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APPLICATION NOTE 21693

# Fast separation of coccidiostats


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## Application benefits

- Sample throughput tripled compared to the legacy methods
- Associated five-fold reduction in cost per sample for optimized method through reduced mobile phase consumption and waste generation

## Goal

To demonstrate how the use of alternate UHPLC stationary phase selectivities can facilitate method speed up

## Introduction

Coccidiostats are used in poultry and other veterinary welfare to control parasitic infections. These compounds are added to some poultry feeds to provide low-level prevention, but there is evidence of the development of resistance by the parasite. Veterinarian organizations are lobbying for more control on their use[1] so the ability to monitor them is desired.

In a previous application note[2] it was shown that a good separation, in fifteen minutes, can be achieved using the Thermo Scientific™ Hypersil GOLD™ Phenyl column chemistry on legacy HPLC equipment. This application note extends and improves that work to a solid core Phenyl-Hexyl column on modern UHPLC equipment.

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One of the key goals for the chromatographer is to achieve a consistent, reproducible separation. The selection of a highly reproducible HPLC column is essential if this goal is to be attained. Based on solid core technology, Accucore HPLC columns allow users of conventional HPLC methods to obtain performance beyond that of columns packed with 5 µm or even 3 µm fully porous particles. High separation efficiencies provide increased peak resolution. An ultra-stable packed bed results in exceptionally robust columns that demonstrate excellent retention and response reproducibility. Accucore columns are available in a wide range of chemistries and particle sizes making them an ideal choice for this type of work.[3]

The Vanquish Flex UHPLC system has the benefit of SmartInject technology and improvements in injection system hardware synchronization. This results in unrivalled retention time precision, providing the user with greater data confidence during method development. The Vanquish Flex system also utilizes Thermo Scientific™ LightPipe™ flow cell technology designed for the diode array detector (DAD), which provides the user with low peak dispersion due to small internal

## Experimental

### Consumables and apparatus

- Accucore Phenyl-Hexyl, 100 × 2.1 mm, 2.6 µm column (P/N 17926-102130)
- LC-MS grade 18 MΩ water from Thermo Scientific™ Smart2Pure™ system (P/N 50129845)
- Fisher Scientific™ LC-MS grade methanol (P/N A456-212)
- Thermo Scientific™ Virtuoso™ 9 mm wide opening, 2 mL screw thread vial and cap kit (P/N 60180-VT400)

### Standards

The compounds used were representative of this class and were purchased from a reputable supplier:

- 4-amino-3,5-dinitrobenzamide [1]
- Nitromid (3,5-dinitrobenzamide) [2]
- Zoalene (3,5-dinitro-o-toluamide) [3]
- Ethopabate [4]

The bracketed number relates to the elution order and peak labelling in the subsequent chromatograms.

### Instrumentation

Analyses were performed using a Vanquish Flex Quaternary UHPLC System consisting of:

- Quaternary Pump F (P/N VF-P20-A)
- System Base Vanquish Flex( P/N VF-S01-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A)
- Active Pre-heater (P/N 6732.0110)
- Diode Array Detector HL (P/N VH-D10-A)
- LightPipe Flow Cell, 10 mm (P/N 6083.0100)
- Thermo Scientific™ Virtuoso™ vial identification system (P/N 60180-VT-100)

### Software

Thermo Scientific™ Chromeleon™ 7.2 SR4

### Sample preparation

Solutions of the compounds were prepared by dissolving a known amount in water/acetonitrile (20:80, v/v) to produce 1 mg/mL primary solutions. A mixed working standard solution and individual working standards were used to assess method development and were prepared in water/acetonitrile (80:20, v/v) at a concentration of 0.1 mg/mL.

### Sample handling

Vial labeling was supported by the Virtuoso vial identification system.

### UHPLC conditions (final method)

| | |
|---|---|
| UHPLC column: | Accucore Phenyl-Hexyl, 2.6 µm, 100 mm × 2.1 mm |
| Mobile phase A: | Water/methanol 95:5, v/v |
| Mobile phase B: | Water/methanol 5:95, v/v |
| On-pump mixing: | Gradient (Table 1) |
| Flow rate: | 0.6 mL/min |
| Column temperature: | 50 °C, still air with eluent pre-heating |
| Injection volume: | 1 µL |
| Mixer: | 50 µL capillary + 150 µL static in combination |
| UV detection: | 268 nm |

Table 1. Gradient elution profile.

| Time (min) | %B |
|---|---|
| 0.00 | 15 |
| 0.07 | 15 |
| 1.47 | 85 |
| 1.48 | 15 |
| 4.70 | 15 |

## Results and discussion

Building on the method outlined in the previous application note[2], the Accucore Phenyl-Hexyl column was configured and the mixed standard analyzed with a flow rate of 0.4 mL/min and a column temperature of 40 °C (Figure 1a). This resulted in all the standards eluting within four minutes and presented a good starting point for further development. This experiment was repeated with a column oven temperature of 50 and 60 °C (Figure 1b, 1c).

