Development and Optimization of a Simple and Sensitive Method for the Determination of Virginiamycin M1 Antibiotic in Aqueous Media by Capillary Electrophoresis

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Development and optimization of a simple and sensitive method for the determination of virginiamycin M1 antibiotic in aqueous media by capillary electrophoresis

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Abstract

Virginiamycin antibiotic is used in Mexico for animal growth promotion, but has been banned in Europe due to risk of resistance development. Given that monitoring of antibiotics is critical, the objective of this work was to develop an analytical method based on capillary electrophoresis and liquid-liquid extraction, to quantify virginiamycin antibiotic in livestock wastes. Linearity, precision, bias and extraction parameters were evaluated following ISO/IEC 17025 procedures. Method and extraction validation was satisfactorily, with average values of absolute recovery $AR = 86\%$, extraction efficiency $EE = 87\%$, and matrix effects $ME = -14\%$, $RSD < 15\%$ for concentrations as low as 50 µg/L.

Keywords: Capillary electrophoresis, Wastewater, Antibiotics, Monitoring, Extraction, Quantification

1 Introduction

Virginiamycin, an antibiotic belonging to streptogramin class, and mixture of two components: factors M1 and S1, acts individually against *Micrococcus aureus* and *Bacillus subtilis*, but in mixtures becomes highly effective against vancomycin and methicillin-resistant infections in humans, especially from *Enterococcus faecium* and *Staphylococcus aureus* (Hammerum et al., 1998). Relevant properties and structure for M1 are depicted in Figure 1. Composed of lactone and containing pyrrolidine and oxazole rings and equal to mikamycin A, ostreogrycin A, streptogramin A and pristinamycin IIA (Goffic et al., 1977), M1 factor assembles a lipophilic, neutral, and low-degradable molecule mainly evacuated in excreta without relevant assimilation in animal tissues.

Mexico and the USA have approved virginiamycin as a feedstuff supplement, alone or combined with other agents in cattle, pig and poultry for disease prevention, increased weight growth and fodder efficiency, at doses ranging from 5 g/ton to 25 g/ton of food (Anadón and Martínez-Larrañaga, 1999). These growth promoters are administered for prolonged periods of time at
sub-therapeutic concentrations. However, with such scheme arises an increased risk of inducing antimicrobial resistance in animal pathogens, which could colonize humans and transfer their resistance to other microorganisms (Wegener, 2003). Evidence of adverse human health consequences include infections that probably would not occur, as with fluoroquinolone-resistant Salmonella cases (Ventola, 2015). Several researchers argue that resistant infections in clinical settings have been due to migration of genetic determinants, between environmental bacteria and human pathogens (Martinez, 2009b; Wright, 2010). Due to incorrect use of veterinary antibiotics, resistant bacteria populate the environment, animals and food products, increasing pathogen exposure and triggering human infection difficult to treat (Martinez, 2009a).

Europe banned use of virginiamycin for animal growth in 1998, due to concerns about impairing the efficacy of streptogramin to treat human infections due to rise of resistance (Aarestrup et al, 2001). However, Mexico and other countries consider virginiamycin safe and authorize it as a growth promoter. Because most of virginiamycin intake is excreted to environment (de Sanidad Inocuidad y Calidad Agroalimentaria, 2016), the risk of resistance development in environmental bacteria arise, justifying an antibiotic monitoring as part of a surveillance program (Sandegren, 2014; Bellanger et al, 2014; Bengtsson-Palme and Larsson, 2015). In fact, different studies revealed selection of resistant bacteria at low concentrations, like those found in aquatic environments receiving agricultural effluents (Hernando et al, 2006; Martinez, 2009b).

To quantify virginiamycin, scientists have preferred chromatography techniques, such as HPLC and GC, but these methods results in high costs and processing time. Capillary electrophoresis (CE) techniques stand out in monitoring applications due to their high efficiency, economy and low volume sample required. In CE, the analyst could use main techniques: (i) capillary
zone electrophoresis (CZE), and (ii) micellar electrokinetic chromatography (MEKC). In MEKC, micelles and additives dissolves in the sample or background buffer (BGB) to allow massive injection and quantify without loss of resolution (Aranas et al., 2009; Wuethrich et al., 2016). To our knowledge, none CE-based method has been published or validated to quantify virginiamycin factor M1 in waste effluents (Flurer, 2001; Tobback et al., 1999). In consequence, in this work the authors developed an ease and economical MEKC method to quantify virginiamycin M1 in livestock effluents. The specific objectives were to assess the effects on analyte separation from: i; addition of NaCl in samples, ii; injection time and iii; buffer composition. Method was validated according to ISO/IEC-17025 recommendations (Magnusson, 2014).

