An improved method for extraction and analysis of tiamulin is presented to address issues that arose during routine analysis of Type C medicated swine feeds under the current U.S. Food and Drug Administration-Center for Veterinary Medicine (FDA-CVM) approved method. The issues included the need for higher sample throughput and the ability to accommodate a wider variety of feed matrices. Changes to the FDA-CVM approved method include reduced sample size and solvent volumes, phosphate buffering of tartaric acid, centrifugation, and use of a new liquid chromatography column and adjusted mobile phase composition. A paired sample study was performed to compare performance of the new and existing methods. The paired sample study showed no statistical difference between sample means of paired sets of 17 samples analyzed by both methods (t = 1.95 at 0.05 significance level, p = 0.068). A recovery study showed the method precision to be 2.06% (coefficient of variation) with an average standard recovery of 95.8%. Ruggedness test results indicated good overall ruggedness of the method.

Tiamulin hydrogen fumarate (C_{32}H_{51}NO_{8}S) is an antibacterial drug added to swine feeds for the control of swine dysentery associated with *Serpulina* (*Treponema*) *hyodysenteriae*. On July 17, 1987, the U.S. Food and Drug Administration-Center for Veterinary Medicine (FDA-CVM) first approved the use of tiamulin in Type A medicated articles for the preparation of Type C medicated feeds (1).

In 1993, 3 FDA-CVM approved methods were published (2) for the analysis of tiamulin hydrogen fumarate in premixes (Method I), in polyvinyl chloride formulations (Method II), and in complete swine meal feeds (Method III). In Method III, the tiamulin fumarate reacts with a 1% sodium carbonate base solution, releasing tiamulin from the hydrogen fumarate salt. The tiamulin, converted to the free base, preferentially partitions into the organic solvent (hexane–ethyl acetate, 3 + 1). A portion of the organic solvent is removed, and the tiamulin is back-extracted from the organic solvent into a 0.1% aqueous tartaric acid solution converting the tiamulin to its tartrate.

On August 20, 1996, the FDA-CVM approved combining separately approved Type A medicated articles containing chlortetracycline hydrochloride and tiamulin to make Type C medicated swine feeds. This feed is used for controlling swine dysentery and treating swine bacterial enteritis caused by *Escherichia coli* and *Salmonella choleraesuis* and bacterial pneumonia caused by *Pasteurella multica*. (3, 4).

In 1997, the Indiana State Chemist Laboratory experienced an increase in the number and variety of swine feed matrices containing tiamulin. The increased popularity of tiamulin in feeds was accompanied by a rise in the number of sample matrices that interfere with the application of the FDA-CVM approved method to accurately analyze tiamulin in a timely manner. Opportunities to improve the FDA-CVM approved method included reducing the large sample sizes and solvent volumes that limited sample throughput, dispersing difficult emulsions at the aqueous to organic layer interface that formed during extraction, increasing the lower back-extraction efficiencies observed for some samples, and eliminating late-eluting matrix components that affected subsequent standard chromatograms and co-eluting matrix components that interfered with tiamulin quantitation in sample chromatograms.

The objective of the present work was to develop improvements to Method III (the FDA-approved method) to increase the sample throughput, accommodate a wider variety of sample matrices, and validate the improved method for regulatory use by the Indiana State Chemist Laboratory.

**METHOD**

**Equipment**

(a) **1-in. riffler.**—Carpco, Jacksonville, FL; Model SS16-25X; used to subsample collected medicated feed samples. A grinding mill (Retsch, Haan, Germany; Model ZM100 or ZM 1000) was used to grind subsamples.

(b) **Liquid chromatography (LC) system.**—Consisting of a quaternary gradient pump [ThermoSeparation Products (TSP), Waltham, MA; P4000], variable loop autosampler (TSP, AS3000), and a photodiode array detector (TSP, UV6000LP). Used for separation and detection of analytes. Photodiode array detection was used for method development, but single-wavelength UV detection is acceptable for routine analysis.
(c) Polypropylene bottles.—Nalgene, Rochester, NY; 250 mL containing a 1-5/16 in. (ca 33 mm) id wide-mouth opening and a 2-3/8 in. (ca 60 mm) diameter base were used for extraction.

(d) Platform orbital shaker.—Innova, Edison, NJ; Model 2300; used for shaking during sample extraction.

