam (2); bowl (3); filter holder with filter (4); pump (5, Borgwaldt Shisha Smoker), and flow diagram of the entire analytical protocol.

For analysis of the bowl water (water of the waterpipe) a 10 ml flask containing 20 μl of the internal standard solution was filled up to the mark, the solution was mixed and then directly injected into the LC–MS/MS sytem.

The pH value of bowl water was determined before and after smoking, using a pH-meter 765 climate from Knick (Knick Elektronische Meßgeräte, Berlin, Germany).

2.4. Instrumental conditions

For sample analysis a Shimadzu LC-20AD prominence (Shimadzu, Duisburg, Germany) HPLC system coupled with an API 4000 Q TRAP mass spectrometer (AB Sciex Instruments, Applied Biosystems, Darmstadt, Germany) was used. The HPLC system comprised two pumps (LC-20AD), a column oven (CTO-20AC HT), a degasser (DGU-20A5), a controller (CBM-20A), and a temperature controlled autosampler (SIL-20ACHT).

Fifteen microliters of the sample extract were injected into the LC–MS/MS system. Chromatography was performed on a Synergi 4u Polar-RP 80A column (150 mm × 4.6 mm, 4 μm particle size, Phenomenex, Aschaffenburg, Germany) at 40 °C with a flow rate of 0.8 ml/min.

Mobile phases A and B consisted of water and 0.1% formic acid in 25% methanol/75% acetonitrile, respectively. HPLC separation was achieved running a gradient under following conditions: 0–2.0 min: 7% B, 2.0–8.0 min: 7–35% B, 8.0–13.0 min: 35–95% B, 13.0–15.0 min: 95% B, 15.0–16.0 min: 95–7% B, 16.0–21.0 min: 7% B. Mass detection conditions were as follows: ionization mode, positive ESI; ion spray voltage, 4500 V; ion source temperature, 550 °C; curtain gas, nitrogen, setting: 25; ion source gas 1 (GS1), nitrogen, setting: 55.0; ion source gas 2 (GS2), nitrogen, setting: 45.0. Compound-dependent parameters were optimized by flow injection analysis. For each analyte, the two most intense ion transition pairs were used under scheduled MRM mode (see Table 1). Parameters for the scheduled mode were: MRM detection window:

Table 1
Analyte-specific parameters and multiple reaction monitoring (MRM) analysis of 33 primary aromatic amines (PAAs) and their corresponding internal standards [27]. PAAs listed in the order of their retention times.

PAA	Purity (%)	Abbreviation	CAS number	IARC group[a]	Internal standard	Retention time (min)	Precursor ion (m/z)	Product ion (m/z)	DP[b] (eV)	EP[c] (eV)	CE[d] (eV)	CXP[e] (eV)
para-Phenylenediamine	98.5	*p*-PDA	106-50-3	3	ANL-d5	2.04	109.1	92.2[f]	66	10	23	4
								65.1[g]	66	10	33	10
meta-Phenylenediamine	≥99.0	*m*-PDA	108-45-2	3	ANL-d5	2.36	109.1	92.2[f]	66	10	23	4
								65.1[g]	66	10	33	10
2,6-Toluenediamine	99.0	2,6-TDA	823-40-5	–	ANL-d5	2.44	123.0	106.1[f]	61	10	23	6
								108.1[g]	61	10	25	6
para-Anisidine	99.5	*p*-ASD	104-94-9	3	ANL-d5	3.91	124.0	93.1[f]	61	10	27	4
								92.0[g]	61	10	25	4
Aniline	≥99.5	ANL	62-53-3	3	ANL-d5	3.98	94.1	77.0[f]	61	10	27	14
								51.1[g]	61	10	43	8
para-Toluidine	99.5	*p*-TOL	106-49-0	–	*o*-TOL-d9	5.21	108.0	93.1[f]	61	10	23	4
								91.1[g]	51	10	27	4
meta-Toluidine	99.0	*m*-TOL	108-44-1	–	*o*-TOL-d9	6.37	108.0	91.1[f]	61	10	27	4
								93.1[g]	61	10	23	4
4,4′-Oxydianiline	99.0	4,4′-ODA	101-80-4	2B	*o*-TOL-d9	6.48	201.1	108.1[f]	81	10	29	20
								80.1[g]	81	10	53	14
ortho-Anisidine	99.5	*o*-ASD	90-04-0	2B	*o*-TOL-d9	6.94	124.1	109.1[f]	51	10	25	6
								80.1[g]	51	10	43	14
4,4′-Methylene-di-aniline	97.8	4,4′-MDA	101-77-9	2B	*o*-TOL-d9	7.05	199.2	106.1[f]	86	10	35	18
								77.0[g]	86	10	69	14
ortho-Toluidine	99.5	*o*-TOL	95-53-4	1	*o*-TOL-d9	7.15	108.0	91.1[f]	61	10	27	4
								93.1[g]	61	10	23	4
meta-Anisidine	98.0	*m*-ASD	536-90-3	–	*o*-TOL-d9	7.49	124.1	92.0[f]	61	10	25	4
								77.0[g]	61	10	31	14
1,5-Diamino-naphthalene	≥98.0	DANP	2243-62-1	3	*o*-TOL-d9	7.52	159.1	115.1[f]	81	10	43	6
								143.1[g]	81	10	31	8
Benzidine	99.9	BNZ	92-87-5	1	BNZ-d8	8.09	185.1	168.1[f]	66	10	27	12
								167.1[g]	66	10	39	12
2,4-Dimethylaniline	98.5	2,4-DMA	95-68-1	3	BNZ-d8	8.23	122.1	107.1[f]	51	10	23	6
								105.1[g]	51	10	23	6
2-Methoxy-5-methylaniline	99.5	2-M-5-MA	120-71-8	2B	BNZ-d8	9.06	138.1	123.1[f]	56	10	21	8
								106.1[g]	56	10	32	5
2,4,5-Trimethylaniline	–[h]	2,4,5-TMA	137-17-7	3	BNZ-d8	9.69	136.2	121.1[f]	71	10	25	6
								91.1[g]	71	10	33	4
4,4′-Methylene-di-*ortho*-toluidine	97.0	4,4′-MDOT	838-88-0	2B	BNZ-d8	9.70	227.2	120.1[f]	91	10	35	6
								77.1[g]	91	10	77	12
ortho-Tolidine	99.5	TLD	119-93-7	2B	BNZ-d8	10.74	213.1	196.1[f]	91	10	29	12
								180.2[g]	91	10	47	12
para-Chloroaniline	99.5	4-CA	106-47-8	2B	4-ABP-d9	11.44	128.1	93.1[f]	61	10	27	6
								75.0[g]	61	10	45	14
2,6-Dimethylaniline	99.5	2,6-DMA	87-62-7	2B	4-ABP-d9	11.50	122.1	105.0[f]	51	10	23	6
								77.1[g]	51	10	35	14
2-Naphthylamine	98.9	2-ANP	91-59-8	1	4-ABP-d9	12.13	144.2	127.0[f]	71	10	35	6
								77.0[g]	71	10	51	12
meta-Chloroaniline	99.9	3-CA	108-42-9	–	4-ABP-d9	12.76	128.1	93.1[f]	61	10	27	6
								75.0[g]	61	10	45	14
1-Naphthylamine	99.9	1-ANP	134-32-7	3	4-ABP-d9	12.80	144.2	127.0[f]	71	10	33	6
								77.0[g]	71	10	51	12
4-Chloro-*ortho*-toluidine	98.5	4-COT	95-69-2	2A	4-ABP-d9	13.06	142.1	107.1[f]	61	10	25	6
								106.1[g]	61	10	39	6

