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Simultaneous determination of various aromatic amines and metabolites of aromatic nitro compounds in urine for low level exposure using gas chromatography–mass spectrometry

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Abstract

A newly developed method permits the simultaneous quantitative determination of various aromatic amines (or metabolites of aromatic nitro compounds, respectively) in human urine in one analytical run. Applying this method it is possible to determine aniline, toluidines, 4-isopropylaniline, *o*-anisidine, 3- and 4-chloroaniline, 4-bromoaniline, amino-nitrotoluenes, aminodinitrotoluenes, 3,5- and 3,4-dichloroaniline, α- and β-naphtylamine and 4-aminodiphenyl. After separation from the urinary matrix by a simple liquid–liquid extraction at pH 6.2–6.4 the analytes are converted into their pentafluoropropionic acid amides. Separation and quantitative analysis is carried out by capillary gas chromatography and mass-selective detection in the single ion monitoring mode. The limits of detection were within the range from 0.05 μg/l (4-aminobiphenyl, *o*-anisidine, 3,5-dichloroaniline) to 2 μg/l urine (4-amino-2,6-dinitrotoluene). The relative standard deviation of the within-series imprecision (determined at spiked concentrations of 2.0 μg/l and 10 μg/l) was between 2.9 and 13.6% depending on analyte and concentration. The relative recovery rates were in the range of 70–121%. The analytes that do not contain a nitro function showed better performance regarding the analytical reliability criteria. In order to determine the suitability of this new method for biological monitoring we analysed 20 12-h urine samples of persons without known exposure to aromatic amines, nitroaromatics or precursors in a pilot study. In these samples various aromatic amines could be clearly identified. The general population renally excretes aniline (median: 3.5 μg/l; 95th percentile: 7.9 μg/l), *o*-(0.12 μg/l; 2.7 μg/l), *m*- (0.17 μg/l; 2.2 μg/l) and *p*-toluidine (0.11 μg/l; 0.43 μg/l), and *o*-anisidine (0.22 μg/l; 0.68 μg/l). Additionally, we found that the persons investigated also excrete 3- (<0.05 μg/l; 0.55 μg/l) and 4-chloroaniline (0.11 μg/l; 0.57 μg/l) as well as 3,5-dichloroaniline (0.18 μg/l; 1.5 μg/l). 3,4-Dichloroaniline was found in some specimens (20%) in concentrations near the limit of detection (<0.05 μg/l; 0.12 μg/l). We did not detect α- or β-naphtylamine, 4-aminobiphenyl or metabolites of explosives in the samples.

Keywords: Aromatic amines; Aromatic nitro compounds

1. Introduction

Aromatic amines, aromatic nitro compounds, and derivatives are important and widely used intermediates in the chemical industry. They serve for the

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preparation of dyes (e.g., *o*-anisidine based azo dyes), pharmaceuticals (e.g., lidocaine), pesticides (e.g., vinclozolin), and plastics (e.g., polyurethanes). Furthermore, the dinitrotoluenes (DNTs) and 2,4,6-trinitrotoluene (TNT) are used in explosives. But normally, this class of chemicals does not occur in final products of the chemical industry. Therefore, it is astonishing that some of these compounds and/or their metabolites have been found in the urine or blood of persons without known exposure [1–7]. Aniline and *o*-toluidine have also been detected in human milk [8]. Recently aniline, toluidines and further aromatic amines were found in indoor and outdoor air [9].

Because of the formation of methaemoglobin the inhalative or dermal intake of aromatic amines can induce cyanosis [10,11]. Furthermore, some of these compounds have been classified as carcinogenic [12,13]. For example, 4-aminodiphenyl (4-ADP), *o*-toluidine (*o*-T), *o*-anisidine (*o*-A), 4-chloroaniline (4-ClA) and DNTs may cause cancer in humans. Aniline (A), *p*-toluidine (*p*-T) and TNT are suspected of having carcinogenic potential, evidenced or indicated by in-vitro and/or animal assays. Most of the amino and nitro aromatics can easily be absorbed through the intact skin [13].

The metabolism of aromatic amines and aromatic nitro compounds is strongly interrelated [14–18]. A simplified metabolic pathway is shown in Fig. 1. The aromatic nitro function can be reduced via nitroso compound and hydroxyl amine to the corresponding amine. Nitro reducing enzymes and intestinal bacteria moreover are involved in this direction of metabolism. The re-reaction, namely the oxidation of the amino function, is also possible and is mediated by the cytochrome P450 system. During this (oxidative) route of metabolism methaemoglobin is formed. The aromatic nitroso compounds may form adducts with the cysteinyl-SH groups of haemoglobin and the homologous aromatic hydroxylamine can act as ultimate carcinogen when developing DNA adducts via the corresponding nitrenium ion [18–20].

The final products of the reductive metabolism pathway, the amino compounds, are renally excreted after partial conjugation. At this point it cannot be distinguished if an aromatic amine detected in urine has been incorporated as aromatic nitro compound or as aromatic amine.

The methods already published for analysis of urinary aromatic amines [4–6,21–24] are not as sensitive as required in detecting these substances in the expected range caused by environmental exposure (ng/l) and/or do not cover all the substances required.

Therefore the aim of this work was to develop a practicable and sensitive method suitable for the determination of aromatic amines in urine samples. The method had to be capable to study the urinary excretion of aromatic amines and/or metabolites of aromatic nitro compounds in urinary samples from the general population.

