SOLUTIONS BY LC Tech

Determination of Aromatic Amines from Azo Colorants According to DIN EN ISO 17234-1

Dr. Hans Rainer Wollseifen (MACHEREY-NAGEL)

Content

1. Introduction

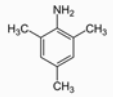
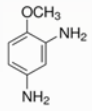
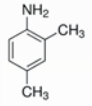
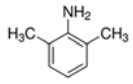
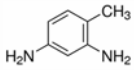
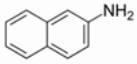
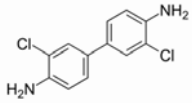
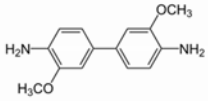
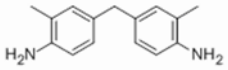
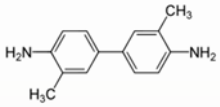
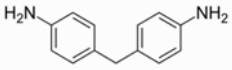
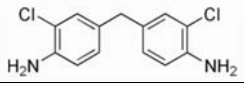
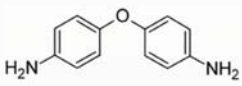
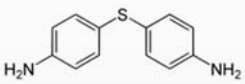
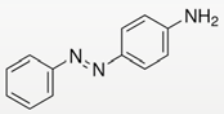
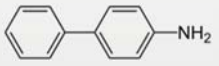
Azo-dyes are characterized by the azo-group-N=N-(nitrogen bond). They are formed through the linking of arylamines and can easily be modified chemically in order to stain typical commodities such as clothing, toys, and shoes.

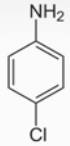
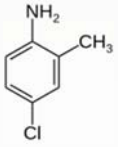
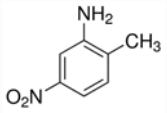
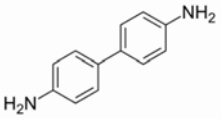
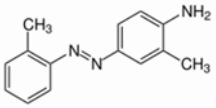
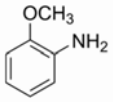
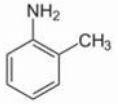
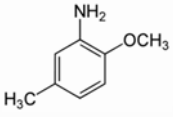
As azo-dyes are in particular colour- and lightfast, they are still very popular in the textile industry for products made of e.g. wool, silk, cotton, polyester and leather, although their use is illegal in many countries.

If the human body gets in contact with azo-dye molecules through skin contact or even oral contact, they get absorbed and degrade under reductive conditions to release any of a group of defined aromatic amines. The absorption rate after oral uptake, especially for infants, that suck on textiles or other dyed products, is much higher than after skin contact.

There is the suspicion, that all azo-dyes, which are cleaved to aromatic amines under reductive conditions, have a carcinogenic potential.

Table 1: Analysed aromatic amines

Analyte	Chemical Formula	Molar Mass [g/mol]	Structural Formula	CAS No.
2,4,5-Trimethylaniline	$C_9H_{13}N$	135,2		137-17-7
2,4-Diaminoanisole	$C_7H_{10}N_2O$	138.2		615-05-4
2,4-Dimethylaniline	$C_8H_{11}N$	121.2		95-68-1
2,6-Dimethylaniline	$C_8H_{11}N$	121.2		87-62-7
2,4-Diaminotoluene	$C_7H_{10}N_2$	122.2		95-80-7
2-Naphthylamine	$C_{10}H_9N$	143.2		91-59-8
3,3-Dichlorobenzidine	$C_{12}H_{10}Cl_2N_2$	253.1		91-94-1
3,3-Dimethoxybenzidine	$C_{14}H_{16}N_2O_2$	244.3		119-90-4
3,3-Dimethyl-4,4-diaminodiphenylmethane	$C_{15}H_{18}N_2$	226.3		838-88-0
3,3-Dimethylbenzidine	$C_{14}H_{16}N_2$	212.3		119-93-7
4,4-Diaminodiphenylmethane	$C_{13}H_{14}N_2$	198.3		101-77-9
4,4-Methylene-bis (2-chloraniline)	$C_{13}H_{12}Cl_2N_2$	267.2		101-14-4
4,4-Oxydianiline	$C_{12}H_{12}N_2O$	200.2		101-80-4
4,4-Thiodianiline	$C_{12}H_{12}N_2S$	216.3		139-65-1
4-Aminoazobenzene	$C_{12}H_{11}N_3$	197.2		60-09-3
4-Aminobiphenyl	$C_{12}H_{11}N$	169.2		92-67-1