Table 1 (*Continued*)

PAA	Purity (%)	Abbreviation	CAS number	IARC group[a]	Internal standard	Retention time (min)	Precursor ion (*m*/*z*)	Product ion (*m*/*z*)	DP[b] (eV)	EP[c] (eV)	CE[d] (eV)	CXP[e] (eV)
3-Aminobiphenyl	97.8	3-ABP	2243-47-2	–	4-ABP-d9	13.22	170.1	153.1[f]	71	10	29	10
								152.0[g]	81	10	39	8
4-Aminobiphenyl	98.5	4-ABP	92-67-1	1	4-ABP-d9	13.22	170.1	153.1[f]	71	10	29	10
								152.0[g]	81	10	39	8
3,4-Dichloroaniline	99.9	3,4-DCA	95-76-1	–	4-ABP-d9	13.90	162.1	127.0[f]	71	10	29	8
								74.0[g]	71	10	71	2
3,5-Dichloroaniline	99.1	3,5-DCA	626-43-7	–	4-ABP-d9	14.14	162.1	127.0[f]	71	10	29	8
								74.0[g]	71	10	71	2
2-Aminobiphenyl	98.5	2-ABP	90-41-5	–	4-ABP-d9	14.17	170.1	153.0[f]	71	10	29	10
								152.1[g]	81	10	39	8
3,4,5-Trichloroaniline	99.0	3,4,5-TCA	634-91-3	–	4-ABP-d9	14.49	196.0	161.0[f]	61	10	31	14
								108.1[g]	61	10	73	6
Aniline-d_5	98.0[i]	ANL-d5	4165-61-1	–	–	3.82	98.9	82.1[f]	61	10	27	14
								56.1[g]	61	10	43	14
ortho-Toluidine-d_9	98.9[i]	*o*-TOL-d9	194423-47-7	–	–	6.82	115.0	98.1[f]	56	10	27	18
								70.2[g]	56	10	41	12
Benzidine-d_8	99.8[i]	BNZ-d8	92890-63-6	–	–	7.88	193.2	174.0[f]	96	10	33	10
								176.2[g]	96	10	29	12
4-Aminobiphenyl-d_9	99.5[i]	4-ABP-d9	344298-96-0	–	–	13.10	179.1	160.2[f]	81	10	41	10
								162.2[g]	81	10	31	10
2,4-Diaminoanisole[j]	97.5	2,4-DAA	615-05-4	2B	(ANL-d5)	(2.95)	139.1	108.1[f]	56	10	23	8
								107.0[g]	56	10	21	6
2,4-Toluenediamine[j]	99.8	2,4-TDA	95-80-7	2B	(ANL-d5)	(2.96)	123.0	106.1[f]	61	10	23	6
								108.1[g]	61	10	25	6

[a] IARC classification groups: 1 = carcinogenic to humans; 2A = probably carcinogenic to humans; 2B = possibly carcinogenic to humans; 3 = not classifiable as to its carcinogenicity to humans.
[b] Declustering potential.
[c] Entrance potential.
[d] Collision energy.
[e] Collision cell exit potential.
[f] Quantification ion.
[g] Confirmation ion.
[h] Stock solution containing 94 ng/μl ± 5%.
[i] Atom % D.
[j] Not included in method running with methanol, but capable for method running with 3% acetic acid or water.

90 s and target scan time: 5 s. Chromatograms were recorded and processed with Analyst 1.5.1 (AB Sciex).

3. Results and discussion

In this study, a multi-analyte method for PAA analysis has been established and applied in the determination of PAA contents of mainstream waterpipe smoke. Since it has been validated for methanol, water and 3% acetic acid, the LC–MS/MS method presented here is not only suitable for the analysis of waterpipe smoke, but also transferable to the testing of compound migrations in food contact materials. In the following sections the individual validation steps and the results for the matrix waterpipe smoke are described.