(e) Centrifuge.—Eppendorf, Hamburg, Germany; Model 5810; used for separation of phases.

Reagents

(a) Tiamulin hydrogen fumarate analytical standards.—Purchased from Sigma Chemical Co. (St. Louis, MO) under the trade name Vetranal®, manufactured by Riedel de Haën as a dry white powder with a 99.5% assay purity. Standard material was used as received.

(b) Sodium carbonate and ammonium carbonate.—Sigma; ACS reagent grade.

(c) ACS reagent grade tartaric acid, potassium dihydrogen phosphate, 85% phosphoric acid, and LC grade acetonitrile, hexane, and ethyl acetate.—Mallinckrodt Chemical Co. (St. Louis, MO).

(d) Methanol.—Burdick & Jackson Chemical Co. (Muskegon, MI); LC grade.

(e) Water.—LC grade; used for preparing buffers and solutions

Preparation

Class B volumetric glassware was used for reagent preparation. Class A volumetric glassware was used for all standard preparations.

(a) 0.1% Tartaric acid (w/v).—Weigh 1.00 ± 0.01 g tartaric acid into 1000 mL volumetric flask, add ca 400 mL water, and dissolve tartaric acid completely. Dilute to volume with water, and mix.
(b) *0.1M Phosphate buffer (w/v).*—Dissolve 13.61 g potassium dihydrogen phosphate (KH$_2$PO$_4$) in 1000 mL water and adjust pH to 2.75 dropwise with 50% phosphoric acid.

(c) *Buffered tartaric acid.*—Weigh 1.00 ± 0.01 g tartaric acid into 1000 mL volumetric flask, add ca 400 mL 0.1M phosphate buffer, and dissolve tartaric acid completely. Dilute to volume with phosphate buffer, and mix.

(d) *Tiamulin standards.*—Prepare stock solution (600 µg/mL) tiamulin by accurately weighing 60.00 ± 0.05 mg tiamulin hydrogen fumarate analytical standard into 100 mL volumetric flask. Dissolve tiamulin and dilute to volume with 0.1% tartaric acid. Prepare working standard (30 µg/mL) by pipetting 5 mL stock solution into 100 mL volumetric flask, and dilute to volume with buffered tartaric acid. Prepare one linearity standard (15 µg/mL) by pipetting 5 mL stock solution into 200 mL volumetric flask, and dilute to volume with buffered tartaric acid. Prepare second linearity standard (60 µg/mL) by pipetting 10 mL stock solution into 100 mL volumetric flask and dilute to volume with buffered tartaric acid. Prepare third linearity standard (90 µg/mL) by pipetting 15 mL stock standard into 100 mL volumetric flask, and dilute to volume with buffered tartaric acid.

(e) *Extraction solvent (hexane–ethyl acetate, 3:1).*—Mix 750 mL hexane and 250 mL ethyl acetate together in tightly capped 1000 mL glass reagent bottle. Prepare fresh daily to prevent changes in composition due to evaporation.

(f) *1% Sodium carbonate (w/v).*—Dissolve 10 g sodium carbonate in 1000 mL water.

(g) *1% Ammonium carbonate solution (w/v).*—Dissolve 10 g ammonium carbonate in 1000 mL water.

(h) *Mobile phase: methanol–acetonitrile–1% ammonium carbonate (39 + 19 + 42).*—Mix 390 mL methanol, 190 mL acetonitrile, and 420 mL 1% ammonium carbonate together in suitable container. Filter through 0.45 µm nylon membrane and degas before use.

**Extraction and Cleanup of Complete Feed (Developed, High Throughput Method)**

Split medicated feed samples into subsamples by using 1 in. riffler. Grind subsamples to pass through 0.75 mm mesh screen by using a grinding mill. Place ground samples in 16 oz glass jars for subsequent extraction, and store at room temperature protected from light.