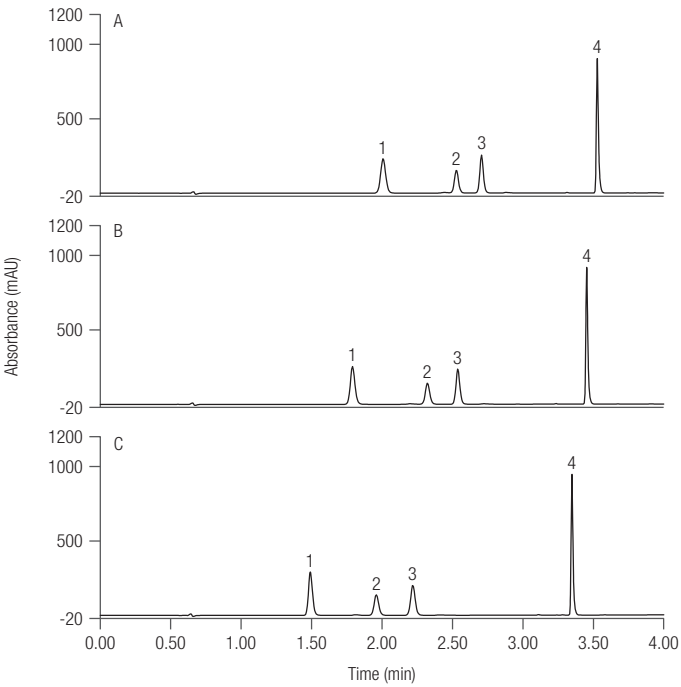


**Figure 1. Chromatograms showing mixed standards analyzed at three column temperatures A) 40 °C, B) 50 °C, and C) 60 °C.**

The elevated temperature shows the expected reduction in retention time and reduction in peak width. There were no overt selectivity changes. Peak resolution, between peaks 2 and 3, was greater than 3.5 for all three temperatures.

A temperature of 50 °C was selected for further work, as some customers have a preference for avoiding higher column temperatures.

Solid core HPLC columns are capable of equivalent efficiencies to much smaller fully porous particles. This allows high efficiency separations at a fraction of the cost in pressure. This, coupled with the ability of the solid core columns to run at optimum (highest) efficiency over a much larger linear velocity range[3] allows the chromatographer to significantly speed up their methods.

While maintaining the column temperature at 50 °C, the effect of flow rate was investigated by injecting the standards mixture at flow rates from 0.4 to 0.6 mL/min. Due to the change in flow rate the gradient was scaled using the Chromeleon UHPLC Speed-Up utility within the software.[4] Figure 2 shows the resulting chromatograms, demonstrating the reduction in retention without significant loss in peak efficiency. A flow rate of 0.6 mL/min was selected to achieve sub-5 minute method time.

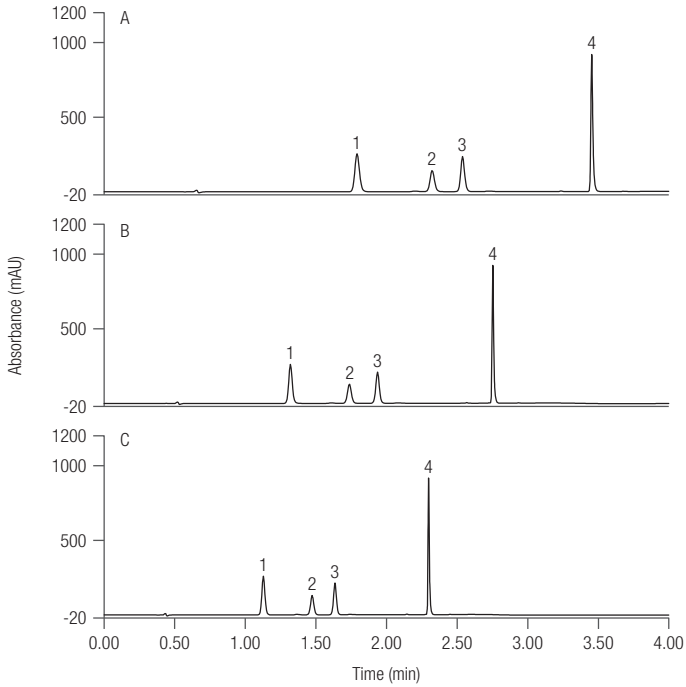


**Figure 2. Chromatogram showing mixed standards analyzed at three flow rates A) 0.4 mL/min, B) 0.5 mL/min, and C) 0.6 mL/min.**

Twenty-four replicate injections were made under the selected conditions (Figure 3 and Table 2). These results show that the method is very stable, with differences between maximum and minimum retention times of less than 0.2 seconds across the replicate injections. This is much better than the 2% RSD criteria usually associated with legacy methods. The separation of all seven components is achieved within 2.5 minutes, though column equilibration extends the method time to just under 5 minutes.