3.1. Method development

Due to the lack of studies in the literature related to this issue we were required to establish a novel and reliable method to sensitively and comprehensively detect the contents of a range of different PAAs in the mainstream waterpipe smoke (Table 1). Fig. 2 depicts the chromatograms obtained from a standard mixture of the entire set of 31 different analytes addressed (concentration of 1 ng/ml each) and from PAAs detected in a waterpipe sample. During method development it became challenging to separate certain pairs of isomers such as *p*- and *m*-PDA, 2,4- and 2,6-TDA, 3,4- and 3,5-DCA, or 3- and 4-ABP (for abbreviations see Table 1). For instance, the solvent methanol and an injection volume of 15 μl proved insufficient for separation of 2,4- and 2,6-TDA. The reduction of the injection volume to 1 μl led to improved separation and peak shapes of the two isomers. To obtain higher analyte signals we used 15 μl of the extraction solution for analysis and excluded 2,4-TDA and 2,4-DAA due to deficient peak shapes. On the other hand, excellent separation could be achieved for 2,4-DAA, 2,4- and 2,6-TDA by applying water or 3% acetic acid as solvent. Irrespective of the kind of solvent (methanol, water, and 3% acetic acid) used, however, 3- and 4-ABP could not be separated at all. Thus the two isomers were analyzed and reported together.

For separation of individual PAAs we tested the following set of HPLC columns: Hypersil GOLD (Thermo Fisher Scientific), Develosil 3u RP-Aqueous (Phenomenex), NUCLEODUR C18 Gravity (Macherey-Nagel), ZORBAX SB-C3 (Agilent Technologies), Synergi 4u Polar (Phenomenex). Best separation was achieved on Synergi 4u Polar, which is made of ether-linked phenyl with polar end-capping and which proved ideal in separating polar and aromatic analytes such as PAAs. By using an HPLC column of 4.60 mm (i.d.) instead of 2.00 mm (i.d.) the injection volume could be increased to 15 μl. Although there was no significant temperature effect on the separation best results were obtained at an oven temperature of 40 °C. Flow and gradient were optimized to obtain best separation and short analysis times.

During optimization a number of different mobile phases and additives were tested. A general trend was the loss of signal intensity particularly for 4-CA and 4-COT when inorganic salts such as ammonium formate or ammonium acetate were added. Addition of formic acid to mobile phases A and B resulted in the faster elution of the amines combined with insufficient peak separation and the loss of signal intensity. Best separation was achieved with water as eluent A and a mixture of acetonitrile/methanol (75:25, v/v) with 0.1% formic acid as eluent B.

In a further experiment we integrated diode array detection (DAD) into the method. Unfortunately DAD was not sensitive enough to achieve the desired performance (<1 ng/ml). Since also loss of sensitivity of the MS/MS signals occurred, DAD was not further used.

In addition to the 31 PAAs which were integrated into the method, we tried to add also the following amines: *ortho*-chloroaniline, 2,3-, 2,4-, 2,5- and 2,6-dichloroaniline, 2,3,4-, 2,4,5- and 2,4,6-trichloroaniline. In the ESI mode detection of these analytes was not possible due to a lack of signal intensity of their mass transitions (positive MRM mode). Reduced signal intensity was observed for those PAAs possessing a chlorine atom in *ortho*-position, irrespective of the total number of chlorine atoms present in the respective molecule. A change of the ionization mode could be a possible way to achieve the performance required (<1 ng/ml), but was not further pursued.

3.2. Method validation

3.2.1. Specificity

Precursor ions of each PAA given in Table 1 were of type $[M+H]^+$ and showed the best sensitivity during the tuning and optimization process. For each PAA two ion transition pairs were recorded. The first transition, which corresponds to the most abundant product ion, was used for quantification, whereas the second was used for confirmation. To examine the purity of the peaks we further determined the peak area ratios of the second ion transition to the first ion transition and compared those between the standards and the samples. The peak area ratios for the standards based on four independent measurements were as follows: 0.686 ± 0.016, 0.373 ± 0.009, 0.500 ± 0.021, 0.510 ± 0.012, 0.436 ± 0.019, 0.401 ± 0.023, 0.247 ± 0.011, and 0.652 ± 0.012 for *m*-PDA, ANL, 4,4′-ODA, *o*-ASD, 2-ANP, 1-ANP 3,5-DCA, and 2-ABP, respectively. The peak area ratios for waterpipe smoke were as follows: 0.674 ± 0.053, 0.371 ± 0.021, 0.496 ± 0.034, 0.454 ± 0.032, 0.383 ± 0.024, and 0.653 ± 0.054 for *m*-PDA, ANL, 4,4′-ODA, 2-ANP, 1-ANP, and 2-ABP, respectively, when using water, and 0.664 ± 0.049, 0.378 ± 0.017, 0.517 ± 0.039, 0.519 ± 0.041, 0.436 ± 0.030, 0.397 ± 0.023, 0.256 ± 0.017, and 0.643 ± 0.046 for *m*-PDA, ANL, 4,4′-ODA, *o*-ASD, 2-ANP, 1-ANP 3,5-DCA, and 2-ABP, respectively, in the absence of water (see Section 3.3).

For the presented method we used four internal standards (aniline-d_5 [ANL-d5], *ortho*-toluidine-d_9 [*o*-TOL-d9], benzidine-d_8 [BNZ-d8], and 4-aminobiphenyl-d_9 [4-ABP-d9], see Table 1). All internal standards applied are commercially available, chemically stable, and provided a sufficient detector response. The assignment of the internal standards occurred according to their ability to represent the individual PAAs at best.