2. Experimental

2.1. Chemicals and methods

Aniline, *o*-anisidine, 4-amino-2-nitrotoluene (4A2NT),

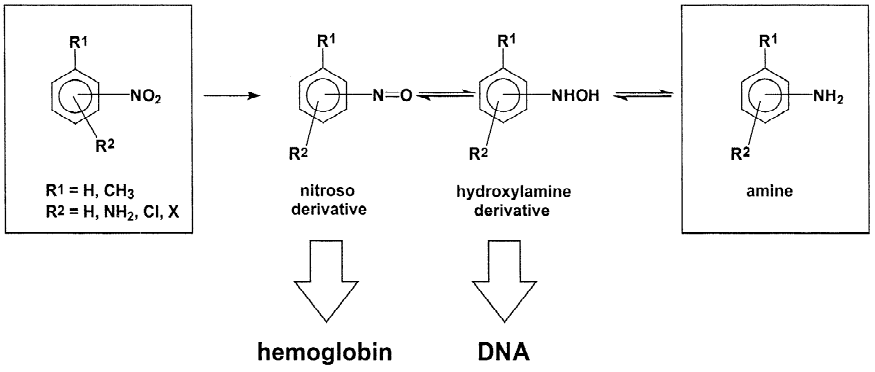

Fig. 1. Simplified metabolic pathway of aromatic amines and aromatic nitro compounds.

2-amino-4-nitrotoluene (2A4NT), 4-amino-5-nitrotoluene (4A5NT), 1-naphtylamine (1-NA), 2-naphtylamine (2-NA), 4-aminodiphenyl (4ADP), and 2-(*N*-morpholino)ethanesulfonic acid (MES) were purchased from Sigma–Aldrich (Deisenhofen, Germany); *o*-toluidine (*o*-T), *m*-toluidine (*m*-T) and pentafluoropropionic anhydride (PFPA) were from Fluka (Deisenhofen, Germany), *p*-toluidine (*p*-T), 3- and 4-chloroaniline (3ClA, 4ClA); 3,4- and 3,5-dichloroaniline (3,4DClA, 3,5DClA), 4-isopropylaniline (4iPA) from Riedel-de Haën (Seelze, Germany). 4-Amino-2,6-dinitrotoluene (4A26DNT) was purchased from Promochem (Wesel, Germany). 2-Amino-6-nitrotoluene (2A6NT), *n*-hexane Unisolv, toluene, methanol, hydrochloric acid (analytical-reagent grade, 37%), sodium hydroxide (NaOH) (analytical-reagent grade), potassium phosphate trihydrate (analytical-reagent grade), orthophosphoric acid (analytical-reagent grade, 85%) and Ultrapure water (equivalent to ASTM type 1) were obtained from Merck (Darmstadt, Germany). Pyridine was from J.T. Baker (Deventer, The Netherlands) and nitrogen 6.0 was from Linde (Nürnberg, Germany). If not noted else all chemicals used were of highest analytical grade available.

To prepare a 10 *M* NaOH solution, 400 g of NaOH was dissolved in 1000 ml water. In portions of 500 ml this solution was twice washed with 150 ml *n*-hexane. For the preparation of 0.5 *M* MES buffer, 107.2 g of MES was dissolved in 1000 ml water. The pH was adjusted to 6 by the addition of 20 ml 10 *M* NaOH. In portions of 500 ml this solution was twice washed with 150 ml *n*-hexane. A 0.01 *M* phosphate buffer was made by dissolving 5.4 g of potassium phosphate trihydrate in 2000 ml water. The pH was adjusted to 8 by the addition of 750 μl of orthophosphoric acid. In portions of 500 ml this solution was also twice washed with 150 ml *n*-hexane.

Statistical analysis was carried out by applying Microsoft Excel 2000 and SPSS 9.01 for Windows.

2.2. Internal standard (I.S.) solution

The stock solution for the I.S. was prepared by dissolving 25 mg 4-amino-5-nitrotoluene in 50 ml methanol (500 mg/l). A 1000 μl volume of this stock solution was diluted to the mark in a 50-ml glass volumetric flask with highly purified water (10 mg/l). The resulting internal standard solution was used for spiking urine samples before sample preparation (see Section 2.4).

2.3. Solutions for calibration and quality control

Starting solutions each containing 500 mg/l of the analytes were prepared by dissolving 25 mg of the analyte in 50 ml methanol. From these starting solutions two standard stock solutions were prepared.

Stock solution A: 1 ml of each starting solution was pipetted into a 100-ml glass volumetric flask and the flask was filled to its nominal value with water (5 mg/l).

Stock solution B: 1 ml of each starting solution was pipetted into a 25-ml glass volumetric flask and the flask was filled to its nominal value with water (20 mg/l).

Six calibration standards with concentrations in the range from 100 ng/l to 100 μg/l were prepared in 100-ml glass volumetric flask from these standard stock solutions by diluting with pooled urine. The calibration standards were aliquoted and stored at −25°C. Under these conditions the calibration standards were stable for at least 6 months.

As no reference material is commercially available, it must be prepared in the laboratory. For this purpose, another pooled urine was spiked with a defined quantity of the aromatic amines (2 μg/l and 10 μg/l; 4A26DNT 10 μg/l and 50 μg/l). Aliquots of this solution were stored at −25°C.

2.4. Sample preparation

A 5-ml volume of each urine sample was spiked with 50 μl of the internal standard solution and 1 ml concentrated hydrochloric acid (37%) was added. Hydrolysis of acid-labile conjugates was achieved by heating the mixture for 1 h at 80°C. Then the samples were cooled in an ice bath before 600 μl of 10 *M* NaOH was added followed by 3 ml of MES buffer and another 550 μl portion of 10 *M* NaOH. The pH of the urine samples should range between 6.0 and 6.4. If necessary the pH has to be adjusted with small amounts of concentrated acetic acid or 10 *M* NaOH. The urine samples were then extracted two times with 5 ml *n*-hexane and the layers were separated by centrifugation at 2500 *g* for 10 min. A

25-μl volume of pyridine (dried over sodium sulfate) and 50 μl of pentafluoropropionic anhydride were added to the combined organic layer. Derivatisation was achieved by heating the vials for 1 h at 80°C in a water bath. To remove excess pentafluoropropionic acid and anhydride the samples were cooled to room temperature and extracted once with 3 ml phosphate buffer (pH 8) for 5 min on a laboratory mixer. The layers were then separated by centrifugation at 2500 *g* for 10 min. The organic solvent was transferred to a 20-ml vial containing 200 μl toluene and then evaporated to 150 μl under a gentle stream of nitrogen. The residue was transferred into a micro insert and finally evaporated to a volume of 40 μl for subsequent quantitative gas chromatography–mass spectrometry (GC–MS) analysis. The sample preparation is summarised in Fig. 2.

