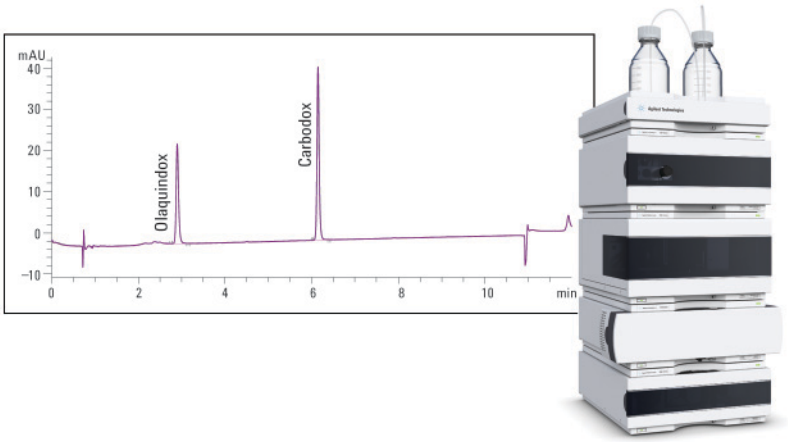
Quantification of growth promoters olaquindox and carbadox in animal feedstuff with the Agilent 1260 Infinity Binary LC system with UV detection

Application Note

Food



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Abstract

Simple, sensitive analytical methods are required for routine monitoring of banned substances, such as antibiotics that are added to animal feed to prevent disease and promote growth. This Application Note demonstrates the quantification of two growth promoters, olaquindox and carbadox in animal feedstuff using the Agilent 1260 Infinity Binary LC system. The 10x higher sensitivity of the 60 mm path length Max-Light flow cell enabled the detection of low levels of the two analytes present in animal feed. The detection and quantification of olaquindox and carbadox at wavelengths showing higher absorption, 375 nm and 306 nm respectively, improved the detection limits of the two analytes. The lower limit of quantification achieved for both molecules was 50 ng/mL. The area response was found to be linear up to 5,000 ng/mL. A chicken feed sample, prepared by a simple extraction procedure using 1:1 methanol/water yielded recoveries > 80% for olaquindox and carbadox.

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Introduction

To comply with the EU regulation[1] that bans the use of antibiotics in animal feedstuff, simple and sensitive HPLC methods are required for routine monitoring. This Application Note describes such a method for the quantification of two antibacterial drugs, olaquindox and carbadox that are expected to be present at very low levels (3 mg/kg and 4 mg/kg, respectively) in animal feed[2]. Although hydrophilic interaction chromatography has been reported for the analysis of these relatively polar compounds[3], we describe a reverse phase HPLC method with UV detection for the quantification of the two analytes.

Experimental

System

The Agilent 1260 Binary LC system consisted of the following modules:

G1312B	Agilent 1260 Infinity Binary Pump
G1379B	Agilent 1260 Infinity Degasser
G1367E	Agilent 1260 Infinity Autosampler
G1330B	Agilent 1260 Infinity Thermostat
G1316B	Agilent 1260 Infinity Thermostatted Column Compartment
G4212B	Agilent 1260 Infinity DAD, with Max-Light 60 mm high-sensitivity flow cell
Software	ChemStation B.04.03

Chromatographic conditions

Column:	*Agilent ZORBAX SB-Aq 3.0 × 100 mm, 3.5 µm (p/n 861954-314)*	
Mobile phases:	*A: Ammonium formate (5 mM)*	
	B: 95% ACN and 5% ammonium formate	
Injection volume:	*5 µL*	
Flow rate:	*0.75 mL/min*	
Gradient:	*Time (min)*	*% Acetonitrile*
	0.0	*2*
	10	*30*
	10.1	*80*
	11	*80*
	11.1	*2*
	Stop time: 12 min	
	Post time: 3 min	
Column temperature:	*40 °C*	
Detection:	*306 nm for carbadox and 375 nm for olaquindox, 4 nm BW; Ref: No; PW > 0.25 s (20 Hz)*	

Samples

Olaquindox and carbadox were purchased from Sigma Aldrich. Stock solutions were prepared in 60/40 acetonitrile/methanol mix and diluted in 1:1 methanol/water. To prepare calibration curves, solutions containing 50, 100, 250, 500, 1000, 2500 and 5000 ng/mL of olaquindox and carbadox in 1:1 methanol/water were used. The method was validated using a solution containing 1000 ng/mL each of olaquindox and carbadox.