3.2.2. Precision

Intra-day and inter-day precision were determined at three concentration levels (1 ng/ml, 10 ng/ml, and 50 ng/ml). In the absence of an analyte-free matrix for waterpipe smoke, standard solutions containing all PAAs, including the internal standards (25 ng/ml), were spiked on clean filter pads (two per analysis) and extracted as described above. For intra-day precision the extracts were analyzed on the same day. For inter-day precision the extracts were analyzed on 5 different days within 2 weeks. The intra-day precision was very good and ranged for the 1 ng/ml spiking solution from 0.6% for *p*-TOL to 8.4% for 3,4,5-TCA, for the 10 ng/ml spiking solution from 0.7% for *o*-TOL to 3.3% for 4,4′-MDOT, and for the 50 ng/ml spiking solution from 0.5% for 4-COT to 2.9% for 2-M-5-MA. The values for inter-day precision showed a greater variation and ranged for the 1 ng/ml spiking solution from 2.6% for *m*-TOL to 16.9% for 4,4′-MDA, for the 10 ng/ml spiking solution from 1.8% for *m*-TOL to 17.9% for 4,4′-MDA, and for the 50 ng/ml spiking solution from 1.3% for *m*-TOL to 17.6% for 4,4′-MDA.

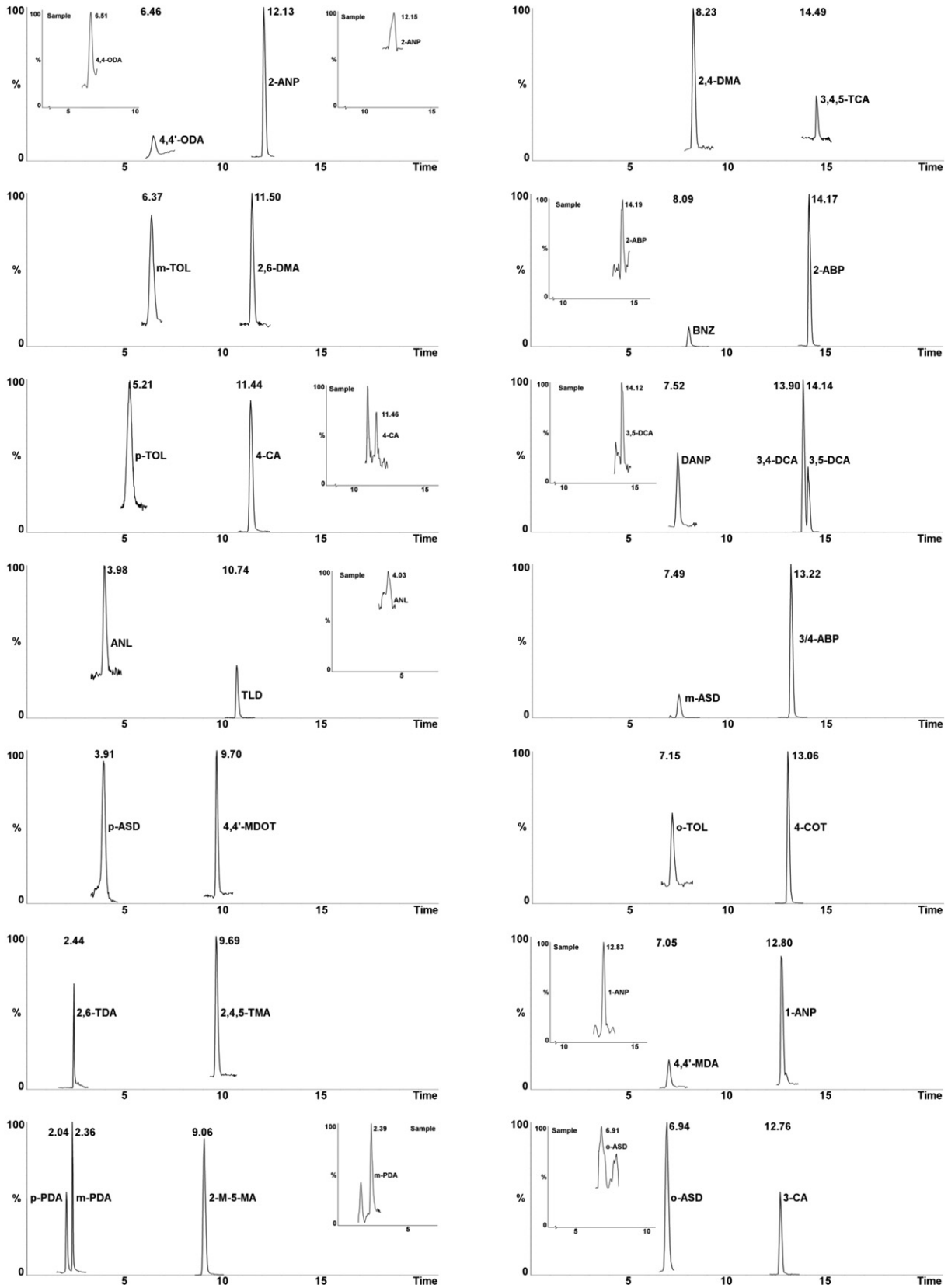

Fig. 2. LC–MS/MS chromatogram of a PAA standard solution containing each analyte in a concentration of 1 ng/ml in methanol and of a waterpipe sample (inlets: PAAs depicted are those for which an individual signal has been received); sums of MRM as listed in Table 1 are shown.