2.5. Calibration procedure

The calibration standards are processed and analysed in the same manner as the assay samples. Calibration graphs were obtained by plotting the quotients of the peak areas for each analyte and that of the I.S. as a function of the concentrations used. These graphs were used to ascertain the unknown concentrations of the aromatic amino compounds in urine samples. Any reagent blank values must be subtracted from the analytical results for the real sample.

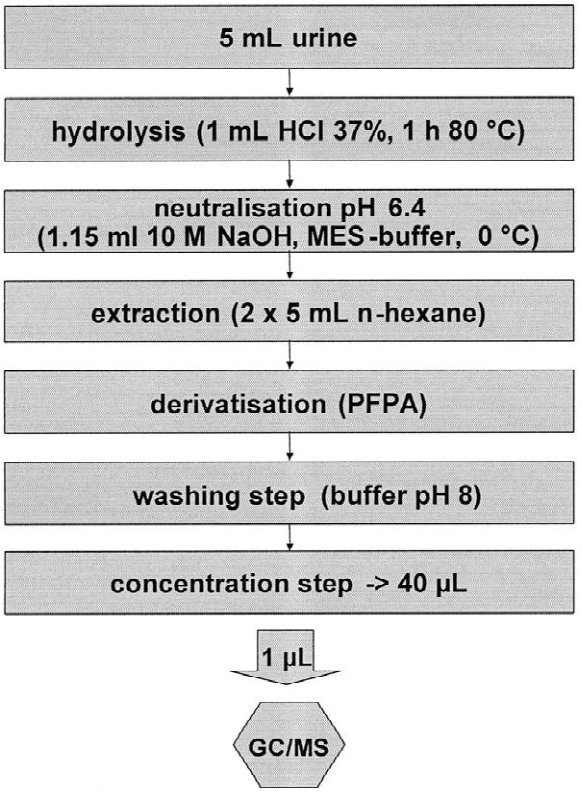

Fig. 2. Sample processing.

2.6. Gas chromatography

Analysis was carried out on a Hewlett-Packard HP 5890 gas chromatograph fitted with a Hewlett-Packard HP 7673A autosampler and a split/splitless injector operating in the splitless mode. The operating temperature of the injector was 260°C. Chromatographic separation was performed on a (35% phenyl)methylpolysiloxane capillary column (60 m× 0.22 mm I.D., 0.25 μm film thickness) purchased from Hewlett-Packard (Waldbronn, Germany). Helium 5.0 was used as carrier gas with constant flow (1.0 ml/min). The initial column temperature of 90°C was raised by a rate of 5°C/min to 180°C and held for 5 min. Then the temperature was increased by a rate of 5°C/min to 200°C and held for 10 min. Finally the temperature was raised by a rate of 15°C/min to 280°C and this temperature was maintained for 25 min. The injection volume was 1 μl.

The retention times for the analytes and I.S. observed under the conditions described can be found in Table 1. A chromatogram of a spiked urine sample (1 μg/l) is illustrated in Fig. 3.

2.7. Mass spectrometry

A Hewlett-Packard HP MSD 5972 mass spectrometer fitted with a quadrupole mass filter was used in electron impact (EI) mode. EI mass spectra were obtained at ionisation energy of 70 eV and electron multiplier voltage was set to +200 V relative (1900 V+200 V). The MS transfer line temperature was maintained at 300°C. For quantitative analysis of the aromatic amine selected ion monitoring (SIM) was used. The retention times together with the ions used for quantification are summarised in Table 1.

Table 1
Parameters, fragments used for quantification and retention times

Parameter	Quantifier ion m/z	Qualifier ion m/z	Retention time (min)	Peak No.
Aniline	239 $[M]^+$	120 $[M-C_2F_5]^+$	11.5	1
o-Toluidine	253 $[M]^+$	134 $[M-C_2F_5]^+$	13.1	2
m-Toluidine	253 $[M]^+$	134 $[M-C_2F_5]^+$	15.0	3
p-Toluidine	253 $[M]^+$	134 $[M-C_2F_5]^+$	15.8	4
o-Anisidine	269 $[M]^+$	150 $[M-C_2F_5]^+$	20.1	5
Isopropylaniline	281 $[M]^+$	266 $[M-CH_3]^+$	23.2	8
3-Chloroaniline	273 $[M(^{35}Cl)]^+$	275 $[M(^{37}Cl)]^+$	19.6	6
4-Chloroaniline	273 $[M(^{35}Cl)]^+$	275 $[M(^{37}Cl)]^+$	20.2	7
4-Bromoaniline	200 $[M-C_2F_5]^+$	318 $[M]^+$	23.0	9
3,5-Dichloroaniline	307 $[M-H]^+$	188 $[M-C_2F_5H]^+$	23.8	10
3,4-Dichloroaniline	307 $[M-H]^+$	188 $[M-C_2F_5H]^+$	24.9	11
4-Amino-5-nitrotoluene (I.S.)	298 $[M]^+$	252 $[M-NO_2]^+$	23.8	–
2-Amino-6-nitrotoluene	281 $[M-OH]^+$	298 $[M]^+$	26.1	12
4-Amino-2-nitrotoluene	281 $[M-OH]^+$	298 $[M]^+$	27.4	14
2-Amino-4-nitrotoluene	281 $[M-OH]^+$	298 $[M]^+$	27.7	15
4-Aminobiphenyl	315 $[M]^+$	168 $[M-COC_2F_5]^+$	30.4	17
4-Amino-2,6-dinitrotoluene	326 $[M-OH]^+$	343 $[M]^+$	31.2	18
α-Naphtylamine	289 $[M]^+$	142 $[M-COC_2F_5]^+$	26.8	13
β-Naphtylamine	289 $[M]^+$	142 $[M-COC_2F_5]^+$	27.7	16

2.8. Collectives and specimen collection

In a pilot study we investigated 20 urine specimens randomly selected from two collectives of the general population (100 residents of rural and 100 residents of urban regions; for a detailed description of collectives see Ref. [25]). These collectives have already been investigated for some aromatic amines