Preparation of animal feed

A chicken feed sample obtained from a local farm was powdered and homogenized in a blender. Approximately 0.1 g of crushed sample was weighed in two microcentrifuge tubes. 50 µL of 1:1 methanol/water was added to the first sample and 50 µL of 10 ppm mix of carbadox and olaqauindox (in 1:1 methanol/water) was added to the second sample. After one hour, 2 mL of 1:1 methanol/water was added to both samples which resulted in the dilution of the sample by a factor of 40. The extraction was carried out by mixing overnight on a rotary mixer. Subsequently, the samples were centrifuged and 1500 µL of the supernatant were transferred to fresh microcentrifuge tubes and centrifuged again. 1000 µL of the supernatant was pipetted into HPLC vials for analysis.

Results and discussion

Olaqindox and carbadox are relatively polar molecules requiring highly aqueous mobile phases for effective chromatography. We applied a reverse-phase method using an Agilent SB-Aq column which is compatible with 100% aqueous mobile phase. This is feasible as the surface chemistry of the column is such that the alkyl phase does not undergo phase collapse even when exposed to 100% water in the mobile phase. After the preliminary method development, a range of parameters were systematically varied to test the robustness of the method. As a readout of the impact of parameter variations on the results we monitored deviations in peak areas and retention times. A maximum deviation of 2% for the retention time and 3% for the area was set as the limit. Table 1 shows the absolute values of the deviations observed for the two compounds as the parameters were varied. It was found that injection volume, gradient slope and detection wavelengths are critical method parameters, and care was taken to keep these parameters constant throughout the study.

	Olaquindox		Carbadox	
Parameter changed	**Deviation in the retention time (%)**	**Deviation in the area (%)**	**Deviation in the retention time (%)**	**Deviation in the area (%)**
Flow + 2%	*1.35*	*0.34*	*0.72*	*0.60*
Flow – 2%	*1.58*	*2.27*	*0.97*	*0.07*
Column temperature + 5%	*0.24*	*1.03*	*0.17*	*1.45*
Column temperature – 5%	*1.28*	*1.68*	*0.96*	*1.08*
Injection volume + 2%	*0.06*	*1.71*	*0.17*	*0.35*
Injection volume – 2%	*0.03*	*1.96*	*0.15*	*4.46*
Gradient slope + 2%	*0.17*	*0.62*	*0.76*	*1.03*
Gradient slope – 2%	*0.61*	*0.56*	*1.34*	*1.07*
Modifier concentration + 10 %	*0.13*	*0.39*	*0.28*	*2.59*
Modifier concentration – 10 %	*0.02*	*0.56*	*0.16*	*2.38*
Wavelength + 1 nm	*0.01*	*1.85*	*0.12*	*2.69*
Wavelength - 3 nm	*0.24*	*0.20*	*0.17*	*0.61*

Table 1
Method robustness: Effect of method parameter changes on peak areas and retention times.

Figure 1 shows the overlay of six replicate chromatograms of the mix of olaquindox and carbadox standards. The compounds elute with excellent reproducibility. The area response was linear from 50 to 5000 ng/mL with the correlation coefficient values being ≥ 0.999, for both the analytes. Table 2 shows the concentration values and the corresponding peak areas that were used to construct the calibration curves for the two compounds shown in Figure 2A and Figure 2B, respectively.

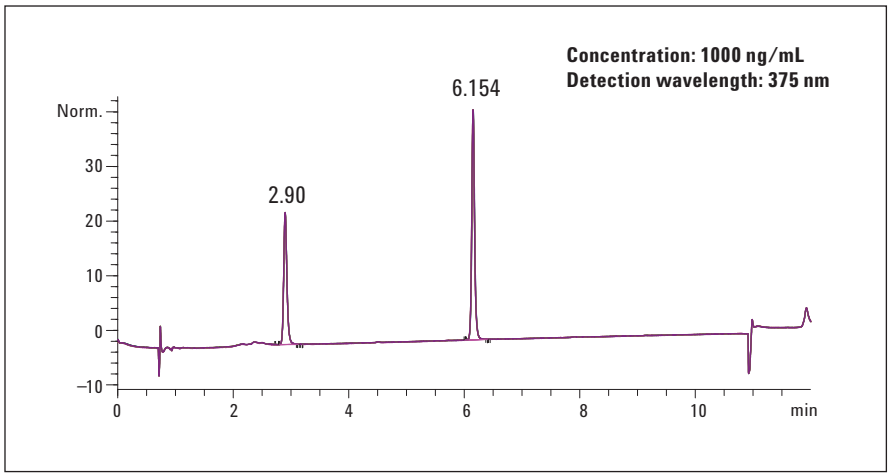

Figure 1
Overlay of six replicate injections of a standard mix of olaquindox and carbadox.
Amount of each analyte injected: 5 ng.

