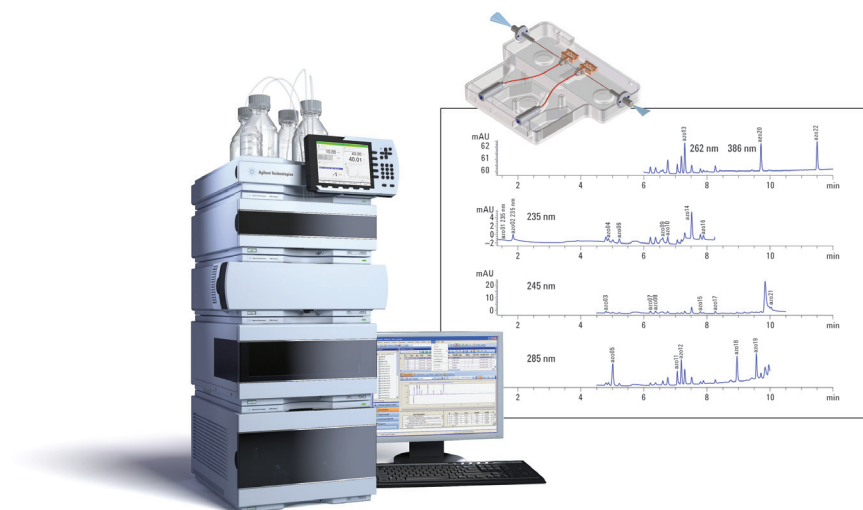


# High Sensitivity UHPLC-DAD Analysis of Azo Dyes using the Agilent 1290 Infinity LC System and the 60 mm Max-Light High Sensitivity Flow Cell

## Application Note

Consumer Products



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### Abstract

In this Application Note, a set of toxic aromatic amines which may be released from certain banned azo colorants are analyzed with the Agilent 1290 Infinity LC System. A 1290 Infinity Diode Array Detector equipped with the Agilent Max-Light high sensitivity flow cell with a 60 mm optical path length is used to obtain highest sensitivity. The results are compared to results obtained with a standard flow cell (10 mm optical path length) and the performance of the high sensitivity method is investigated.



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## Introduction

Azo dyes are colorants widely used in consumer products such as leather, textiles, and cosmetics. These products contain an azo group that can undergo reductive cleavage, leading to the formation of aromatic amines of which have known mutagenic and/or carcinogenic properties. The use of certain azo dyes is prohibited in Europe, US, and many other countries. In the European Union a directive of 2002 describes the restrictions on the marketing and use of certain azo dyes<sup>1</sup> and official analytical methods have been published<sup>2,3</sup>. The directive defines a limit value of 30 ppm (mg/kg sample) for a set of 22 potentially carcinogenic amines. If the detected amount is above this value, it is assumed that a certain azo colorant was used.

The determination of azo dyes involves a chemical reduction of the dye into the amines followed by HPLC-DAD or LC/MS analysis. Although LC/MS is a suitable technique to determine these amines at low levels<sup>4</sup>, the use of DAD for this analysis is still widespread. This is mainly due to the lower cost of purchase and operation compared to MS instrumentation. The absence of the mass selectivity of an MS system necessitates the complete chromatographic separation of all compounds under investigation. This is not straightforward within an acceptable analysis time and a recent application note describes the use of the Agilent Method Development System and the Agilent Method Scouting Wizard software to develop and optimize the separation<sup>5</sup>.

The results describe the application of the previously developed method with the Agilent 1290 Infinity Diode Array Detector (DAD) equipped with a high sensitivity flow cell to perform trace analysis of the 22 restricted amines. Detection limits are typically around 0.2-1 ng/mL for standard solutions. This is significantly lower than the requested quantification limit to meet the European regulations. Performance parameters such as repeatability of injection, detection limits, and linearity are evaluated.

## Experimental

### Standard solutions

A standard stock solution of 20 amines in acetonitrile (Azodyes-Mix 1, Dr. Ehrenstorfer, Augsburg, Germany) was mixed with a stock solution of azo compounds 04 and 20 (Sigma-Aldrich, Bornem, Belgium, see Table 1) to make up a 22 component standard mixture. This mixture was diluted in 0.1% formic acid in methanol/water 10/90 to the appropriate concentration.