Wipe exterior of 250 mL polypropylene extraction bottles carefully with antistatic cloth before weighing each sample. Weigh ca 10 g medicated feed sample, containing 35 g/ton tiamulin, accurately (to 4 decimal places) into 250 mL polypropylene extraction bottle. Using Class A volumetric pipet, add 50 mL extraction solvent. Using Class B graduated cylinder, add 50 mL 1% sodium carbonate. Cap bottle and shake for 3 h on orbital shaker at 250 rpm. After shaking, centrifuge bottles at 1467 × g for 15 min to break up emulsions. Using

![Figure 3](image.png)

**Figure 3.** Method comparison plot for paired sample study. The solid line represents perfect correlation between the 2 methods. Each sample is represented by a diamond where the FDA method is the x-value and the high throughput method result is the y-value.
15 mL polypropylene transfer pipet, transfer ca 35–40 mL upper solvent layer (hexane–ethyl acetate, 3 + 1) to 50 mL centrifuge tube, taking care to avoid transferring any emulsion if possible. Cap centrifuge tube and centrifuge at 1467 \( \times g \) for 5 min. Using a pipet, carefully transfer ca 30–35 mL organic layer to second 50 mL centrifuge tube, leaving any undisturbed precipitate in bottom of first tube. Cap second centrifuge tube and centrifuge at 1467 \( \times g \) once more for 5 min to settle any solids to bottom of tube. Using Class A volumetric pipet, carefully pipet 25 mL organic solvent from second tube into third 50 mL centrifuge tube. Using Class A volumetric pipet, pipet 5 mL buffered tartaric acid to third centrifuge tube containing organic solvent. Tightly cap third tube and shake vigorously by hand to mix contents together once every 5 min for 15 min. To facilitate separation of phases, centrifuge third tube at 1467 \( \times g \) for 5 min. If emulsions are still present at aqueous to organic interface, centrifuge samples at 1811 \( \times g \) for additional 10 min. Using glass transfer pipet, carefully remove ca 2 mL lower tartaric acid layer and filter through 0.45 \( \mu \)m membrane syringe filter (Millex LCR, Bedford, MA) into autosampler vial for LC analysis.

**Determinative Procedure**

(a) **LC conditions.**—Waters X-Terra \textsuperscript{TM} RP \textsubscript{18}, 5 \( \mu \)m, 4.6 \( \times \) 250 mm analytical column (Part No. 186000496, Waters, Milford, MA) for LC analysis. Condition column by programming from storage solvent to mobile phase; then pump mobile phase through column at flow rate of 1.5 mL/min for 30 min. The column back pressure should be near 3500 psi. Use 30 \( \mu \)L injection volume and bracket every 2 sample injections with working standard injections. Determine tiamulin peak areas by using UV absorbance detection at 254 nm. The retention time of tiamulin should be ca 18–20 min.

(b) **Calculations.**—Calculate tiamulin concentration (g/ton) in sample by comparing peak areas of standards and samples by using the following equation:

\[
\text{Concentration, g/ton} = \times 907184.74 \times P
\]

where \( R_u \) is average response (peak area) for unknown feed sample, \( R_s \) is average response (peak area) for 2 bracketing standards, \( W_u \) is weight (g) of feed sample, \( W_s \) is weight (g) of standard used to prepare stock solution, 10 is total dilution (mL) of sample, 2000 is total dilution (in mL) of original standard weighed to prepare working (bracketing) standard, 907 184.74 is unit conversion from mass fraction to g/ton, and \( P \) is purity of reference standard expressed as mass fraction (e.g., 99.5\% = 0.995).

**Results and Discussion**

**Chromatography Study**

Several experiments were performed to determine the cause of baseline anomalies found in sample and standard chromatograms. Numerous Type C medicated feed samples containing tiamulin were extracted by the FDA-approved method. The sample components were separated on a Waters X-Terra RP \textsubscript{18} column, and on a Hypersil ODS 5 \( \mu \)m, 4.6 \( \times \) 250 mm analytical column (Cat. No. Z226343, Supelco, Inc., Bellefonte, PA).

The FDA-approved method uses the Hypersil ODS column with a mobile phase consisting of methanol–acetonitrile–1\% ammonium carbonate (60 + 30 + 25, v/v), prepared by combining 600 mL methanol, 300 mL acetonitrile, and 250 mL 1\% (w/v) ammonium carbonate, and then filtering and degassing before use. The pH of the mobile phase is ca 9.5, which is more basic than the recommended pH.
The stability of the Hypersil ODS column. The retention time of tiamulin under these conditions was approximately 7 min.