Analyte	Chemical Formula	Molar Mass [g/mol]	Structural Formula	CAS No.
4-Chloroaniline	$ClC_6H_4NH_2$	127.6		106-47-8
4-Chloro-o-toluidine	C_7H_8ClN	141.6		95-69-2
5-Nitro-o-toluidine	$C_7H_8N_2O_2$	152.2		99-55-8
Benzidine	$C_{12}H_{12}N_2$	184.2		92-87-5
o-Aminoazotoluene	$C_{14}H_{15}N_3$	225.3		97-56-3
o-Anisidine	C_7H_9NO	123.2		90-04-0
o-Toluidine	C_7H_9N	107.2		95-53-4
p-Cresidine	$C_8H_{11}NO$	137.2		120-71-8

2. Method Development

2.1 Reagents and Materials

- Analytical standards of amines (CAS-Number)
  - 4-Aminobiphenyl (92-67-1)
  - Benzidine (92-87-5)
  - 4-Chloro-2-methylaniline (95-69-2)
  - 2-Naphthylamine (91-59-8)
  - o-Aminoazotoluene (97-56-3)
  - 5-Nitro-o-toluidine (99-55-8)
  - 2,4-Diaminoanisole (615-05-4)
  - 4,4-Diaminodiphenylmethan (101-77-9)

  - 3,3-Dichlorobenzidine (91-94-1)
  - o-Dianisidine (119-90-4)
  - o-Tolidine (119-93-7)
  - 4,4-Methylene-di(o-toluidine) (838-88-0)
  - 2-Methoxy-5-methylaniline (120-71-8)
  - 4,4-Methylene-bis(2-chloroaniline) (101-14-4)
  - 4,4-Diaminodiphenyl (101-80-4)
  - 4,4-Thiodianiline (139-65-1)
  - o-Toluidine (95-53-4)
  - 2,4-Diaminotoluene (95-80-7)
  - 2,4,5-Trimethylaniline (137-17-7)
  - o-Anisidine (90-04-0)
  - 4-Aminoazobenzene (60-09-3)
  - 2,4-Dimethylaniline (95-68-1)
  - 2,6-Dimethylaniline (87-62-7)
- Mixture of amines for methodology control, 25 µg/mL of each amines in methanol
- Methyl tert-butyl ether (MTBE)
- CHROMABOND® XTR, alkaline activated cartridges (15 mL, 3.0 g)
- Kieselguhr phase for liquid-liquid extraction (15 mL, 3.0 g)
- Methanol
- Sodium dithionite
- n-Hexane
- Citrate buffer solution, 0.06 mol/L, pH = 6.0, preheated up to 70 ± 5 °C
- Methanolic sodium hydroxide solution, 20 %

2.3 Sample Preparation

The sample preparation should be performed with n-hexane for leather samples according to the degreasing process of the DIN 17234-1:2015. After that, the sample would be extracted with preheated citrate buffer for 25 minutes in a waterbath at 70 C.

The reductive cleavage of azo colorants to amines would be achieved with dithionite solution in heat (70 °C) for ten minutes and would be stopped by cooling with cold water.

In the following a liquid-liquid extraction of the sample extract is performed on Kieselguhr phase with MTBE. After sample application on the Kieselguhr column the leather residue should be washed with a methanolic sodium hydroxide solution and MTBE and the washing mixture should also be applicated onto the Kieselguhr column.

For this application note we started with the control of the analytic system as described in point 9.4 within DIN 17234-1:2015.

2.4 Instrumentation

2.4.1 FREESTYLE System

The FREESTYLE system consists of the xyz-robotic platform FREESTYLE BASIC, equipped with the SPE as well as the EVAporation module. Additionally a HPLC Direct Injection module may be directly connected with any brand of HPLC system.

In the following the required items for a processing are listed together with their corresponding part numbers.