Peak Code	Name	CAS no.	FW
AZO 01	4-Methoxy-1,3-phenylenediamine	615-05-4	138
AZO 02	2,4-Diaminotoluene	95-80-7	122
AZO 03	4-Aminophenylether	101-80-4	200
AZO 04	o-Anisidine	90-04-0	123
AZO 05	4,4'-Benzidine	92-87-5	184
AZO 06	o-Toluidine	95-53-4	107
AZO 07	Bis-(4-aminophenyl)-methane	83712-44-1	198
AZO 08	4-Chloroaniline	106-47-8	127
AZO 09	2-Methoxy-5-methylaniline	120-71-8	137
AZO 10	2-Methyl-5-nitroaniline	99-55-8	152
AZO 11	3,3'-Dimethoxybenzidine	119-90-4	244
AZO 12	3,3'-Dimethylbenzidine	119-93-7	212
AZO 13	4-Aminophenylthioether	139-65-1	216
AZO 14	2-Naphthylamine	91-59-8	143
AZO 15	4-Chloro-2-methylaniline	95-69-2	141
AZO 16	2,4,5-Trimethylaniline	137-17-7	135
AZO 17	4,4'-Diamino-3,3'-dimethyldiphenyl methane	838-88-0	226
AZO 18	4-Aminobiphenyl	92-67-1	169
AZO 19	3,3'-Dichlorobenzidine	91-94-1	252
AZO 20	4-Aminoazobenzene	60-09-3	197
AZO 21	4,4'-Methylene-bis(2-chloroaniline)	101-14-4	266
AZO 22	4-Amino-2',3-dimethylazobenzene	97-56-3	225

**Table 1**  
Investigated azo dye derived amines listed in the European Parliament and Council Directive No. 2002/61/EC.

## Results and discussion

In order to illustrate the influence of the two DAD flow cells a 200 ng/mL standard solution was analyzed with both configurations. Theoretically, the Agilent Max-Light Cartridge High Sensitivity Cell should increase the sensitivity by a factor of 5–6. Figure 1 clearly shows the gain in sensitivity with the longer optical path length.

## Equipment

An Agilent 1290 Infinity LC system with the following configuration was used:

G4220A	Agilent 1290 Infinity Binary Pump with integrated vacuum degasser
G4226A	Agilent 1290 Infinity Autosampler
G1330B	Agilent 1290 Infinity Thermostat
G1316C	Agilent 1290 Infinity Thermostatted Column Compartment
G4212A	Agilent 1290 Infinity Diode Array Detector
G4212-60007	Agilent Max-Light Cartridge High Sensitivity Cell (60 mm optical path length)
G4212-60008	Agilent Max-Light Cartridge Standard Cell (10 mm optical path length)

## Chromatographic Conditions

Method parameters:

Column: Agilent ZORBAX StableBond C18 RRHT, 100 mm L × 4.6 mm id, 1.8 μm d<sub>p</sub> (p/n 828975-902)

Mobile phase: A = 20 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 4.60  
B = methanol/acetonitrile 50/50 v/v

Flow rate: 1.6 mL/min

Gradient: 1–12 min 5–80% B  
12–12.1 min 80–98% B  
12.1–14 min 98% B  
14–15.5 min 5% B (post-time)

Temperature: 36 °C

Injection: 20 μL, needle wash (4 s, flushport, mobile phase B)

Detection DAD:

• Peak width >0.012 min (20 Hz)