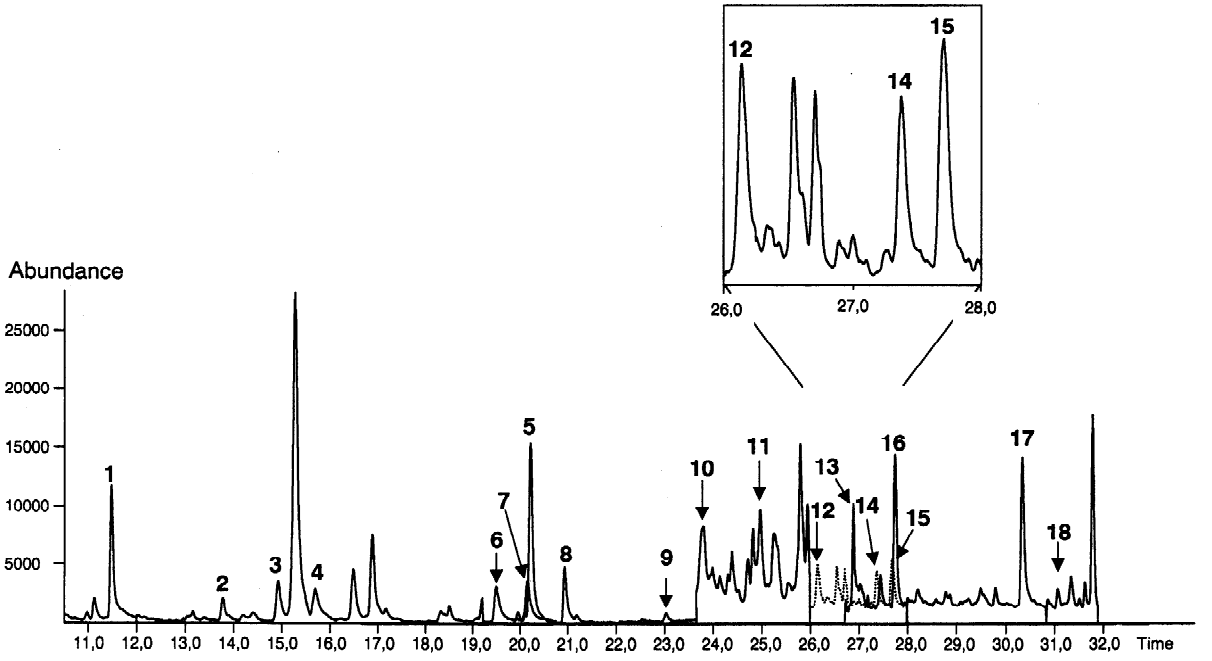

Fig. 3. SIM chromatogram of a processed urine sample spiked with 1 μg/l (4A26DNT 5 μg/l); peak annotation see Table 1.

(aniline, toluidines, 3,5-DClA [22]). As the number of specimen analyzed is low, the results given in Section 3 should be regarded as preliminary trends and may not stand for the complete collectives or the general population. The specimen taken from the two collectives have to be regarded as spot checks and should rather be characterised as specimen from persons without known exposure to aromatic amine or metabolic precursors.

All samples were collected in 1998 as 12-h urine and stored in sealable plastic bottles at −25°C until processing. It is noteworthy, that the specimen investigated in this study have been severalfold thawed and refrozen for other purposes prior to processing.

3. Results and discussion

Most of the various amino and nitro compounds are incorporated via different routes in man (oral, inhalative, dermal). Therefore, the best way to determine the body load of exposed and non-exposed persons is the biological monitoring of these substances or their metabolites in body fluids [26]. The interrelated metabolism of aromatic nitro and amino compounds enables the monitoring of these two groups of chemicals in one analytical run via the determination of their (common) amino metabolites in urine. The method presented here is an improvement of two methods previously applied in biological monitoring of aromatic amines [24,25].

As a mass-selective detector was used for quantification, the method proved to be much more specific than methods described in the literature using nitrogen–phosphorus detection, electron-capture detection (ECD) or UV detection after high-performance liquid chromatographic separation.

3.1. Sample preparation and clean up procedure

Because most of the aromatic amines in urine are partially conjugated the samples were treated with conc. HCl at 80°C for 1 h. Besides the cleavage of conjugates acidic hydrolysis also may lead to a conversion of metabolites of dicarboximide pesticides (vinclozolin, procymidone, iprodione, chlozolinate) to their corresponding amine, 3,5-dichloroaniline [6].

After hydrolyses the pH is set to 6.2–6.4 by adding 10 *M* NaOH and MES buffer. During this step the specimens were cooled in an ice bath to prevent oxidative decomposition of the aromatic amines in local hot spots. *n*-Hexane as extraction solvent and a pH value of 6.2–6.4 are key steps in the sample preparation procedure. Experiments have shown the non-polar organic solvent ensures that only minor amounts of matrix components are extracted together with the analytes. The pH of 6.4 is an optimum for 4-amino-2,6-dinitrotoluene. Compared with the remaining analytes 4A26DNT is relatively polar, has the lowest pK_a value and is in this class of analytes the most difficult to extract.

3.2. Gas chromatography

The conditions described in Section 2.6 were optimised for the quantification of the analytes that do not contain the NO_2 moiety. Analytes and solvent were first condensed in the capillary column and the solvent was then released by a rapid increase of the temperature. This procedure leads to an optimal peak shape for most of the analytes but also to an increasing peak width of the amino nitro compounds. The peak shape of the latter can be improved by a starting temperature above 120°C and a flow-rate of at least 1.1 ml/min. But the latter conditions may worsen the peak shape of the analytes without any nitro function.

However, separation of all analytes from interfering and coeluting compounds from the matrix is very sophisticated. Especially the quantification of *m*- and *p*-toluidine may be affected by a coeluting substance deriving from urine but not from the reagents used. This unknown substance decomposes in the ion source to the same mass fragments in almost the same mass ratio as the toluidines. Even capillary columns with the same specification may differ in their efficiency of separation in a manner that *m*- or *p*-toluidine peaks overlap or even coincide with the interfering peak.