Concentration (ng/mL)	Olaquindox Average area (n=6)	RSD area	Carbadox Average area (n=6)	RSD area
50	*4.87*	*1.34*	*16.40*	*0.65*
100	*9.51*	*2.47*	*32.93*	*0.53*
250	*21.93*	*1.42*	*82.62*	*0.32*
500	*45.31*	*0.89*	*164.28*	*0.22*
1000	*93.88*	*0.35*	*331.95*	*0.18*
2500	*220.05*	*0.16*	*839.69*	*0.13*
5000	*460.21*	*0.07*	*1672.21*	*0.08*

Table 2
Analyte concentrations and the corresponding area responses.

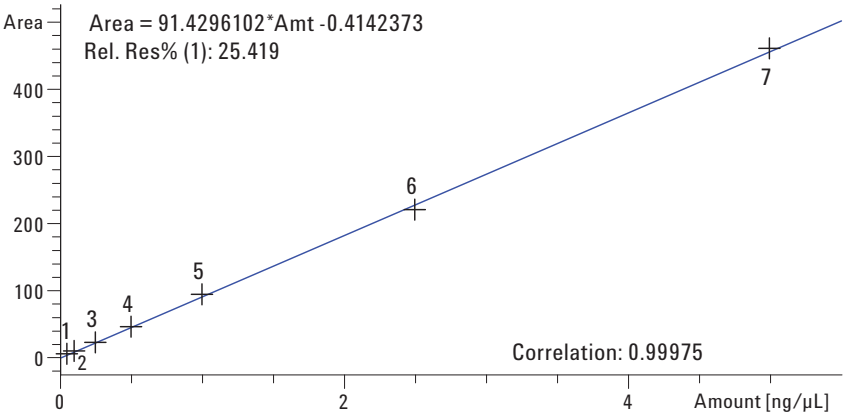

Figure 2A
Calibration curve for olaquindox.

We calculated the signal/noise levels by dividing the peak heights by the peak to peak noise between 6.3 and 7.3 min at the 50 ng/mL level (Figure 3). Signal/Noise was 31.1 and 92.7 for olaquindox and carbadox respectively. Usually the S/N value at the limit of quantification is 10, for the present method 50 ng/mL with the higher S/N values was assigned as the limit of quantification for both the analytes.

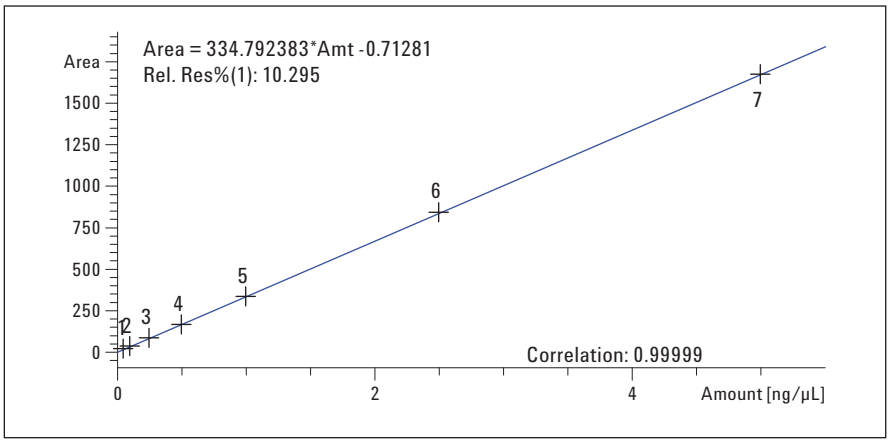

Figure 2B
Calibration curve for carbadox.

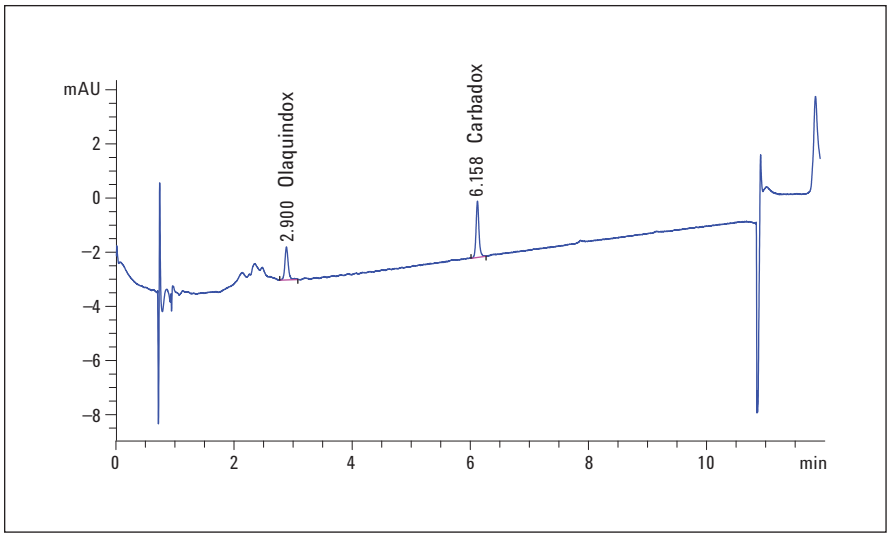

Figure 3
Chromatogram of the 50 ng/mL calibration standard. Amount of each analyte injected: 250 pg.