1.	1 x FREESTYLE BASIC, 6 solvents	P/N	12663-12
2.	1 x FREESTYLE SPE Module	P/N	12668
3.	1 x FREESTYLE EVAporation Module	P/N	13841
4.	1 x HPLC Direct Injection Module (for UHPLC)	P/N	16278
5.	1 x Sample Loop, stainless steel, 20 µL	P/N	14485
6.	3 x Reusable Needles, stainless steel	P/N	13382
7.	1 x Chiller/circulating cooler	P/N	12060
8.	3 x Frame, 100 mm	P/N	11915
9.	2 x Tray for 30 Pcs., 16 mL vials	P/N	11933
10.	1 x Tray, 60 positions, 1 mL vials	P/N	11920
11.	1 x Injection Vials, 1 mL	P/N	V0001
12.	1 x Crimp Seal R11 for 1 mL	P/N	V0001-B
13.	1 x Screw-Thread Vial, 16 mL	P/N	V0016
14.	1 x Screw Cap with Hole, black for 16 mL vials	P/N	V0016-SL
15.	1 x Seal G18 for 16 mL Vials	P/N	V0016-D
16.	2 x Special Rack for up to 18 SPE Columns	P/N	13946
17.	4 x Column Adapter for 15 mL SPE Cardridges (10 pcs/pck)	P/N	13414
18.	4 x Reusable Caps for Standard 15 mL Cartridges (10 pcs/pck)	P/N	13392

2.3 Analytical Set-up for HPLC-MS/MS

2.4.2 HPLC System and Settings

- Infinity II 1290 Agilent (Modules G7116B, G7167B, G7120A)
- API 5500 Triple Quad, Turbo Spray (ESI)
- Scan type: SMRM
- MRM detection window: 60 sec
- Polarity: positive
- Curtain gas: 35 psig
- Ion spray voltage: 5500 V
- Temperature: 450 °C
- Gas 1 (nebulizer): 45 psig
- Gas 2 (turbo gas): 45 psig
- CAD gas: medium

2.4.3 Chromatographic Conditions

- Column: EC 100/3 NUCLEODUR® π2, 3.0 µm (REF 760636.30)
- Eluent A: 0.1 % Formic acid in water
- Eluent B: 0. 1% Formic acid in methanol
- Gradient: in 10 min from 5 % to 95 % B, hold for 4.0 min, in 1 min to 5 % B, hold 5 % B for 5 min
- Flow rate: 0.56 mL/min
- Temperature: 35 °C
- Injection volume: 20 µL (Concentration: 5 ng/mL in water each amine)

2.4 Analytical Set-up for HPLC-UV

2.4.4 HPLC System and Chromatographic Conditions

- Ultimate 3000 Thermo scientific
- Column: EC 150/4.6 NUCLEODUR® Sphinx RP, 3.0 µm (REF 760805.46)
- Eluent A: 0,575 g Ammonium dihydrogen phosphate + 0,7 g Sodium
- Hydrogen phosphate in water, pH = 6.9
- Eluent B: Methanol
- Gradient: from 10% B in 40 min up to 50 % B, in 20 min up to 95 % B für, from 95 % down to 10 % B in 5 mi, hold 10 % B for 10 min
- Flow rate: 1.00 mL/min
- Temperature: 40 °C
- Injection volume: 20 µL (Concentration: 25 µg/mL in methanol/water (1 + 1, v + v))
- Detection: 240 nm, 280 nm, 305 nm

2.5 Software Protocol

In the following, the FREESTYLE method protocol for the SPE as well as the evaporation step (EVA) is shown. It was used for the CHROMABOND® XTR, alkaline activated cartridges and LC-MS/MS detection.

