

Determination of unsulfonated aromatic amines in the color additives FD&C Yellow No. 5, FD&C Yellow No. 6, and D&C Red No. 33 using liquid chromatography/mass spectrometry

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Introduction

FD&C Yellow No. 5 (**Y5**, Tartrazine, trisodium salt of 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-4-[4-sulfophenyl-azo]-1H-pyrazole-3-carboxylic acid, **Figure 1**) is permitted for use in coloring foods, drugs, and cosmetics.¹

FD&C Yellow No. 6 (**Y6**, C.I. Food Yellow 3, disodium salt of 6-hydroxy-5-[(4-sulfophenyl)azo]-2-naphthalenesulfonic acid, **Figure 1**) is also permitted for use in coloring foods, drugs, and cosmetics.²

D&C Red No. 33 (**R33**, disodium salt of 5-amino-4-hydroxy-3-(phenylazo)-2,7-naphthalenedisulfonic acid, **Figure 1**) is permitted for use in coloring drugs and cosmetics.³

Y5, **Y6**, and **R33** are subject to batch certification by FDA to ensure compliance with their Code of Federal Regulations (CFR) listing requirements, which include ppm- and ppb-level specifications for aniline (**ANL**), benzidine (**BNZ**), 4-aminobiphenyl (**4-ABP**), and 4-aminoazobenzene (**4-AAB**) (**Table 1**).¹⁻³

Currently, the unsulfonated aromatic amines are determined by a labor-intensive method that includes chloroform extraction from the color additives, diazotization and coupling with 4,5-dihydro-5-oxo-1-(4-sulfophenyl)-1H-pyrazole-3-carboxylic acid, and analysis of the coupling products by reversed-phase high-performance liquid chromatography (HPLC).⁵

The present study reports the development of a new method for the direct determination of the unsulfonated aromatic amines in **Y5**, **Y6**, and **R33** following a one-step solid phase extraction (SPE). The analytes are separated by ultra-performance liquid chromatography (UPLC) and quantitated by tandem mass spectrometry (LC-MS/MS). 2-Aminobiphenyl (**2-ABP**) is used as an internal standard.

Preliminary results using the new LC-MS/MS method are presented for **Y5** and **Y6**. Work on **R33** is in progress.

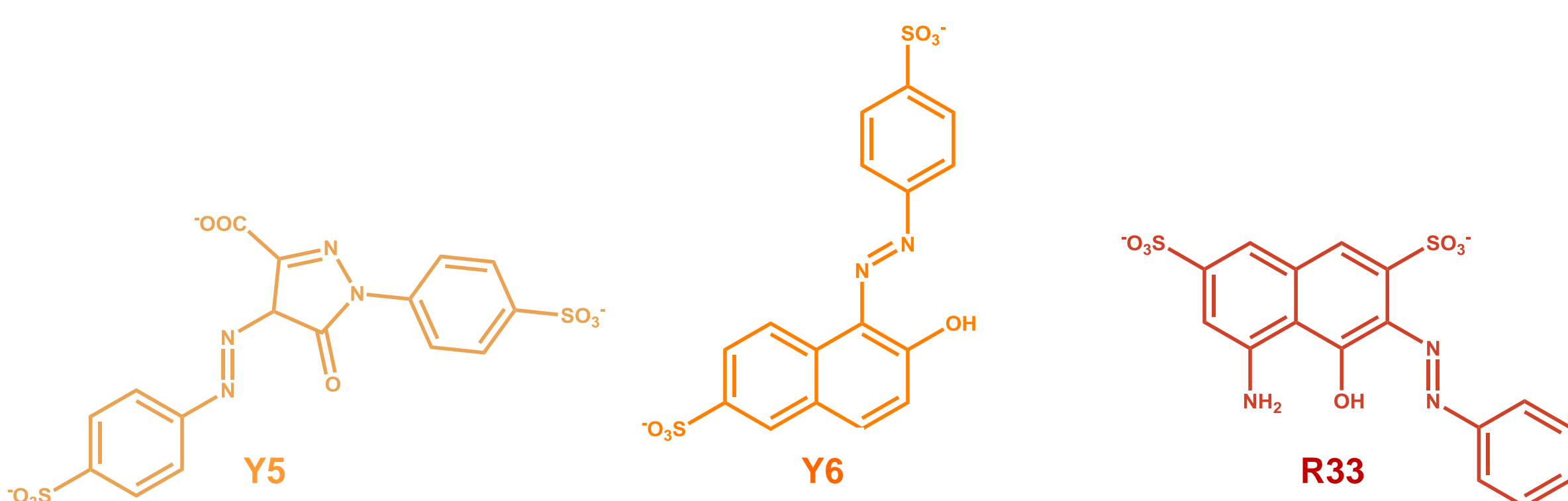


Figure 1 The three dye anions **Y5**, **Y6**, and **R33**.