3.3. Reliability

3.3.1. Calibration graphs

The calibration graphs were linear for the investigated range. Aqueous calibration standards (100 ng/

l–100 μg/l) delivered correlation coefficients higher than 0.98.

Calibration curves were also generated from pooled urine standards. The pooled urine contained aniline, *o*-, *m*- and *p*-toluidine as well as *o*-anisidine, 4-chloroaniline, 3,4- and 3,5-dichloroaniline. Thus the corresponding urinary calibration curves showed a positive intercept for these analytes.

The associated correlation coefficients of the urinary calibration were higher than 0.995 in all cases. Depending on the analyte calibration curves generated from both urinary and aqueous standard solutions differed slightly or offered nearly the same slope of regression. In particular, the analytes having a poor absolute recovery (aminodinitrotoluene and aminonitrotoluenes, Table 2) differed between aqueous and urinary calibration.

For these reasons and to ensure a better separation of aqueous and organic layers urinary calibration was used for quantification. Various calibration functions from urinary and aqueous standards can be seen in Table 3.

Table 2
Precision data (LOD=limit of detection)

Parameter	Relative/ recovery (%)	Spiked concentration (μg/l)	Imprecision (%)		Losses due to processing (%)	LOD (ng/l)
			Between-day (n=6)	Within series (n=8)		
Aniline	100	2.0	7.5	7.5	0	50
	101	10	13.7	8.3		
o-Toluidine	106	2.0	14.1	7.3	10	50
	95	10	10.6	7.0		
m-Toluidine	102	2.0	16.2	9.7	0	50
	121	10	11.2	7.7		
p-Toluidine	110	2.0	14.4	3.0	15	50
	105	10	13.8	3.1		
o-Anisidine	81	2.0	13.6	9.8	0	50
	100	10.0	14.6	9.8		
3-Chloroaniline	96	2.0	13.5	2.2	0	50
	103	10	12.7	3.4		
4-Chloroaniline	96	2.0	11.8	2.5	0	50
	105	10	12.9	3.3		
Isopropylaniline	119	2.0	16.6	3.3	0	100
	125	10	10.8	1.8		
4-Bromoaniline	100	2.0	16.5	2.0	30	200
	111	10	14.9	5.5		
3,5-Dichloroaniline	100	2.0	11.8	7.4	7	50
	101	10	9.9	5.1		
3,4-Dichloroaniline	94	2.0	12.4	5.0	5	50
	109	10.0	10.9	3.3		
2-Amino-6-nitrotoluene	80	2.0	13.9	9.1	55	400
	95	10	14.3	9.6		
4-Amino-2-nitrotoluene	80	2.0	14.8	7.7	58	400
	109	10.0	11.0	8.6		
2-Amino-4-nitrotoluene	70	2.0	12.7	6.6	57	400
	88	10.0	14.7	8.6		
4-Aminobiphenyl	100	2.0	15.1	5.9	0	50
	108	10.0	9.8	2.7		
4-Amino-2,6-dinitrotoluene	78	10.0	14.8	13.6	65	2000
	96	50.0	14.0	13.0		
α-Naphtylamine	90	2.0	18.6	8.2	0	150
	70	10.0	12.6	2.8		
β-Naphtylamine	90	2.0	13.0	6.3	0	75
	90	10.0	12.9	2.6		

Table 3
Calibration functions of aqueous and urinary calibration

Parameter	Aqueous calibration		Urinary calibration	
	Function	R^2	Function	R^2
Aniline	$0.065x+0.0082$	0.9991	$0.0109x+0.056$	0.9988
o-Toluidine	$0.0105x+0.0031$	0.9999	$0.0129x+0.0041$	0.9997
m-Toluidine	$0.0178x+0.0169$	0.9998	$0.0180x+0.0144$	0.9998
p-Toluidine	$0.0183x+0.0212$	0.9997	$0.0184x+0.0200$	0.9997
o-Anisidine	$0.0066x+0.0032$	0.9998	$0.0123x+0.0101$	0.9992
Isopropylaniline	$0.0443x+0.1154$	0.9980	$0.0404x+0.0097$	0.9999
3-Chloroaniline	$0.0181x+0.0193$	0.9991	$0.0170x+0.0100$	0.9999
4-Chloroaniline	$0.0200x+0.0265$	0.9988	$0.0185x+0.0185$	0.9998
4-Bromoaniline	$0.0069x+0.0144$	0.9987	$0.0059x+0.0008$	0.9999
3,5-Dichloroaniline	$0.0174x+0.0019$	0.9997	$0.0133x+0.0110$	0.9999
3,4-Dichloroaniline	$0.0161x+0.0212$	0.9995	$0.0123x+0.0008$	0.9999
2-Amino-6-nitrotoluene	$0.0015x+0.0009$	0.9998	$0.0010x+0.0003$	0.9999
4-Amino-2-nitrotoluene	$0.0029x+0.0008$	0.9999	$0.0018x+0.0007$	0.9996
2-Amino-4-nitrotoluene	$0.0070x+0.0094$	0.9985	$0.0020x+0.0011$	0.9997
4-Aminobiphenyl	$0.0424x+0.1002$	0.9983	$0.0313x+0.0051$	0.9999
4-Amino-2,6-dinitrotoluene	$0.0059x+0.0241$	0.9750	$0.0024x+0.0015$	0.9951
α-Naphtylamine	$0.0274x+0.0269$	0.9997	$0.0256x+0.0046$	0.9999
β-Naphtylamine	$0.0410x+0.0796$	0.9988	$0.0301x+0.0043$	0.9999

3.3.2. Precision

In order to assess the within-series imprecision, pooled urine with spiked concentrations of 2 and 10 μg/l (4A26DNT 10 and 50 μg/l, respectively) was analysed nine times. Depending on the analyte the relative standard deviations (RSDs) were in the range of 1.8–13.6%.