2 Methods

2.1 Reagents and Solutions

Antibiotic standard solutions were prepared weekly using virginiamycin M1 (Sigma Aldrich, purity 95 %, CAS 21411-53-0) with methanol (J. T. Baker) as diluent and stored at 275.15 K. Working solutions were prepared diluting standard solutions with NaCl (J. T. Baker) 0.5% w/w. Background buffer (BGB) was prepared mixing quantities of sodium dodecyl sulfate solutions (SDS, CE grade), phosphoric acid (85% w/w), monosodium phosphate, and β-cyclodextrin (Sigma Aldrich). All solutions and samples were filtered using 0.45 um nylon filters (Millipore).

2.2 Capillary Electrophoresis Procedures

An MDQ P/ACE Capillary Electrophoresis System (Beckman Coulter) was used to separate samples. A capillary tube filled with the sample and BGB
are subjected at a constant voltage and current, separating molecules at different electrophoretic velocities and read in a capillary window by a diode array detector (DAD). The analytical method is based on Micellar Electrokinetic Chromatography (MEKC), with BGB consisting of SDS, methanol, phosphates and β-cyclodextrin as partition modifier. The MEKC-CE technique was introduced by Terabe et al. (1984) to analyze neutral compounds typically impossible to separate by capillary zone electrophoresis (CZE). The conditions and compositions depend on analyte and capillary selected. To obtain the best results, buffer and instrument conditions were optimized and validated using different approaches, as summarized in Figure 2.

A bare-fused silica capillary covered with polyamide (Polymicro Technologies) was used for separation using negative voltages. For this study, capillary length-to-window of 50 cm and diameter of 50 µm was used to enhance resolution. However, different lengths of capillary tubes could be used, resulting in variable migration times but with no significant loss of efficiency at total lengths greater than 40 cm. Capillary conditioning was required for precision, with 2 min rinses of HCl 0.5 M, NaOH 0.5 M and BGB between runs. After conditioning, up to \(\approx 15\) cm of sample plug was injected. Both ends of capillary were placed in buffer vials and a negative voltage from 12 kV to 25 kV was applied. Temperature was held constant in the range of 293.15 K to 298.15 K. The instrument injects using as low as 5 µL of sample volume, generally by hydrodynamic pressure. Samples and calibrators were diluted with NaCl solution from 0.25 to 1.0% w/w and 10% acetonitrile. Absorbance measures at the capillary window was read at 220 nm, recording an electropherogram with analyte and sample components signals. To quantify the analyte, a standard curve was built, correlating concentration against analyte peak area. Working range was selected as the linear portion of calibrated curve. Finally, assessment
of separation efficiency was realized comparing values of the number of theoretical plates $N$, resolution $R_s$ and peak height $H$. While $H$ is given directly, $N$ and $R_s$ were calculated according to equations 1 and 2, where $t_m$ stands for migration time and $w_h$ for peak width at half height.

$$N = 5.545 \left( \frac{t_m}{w_h} \right)^2 \quad (1)$$

$$R_s = \frac{t_{m2} - t_{m1}}{0.5 \times (w_{h1} + w_{h2})} \quad (2)$$

### 2.3 Development of Analytical Method

To determine the most significant factors of running buffer that affects analyte separation, an experimental design approach was chosen, as it enables lower time consumption and best data analysis. A screening method based on a $2^{4-1}$ reduced factorial design was set-up to identify the most significant buffer variables at two levels: A-pH; B-SDS; C- phosphate and D-β-cyclodextrin, as shown in Table 1. Response variables $H$ and $N$ were computed using Design Expert 7.0 software. Other variables, were optimized modifying one variable at a time with a constant running buffer. The selected variables were: A-NaCl percent added to standard; B-separation voltage; C-injection time and D-cartridge temperature. Selection of the best conditions was done comparing electropherograms and peak efficiencies.

### 2.4 CE Method Validation

The CE method was validated by evaluating precision, linear range, limit of detection (LOD), limit of quantitation (LOQ) and bias. An Analysis of Variance (ANOVA) was used to compare variation within groups at 95 percent confidence. The LOD is the lowest concentration that could be detected with
enough confidence; it was approximated by diluting a calibrator solution until
signal of analyte is similar to noise, then analyzed ten times and calculated
as $LOD = 3 \times S'_o$, where $S'_o = \frac{S}{\sqrt{n}}$ explains data dispersion. The limit of
quantification (LOQ) was then estimated as $LOD \times 5$.