Table 1 Unsulfonated aromatic amines, CFR specifications, and MS parameters used in the LC-MS/MS method.

Amines	CFR specifications (ppb)			Precurs or ions	Daughter ions	Cone voltage (eV)	Collision energy (eV)	Structure
	Y5	Y6	R33					
ANL	100	250	25x10 ³	94.0	77.0	40	15	
BNZ	1	1	20	184.9	168.0	45	18	
4-ABP	5	15	275	170.0	76.9	50	19	
4-AAB	75	50	100	198.0	153.0	38	18	
2-ABP	N/A			170.0	153.0	50	19	

Electrospray MS parameters

- Capillary voltage, 3 kV
- Source temperature, 125 °C
- Desolvation temperature, 450 °C
- Desolvation gas flow, 700 L/h
- Cone gas flow, 60 L/h
- Dwell time, 0.1 sec

Materials & Methods

Materials

- Strata SCX cation-exchange cartridges (500 mg/6mL)
- Whatman 0.2 µm PTFE syringeless filters
- SPE manifold system

Analytical reference materials:

- ANL** (99.8%)
- 4-AAB** (97.8%)
- BNZ** (99.9%)
- 2-ABP** (98%)
- 4-ABP** (99.9%)

LC-MS/MS system

- Waters Acquity UPLC system with tandem quadrupole (TQD) mass spectrometer
- XBridge Phenyl column (2.1x75 mm id, 2.5 µm particles)

Chromatography Conditions:

- Eluents: (A) 0.1% formic acid/water, pH ~ 2.7 (B) acetonitrile
- Gradient: Linear segments of 90-70% A in 1 min, 70-60% A in 0.1 min, 60-50% A in 1.9 min, 50-90% A in 0.1 min, hold at 90% A for 0.9 min
- Flow rate: 0.6 mL/min
- Column temperature: 40°C ± 5 °C
- Injection volume: 10 µl
- Run time: 4 min

Sample preparation

Calibration and internal standard solutions:

- 0.5 µg/mL **ANL**, 0.1 µg/mL **4-ABP** and **4-AAB**, and 0.01 µg/mL **BNZ** in acetonitrile
- 0.05 µg/mL of **2-ABP** in acetonitrile

Calibration standards:

- 2.0 g **Y5** matrix in 150 mL 5% formic acid/water spiked with 0.1, 0.2, 0.6, 0.8, 1.0, and 1.5 mL standard solutions
- 0.6 mL **2-ABP** also added

Survey samples:

- 2 g **Y5** and **Y6** samples in 150 mL 5% formic acid/water
- 0.6 mL **2-ABP** also added

SPE extraction

- Condition: 5 mL methanol
- Equilibrate: 5 mL 5% formic acid/water
- Load: 150 mL standard or sample solutions
- Wash: 5 mL 5% formic acid/water
- Wash: 5 mL water
- Wash: 5 mL 90% methanol/water
- Dry: 10 min under vacuum
- Elute cartridge with 3.0 mL 5% ammonium hydroxide/methanol into 5 mL volumetric flasks
- Add 100 µL conc. formic acid, diluted to volume with water, and filter into LC vials for LC-MS/MS analysis

Results and Discussion

ANL, **BNZ**, **4-ABP**, and **4-AAB** were quantified by using six-point calibration curves prepared according to the internal-standard procedure that involved analyzing separate test portions of a batch of **Y5** spiked with the analytes. The matrix batch had been previously found by the current method to be free of the analytes.

The data points ranged from 25-375 ppb for **ANL**, 0.5-10 ppb for **BNZ**, and 5-75 ppb for **4-ABP** and **4-AAB**. The instrument response was linear over these ranges (see **Figure 2**). The limit of detection (LOD) and the limit of quantification (LOQ), calculated from the calibration data, are shown in **Table 2**.

The precision of the method, expressed as relative standard deviation (RSD), was determined at three different concentration levels by analyzing three separately prepared solutions (see **Table 2**).

Figure 3 shows the multiple reaction monitoring (MRM) ion chromatograms of a **Y5** sample fortified with 30 ppb **ANL**, 3 ppb **BNZ**, 15 ppb **4-ABP**, 15 ppb **4-AAB**, and 15 ppb **2-ABP**.