In order to obtain the between-day imprecision, spiked samples of pooled urine with two different concentrations (2 μg/l and 10 μg/l; 4A26DNT 10 μg/l and 50 μg/l, respectively) were processed and analysed on at least 6 different days. The RSDs were varied in the range 7.5–18.6%. The detailed precision data for each analyte are presented in Table 2.

3.3.3. Recovery of spiked concentrations and quality control

Accuracy was examined by carrying out recovery experiments. In order to determine the rate of recovery, urine was pooled. Spiking this urine with two different amounts of analytes resulted in two solutions containing 2 and 10 μg analyte per litre urine (plus the blank value). Aliquots were analysed eight times and the results were calculated from urinary calibration curves. Relative recovery rates depend on the analyte and were found to be between 70 and 125%. For the detailed data see Table 2.

In order to determine absolute recovery rates or the losses due to processing aqueous solutions were used. These samples were spiked with 10 μg/l of aromatic amines and processed. Similarly, 10 ml of a *n*-hexane solution containing the same amounts of aromatic amines as the aqueous solutions were treated with derivatisation reagents and concentrated in the same manner as described. By comparing the absolute peak areas for the aromatic amines in the processed aqueous sample and the hexane solution, absolute recovery between 35 and 100% was found. It should be noted that these recovery experiments do not cover losses during the derivatisation step and the concentration step.

In order to investigate the influence of individual urine specimen additionally five different individual samples, with different blank amine concentrations and different creatinine contents (0.50–1.74 g creatinine per litre) were analyzed. Subsequently these samples were spiked with 10 μg/l of aromatic amines and analysed once again. Relative individual recoveries were in the same order of magnitude or better as obtained in the recovery experiments (Table 2, second row). No correlation to individual creatinine levels was observed. These experiments in combination with the comparison of aqueous and urinary calibration (Section 3.3.1) confirm that no

relevant interference from different urinary matrices affected the accuracy of our method or have been levelled by the internal standard, respectively.

The method as described worked well with a single internal standard (4A5NT). However, the precision might be further improved by the use of auxiliary internal standards, e.g., deuterated analogues of the analytes.

As no reference material was commercially available, it was prepared in our laboratory. For this purpose, pooled urine from non-smoking persons without known exposure to aromatic amines was spiked with defined quantities of the aromatic amines. Aliquots of these solutions were stored in the deep-freezer. The concentrations of this quality control material were 2 and 10 μg/l (4A26DNT: 10 μg/l and 50 μg/l), respectively. This quality control material must be included in each analytical series.

3.3.4. Detection limit

Based on a signal-to-noise ratio of 3:1, the resulting detection limits of the aromatic amines measured with this method were in the range 50–2000 ng/l urine. These data are shown in Table 2. As for numerous aromatic amines the limit of detection lies below the background levels detection limits in those cases have been calculated from the calibration graphs. Therefore, the surroundings of the actual peak were checked for the degree of background noise. The threefold background noise was calculated and then taken as theoretical peak height. With this peak height the theoretical peak area at the (theoretical) limit of detection was calculated assuming an ideal Gaussian peak shape.

In the case of aniline the calculated limit of detection (50 ng/l) cannot be checked in practice, as it lies below the blank reagent value. For aniline a limit of determination is given with a value of 400 ng/l. This value represents the twofold maximum blank reagent value determined during validation of the method.

3.3.5. Sources of error

Aniline could be clearly identified in extracts from blank water. Experiments have shown that aniline concentration could be decreased to values between 100 and 200 ng/l, when the solutions used (NaOH solution and buffers) were purified as described in Section 2. Water, prepared with a Millipore technique may contain aniline sourced from the purifier resin. Hence the use of tap water or purchasable water is recommended. In Fig. 4 a chromatogram is given representing the aniline blank value (determined concentration 0.1 μg/l), an aqueous standard (0.1 μg/l) and a urinary specimen (determined concentration 2.6 μg/l).

For these reasons a blank aqueous specimen including the internal standard has to be processed together with the calibration standards. The determined aniline concentration in this blank ''standard''

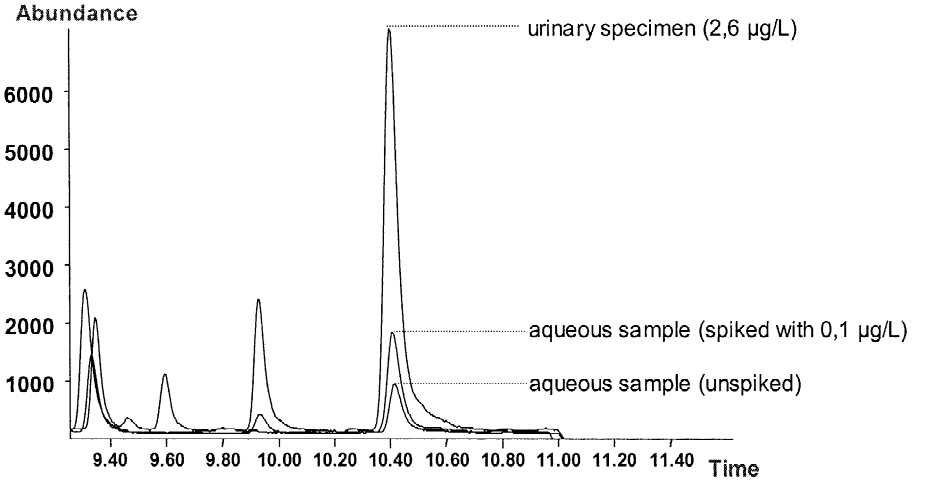

Fig. 4. SIM chromatogram of a processed urinary specimen, an aqueous standard (0.1 μg/l) and an unspiked aqueous sample.

must be subtracted from each result calculated in accordance with Section 2.5. However, applying the measures for the reduction of external aniline contamination it is possible to determine aniline concentrations accurately above 400 ng/l urine. This limit of determination lies twofold above the maximum blank reagent value observed in aqueous samples and approx. 10-fold below the aniline median measured in the urine of persons without known exposure (Table 4). Furthermore, no other contamination of reagents used was observed.