The typical baseline chromatograms encountered in the FDA-approved method by using the Hypersil column are depicted in Figure 1. In the first injection (labeled “1”), the tiamulin (denoted as “A”), extracted from a feed sample, elutes with unresolved matrix components and causes a characteristic hump and elevated baseline. When matrix components fail to completely elute from the column, the instrument zeroes the baseline against the eluting components at the beginning of the following injection. As the matrix components finally elute, the baseline drops as depicted by the subsequent standard chromatogram (labeled “2”) in Figure 1.

For the newly developed (high throughput) method, the original Hypersil ODS column was replaced with a newer Waters X Terra RP18 column. The Waters column incorporates methylsiloxane groups into the particle bed in place of the siloxane groups as found in the Hypersil column. The embedded nonpolar methyl groups reduce the number of hydroxy groups at the bed surface that are subject to attack by high pH mobile phases, making the particles more stable in higher pH mobile phases. The composition of the mobile phase was adjusted to resolve the tiamulin peak from the sample matrix components. A final mobile phase with a composition of methanol–acetonitrile–1% ammonium carbonate (39 + 19 + 42) was selected.

The effect of the new column and adjusted mobile phase composition on the tiamulin chromatograms is depicted in Figure 2. In the sample injection (labeled “1”), the tiamulin (denoted “A”) has been resolved from the previously co-eluting matrix components. Also, the late eluting matrix components evident in Figure 1 were absent from the chromatogram for the subsequent standard injection (labeled “2”) in Figure 2. The retention time increased from 7 to 18 min.

### Linearity

The linearity of the calibration over the range of interest was established by using 15, 30, 60, and 90 μg/mL standards. Standard data were collected from 7 separate determinations over a 6-month period by using the new chromatographic conditions. The linearity of the calibration was derived by plotting peak areas versus the standard concentration. The average calibration line fit the linear regression model, $y = 9813x + 2807$, with a coefficient of determination ($R^2$) of 0.999977. Because the 95% confidence limits of the $y$-intercept include the origin, quantitation of the samples against a single concentration standard is considered valid. The coefficient of variation (CV) about the standard curve varied from 2.31 to 3.14%.

### Limit of Quantitation (LOQ)

The LOQ was estimated from the set of 7 calibration curves used to determine method linearity. The LOQ may be calculated as $10 \times \text{standard deviation (SD)}/\text{slope}$, where SD is the SD of the $y$-intercepts of the 7 calibration curves, and slope is the mean slope of the 7 calibration curves. The LOQ was calculated to be 6.25 μg/mL, which corresponds to 5.67 g/ton tiamulin in the feed samples.

### High Throughput Extraction

An improved method was developed to increase sample throughput by reducing the sample size and solvent volumes by four-fifths of the amounts used in the FDA-approved method. For extraction of 35 g/ton feed samples, the sample size was reduced from 50 to 10 g, and the volumes of 1% sodium carbonate and hexane–ethyl acetate (3 + 1) were reduced from 250 to 50 mL each. During the back-extraction step, the volumes of hexane–ethyl acetate extract (3 + 1) and 0.1% tartaric acid were reduced by two-thirds from 75 and 15 mL to 25 and 5 mL, respectively.
Table 1. Tiamulin recovery and precision from control feed sample

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tiamulin recovery, %</th>
<th>Tartaric acid, pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonspiked medicated feed (n = 2)</td>
<td></td>
<td>2.98</td>
</tr>
<tr>
<td>Spiked medicated feed</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50% spike (n = 6)</td>
<td>98.7 ± 1.40(^b)</td>
<td>2.97</td>
</tr>
<tr>
<td>CV</td>
<td>1.42%</td>
<td>0.4%</td>
</tr>
<tr>
<td>100% spike (n = 6)</td>
<td>95.8 ± 2.00(^b)</td>
<td>2.96</td>
</tr>
<tr>
<td>CV</td>
<td>2.06%</td>
<td>0.57%</td>
</tr>
<tr>
<td>150% spike (n = 6)</td>
<td>94.4 ± 2.30(^b)</td>
<td>2.97</td>
</tr>
<tr>
<td>CV</td>
<td>2.47%</td>
<td>0.33%</td>
</tr>
</tbody>
</table>

\(^a\) NDI, no detectable interference.
\(^b\) Based on standard deviation.
\(^c\) CV, coefficient of variation.