		SPE Column: 15mL_mit_	
Extension cannula:		yes	
Processing speed selection:		Standard (organic solvents)	
Rinsing intensity:		Standard rinsing cycle	wo. Extra Cleaning after Load
Use pressure limitation function during loading and washing:		no	
Step: Load		Basic type: Load - Transfer Sample-Aliquot over sample loop	Step: - ID: 892
Volume: 4 ml	Suction Speed: 10 ml/min	Dispensing Speed: 10 ml/min	
Vial Type: Type1@60	Waiting Time after Dosage: 300 sec.	Waiting Time after Step: 600 sec.	
			Dispense: into Waste
without rinsing of vial			
Step: Eluting_MTBE		Basic type: Eluting	Step: - ID: 893
Volume: 4.4 ml	Suction Speed: 20 ml/min	Dispensing Speed: 10 ml/min	Port: 7 MTBE
	Repetitions: 4		
	Waiting Time after Dosage: 30 sec.	Waiting Time after Step: 30 sec.	
			Dispense: direct into chamber

EVA:	Temperature water heating 35 °C		Temperature bottom cone 40 °C
	Sample input: Online from GPC or SPE process		
	Batch volume = limit from where concentration starts: 5 ml (fix) + Waiting time: 0 min.		
	Vacuum during GPC online sample input: 200 mbar		
	Phase 1: Concentrate to level: 1 ml		
	Vacuum absolute: 500 mbar		
	Rinsing volume after phase 1: 2 ml	Rinsing steps: 1 x	Solvent from Port: 7 MTBE
	Phase 2: Concentrate to level: 1 ml		
	Vacuum absolute: 250 mbar		
	Rinsing volume after phase 2: 2 ml	Rinsing steps: 1 x	Solvent from Port: 1 methanol
	Time control for vacuum process: no		
	to dryness: no		
	Nitrogen blow-down: no		
	Remove Aliquot: no		
	Solvent exchange: yes		
	At reach of level: 3 ml		
	Solvent addition per exchange: 1 ml	Solvent from Port: 1 methanol	
	Number of solvent exchanges: 3	End volume after exchange: 0.5 ml	
	Vacuum starts at level: 250 mbar abs.	Gradient of vacuum : 0 mbar/min	Vacuum end at level: 250 mbar abs.
	Rinsing, filling up, mixing and transfer into vials:		
	Rinsing volume at the end: no		
	Fill up to volume: 1 ml		Way of mixing: with gas / air, Volume = 2 ml
	of Port: 8 water		
	Concentrate: into vials / Direct Injection HPLC		
	Nr.: 1 1 [each]	Type: Type1@1 ml	Volume per vial: 0.8 ml
	Fill Quantitativ: no		
	1. Cleaning cycle		
	Rinsing volume: 5 ml	Rinsing steps: 2 x	Solvent from Port: 7 MTBE
	2. Cleaning cycle		
	Rinsing volume: 5 ml	Rinsing steps: 1 x	Solvent from Port: 1 methanol

Figure 1: Method report for automated FREESTYLE SPE-EVAporation

3. Results

3.1 Recovery Rates

The recovery rates were achieved with the LC-MS/MS approach.

Table 2: Recovery data for automated analysing of the 23 amines using CHROMABOND® XTR, alkaline activated

Analyte	CAS No.	Recovery Rate with CHROMABOND® XTR Alkaline Activated (15 mL, 3.0 g) in %	Standard Deviation in %	Recovery Rate Required in ISO 17234-1:2015-07
4-Aminobiphenyl	92-67-1	83.67	4.34	70.00
Benzidine	92-87-5	78.33	5.93	70.00
4-Chloro-2-methylaniline	95-69-2	109.04	3.53	70.00
2-Naphthylamine	91-59-8	85.95	4.76	70.00
o-Aminoazotoluene	97-56-3	82.49	0.94	70.00
5-Nitro-o-toluidine	99-55-8	84.03	5.17	70.00
2.4-Diaminoanisole	615-05-4	82.19	17.25	20.00
4.4-Diaminodiphenyl-methan	101-77-9	85.30	5.56	70.00
3.3-Dichlorobenzidine	91-94-1	83.23	3.09	70.00
o-Dianisidine	119-90-4	105.43	4.38	70.00
o-Tolidine	119-93-7	83.68	4.93	70.00
4.4-Methylene-di (o-toluidine)	838-88-0	82.47	5.39	70.00
2-Methoxy-5-methylaniline	120-71-8	107.65	2.30	70.00
4.4-Methylene-bis (2-chloroaniline)	101-14-4	82.25	4.04	70.00
4.4-Diaminodiphenyl	101-80-4	82.73	5.16	70.00
4.4-Thiodianiline	139-65-1	78.77	2.66	70.00
o-Toluidine	95-53-4	131.92	3.65	50.00
2.4-Diaminotoluene	95-80-7	109.52	1.69	70.00
2.4.5-Trimethylaniline	137-17-7	85.33	7.43	50.00
o-Anisidine	90-04-0	115.02	2.08	70.00
4-Aminoazobenzene	60-09-3	82.86	4.14	70.00
2.4-Dimethylaniline	95-68-1	123.81	4.60	50.00
2.6-Dimethylaniline	87-62-7	132.13	5.54	50.00