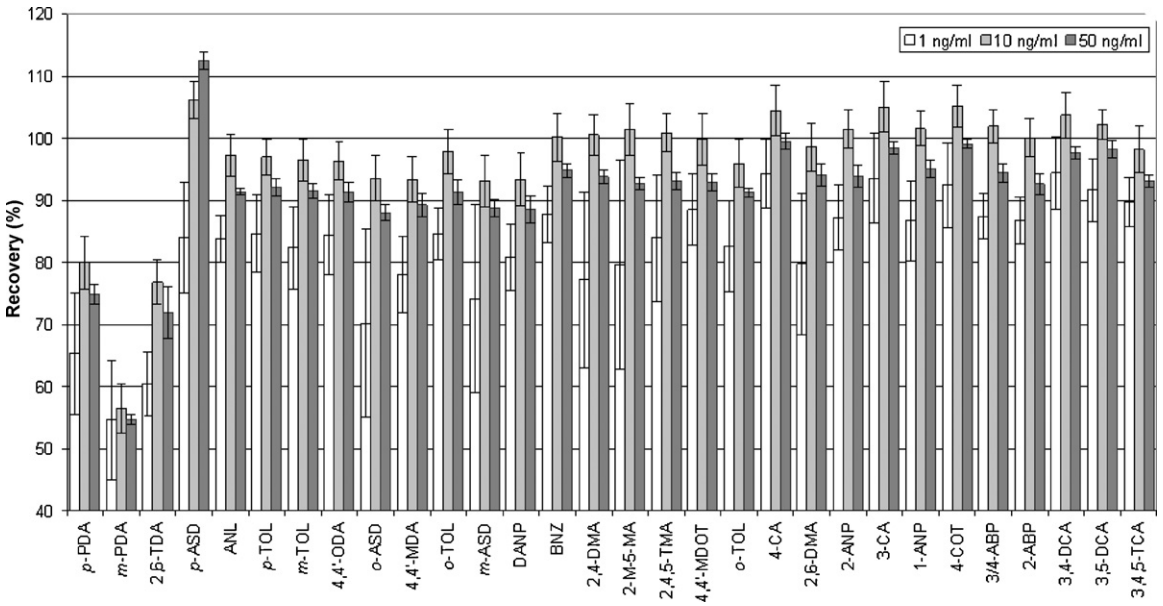

Fig. 3. Method recovery of 31 PAAs dissolved in methanol, relative standard deviation (RSD, error bars) ranged from 1 to 17%.

3.2.3. Recovery

Recovery was determined by spiking known amounts of each analyte (1 ng/ml, 10 ng/ml and 50 ng/ml) and internal standards (25 ng/ml) on clean filter pads (two per analysis) followed by extraction as mentioned above. The extracts were analyzed by LC–MS/MS and the recovery rates were calculated (Fig. 3).

For extraction we tested three different solvents, i.e. methanol, water and 3% acetic acid. These solvents can be directly injected into the LC–MS/MS without prior sample work-up, thus they have been frequently used also by others [22–24]. We found that water and 3% acetic acid were not suitable for our purpose. When using water there was an insufficient extraction efficiency for analytes with a higher partition coefficient (Table 2). For instance, we found an extraction efficiency of nearly 100% for 2,6-TDA with a log *P* value of −0.36, whereas only 13% was achieved for 2-ABP with a log *P* value of 2.86 (data not shown).

When using 3% acetic acid as extraction agent some of the analytes became degraded (i.e. *p*-PDA, 2,4-DAA, DANP, 1-ANP, 3,5-DCA, 3,4,5-TCA, data not shown). Similar findings were reported by other authors during recent years. Li et al. [25] described a poor recovery of PAAs (e.g. 1-ANP) when using acidic or basic extraction agents (pH 5.0 or 9.0). They attributed this to a possible deactivation of the sorbent in the SPE cartridges at unphysiologic pH values. Since we had not performed an SPE clean-up step but still observed degradation of some of the PAAs, it is also conceivable that the analytes itself become degraded. In water (pH 7.0) a sufficient recovery was reported [25]. We also confirmed the stability of PAAs in water. In addition Mortensen et al. [23] reported that *p*-PDA is, as a standard, not stable in 3% acetic acid for a longer time either. In 2009 Saha et al. [11] demonstrated that benzidine (BNZ) was degraded in an acidic cigarette extract during a period of 8 h by more than 65%. In contrast to this, no degradation occurred and adequate recovery rates were observed when methanol was applied as extraction agent (Fig. 3). There is also no additional sample clean-up necessary, and the extract can be directly injected into the LC–MS/MS system.

3.2.4. Limit of detection (LOD) and limit of quantification (LOQ)

LOD and LOQ were determined according to the German Industrial Norm (DIN) 32645 [28]. For this purpose, calibration curves were constructed and the LOD and LOQ were calculated for each analyte using the equations given in DIN 32645. The results obtained for the solvents methanol, water and 3% acetic acid are summarized in Table 2. For methanol we also specified the LOQ for a single waterpipe smoking session. The LOD values for methanol were found in the range of 0.45 ng/session (2-ABP) to 4.50 ng/session (3,4,5-TCA). Taking into account the injection volume the instrumental limits of detection were in the range of 0.135 pg absolute (2-ABP) to 1.35 pg absolute (3,4,5-TCA), thereby demonstrating that the method developed met the sensitivity required.

The 10-point calibration curves showed excellent linearity between the LODs and 50 ng/ml for each PAA, with typical values for correlation coefficients (r^2) between 0.990 and 0.997 (Table 2). The calibration curves were weighted by $1/x$. The internal standard concentration was set to 25 ng/ml for each deuterated amine.