The method works without any drying step. As the derivatisation procedure requires the absence of water it is very important to separate exactly aqueous and organic layer during the extraction and washing steps. Due to the relatively high derivatisation temperature of 80°C (boiling point *n*-hexane: 69°C!) it is strongly recommended to use glass vials that are pressure stable and gas proof.

Because of the volatility of aniline and toluidines specimen should not run dry during the step of concentration under a stream of nitrogen. Also for that reason toluene was used as keeper.

The samples fully processed for GC injection (toluene solution) were at least stable for 2 days in the refrigerator (5°C). It was observed that longer storage periods may result in partial decomposition of analytes and internal standard.

However, due to the ubiquitous airborne occurrence of some aromatic amines [9] contact to air of all chemicals and solutions must be reduced to a minimum, particularly in the case of the derivatisation reagent PFPA.

We observed an interfering compound from urine that may affect the accurate quantification of *m*- or *p*-toluidine (Section 3.2, Figs. 3 and 5). It is even possible that this interference co-elutes with one of the toluidines. This should be taken into consideration when first applying the method and if necessary, the GC temperature program has to be readapted.

3.4. Results of biological monitoring

In all urinary samples investigated aniline was found. Its concentrations were in the range of 0.4–8.8 μg/l urine with a median value of 3.5 μg/l and a 95th percentile of 7.9 μg/l. We also found a baseline excretion of the three isomeric toluidines. At least in 75% of the samples we could quantify toluidines with median values of 0.12 μg/l (*o*-T), 0.17 μg/l (*m*-T) and 0.11 μg/l (*p*-T). In a former study [24] we analysed 200 urine specimens from the general population with regard to their content of aromatic amines (100 persons living in a rural region and 100 persons living in an urban area in Germany). The values measured with the new method differ slightly from those of our former findings. The aniline concentrations averaged higher (former study, aniline: median 1.5 μg/l, 95th 7.4 μg/l), when applying the new method and the toluidines and 3,5-dichloroaniline concentrations were lower to

Table 4
Results from pilot study: 4-ABP, α- and β-naphtylamine as well as TNT- and DNT-metabolites have not been found in the general population

	Persons without known exposure				
	n	Median (μg/l)	95% (μg/l)	Range (μg/l)	Positive results (%)
Aniline	20	3.5	7.9	0.4–8.8	100
o-Toluidine	20	0.12	2.7	<0.05–3.1	80
m-Toluidine	20	0.17	2.2	<0.05–2.8	95
p-Toluidine	20	0.11	0.43	<0.05–0.55	75
3,5-Dichloroaniline	20	0.18	1.5	<0.05–2.0	90
o-Anisidine	20	0.22	0.68	<0.05–4.2	95
3-Chloroaniline	20	<0.05	0.55	<0.05–2.5	50
4-Chloroaniline	20	0.11	0.57	<0.05–1.1	90
3,4-Dichloroaniline	20	<0.05	0.12	<0.05–0.15	20

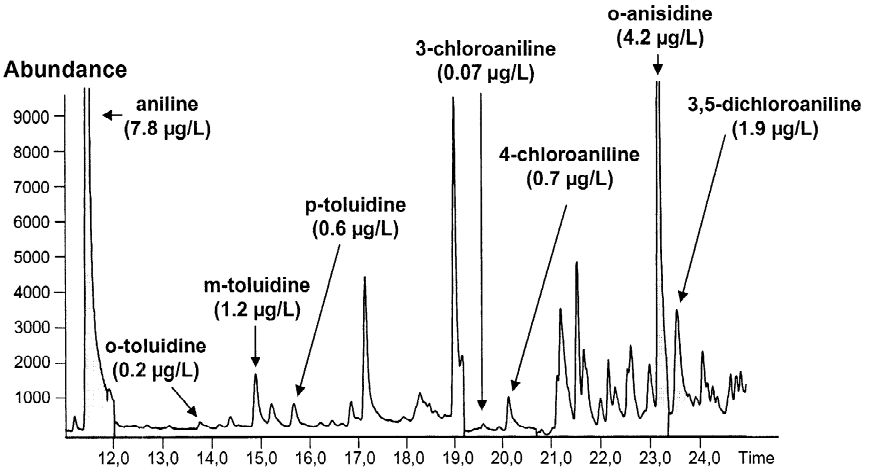

Fig. 5. SIM chromatogram of a processed urine specimen from a person without occupational exposure to aromatic amines or aromatic nitro compounds (LOD=limit of detection).

some extent (*o*-T: 0.44 μg/l, 2.6 μg/l; *m*-T: 0.28 μg/l, 3.9 μg/l; *p*-T: 1.2 μg/l, 11.4 μg/l; 3,5-DClA: 0.54 μg/l, 3.9 μg/l). We attribute these differences to the long period of storage (more than 3 years). Moreover, the specimens have been severalfold thawed and refrozen for other purposes.

Comparing these results to others quoted in the literature we ascertained a good match with the findings of Riffelmann et al. [4] [controls (mean): aniline=0.8 μg/l, *o*-T=0.85 μg/l, *m*-T=0.35 μg/l, *p*-T=1.75 μg/l; *n*=16]. The values found by Teass et al. [5] [controls (mean): aniline=2.7 μg/l, *o*-toluidine=1.1 μg/l, *n*=31] are in a similar range of concentration but exceed Riffelmann et al.'s and our results to some extent. However, those values of Teass et al. cannot be taken for direct comparison. In this study controls were chosen from the white collar staff of a plant producing aniline and *o*-toluidine. Therefore exposure of controls to aniline and *o*-toluidine sourced from production is obvious.

Additionally we obtained in 90% of the specimen positive results for 3,5-dichloroaniline with an median value of 0.18 μg/l and a 95th percentile of 1.5 μg/l. The maximum value analysed was 2.0 μg/l. Taking into account the possible losses during storage and our former findings [25] these results are in good conformity with a study recently published by Wittke et al. [22]. Wittge et al. found an average 3,5-dichloroaniline concentration of 0.76 μg/l examining 5 persons without known exposure applying gas chromatography and electron capture detector. In 1995 Will [6] investigated four urine specimens of the general population in a pilot study using GC–ECD and HPLC–electrochemical detection. He identified 3,5-DClA with an average value of 6.7 μg/l. These results differ by an order of magnitude from Riffelmann et al.'s and our results. The small number of samples and the less specific detector applied by Will might explain these differences.