• Wavelength

A=Time programmed

0–5.6 min Signal 210/5 nm, Reference off

5.6–8.6 min Signal 262/10 nm, Reference off

8.6–14 min Signal 386/15 nm, Reference off

B = Signal 235/20 nm, Reference off

C = Signal 245/10 nm, Reference off

D = Signal 285/30 nm, Reference off

• Spectra acquisition On, 190–400 nm

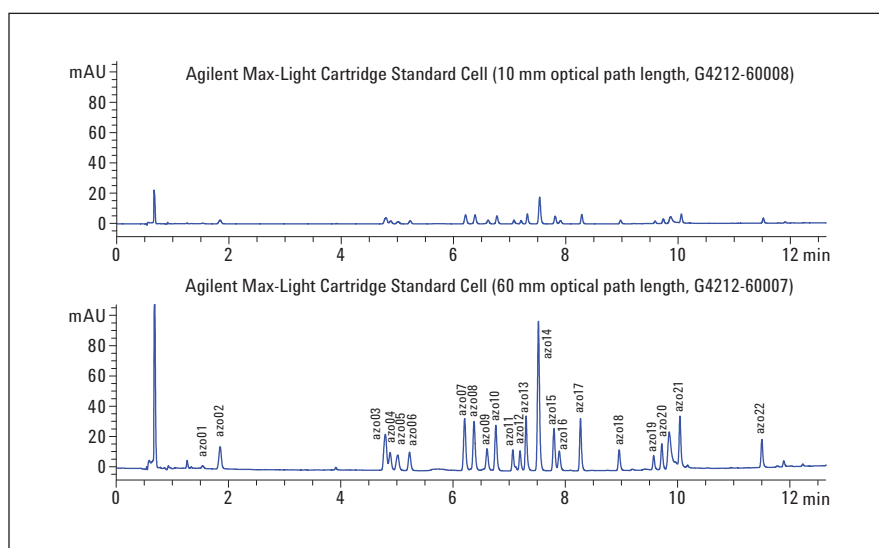
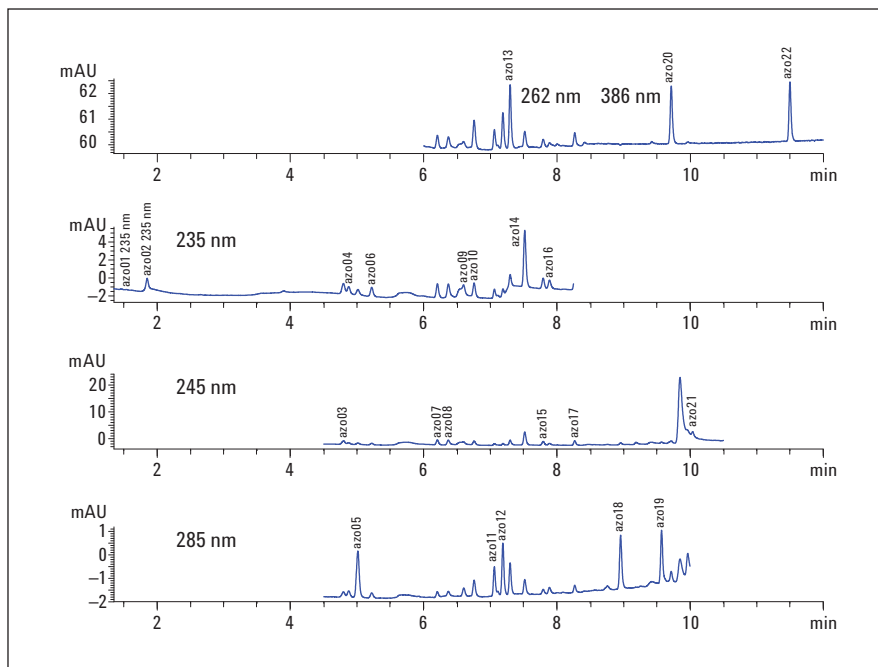


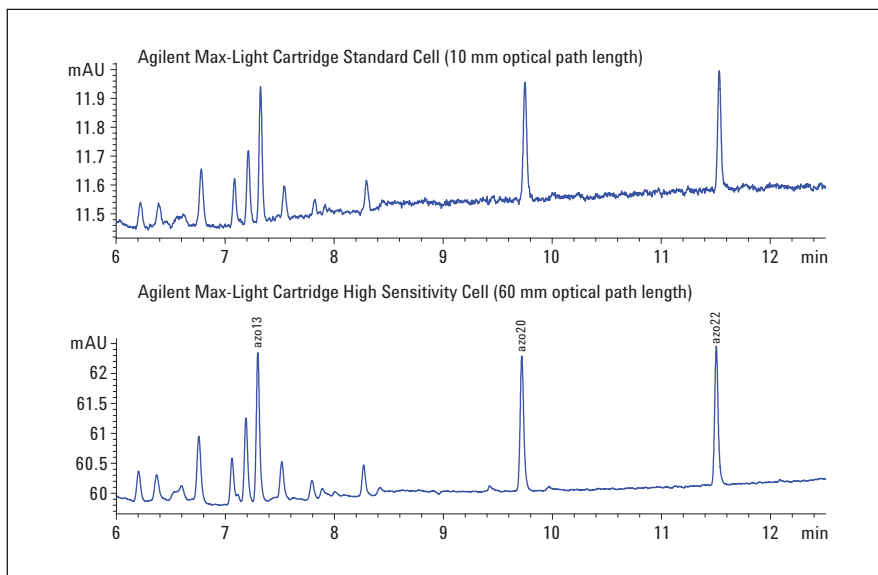
Figure 1

Comparison of the standard (10 mm) and high sensitivity (60 mm) flow cell for a 200 ng/mL standard mixture of the 22 azo derived amines. Detection wavelength: 245 nm.

The sensitivity of the method was further optimized by using various detection wavelengths for the specific amines. Channel A was time-programmed and three other channels were used to cover all 22 amines. The result for a low level standard (10 ng/mL) is shown in Figure 2. The large system peak is present at 245 nm close to the retention time of azo 21. This demonstrates that the quality of the solvents and material used with the high sensitivity cell is of utmost importance because interferences are enlarged in the same order as the analyte peaks. The influence of the flow cell at this concentration level is demonstrated for Channel A in Figure 3. The increased signal-to-noise ratio with the high sensitivity flow cell is obvious.