3.2.5. Estimation of uncertainty

In accordance to the Joint Committee for Guides in Metrology (JCGM) [30], the combined uncertainty associated to the results produced by an analytical method can be determined by taking the individual sources of uncertainty into consideration. In a recent review [31] the main sources of uncertainty in a chromatographic analysis were listed as the uncertainties associated with the amount of sample used for a determination (e.g. weighing of tobacco), the calibration, the recovery, the repeatability (e.g. the whole smoking process) and the analyte concentration. In the present study the uncertainties associated with repeatability and analyte concentration were the main contributors to the combined uncertainty and were calculated according to Konieczka and Namieśnik [31]. Hence the uncertainties associated with the amount of sample used for determination and with calibration can be neglected. The uncertainty associated with recovery was calculated from the relative standard deviation of the average percent recovery [32] and was also negligible. The values for recovery were not included into calculation of the final results (Table 3), since the overall matrix effect remains unknown. The expanded uncertainties, using a coverage factor of two ($k=2$) for waterpipe smoking were calculated as follows: 2.3 ng/session (*m*-PDA), 4.0 ng/session (ANL), 5.3 ng/session (4,4′-ODA), 1.3 ng/session (2-ANP), 4.6 ng/session (1-ANP), and 1.0 ng/session (2-ABP) with water and 3.7 ng/session (*m*-PDA),

Table 2
Log *P* values, linear regression data, limit of detection (LOD) and limit of quantification (LOQ) of 33 PAAs.

PAA[a]	log *P*[b]	Correlation coefficient[d]	LOD [ng/ml] (methanol)	LOQ [ng/ml] (methanol)	LOQ [ng/session] (methanol)	LOD [ng/ml] (water)	LOQ [ng/ml] (water)	LOD [ng/ml] (3% acetic acid)	LOQ [ng/ml] (3% acetic acid)
p-PDA	−0.36	0.993	0.037	0.104	5.22	0.046	0.128	0.097	0.268
m-PDA	−0.25	0.994	0.022	0.064	3.18	0.014	0.040	0.032	0.091
2,6-TDA	−0.36	0.992	0.045	0.127	6.34	0.018	0.052	0.039	0.111
p-ASD	0.95	0.993	0.034	0.097	4.87	0.024	0.069	0.020	0.057
ANL	0.90	0.996	0.031	0.088	4.40	0.040	0.114	0.066	0.179
p-TOL	1.39	0.994	0.032	0.091	4.53	0.038	0.107	0.024	0.070
m-TOL	1.40	0.996	0.023	0.067	3.37	0.036	0.103	0.035	0.098
4,4′-ODA	1.34	0.991	0.049	0.136	6.82	0.069	0.188	0.032	0.090
o-ASD	1.18	0.997	0.026	0.075	3.76	0.032	0.090	0.026	0.075
4,4′-MDA	1.59	0.991	0.049	0.137	6.83	0.047	0.130	0.026	0.075
o-TOL	1.43	0.996	0.018	0.051	2.55	0.031	0.089	0.032	0.091
m-ASD	0.93	0.996	0.025	0.073	3.63	0.040	0.112	0.027	0.079
DANP	0.89	0.992	0.059	0.161	8.07	0.037	0.103	0.134	0.343
BNZ	1.34	0.990	0.028	0.080	3.98	0.043	0.119	0.069	0.185
2,4-DMA	1.68	0.996	0.019	0.056	2.80	0.033	0.093	0.021	0.060
2-M-5-MA	1.74	0.997	0.021	0.062	3.11	0.024	0.069	0.018	0.051
2,4,5-TMA	2.29	0.995	0.033	0.095	4.75	0.022	0.065	0.021	0.060
4,4′-MDOT	3.50[c]	0.991	0.018	0.052	2.61	0.034	0.097	0.020	0.057
TLD	2.34	0.990	0.019	0.056	2.79	0.016	0.047	0.016	0.047
4-CA	1.83	0.995	0.023	0.068	3.39	0.025	0.073	0.028	0.079
2,6-DMA	1.84	0.995	0.028	0.080	3.98	0.028	0.081	0.028	0.081
2-ANP	2.34	0.994	0.012	0.036	1.82	0.023	0.065	0.028	0.081
3-CA	1.88	0.994	0.024	0.069	3.46	0.043	0.121	0.034	0.096
1-ANP	2.25	0.993	0.045	0.066	3.28	0.026	0.076	0.063	0.170
4-COT	2.28	0.994	0.021	0.061	3.03	0.026	0.075	0.031	0.088
3/4-ABP	2.69 (3-ABP) 2.86 (4-ABP)	0.995	0.022	0.066	3.30	0.042	0.121	0.037	0.108
3,4-DCA	2.68	0.996	0.018	0.052	2.61	0.032	0.092	0.024	0.071
3,5-DCA	2.90	0.991	0.026	0.075	3.77	0.044	0.122	0.030	0.085
2-ABP	2.84	0.995	0.009	0.025	1.27	0.023	0.067	0.023	0.067
3,4,5-TCA	3.32	0.992	0.090	0.238	11.9	0.108	0.280	0.102	0.244
2,4-TDA[e]	0.14	0.998[f]	–[e]	–[e]	–[e]	0.033	0.093	0.029	0.082
2,4-DAA[e]	0.23[c]	0.998[f]	–[e]	–[e]	–[e]	0.038	0.106	0.042	0.121

[a] Abbreviations see Table 1.
[b] LOGKOW© databank, http://logkow.cisti.nrc.ca/logkow/index.jsp, Sangster Research Laboratories, Canada, 2010-11-10.
[c] See Ref. [29].
[d] $n = 6$ (methanol).
[e] Not included in method running with methanol.
[f] $n = 2$ (3% acetic acid).

Table 3
Results for the determination of PAAs in waterpipe mainstream smoke and comparison to literature data of 2R4F reference cigarettes.