To quantify virginiamycin, a linear regression of measured absorbance
against concentration was built using seven calibrators determined by tripli-
cates at analyte concentrations of 0.05, 0.1, 0.2, 0.4, 0.8, 1.6 and 3.2 mg/L.
Precision was evaluated analyzing single and intra-day repeatability of the
smaller calibrator along with two midpoints, running five and three repeti-
tions respectively. For inter-day repeatability, analysis was done at three days
at constant conditions. One-factor ANOVA was run to test for variability.
Repeatability limit ($r$) was calculated as $r = 2.8 \times S_r$, where $S_r$ is the stan-
dard deviation of repeatability. Intermediate precision was obtained by the
square root of the sum of the squares of within-group and between-group
precision. Then standard deviation of the repeatability $S_r$ was obtained by
calculating the square root of the within-group mean square term, which rep-
resents the within-group variance as $S_r = \sqrt{MS_i}$. Contribution to the total
variance of the clustering factor $S_i$ is also obtained from the ANOVA test:
$S_i = \sqrt{\frac{MS_e - MS_i}{n}}$. The intermediate precision $S_I$ was calculated by combi-
ing both terms: $S_I = \sqrt{S^2_r + S_i^2}$. Finally, bias was measured in relative terms
as a percentage of recovery $R' = \frac{\bar{x} - \bar{x}_{add}}{x_{add}} \times 100$ using blank and spiked samples,
where $\bar{x}'$ is the average spiked value, $\bar{x}$ is the average value with the added
concentration and $x_{add}$ is the added standard (Magnusson, 2014).

### 2.5 Sample Treatment and Extraction Validation

Immediately after sampling, waste water was acidified to pH 4 with sulfuric
acid 85% w/w, filtered and stored at 275.15 K. Antibiotic extract was obtained
using a liquid-liquid biphasic systems as described by Díaz-Quiroz et al (2020).

Such procedure is based on the formation of an aqueous two-phase system (ATPS) comprised of citrate salt, acetonitrile and water. The system produce an organic layer rich on acetonitrile in top phase to which virginiamycin migrates. The bottom phase forms an aqueous phase rich in citrate salt, where majority of interferences are removed from the sample. For the ATPS sample clean up, a diagram phase and tie line at constant pH and temperature was used at an appropriate composition. Selected system was prepared at 298.15 K with 800 µL of sample volume, 0.6 g of citrate salt and 150 µL of acetonitrile.

To form the two phases, the substances in the vials were dissolved aided by a sonication bath for 5 min. Then, vials were centrifuged for 3 minutes and an aliquot of 10 µL was transferred to a microvial. Finally, 90 µL of diluent was added, vorterized and ready for injection in the CE system. Fortified samples were prepared in the same fashion, but adding antibiotics to diluent. Performance was verified using the parameters of extraction efficiency $EE$, matrix effects $ME$ and recovery $AR$, as disclosed in following equations:

$$EE = \left( \frac{C}{B} \right)$$  \hspace{0.5cm} (3)

$$ME = \left( \frac{B}{A} - 1 \right)$$  \hspace{0.5cm} (4)

$$AR = \left( \frac{C}{A} \right)$$  \hspace{0.5cm} (5)

Each variable represents the peak area of virginiamycin M1 in the conditions: $A$, in the standard solution; $B$, spiked in sample after extraction; $C$, spiked in sample before extraction. Matrix effects indicate if suppression of
signal occurs (negative values), or if the signal has been enhanced (positive values).

3 Results

3.1 Method development

Initially, the MEKC method was initiated with the injection of a standard virginiamycin M1 solution dissolved in distilled water. A typical electropherogram for the separation is presented in Figure 3 at a concentration of 20 mg/L, at \(t_m\) around 13 min.

Then, a factorial design was employed to determine which BGB conditions affect \(H\) and \(N\). The results are presented in Table 2, where \(H\) and \(N\) differ significantly for several treatments. All experiments were performed at 298.15 K, -14 kV and standard injection of 60 s at 13.79 kPa (2 psi), corresponding to an injection of about 15 cm of capillary length. Enhanced behavior at lower pH probably is due to reduction of electroosmotic flow at pH < 4. Likewise, micelle properties depend on pH; hydronium ions decrease electrostatic repulsion of the charge heads by decreasing the charge density on the surface of the micelle, thus increasing hydrophobic affinity for virginiamycin and granting efficient separations. Regarding buffer composition, the treatments with lower levels (–) of: sodium phosphate, SDS and β-cyclodextrin, allowed better separations. Behavior of the different variables are better understood using a 3D plot, as in Figure 4. Reviewing those surfaces, clearly β-cyclodextrin addition proved detrimental to separation and therefore optimum experimental conditions closely fluctuate around pH 2, 35 mM monosodium phosphate and 60 mM SDS.