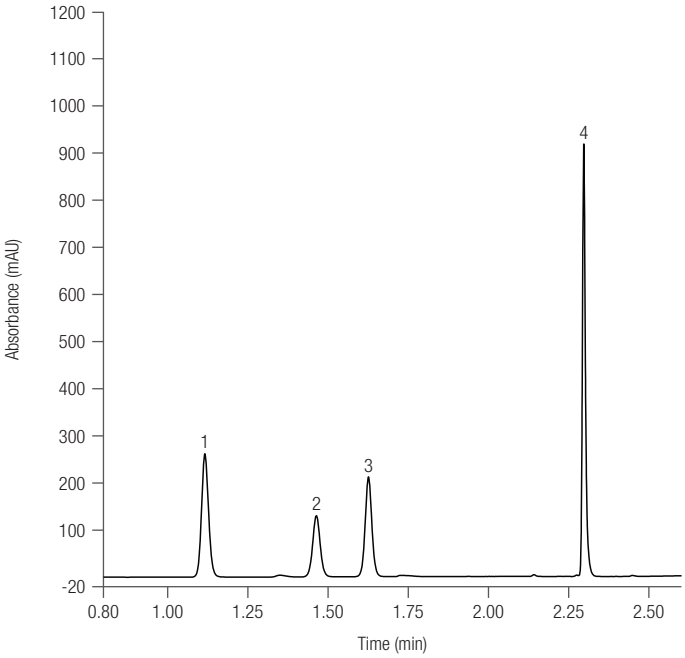


Figure 3. Overlay of 24 replicate injections of standards mixture at 50 °C and a flow rate of 0.6 mL/min.

Table 2. Peak summary data for twenty four replicate injections of standards mixture at 60 °C and a flow rate of 0.6 mL/min.

| Parameter | Peak 1 | Peak 2 | Peak 3 | Peak 4 |
|---|---|---|---|---|
| Maximum RT (min) | 1.117 | 1.465 | 1.628 | 2.298 |
| Average RT (min) | 1.115 | 1.463 | 1.626 | 2.296 |
| Minimum (min) | 1.114 | 1.461 | 1.624 | 2.295 |
| Standard Deviation (min) | 0.001 | 0.001 | 0.001 | 0.001 |
| RSD % | 0.08 | 0.07 | 0.06 | 0.04 |

When compared to typical legacy methods (15 min, 1.0 mL/min), this method development has provided a three-fold increase in sample throughput and a five-fold reduction in mobile phase consumption / waste generation (Figure 4).

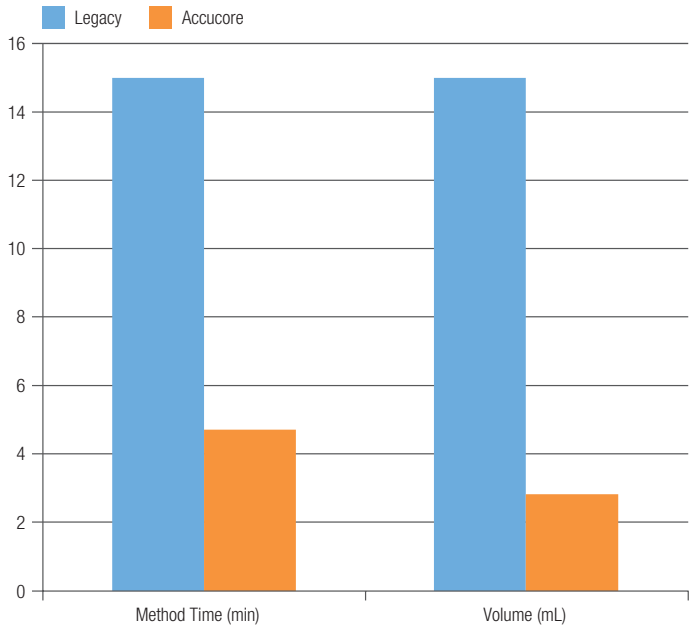


Figure 4. Relative differences in method time and mobile phase consumption between typical legacy methods and this improved method.

## Conclusions

A high-throughput application has been developed showing the separation of four coccidiostats in under 5 minutes. When compared to the legacy method on a 150 × 4.6 mm column, this application demonstrates the following:

- Three-fold increase in method throughput, allowing more samples to be analyzed in a given time, or more rapid availability of results.
- Five-fold reduction in cost per sample through reduced mobile phase consumption and waste generation

## References

1. FVE position paper on coccidiostats or anticoccidials http://www.fve.org/uploads/publications/docs/040_016_coccidiostats_adopted.pdf
2. Improved analysis of veterinary drug coccidiostats using a Thermo Scientific Hypersil GOLD Phenyl HPLC column https://appslab.thermofisher.com/App/521/improved-analysis-veterinary-drug-coccidiostats-using-a-thermo-scientific-hypersil-gold-phenyl-hplc-column
3. Accucore HPLC Columns Technical Guide
4. https://tools.thermofisher.com/content/sfs/brochures/TG-20666-Accucore-HPLC-Columns-TG20666-EN.pdf

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