Table 3 summarizes preliminary results obtained from analyses of **ANL**, **BNZ**, **4-ABP**, and **4-AAB** in four certified lots of **Y5** and **Y6** from two domestic (C, F) and ten foreign (A, B, D, E, G, and H) manufacturers. The results obtained by the LC-MS/MS method are compared with those obtained by the current method.

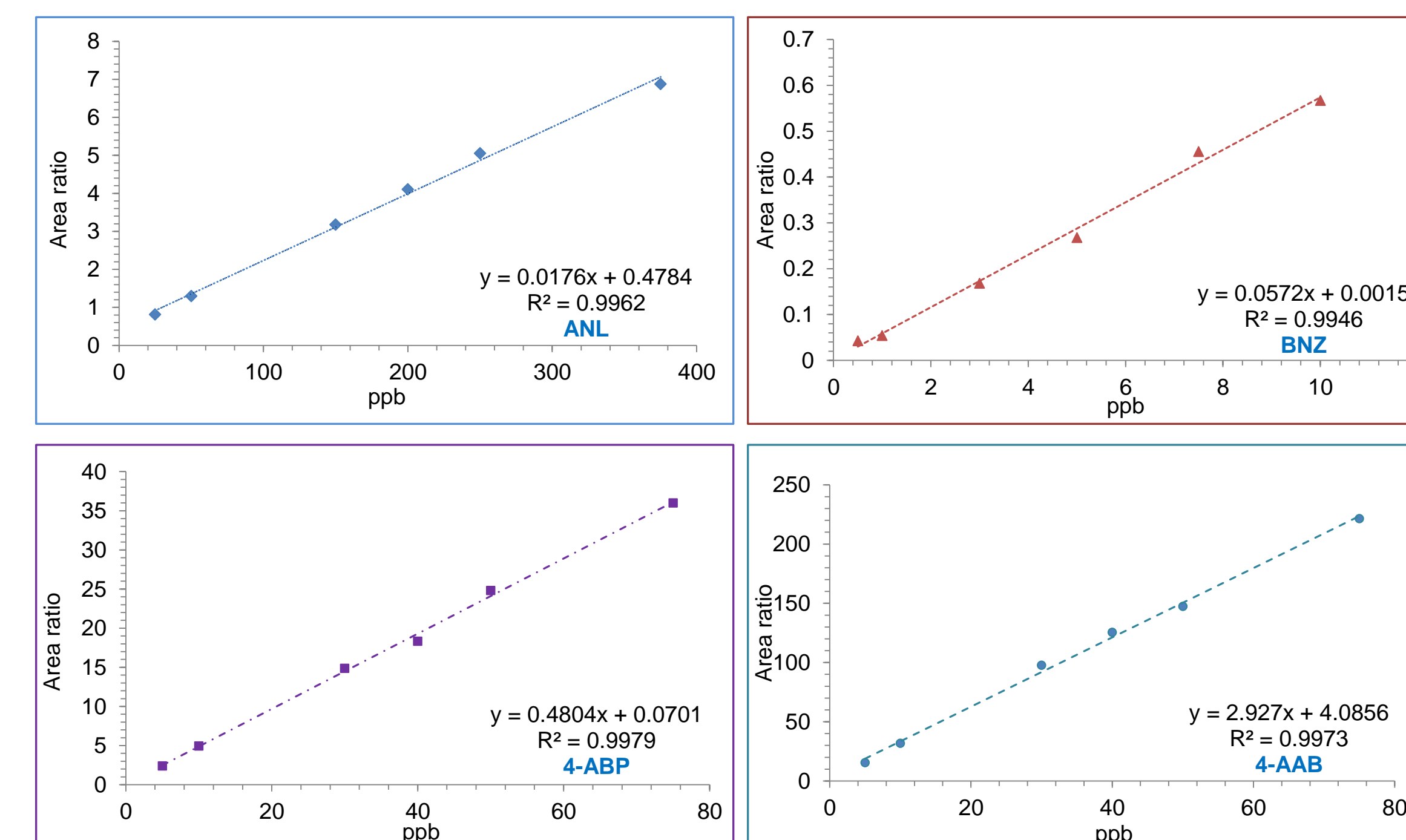


Figure 2 Calibration curves for the quantitative determination of (A) **ANL**, (B) **BNZ**, (C) **4-ABP**, and (D) **4-AAB** in the color additives **Y5** and **Y6** using LC/MS.

Table 2 Validation data for the LC-MS/MS method.