Table 3: Recovery data for automated analysing system of the 23 amines using Kieselguhr phase

Analyte	CAS No.	Recovery Rate with Kieselguhr Phase	Standard Deviation in %	Recovery Rate Required in ISO 17234-1:2015-07
4-Aminobiphenyl	92-67-1	74.50	4.59	70.00
Benzidine	92-87-5	85.33	3.75	70.00
4-Chloro-2-methylaniline	95-69-2	40.52	8.11	70.00
2-Naphthylamine	91-59-8	66.73	5.23	70.00
o-Aminoazotoluene	97-56-3	70.93	4.47	70.00
5-Nitro-o-toluidine	99-55-8	74.01	5.48	70.00
2.4-Diaminoanisole	615-05-4	64.47	18.39	20.00
4.4-Diaminodiphenylmethan	101-77-9	74.09	5.52	70.00
3.3-Dichlorobenzidine	91-94-1	72.82	4.10	70.00
o-Dianisidine	119-90-4	60.47	6.05	70.00
o-Tolidine	119-93-7	76.28	5.31	70.00
4.4-Methylene-di (o-toluidine)	838-88-0	77.86	4.60	70.00
2-Methoxy-5-methylaniline	120-71-8	44.02	7.94	70.00
4.4-Methylene-bis (2-chloroaniline)	101-14-4	76.95	4.28	70.00
4.4-Diaminodiphenyl	101-80-4	76.89	4.82	70.00
4.4-Thiodianiline	139-65-1	71.03	9.33	70.00
o-Toluidine	95-53-4	30.08	9.70	50.00
2.4-Diaminotoluene	95-80-7	41.34	7.16	70.00
2.4.5-Trimethylaniline	137-17-7	82.37	19.25	50.00
o-Anisidine	90-04-0	39.13	7.49	70.00
4-Aminoazobenzene	60-09-3	77.54	4.48	70.00
2.4-Dimethylaniline	95-68-1	32.72	8.74	50.00
2.6-Dimethylaniline	87-62-7	29.73	10.10	50.00