We did not find 4-aminobiphenyl in urine. Also α- and β-naphtylamine as well as the metabolites of the dinitrotoluenes and the metabolite of TNT (4A26DNT) have not been detected. These findings are in accordance with our prior investigations [24]. In that former study we detected 4-ABP in only one of the samples (*n*=200) similar to Riffelmann et al.'s findings.

But Riffelmann et al. found β-naphtylamine in the urine of occupationally non-exposed person (1.8 μg/l average six smokers, six non-smokers). However, these values seem to be very high (GC–ECD) and could not be confirmed to this extent by Grimmer et al. [23]. Using a very expensive procedure (200 ml urine extracted with 100 ml benzene, concentrated to 50 μl) Grimmer et al. detected β-naphtylamine in smokers (mean: 84 ng/24 h, maximum 275 ng/24 h, *n*=12) as well as in non-smokers (mean: 120 ng/24 h, maximum 1073 ng/24 h, *n*=14) and passive

smokers (mean: 95 ng/24 h, maximum 1068 ng/24 h, $n=22$). The authors provided the information that these findings were solely possible by adding *p*-toluidine (10 μg) to the freshly collected urine to ''prevent decomposition'' of the aromatic amines analysed. It remains to be elucidated if this should be the reason why we did not detect β-naphtylamine in any specimen and if the addition of a scavenger could prevent a decomposition of analytes during storage. Furthermore, microbial degradation of aniline derivatives should be taken into account to explain the increased aniline values and the decreased amounts of the toluidines and 3,5-dichloroanilines [35,36].

In addition to the parameters investigated with our former method the new method allowed the quantification of *o*-anisidine, 3,4-dichloroaniline, 3- and 4-monochloroaniline, 4-bromoaniline, and *p*-isopropylaniline.

In our pilot study ($n=20$, new method) we did not detect 4-bromoaniline or *p*-isopropylaniline in any specimen. In addition to the mentioned parameters we could quantify the aromatic amines *o*-anisidine, 3,4-dichloroaniline, 3- and 4-monochloroaniline in several specimens. The results are given in Table 4.

The sources of this inner burden are rather unknown. But it can be assumed that the exposure is widespread. In the case of aniline smoking habits definitely influences the urinary concentration [25], but only incrementarily. Aniline and the toluidines may have their origin in airborne ubiquitous contamination; in addition *m*-T is a metabolite of phenmedipham [27] and *p*-T a metabolite of tolylfluanid. Recently aniline and the toluidines have been found in indoor and outdoor air in Italy by Palmiotto et al. [9]. This working group detected indoor aniline in concentrations from 53 ng/m^3 (office of non-smokers) up to 2 μg/m^3 (discotheque), but these values were not related to smoking. Outdoor concentrations of aniline have also been measured in different urban as well as in rural areas of Italy (3–400 ng/m^3), whereas aniline concentration was extremely variable. Additionally, Palmiotto et al. detected toluidines in various concentrations in indoor and outdoor air, but definite toluidine values were not given in this publication. Finally the uptake of nitrobenzene also results in elevated urinary aniline concentrations.

Another source may be the exposure to pesticides via diet [28,29], colourants in food or textiles [28,30]. With the exception of *o*-anisidine all aromatic amines quantified in the general population are also metabolites of different pesticides. Some of these pesticides were periodically detected in different foods [29]. Examples of pesticides that may release the detected aromatic amines or metabolites during the hydrolysis in the sample treatment are given in Table 5. *o*-Anisidine is used as an intermediate for a number of azo and naphthol pigments and dyes which are used for printing (90%) and for paper (3%) and textile (7%) dyeing. It may be released from textiles and leather goods coloured with those azo dyes.

However, it is beyond the scope of this article to discuss in detail all possible sources of environmental exposure which may cause the baseline excretion of aniline, the toluidines, *o*-anisidine and the chlorinated anilines.

Table 5
Pesticides and their possible metabolites [31–34]

Possible metabolite	Pesticide
Aniline	Carboxin, carbetamid, chlorbromuron, desmedipham, dichlofluanid, fenfuram, fenuron, pencycuron, propachlor, propham, siduron
m-Toluidine	Phenmedipham
p-Toluidine	Tolylfluanid
3,5-Dichloroaniline	Chlozolinate, iprodion, procymidone, vinclozolin
3-Chloroaniline	Barbam, chlorpropham, chlorbufam, dimefuron
4-Chloroaniline	Buturon, diflubenzuron, dimilin, monulinuron, monuron, urox
Isopropylaniline	Isoproturon
4-Bromoaniline	Metobromuron
3,4-Dichloroaniline	Benzoylprop-ethyl, diuron, linuron, neburon, propanil

4. Conclusions

We presented a precise, accurate, specific, easy to handle and very sensitive method for the quantitative determination of various aromatic amines in urine samples using mass-selective detection after capillary gas chromatography. Within-series and between-day imprecision as well as recovery were found to be good. Merely the analytes that contain a nitro function showed lower performance regarding the analytical reliability criteria. The method proved to be practicable in routine analysis. The excellent limit of detection (down to 50 ng/l) enables the biological monitoring of 16 aromatic amines in urine of persons occupationally as well as environmentally exposed.

Applying this method we found that the general population renally excretes aniline, all isomers of toluidine, *o*-anisidine, and 3,4- and 3,5-dichloroaniline, 3- and 4-monochloroaniline. α- and β-naphtylamine, 4-aminodiphenyl and metabolites of explosives or certain pesticides (e.g., *p*-isopropylaniline, 4-bromoaniline) have not been detected in the urine of the general population.

Sources of this inner burden are widespread. Active and passive tobacco smoking (inhalative), uptake of intact pesticides or their metabolites via diet are possible routes of incorporation (oral). The release from (azo) dyes in textiles, leather or other subjects must also be taken into consideration (dermal).

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