**Paired Sample Study**

The purpose of the paired sample study was to determine whether changes made to the FDA-approved method extraction would negatively affect the tiamulin sample results. This study compared the results of 17 Type C medicated swine feed samples guaranteed to be fortified with tiamulin at 35 g/ton. The high throughput method extraction was performed without the benefit of buffering the tartaric acid, an adaptation that was added later. The improved chromatographic conditions described in the high throughput method were used for LC analysis of samples for both extractions, because the original chromatographic conditions did not adequately resolve tiamulin in many samples.

A 2-method comparison plot shown in Figure 3 was used to graphically display the results of the paired sample study. The solid line represents perfect correlation between the 2-method extractions. The plot shows that the high throughput extraction yielded results higher than the FDA-approved method extraction for most of the samples studied. For some of the samples, there was a large difference between results for the 2 extractions. However, large variations in extraction efficiencies for some samples had been observed in past determinations, so this result was not surprising. As indicated earlier, buffering of the tartaric acid was later added to the back-extraction to reduce the large variation in back-extraction efficiency for the few problem samples.

A paired t-test for the 2 sample means was also used to compare extraction method performances. For the t-test, the null (H\(_0\)) hypothesis was that there is no difference between the 2 means for the different extractions. The sample mean for the FDA-approved method extraction was 30.20 g/ton with SD of 4.60 g/ton. The sample mean for the high throughput method was 33.79 g/ton with SD of 5.09 g/ton. Although the SD appears slightly larger for the high throughput method, the SDs did not differ significantly according to F-test results.

For the paired t-test, a 2-tailed t-statistic of 1.95 was calculated (p = 0.068), which is close to the 2-tailed critical value of 2.12 for 16 degrees of freedom at a significance level of 0.05. In this case, the null hypothesis was not rejected at the 5% significance level. The 95% confidence intervals for the data set were calculated further to determine how closely the high throughput method compares with the FDA-approved method. Based on these results, at the 95% confidence level, the true amount of tiamulin recovered by the high throughput method lies between 0.31 g/ton less than and 7.49 g/ton more than the FDA-approved method.

**Buffered Tartaric Acid**

It was observed that certain samples generated difficult emulsions, higher final tartaric acid pH, and lower sample recovery of tiamulin believed to be a result of poor back-extraction efficiency. When the sample results were plotted against the final pH of the tartaric acid extract, it was clear that the recovery of tiamulin depended on pH. Experiments were performed in which the initial pH of the tartaric acid was varied between 2.71 and 5.5. The amount of tiamulin back-extracted from the organic phase of the same sample into each of the tartaric acid solutions was quantitated by LC, and the final tartaric acid pH was measured by a calibrated pH meter. Figure 4 shows that, above a final tartaric acid pH of 3.5 or 4.0, the recovery of tiamulin back-extracted from the organic phase begins to decrease significantly.

A comparative review of the feed sample labels identified whey as the only ingredient specific to several of the problem feed samples with elevated final pH and low tiamulin recovery. The effects of whey on emulsion formation and sample recovery were examined by adding whey to medicated feed samples that did not contain whey. Results showed no conclusive evidence to suggest a correlation between added whey and emulsion formation or low recovery.

Another possibility was that a small amount of the 1% sodium carbonate base was entrained into the organic layer during extraction, which later reacted with the 0.1% tartaric acid during back-extraction. However, attempts to remove water by passing the organic phase through dried sodium sulfate before back-extraction were not successful. No significant difference was found in results between identical sample extracts, whether passed through sodium sulfate or not.

In an attempt to control the pH of the aqueous tartaric acid during back-extraction, the tartaric acid was dissolved in a phosphate buffer (pH 2.75). Several experiments showed that buffering the 0.1% tartaric acid dramatically improved the back-extraction efficiency compared with nonbuffered 0.1% tartaric acid. In tests with a problematic sample matrix, the final pH of the buffered extract averaged 0.85 pH units lower than that of the unbuffered 0.1% tartaric acid, and the amount of tiamulin recovered increased by 49%. In further tests with increased concentrations of buffer, optimum recovery was reached at 0.1M KH\(_2\)PO\(_4\), and higher buffer concentrations negatively impacted the tiamulin partition from the organic phase. The effect of buffer concentration on tiamulin recovery during back-extraction is depicted in Figure 5.
Recovery

A recovery study was performed by using control (no tiamulin added) feed samples fortified by standard addition at 50, 100, and 150% of the typical label concentration for Type C medicated feeds (35 g/ton). Six replicate extractions at each standard level were assayed by using the high throughput method with buffered tartaric acid. Two additional unfortified control feed samples (blanks) were analyzed to determine the potential for chromatographic interferences. The control feed sample chosen was a Type C medicated pig starter feed containing chlortetracycline, sulfathiazole, and penicillin (Table 1).