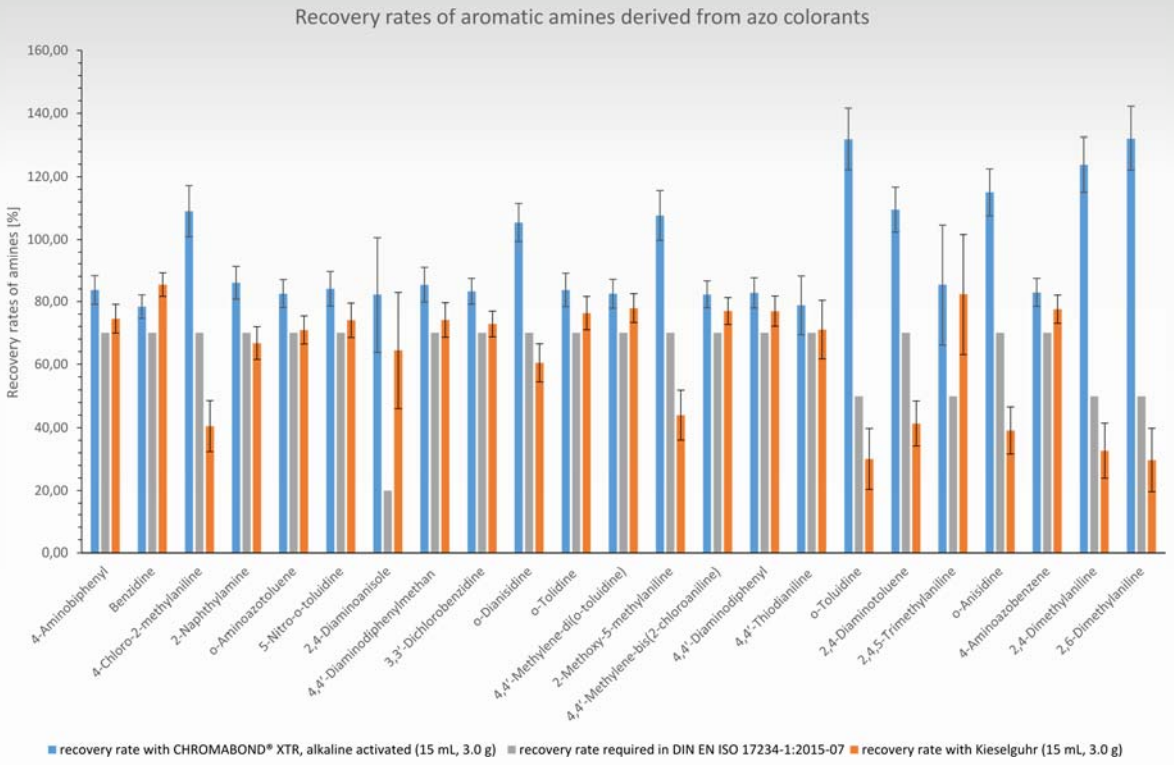


Figure 2: Comparison of recovery rates

3.2 Chromatograms

In Fig. 3 an exemplary LC-MS/MS chromatogram of the amine mix under the given chromatographic conditions with 23 compounds is shown.