Analytes	Retention time (min)	Equation	R ²	Linear range (ppb)	LOD (ppb)	LOQ (ppb)	RSD (%)
ANL	0.49	y=0.0176x+0.478	0.996	25.0-375	0.10	0.31	7.8-9.8
BNZ	0.52	y=0.0572x+0.0015	0.995	0.5-10	0.12	0.37	9.1-15.0
4-ABP	1.69	y=0.4804x+0.0701	0.998	2.5-75	0.077	0.23	2.6-13.6
4-AAB	2.45	y=2.927x+4.0856	0.997	2.5-75	0.076	0.23	2.5-12.7

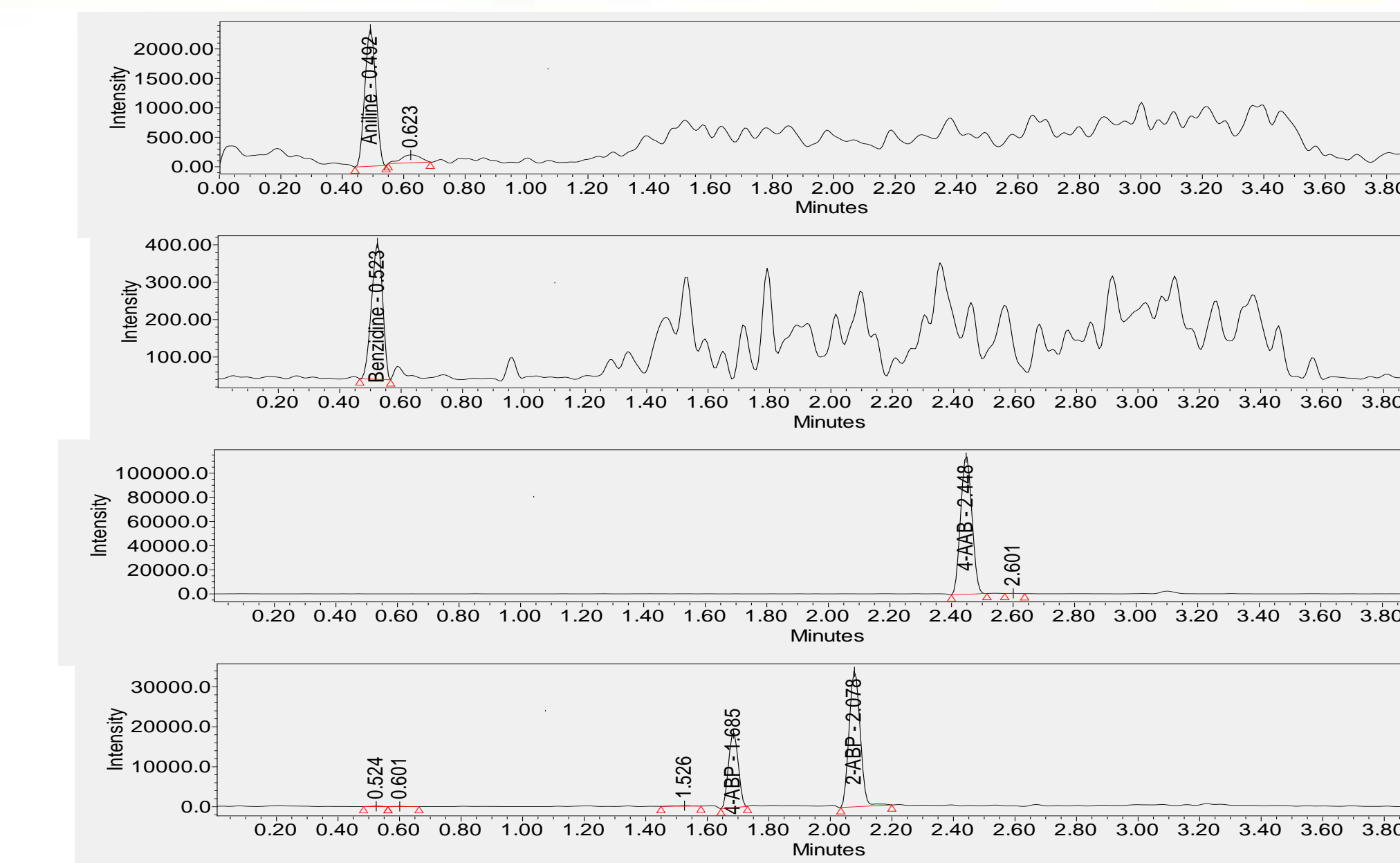


Figure 3 MRM ion chromatograms (A) **ANL**, transition m/z 94 → 77, (B) **BNZ**, transition m/z 184.9 → 168 (C) **4-AAB**, transition m/z 198 → 76.9, and (D) **4-ABP** and **2-ABP**, transitions m/z 170 → 153 for extracted **Y5** sample fortified with 30 ppb **ANL**, 3 ppb **BNZ**, 15 ppb **4-AAB**, and 15 ppb **4-ABP** and **2-ABP** (IS).