**Figure 2**  
Analysis of a 10 ng/mL standard mixture with the high sensitivity flow cell.



**Figure 3**  
Comparison of the channel A signal on the standard (10 mm) and high sensitivity (60 mm) flow cell for a 10 ng/mL standard mixture.

The calculated signal-to-noise ratios for both detection cells are summarized in Table 2.

	S/N 10 mm	S/N 60 mm	Detection WL
Azo 01	Not detected	4	235
Azo 02	11	38	235
Azo 03	15	55	245
Azo 04	9	26	235
Azo 05	22	50	285
Azo 06	10	32	235
Azo 07	21	74	245
Azo 08	22	74	245
Azo 09	14	44	235
Azo 10	16	51	235
Azo 11	17	31	285
Azo 12	27	55	285
Azo 13	19	109	262
Azo 14	64	195	235
Azo 15	17	58	245
Azo 16	10	30	235
Azo 17	21	71	245
Azo 18	26	58	285
Azo 19	26	58	285
Azo 20	17	99	386
Azo 21	17	59	245

**Table 2**  
**Comparison of signal-to-noise ratios obtained with the standard (10 mm) and high sensitivity (60 mm) flow cell for a 10 ng/mL standard mixture.**

The repeatability of injection of the developed method was investigated at two concentration levels (100 and 500 ng/mL) by six consecutive injections. The linearity was calculated by single injections of various standard solutions. Table 3 shows these data together with the detection limits obtained with both flow cells.

	DAD WL	Repeatability of injection, n = 6 (RSD%)			LOD (ng/mL)		Linearity	
		tR	Area 100 ng/mL	Area 500 ng/mL	10 mm	60 mm	Range (ng/mL)	R <sup>2</sup>
Azo 01	235	0.04	1.81	0.41	20	5	5-500	0.9887
Azo 02	235	0.03	0.31	0.05	5	1	2-500	0.9999
Azo 03	245	0.03	0.04	0.02	2	0.5	2-500	1.0000
Azo 04	235	0.03	0.44	0.06	2	0.5	2-500	0.9999
Azo 05	285	0.03	0.07	0.02	1	0.2	2-500	1.0000
Azo 06	235	0.03	0.05	0.06	1	0.5	2-500	0.9999
Azo 07	245	0.03	0.41	0.03	1	1	2-500	0.9999
Azo 08	245	0.03	1.86	0.06	1	1	2-500	0.9999
Azo 09	235	0.03	0.66	0.07	5	5 (1)	5-500	0.9995
Azo 10	235	0.03	0.37	0.03	1	0.2	2-500	0.9999
Azo 11	285	0.03	0.39	0.05	1	0.2	2-500	0.9989
Azo 12	285	0.03	0.37	0.03	1	0.2	2-500	1.0000
Azo 13	262	0.04	0.08	0.03	1	0.2	2-500	0.9999
Azo 14	235	0.03	0.18	0.02	5	0.2	2-500	1.0000
Azo 15	245	0.03	0.12	0.02	1	0.5	2-500	0.9999
Azo 16	235	0.03	0.29	0.06	1	0.5	2-500	0.9998
Azo 17	245	0.03	0.18	0.06	1	0.5	2-500	1.0000
Azo 18	285	0.03	0.14	0.14	1	0.2	2-500	0.9999
Azo 19	285	0.03	0.24	0.04	1	0.2	2-500	1.0000
Azo 20	386	0.03	0.18	0.06	1	0.2	2-500	1.0000
Azo 21	245	0.03	0.34	0.12	2	2 (1)	2-500	0.9999
Azo 22	386	0.02	0.12	0.11	1	0.2	2-500	1.0000

(1) Interfering peak

**Table 3**  
Performance of the developed method (60 mm flow cell unless specified otherwise).

## **Conclusion**

The use of the Agilent Max-Light High Sensitivity Cell in the Agilent 1290 Infinity DAD significantly increases the sensitivity for the azo colorant derived amines and enables the detection of levels as low as 0.2 ng/mL (4 pg on-column). The repeatability of injection and linearity of the method were acceptable and the improved sensitivity compared to the Agilent Max-Light Cartridge Standard Cell is demonstrated.

## **References**

1. European Parliament and Council Directive No. 2002/61/EC (19 July 2002).
2. European Standard No. EN 14362-1:2003 (October 2003).
3. European Standard No. EN 14362-2:2003 (October 2003).
4. S. S. Lateef, Agilent Technologies, Application Note 5990-5731EN (May 2010).
5. G. Vanhoenacker, F. David, P. Sandra, Application note method development system for Azo dyes, submitted

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