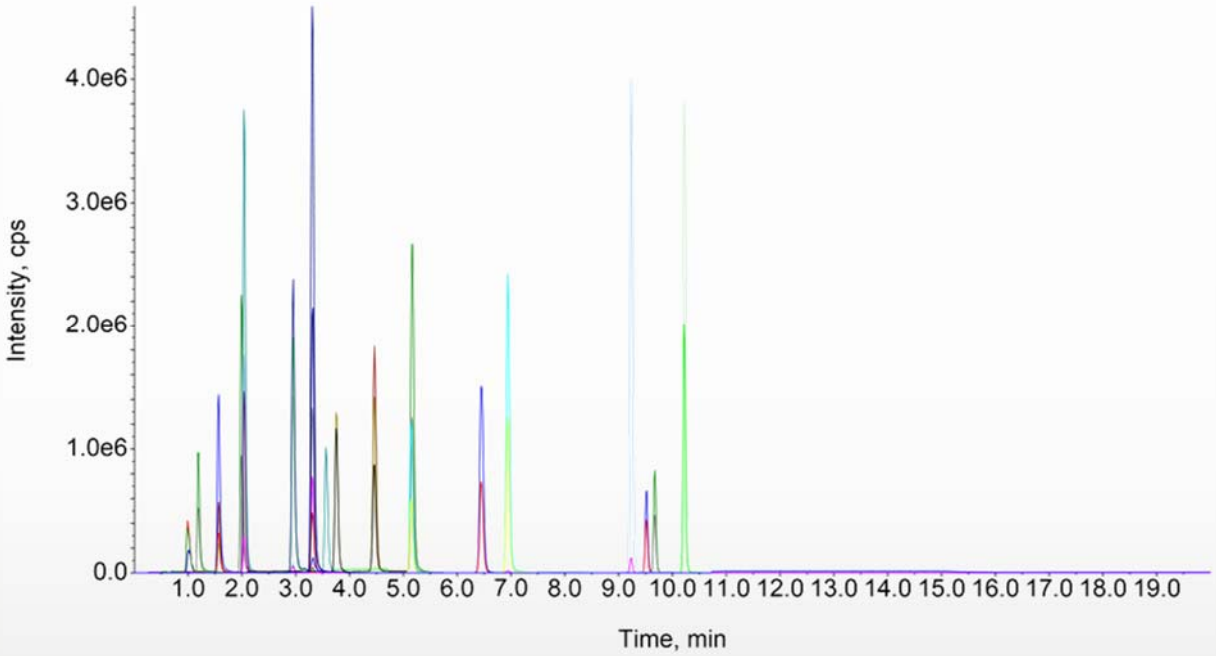


Figure 3: Chromatogram of 23 amines

Table 4: LC-MS/MS parameters

Number	Name	CAS-Number	RT (Min)	[M+H]+	Q1	Q2
1	4-Aminobiphenyl	92-67-1	6.97	170.1	152.1	153.0
2	Benzidine	92-87-5	1.60	185.0	99.9	167.1
3	4-Chloro-2-methylaniline	95-69-2	6.52	142.0	107.1	106.1
4	2-Naphthylamine	91-59-8	5.23	144.1	127.1	77.0
5	o-Aminoazotoluene	97-56-3	10.24	226.1	91.1	121.1
6	5-Nitro-o-toluidine	99-55-8	6.97	151.0	77.0	98.1
7	2,4-Diaminoanisole	615-05-4	2.59	139.1	124.1	93.0
8	4,4′-Diaminodiphenylmethan	101-77-9	1.64	199.1	106.1	77.1
9	3,3′-Dichlorobenzidine	91-94-1	9.53	253.0	216.9	181.9
10	o-Dianisidine	119-90-4	3.80	245.1	230.0	186.9
11	o-Tolidine	119-93-7	2.07	213.1	180.0	123.1
12	4,4′-Methylene-di (o-toluidine)	838-88-0	3.41	227.1	120.1	77.0
13	2-Methoxy-5-methylaniline	120-71-8	3.29	138.1	123.1	65.0
14	4,4′-Methylene-bis (2-chloroaniline)	101-14-4	9.69	267.0	230.9	140.1
15	4,4'-Diaminodiphenyl	101-80-4	1.25	201.1	108.1	80.1
16	4,4′-Thiodianiline	139-65-1	5.24	217.09	124.1	80.0
17	o-Toluidine	95-53-4	2.07	108.1	91.1	93.1
18	2,4-Diaminotoluene	95-80-7	3.41	123.1	106.0	77.1
19	2,4,5-Trimethylaniline	137-17-7	4.21	136.2	121.2	91.0
20	o-Anisidine	90-04-0	2.10	124.1	109.1	80.1
21	4-Aminoazobenzene	60-09-3	9.24	198.1	77.1	93.1
22	2,4-Dimethylaniline	95-68-1	4.55	122.1	107.1	77.0
23	2,6-Dimethylaniline	87-62-7	3.07	122.1	107.1	77.0

In Fig. 4 an exemplary LC-UV chromatogram of the amine mix under the given chromatographic conditions with 19 compounds is shown.