Analysis of spikes samples

A chicken feed sample was obtained from a local farm. Two aliquots of the sample each weighing 0.1 g, were placed in two microcentrifuge tubes. One of the aliquots was extracted as described in the experimental section and analyzed. No olaquindox peak was found in this sample. A very small peak seen at the retention time for carbadox could not be confirmed by spectral matching and is probably an unidentified interfering compound. Figure 4A shows the unidentified interferent peak at 306 nm in this unspiked chicken feed sample. Then, 500 ng of each analyte were spiked into the second aliquot of the sample. After extraction and analysis, 439.2 ng of olaquindox and 482.8 ng of carbadox were calculated to be present in 0.1 g of the spiked sample, yielding recoveries of 87.8% and 83.8% for olaquindox and carbadox respectively. Figure 4B shows the olaquindox and carbadox peaks at 375 nm.

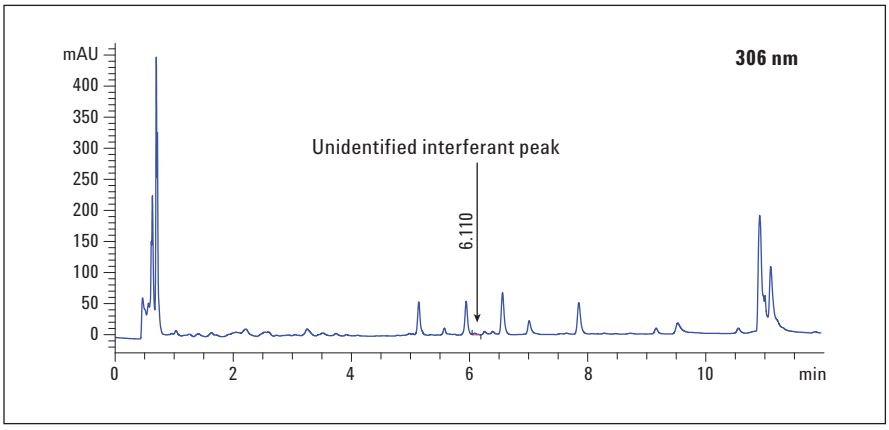

Figure 4A
Chromatogram of the unspiked chicken feed sample.

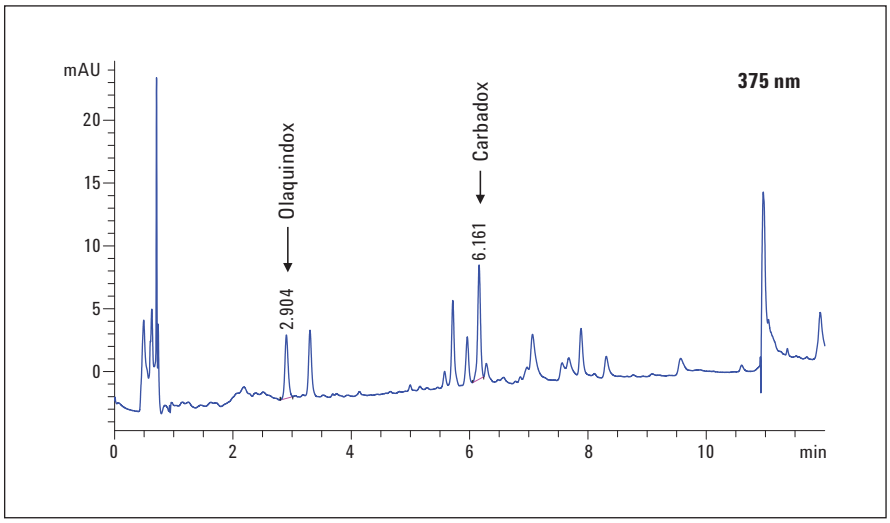

Figure 4B
Chromatogram of the spiked chicken feed sample.

Conclusions

In this Application Note, we describe the detection and quantification of two synthetic antibiotics, olaquindox and carbadox, used as growth promoters in animal feed. The Agilent SB-Aq column enables the chromatography of these relatively polar compounds using highly aqueous mobile phase. The higher sensitivity of the 60 mm path length Max-Light flow cell helps in the quantification of very low levels of the two analytes in animal feedstuff. No olaquindox or carbadox was found in a chicken feed sample obtained from a local farm.

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