Table 3 Survey of **ANL**, **BNZ**, **4-ABP**, and **4-AAB** in certified lots of **Y5** and **Y6**.

Color additive ^a	Manufacturer	ANL		BNZ		4-ABP		4-AAB	
		LC-MS/MS	Current ^b	LC-MS/MS	Current	LC-MS/MS	Current	LC-MS/MS	Current
Y5 ^c	A	<LOQ ^d	11	NF ^e	<1	<LOQ	<5	<LOQ	<5
Y5	B	<LOQ	<1	NF	<1	<LOQ	<5	<LOQ	<5
Y6	C	<LOQ	8	NF	<1	NF	<5	<LOQ	<5
Y6	D	409	107	NF	<1	<LOQ	<5	<LOQ	<5
Y5	E	142	128	NF	NF	<LOQ	<5	<LOQ	<5
Y5	B	79	213	NF	NF	<LOQ	13	<LOQ	57
Y5	B	77	225	NF	NF	<LOQ	8	<LOQ	184
Y5	B	62	160	NF	NF	<LOQ	6	<LOQ	58
Y6	F	399	365	NF	NF	1.37	<5	<LOQ	<5
Y6	G	271	273	NF	NF	<LOQ	<5	<LOQ	<5
Y6	G	115	153	NF	NF	<LOQ	<5	298	163
Y6	H	12	68	NF	NF	0.85	<5	334	325

^a Batches certified in 2014
^b HPLC with PDA detection
^c Matrix sample
^d Limit of quantitation
^e Not found

Conclusions

Our new LC-MS/MS method for the determination of **ANL**, **BNZ**, **4-ABP**, and **4-AAB** in **Y5** and **Y6** may be applicable for use in routine batch certification and can replace the currently used, cumbersome HPLC method.

The new method is rapid (4 min total analysis cycle), requires only a single cleanup/extraction step, is more accurate, with significantly lower quantitation limits, and is simpler to implement compared to the current method.

In our preliminary survey of **Y5** and **Y6** samples, **BNZ**, **4-ABP**, and **4-AAB** either were not found or the results were below our quantitation limits. The results for **Y6** are similar to those obtained by the current method. The results for **ANL** and **Y5** by both methods are inconsistent and will be investigated further.

For **R33**, the same SPE extraction and LC-MS/MS method parameters will be used. However, a different internal standard will be required because **2-ABP** is a GMP-regulated impurity in **R33**. New calibration curves will be required for analytes present in **R33** at higher levels.

A comprehensive survey of **Y5**, **Y6**, and **R33** samples submitted for certification is in progress.

References

- Code of Federal Regulations, Title 21, Sections 74.705, 74.1705, and 74.2705, U.S. Government Printing Office, Washington, DC, 2014.
- Code of Federal Regulations, Title 21, Sections 74.706, 74.1706, and 74.2706, U.S. Government Printing Office, Washington, DC, 2014.
- Code of Federal Regulations, Title 21, Sections 74.1333 and 74.2333, U.S. Government Printing Office, Washington, DC, 2014.
- (a) Hand, R. L.; Nelson, R. F.; *J. Am. Chem. Soc.* **1974**, *96*, 850-860. (b) Zollinger, H. "Azo and Diazo Chemistry"; Interscience: New York, 1961; Chapter 8. (c) Lippmaa, M.; Vermin, G.; Metzger, J. *Helv. Chim. Acta* **1980**, *63*, 456-466. (d) Hegarty, A. F. "Chemistry of Diazonium and Diazo Groups"; Patai, S. Ed.; Wiley: New York, 1978; Chapter 12.
- (a) Bailey, J. E., Jr.; Bailey, C. J. *Talanta* **1985**, *32*, 875-882. (b) Richfield-Fratz, N.; Bailey, J. E., Jr.; Bailey, C. J. *J. Chromatogr.* **1985**, *331*, 109-123. (c) Bailey, J. E., Jr. *Anal. Chem.* **1985**, *57*, 189-196.