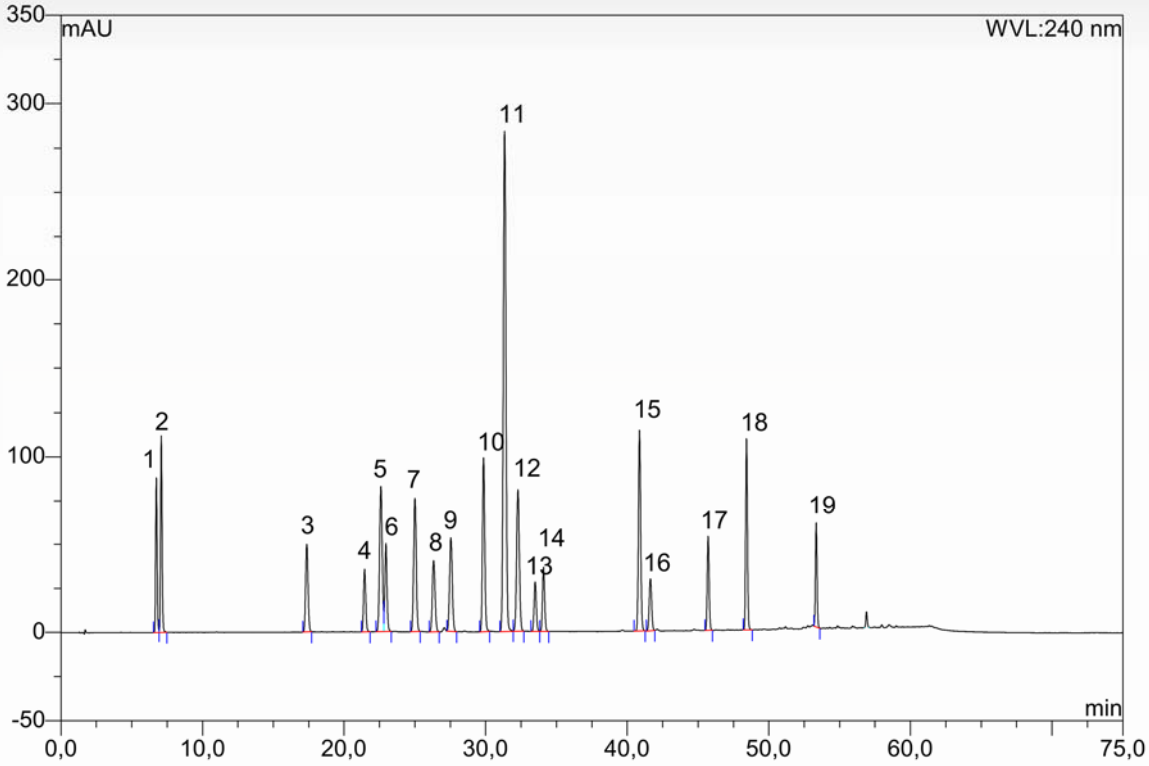


Figure 4: UV-chromatogram of 19 amines, detection wavelength 240 nm

Table 5: LC-UV parameters

Number	Name	CAS-Number	RT (Min)
1	2,4-Diaminotoluene	95-80-7	6.74
2	2,4-Diaminoanisole	615-05-4	7.09
3	o-Toluidine	95-53-4	17.36
4	Benzidine	92-87-5	21.45
5	4,4-Diaminodiphenyl	101-80-4	22.60
6	4-Chloroaniline	106-47-8	22.95
7	5-Nitro-o-toluidine	99-55-8	25.00
8	2,6-Dimethylaniline	87-62-7	27.54
9	2,4-Dimethylaniline	95-68-1	26.33
10	4,4-Diaminodiphenylmethan	101-77-9	29.85
11	2-Naphthylamine	91-59-8	31.33
12	4-Chloro-2-methylaniline	95-69-2	32.28
13	o-Tolidine	119-93-7	33.49
14	o-Dianisidine	119-90-4	34.09
15	4,4-Methylene-di(o-toluidine)	838-88-0	40.86

Number	Name	CAS-Number	RT (Min)
16	4-Aminobiphenyl	92-67-1	41.63
17	4-Aminoazobenzene	60-09-3	45.71
18	4,4-Methylene-bis(2-chloroaniline)	101-14-4	48.42
19	o-Aminoazotoluene	97-56-3	56.89

4. Conclusion

In this application note, aromatic amines were tested on two different analytical systems and with two different cartridges.
Both approaches showed in general good recoveries and standard deviation, whereupon the combination of the CHROMABOND® XTR, alkaline activated cartridge and LC-MS/MS detection achieved the best results.

The recoveries are far beyond the recovery rates, that are required in DIN EN ISO 17234-1 and also the standard deviation is < 10 % except one problematic amine.
As seen in Fig. 2, the recoveries are much higher than with the Kieselguhr phase. In addition to that, the LC-MS/MS analysis detected 23 of the 24 aromatic amines.
The usage of the CHROMABOND® XTR, alkaline activated cartridge is also more user-friendly, because the normal Kieselguhr phase still has to be activated manually and has an additional conditioning step.

As the system can work fully unattended over night or during the weekend it is a great support for any routine lab. In combination with the HPLC Direct Injection module, the level of automation is extremely high, as the user just has to put the extracts into the FREESTYLE system and gets the chromatograms in the end without any intermediate manual step.

Contact

LCTech GmbH
Daimlerstraße 4
84419 Obertaufkirchen
Germany

Tel.: +49 8082 2717-0
Fax: +49 8082 2717-100
E-Mail: info@LCTech.de

www.LCTech.de
www.LCTech-online.com

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