PAA[a]	Waterpipe (with water) [ng/session] ($n = 3$) (SD)	Waterpipe (without water) [ng/session] ($n = 3$) (SD)	Filter effect of water [%]	2R4F reference cigarette[b] [ng/cigarette] (SD)
m-PDA	6.50 (0.3)	10.3 (2.6)	37	ND
ANL	31.3 (2.2)	51.6 (4.4)	39	251.60 (18.09)
p-TOL	n.d.	n.d.	–	29.68 (3.23)
m-TOL	n.d.	n.d.	–	46.26 (4.71)
4,4′-ODA	28.0 (1.6)	47.1 (9.9)	41	ND
o-ASD	BLQ	5.03 (0.3)	–	ND
o-TOL	n.d.	n.d.	–	42.42 (2.72)
BNZ	n.d.	n.d.	–	0.09 (0.02)
2,4-DMA	n.d.	n.d.	–	15.12 (2.16)
TLD	n.d.	n.d.	–	n.d.
4-CA	BLQ	BLQ	–	ND
2,6-DMA	n.d.	n.d.	–	3.93 (0.53)
2-ANP	2.84 (0.3)	3.15 (0.4)	10	8.60 (0.68)
1-ANP	6.20 (0.6)	10.9 (2.1)	43	17.00 (1.26)
3,5-DCA	BLQ	6.34 (0.9)	–	ND
3/4-ABP	n.d.	n.d.	–	4.55[c]
2-ABP	3.33 (0.2)	3.71 (0.3)	10	ND
Total amines	78	138	–	468

SD, standard deviation; n.d., not detected; BLQ, below limit of quantification; ND, not determined.
[a] Abbreviations see Table 1.
[b] See Ref. [10].
[c] Combined values for 3- and 4-ABP (2.95 ± 0.30 and 1.60 ± 0.13 ng/cigarette, respectively).

6.0 ng/session (ANL), 13 ng/session (4,4′-ODA), 2.7 ng/session (*o*-ASD), 1.3 ng/session (2-ANP), 5.2 ng/session (1-ANP), 1.0 ng/session (2-ABP), and 2.8 ng/session (3,5-DCA) in the absence of water. However the results compiled in Table 3 were expressed only with the standard deviation, to prevent misunderstandings and for comparability reasons, since the expression of standard deviations is common for smoke analysis.

3.3. Waterpipe smoke

PAAs were determined in the waterpipe mainstream smoke based on three standard smoking experiments. The TPM was collected on glass fiber filters and extracted as mentioned above. In addition, we performed three smoking experiments without water in the bowl and three control experiments (without tobacco) and measured the PAA contents in the smoke. The results obtained are compiled in Table 3.

For three replicate smoking sessions with water we determined an average TPM of 2.28 ± 0.15 g, whereas for smoking sessions without water 2.07 ± 0.19 g were detected. Charcoal and tobacco consumption were 7.49 ± 0.06 g and 3.60 ± 0.24 g with water and 7.57 ± 0.06 g and 3.96 ± 0.29 g without water. In another experiment we determined the pH value of the bowl water at 3.72. The pH value of the distilled water was 6.90 and for the smoking blanks 5.73. It is likely that the pH shift in the water was caused by dissolving carbon dioxide present in the air or smoke. At the same time, decreasing pH values (acidification of the water) might be the reason for the filter capacity of the water with respect to the PAAs present in the smoke (see below).

In the waterpipe smoke we detected only 9 PAAs, including aniline (ANL) and the two naphthylamines (ANPs). The remaining PAAs targeted were not detectable and therefore only those also detected in the cigarette smoke were listed in Table 3. ANL and 4,4′-ODA revealed with highest concentrations of 31.3 ± 2.2 and 28.0 ± 1.6 ng/session, respectively. Smoking blanks were without interferences or detectable signals (data not shown). Experiments carried out without water showed consistently higher values for the PAAs, thus indicating a filtering effect of the bowl water. As a result, *o*-ASD and 3,5-DCA could be detected quantitatively. For ANL, 4,4′-ODA, *m*-PDA and 1-ANP roughly 40 percent and for 2-ANP and 2-ABP roughly 10 percent were retained by the water. These findings are consistent with the reported log *P* values (see Table 2). By contrast, detection of PAAs in the bowl water was not achievable due to too low concentrations.

In comparison with the smoke of the 2R4F reference cigarette, concentrations of PAAs in the waterpipe smoke were lower. For instance, ANL and 1-ANP levels were 8.1- and 2.7-fold lower in waterpipe smoke, respectively. Nine other PAAs, which have been found in the cigarette smoke, could not be detected in the waterpipe smoke at all. However, several parameters could be responsible for the differences between cigarette and waterpipe smoke. First, the compositions of waterpipe and cigarette tobacco differ greatly. Waterpipe tobacco contains tobacco and many other additives such as humectants (glycerol or propylene glycol), molasses, fruits and flavorings resulting in a sticky mass. Cigarette tobacco on the other hand is much more dry. Second, the process of heating is different, too. During the smoking process cigarette tobacco burns directly whereas waterpipe tobacco does not burn in a self sustaining manner and requires an external heat source such as charcoal. Third, in the cigarette the temperature of the burning tobacco rises to 900 °C, whereas waterpipe tobacco is only heated up to 200 °C, thus resulting in a completely different reaction pattern. This difference may explain the observed differences in the formation of PAAs.

With regard to PAAs, waterpipe smokers are exposed to lower concentrations than cigarette smokers. Nevertheless the presence of the carcinogen 2-ANP and of ANL and 1-ANP confirm that waterpipe smoking indeed can be considered as health hazard.