Statistical information to estimate error and significance was done with aid of ANOVA. It was performed only for peak height \(H\), because it is not
affected by small variations of \( t_m \) or capillary length. According to Table 3, the
table has statistical significance and \( pH \) sets the main influence on response.
Goodness of fit test also suggests adequate model adjustment and precision,
with \( \text{Adj.} R^2 > 0.8 \) and \( \text{RSD} < 15\% \). Regarding optimization of \( \text{NaCl} \) added
to sample and injection volume, addition of \( \text{NaCl} \) from 0.1\% to 1\% to standard
solution increased \( N \) massively; as for the standard peak \( N \) rises up to \( 7 \times 10^5 \).
In this MEKC method, online concentration was based on salt addition to
samples, in a manner ionic strength scale up two to three times higher than
BGB (Palmer et al., 1999). It causes an analyte sweeping at the interphase of
sample plug and BGB, allowing increased sample injection without affecting
negatively the analyte separation. The salt comprised \( \text{NaCl} \) or \( \text{KLi} \), enabling
injection of sample plug up to 15 cm. Interferences from samples will affect the
volume injected, reducing \( N \) and \( H \) but in the method conditions, injection
length up to 15 cm did not affect the separations. However, injections higher
than 15 cm resulted in gradual peak broadening and loss of \( N \). Considering
typical injection in CE fluctuates around 1 cm, then online concentration up to
15 folds of analyte could be attained. Finally, temperature between 295.15 K
and 230.15 K and voltages between 12 kV and 20 kV performed satisfactorily.

3.2 Method Validation
Validation parameters evaluated were precision, working range, bias, \( \text{LOD} \) and
\( \text{LOQ} \). Linear regression and goodness of fit are presented in Table 4. Working
range was validated from 0.05 mg/L to 3.2 mg/L. Repeatability and interme-
diate precision were 9.57\% and 11.57\% RSD respectively along the working
range. Bias was calculated as \( R\% = -8.51 \) and \( RSD\% = 12.16 \), which repre-
sent acceptable values. \( \text{LOD} \approx 0.093 \text{mg/L} \) and \( \text{LOQ} \approx 0.047 \text{mg/L} \) were also
estimated.
3.3 Extraction Validation

Figure 5 shows an electropherogram for blank and spiked sample at 5 mg/L. As noted, not major signal suppression appears at 1:9 weight ratio dilution, though spiked samples have to be analyzed for every run. Further, resolution $R_s \geq 1.5$ seems reasonable to detect and quantify virginiamycin M1. To verify the identity of analyte, Figure 6 shows EG’s of samples fortified before and after extraction, at 50 µg/L and 500 µg/L respectively. Virginiamycin peak appears labeled and their vicinity is spanned in the bottom EG, showing the identity of virginiamycin unambiguously. Extraction data is presented in Table 5 for three concentrations. The computed parameters were: $EE = 0.87$, $ME = -0.14$ and $AR = 0.86$. Since signal suppression stand low, no significant matrix effects reduce analyte signal. Also, average values of $EE$ and $AR$ found satisfactory, allowing most analyte recovered from extraction procedure.

4 Conclusions

Data confirms extraction of virginiamycin as an efficient process, with satisfactory recovery and minimal matrix effects at a 1:9 sample dilution ratio. The method proves fast and reliable analysis for concentrations as low as 50 µg/L.
5 Tables

Table 1 Two-levels screening factorial design

| Factor                        | (-1) | (0) | (+) |
|-------------------------------|------|-----|-----|
| A: pH                         | 2    | 3   | 4   |
| B: Sodium dodecyl sulphate    | 35   | 50  | 70  |
| C: Sodium phosphate\(^1\)     | 30   | 60  | 90  |
| D: \(\beta\)-ciclodextrin     | 0    | 5   | 10  |

Except for pH, treatment levels are concentrations expressed in mM
\(^1\)Sodium phosphate monohydrate