The standard spiking solutions were prepared by dissolving tiamulin hydrogen fumarate in the extraction solvent. To facilitate dissolution of the tiamulin hydrogen fumarate, 50 mL extraction solvent was mixed with 2 mL concentrated ammonium hydroxide. After allowing the 2 solution phases to separate, the upper organic (hexane—ethyl acetate) layer was used to dissolve 25 mg tiamulin powder in a 25 mL Class A volumetric flask. This stock solution was then diluted to the appropriate concentrations in three 10 mL Class A volumetric flasks with extraction solvent. Each set of sample replicates received a 1 mL spike of the appropriate level spiking standard before extraction.

A weighted linear regression model describes the best curve for depicting recovery of tiamulin by the high throughput method. A linear response was established by plotting the average of 6 replicate measurements of tiamulin at each level against the actual quantity of tiamulin added to each sample (g/ton). The weighted model was chosen because the SDs at the replicate levels were proportional to the magnitude of the value being measured and a plot of their residuals showed a heteroscedastic relationship. The average recovery line fit the weighted linear regression model, \( y = 0.9252x + 1.0924 \), with a coefficient of determination of 0.9977. With this model, the average amount of tiamulin recovered by the high throughput method was 33.65 g/ton, or 95.6% of the spiked amount. Based on the results, there is a 95% confidence that the true amount of tiamulin recovered by the high throughput method lies between 32.78 and 34.52 g/ton, which is 93.2–98.1% of the medicated level guaranteed in Type C feeds.

Precision

Precision was determined by tracking the performance of a medicated feed sample used for quality control (QC) of routine analyses. Between- and within-day variance of the QC sample were calculated by using analysis of variance (ANOVA; 5). Two separate weighings of the QC sample were determined by the high throughput method on each day for 23 nonconsecutive days. Table 2 shows the results of historic method performance before and after implementation of improved chromatographic conditions.

With the new chromatographic conditions, the high throughput method performance showed marked improvement in method precision. By comparison, the overall (combined within-day and between-day) precision of the FDA-approved method was 7.3% CV. The FDA-approved method results are based on 16 data points over a 5-year period by using a QC sample fortified at 35 g/ton. Likewise, results of the recovery study demonstrate the within-day variation among replicate fortified feed samples averaged 2.06%. These results are based on 6 replicates at the FDA-approved tiamulin medication level of 35 g/ton.

Method Ruggedness

A ruggedness study was performed to examine how sensitive the method is to minor changes in procedure. A 2\(^5\) partial factorial design was adopted where combinations of 6 factors were varied by ±10% from the high throughput method levels in a series of 8 experiments. The sample study design conforms to the formal ruggedness study design described by Youden and Steiner (6). Whereas Youden recommends variation from the norm in only one direction, this study incorporated ±10% variations from the normal method levels to examine the effect of changes in both directions.

The study examined the variability caused by changes in the concentration of the base in the aqueous layer (CB) used for extraction, the ratio of hexane to ethyl acetate (HE) in the organic layer, the ratios of aqueous to organic layer during initial extraction (AO), the concentration of tartaric acid (CT) in the back-extraction, the concentration of phosphate buffer for the tartaric acid solution (CB), and the initial pH of the buffered tartaric acid solution (PH). The below and above conditions for the study are presented in Table 3. The study used a medicated swine feed found historically to contain tiamulin at 26.6 g/ton.

For each of the 6 factors, the difference between levels was calculated by subtracting the average of the 4 above-condition results from the average of the 4 below-condition results. The absolute values of all 6 differences were then rank-ordered from smallest to largest (Table 3).

Historic results after implementing the improved chromatography from a QC sample were used to judge the typical variability associated with the method. The sample deviation, s, obtained for 8 replicate measurements at the fortified level was 0.8373. When the absolute value of each factor difference exceeds or differs to the formal ruggedness study design described by Youden and Steiner (6). Whereas Youden recommends variation from the norm in only one direction, this study incorporated ±10% variations from the normal method levels to examine the effect of changes in both directions.