4. Conclusion

The aim of this study was to develop a sensitive and robust analytical method for a range of PAAs and to detect and quantify these analytes in the mainstream waterpipe smoke. The major advantages of the presented approach are the following: (I) direct determination of PAAs without any further sample clean-up became feasible, thus enabling a short and comfortable sample preparation, especially when compared to GC–MS which – in most cases – requires chemical derivatization, (II) detection of 31 respectively 33 individual PAAs becomes possible in one single LC run, (III) use of internal standards improved the robustness and accuracy of the method, and (IV) low limits of detection, good recovery, an excellent reproducibility, and the opportunity to use a range of different solvents for extraction (e.g. methanol, water, and 3% acetic acid) make the method attractive and suitable also for other analytical challenges.

To our knowledge this is the first qualitative and quantitative analysis of the contents of PAAs in waterpipe smoke. The results clearly demonstrate that certain PAAs are present in the smoke in considerable amounts, thereby conferring a health hazard to waterpipe smokers. Additionally we show that the bowl water in the pipe leads to some degree of absorption of the compounds present in the smoke.

Acknowledgements

The authors would like to thank Timo Otter for excellent technical assistance at the LC–MS/MS system. This work was supported by an internal grant of the German Federal Institute for Risk Assessment (BfR).

References

[1] D. Jackson, P. Aveyard, BMC Public Health 8 (2008) 174.
[2] S. Poyrazoglu, S. Sarli, Z. Gencer, O. Günay, Ups. J. Med. Sci. 115 (2010) 210.
[3] BZgA, On the affinity of adolescents against drugs. (Report of the Federal Centre for Health Education (BZgA) in German language: Die Drogenaffinität Jugendlicher in der Bundesrepublik Deutschland 2008.), http://www.bzga.de, 2008.
[4] J. Rakower, B. Fatal, Brit. J. Cancer 16 (1962) 1.
[5] E. Sepetdjian, A. Shihadeh, N.A. Saliba, Food Chem. Toxicol. 46 (2008) 1582.
[6] M. Al Rashidi, A. Shihadeh, N.A. Saliba, Food Chem. Toxicol. 45 (2008) 3546.
[7] R.R. Baker, in: D.L. Davis, M.T. Nielsen (Eds.), Tobacco: Production, Chemistry, and Technology, Blackwell, Oxford, 1999 (Chapter 12).
[8] Y. Masuda, D. Hoffmann, Anal. Chem. 41 (1969) 650.
[9] G. Pieraccini, F. Luceri, G. Moneti, Rapid Commun. Mass Spectrom. 6 (1992) 406.
[10] C.J. Smith, G.L. Dooly, S.C. Moldoveanu, J. Chromatogr. A 991 (2003) 99.
[11] S. Saha, R. Mistri, B.C. Ray, J. Chromatogr. A 1216 (2009) 3059.
[12] IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 99, Some Aromatic Amines, Organic Dyes, and Related Exposures, 2010.
[13] D. Hoffmann, Y. Masuda, E.L. Wynder, Nature 221 (1969) 254.
[14] G. Grimmer, D. Schneider, K.W. Naujack, G. Dettbarn, J. Jacob, Beitr. Tabakforsch. 16 (1995) 141.
[15] M.F. Borgerding, J.A. Bodnar, H.L. Chung, P.P. Mangan, C.C. Morrison, C.H. Risner, J.C. Rogers, D.F. Simmons, M.S. Uhrig, F.N. Wendelboe, D.E. Wingate, L.S. Winkler, Food Chem. Toxicol. 36 (1997) 169.
[16] Official Method, Health Canada, No. T-102, 31 December 1999, www.hc-sc.gc.ca.
[17] J.B. Forehand, G.L. Dooly, S.C. Moldoveanu, J. Chromatogr. A 898 (2000) 111.
[18] R. Stabbert, K.H. Schäfer, C. Biefel, K. Rustemeier, Rapid Commun. Mass Spectrom. 17 (2003) 2125.
[19] P.X. Chen, S.C. Moldoveanu, Beitr. Tabakforsch. 20 (2003) 448.
[20] M. Intorp, S. Purkis, Beitr. Tabakforsch. 24 (2010) 78.
[21] H. Kataoka, M. Miyake, K. Mitani, J. Sep. Sci. 30 (2007) 90.
[22] C. Brede, I. Skjevrak, H. Herikstad, J. Chromatogr. A 983 (2003) 35.
[23] S.K. Mortensen, X.T. Trier, A. Foverskov, J.H. Petersen, J. Chromatogr. A 1091 (2005) 40.
[24] M. Aznar, E. Canellas, C. Nerin, J. Chromatogr. A 1216 (2009) 5176.
[25] R. Li, Y. Zhang, C.C. Lee, R. Lu, Y. Huang, J. Chromatogr. A 1217 (2010) 1799.

[26] A. Shihadeh, S. Azar, C. Antonios, A. Haddad, Pharmacol. Biochem. Behav. 79 (2004) 75.
[27] IARC Monographs on the Evaluations of Carcinogenicity to Humans, vol. 1–100, International Agency for Research on Cancer, Lyon, 2010.
[28] DIN 32645: 2008-11, Chemical Analysis—Decision Limit, Detection Limit and Determination Limit Under Repeatability Conditions—Terms, Methods, Evaluation, Beuth Publishing, Berlin, 2008.
[29] R. Franke, A. Gruska, A. Giuliani, R. Benigni, Carcinogenesis 22 (2001) 1561.
[30] Evaluation of measurement data—Guide to the expression of uncertainty in measurement, Joint Committee for Guides in Metrology, JCGM 100, 2008.
[31] P. Konieczka, J. Namieśnik, J. Chromatogr. A 1217 (2010) 882.
[32] N. Ratola, L. Santos, P. Herbert, A. Alves, Anal. Chim. Acta 573–574 (2006) 202.