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Based on the ruggedness results, the HE is the chief factor that contributes significantly to the sample result. Therefore, it is important to maintain tighter than ±10% control over this

<table>
<thead>
<tr>
<th>Table 2. Effect of chromatography changes on method precision</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard deviation</td>
</tr>
<tr>
<td>--------------------</td>
</tr>
<tr>
<td>Within-day</td>
</tr>
<tr>
<td>Between-day</td>
</tr>
<tr>
<td>Overall CV(^b)</td>
</tr>
</tbody>
</table>

\(^a\) The within-day variability was greater than the between-day variability.

\(^b\) Combined (within- and between-day) coefficient of variation.
factor during sample extraction and analysis. The HE is important in maintaining a proper solvent polarity for extraction of the tiamulin as the free base. The typical solvent polarity for hexane–ethyl acetate (3 + 1) is 1.100. A 10% variation in the volume of hexane (–30 mL) results in a slight variation in the solvent polarity (from 1.023 to 1.189). Based on the ruggedness study results, the minor adjustments to the solvent polarity can affect the final sample result by as much as 2.84 g/ton, more than twice the SD for the method. Because both solvents can evaporate on exposure to air (e.g., during mixing), it is recommended the hexane–ethyl acetate organic solvent be prepared fresh daily and kept in a tightly closed container. During preparation, the volumes of hexane and ethyl acetate should be kept within Class B tolerances of their target volumes.

The CT almost significantly affected the tiamulin results in the ruggedness study. However, the results are slightly above the significance threshold and are not considered sufficient evidence to warrant concern, because this concentration is controlled more tightly than ±10%.

Conclusions

The main objective of the present work was to adapt the extraction and chromatography of the FDA-approved method to accommodate the wider variety of feed sample matrices being tested, to increase sample throughput of the existing method, and to validate the improved method for regulatory use by the Indiana State Chemist Laboratory. To accomplish this, we reduced the sample size and extraction volumes, which enabled an increase in sample throughput by allowing more samples to be extracted simultaneously and by reducing the bulkiness of the method. This also decreased the amount of waste generated by the method. Centrifugation was added to the initial extraction step to facilitate phase separation and to shorten extraction times. Incorporation of buffered tartaric acid resulted in a more robust and efficient back-extraction. Finally, a new LC column and adjusted mobile phase composition were added to resolve the tiamulin peak from matrix components and to eliminate late-eluting matrix components. In a paired sample study, the high throughput method extraction compared favorably with the FDA-approved method. The high throughput method was successfully validated in-house with good linearity ($R^2 = 0.99998$), recovery (95.8%), precision (2.06% RSD), and ruggedness (with the hexane–ethyl acetate ratio control recommended).

Acknowledgments

We thank Beth Anne Sturgeon (Office of Indiana State Chemist, West Lafayette, IN) for preparing and grinding all study samples.

References


Table 3. Ruggedness test conditions and results

<table>
<thead>
<tr>
<th>Factor</th>
<th>Below conditions (+)</th>
<th>Above conditions (−)</th>
<th>Factor differencea</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous organic (AO)</td>
<td>0.9:1</td>
<td>1.1:1</td>
<td>−0.026</td>
</tr>
<tr>
<td>Concentration of base (CB)</td>
<td>0.9% Na₂CO₃</td>
<td>1.1% Na₂CO₃</td>
<td>0.039</td>
</tr>
<tr>
<td>Initial pH of tartaric acid (PH)</td>
<td>2.5</td>
<td>2.9</td>
<td>−0.598</td>
</tr>
<tr>
<td>Buffering of tartaric acid (BT)</td>
<td>0.09M KH₂PO₄</td>
<td>0.11M KH₂PO₄</td>
<td>−1.022</td>
</tr>
<tr>
<td>Concentration of tartaric acid (CT)</td>
<td>0.09%</td>
<td>0.11%</td>
<td>1.307b</td>
</tr>
<tr>
<td>Hexane to ethyl acetate (HE)</td>
<td>2.7:1</td>
<td>3.3:1</td>
<td>−2.837b</td>
</tr>
</tbody>
</table>

a Factor differences determined by average of 4 above (−) conditions minus the average of 4 below (+) conditions (expressed in g/ton).
b Absolute value exceeds 1.294 (or $\sqrt{25}$).