Table 2 Results of screening factorial design.
Dependent variables, peak height \(H\) and theoretical plate numbers \(N\)

| Run | A  | B     | C     | D     | \(H\)  | \(N\)  |
|-----|----|-------|-------|-------|-------|-------|
| 1   | 3  | 90    | 50    | 5     | 4152  | 61134 |
| 2   | 4  | 120   | 70    | 10    | 2014  | 12314 |
| 3   | 4  | 60    | 30    | 10    | 1661  | 5639  |
| 4   | 3  | 90    | 50    | 5     | 4315  | 60415 |
| 5   | 4  | 120   | 30    | 0     | 2779  | 35468 |
| 6   | 3  | 90    | 50    | 5     | 3833  | 54890 |
| 7   | 3  | 90    | 50    | 5     | 3476  | 46119 |
| 8   | 2  | 120   | 70    | 0     | 4521  | 60757 |
| 9   | 2  | 60    | 70    | 10    | 4177  | 58972 |
| 10  | 2  | 60    | 30    | 0     | 5586  | 70000 |
| 11  | 4  | 60    | 70    | 0     | 2430  | 27863 |
| 12  | 2  | 120   | 30    | 10    | 4278  | 56884 |
| 13  | 3  | 90    | 50    | 5     | 5000  | 6000  |
Table 3  ANOVA data for screening factorial design of analyte peak height $H$

| Source      | df | F-value | p-value |
|-------------|----|---------|---------|
| Model       | 5  | 10.97   | 0.0056  |
| A           | 1  | 47.55   | 0.0005  |
| B           | 1  | 0.0349  | 0.8581  |
| C           | 1  | 0.6855  | 0.4394  |
| D           | 1  | 5.15    | 0.0637  |
| AB          | 1  | 1.41    | 0.2801  |
| Curvature   | 1  | 6.56    | 0.0428  |
| Lack of Fit | 2  | 0.265   | 0.7797  |

| Adeq. Precision | $R^2$ | Adj.$R^2$ | RSD% |
|-----------------|-------|-----------|------|
| 9.9766          | 0.9014| 0.8192    | 13.38|

Table 4  Virginiamycin standard calibration in dH2O and extract from wastewater samples. Addition of ten percent v/v acetonitrile and 0.25 percent w/v NaCl was added to all calibrators as diluent.

| Calibration | Best-Fit Values | Std Error | Goodness of Fit |
|-------------|-----------------|-----------|-----------------|
| dH$_2$O     | Slope  | Y-intercept | Slope  | Y-intercept | R squared | $Sy.x^1$ |
|             | 4075   | -109.1     | 117.2  | 54.04      | 0.9955    | 98.9     |
| Extract$^2$ | 3618   | -189.1     | 149.53 | 86.63      | 0.9904    | 123.86   |

$1 Sy.x = \sqrt{\frac{\sum(residual^2)}{n-k}}$

$2$The extract was reconstituted with diluent in a 1:9 weight ratio.

Table 5  Validation of Virginiamycin M1 extraction using external standard calibration. Values are average of three replicates.

| $Y(\mu g/L)$ | $\hat{Y}(\mu g/L)$ | RSD $\times 100$ |
|--------------|---------------------|------------------|
| 50           | 44.29               | 14.39            |
| 250          | 221.1               | 2.09             |
| 500          | 506                 | 5.96             |
Fig. 1 Virginiamicyn M1 molecule. Relevant properties calculated by OPERA: \( \text{LogP} = 2.49 \), \( \text{Solubility} = 2.37 \times 10^{-3} \, \text{mol/L} \), \( \text{Biodegr. HalfLife} = 3 \, \text{days} \)
Fig. 2 Flowchart of the general method development of Virginiamycin M1 analyzed by capillary electrophoresis in porcine wastewater
Fig. 3 Typical electropherogram of virginiamicyn (1) standard analyzed by CE-MEKC at 20 mg/L. Conditions: 15 kV, 25 °C, capillary 40 cm length and 50 µm ID, reverse polarity.
Sample: 0.25 percent w/v NaCl in distilled water. Buffer: SDS 35 mM, sodium phosphate 30 mM, methanol 20 percent v/v, pH 2 acidified with HCl
Software: Karat 32
Fig. 4  Response surface plots for the reduced factorial design in the analysis of viginiamycin M1. Independent variables are pH and molar concentrations of SDS, sodium phosphate and β-cyclodextrin. Dependent variable is analyte peak height $H$. Concentrations of phosphate and cyclodextrin corresponds to following plots, coded in parenthesis: A, 35 mM phosphate without β-cyclodextrin (−, −); B, 70 mM phosphate and 10 mM β-cyclodextrin (+, +); C, 70 mM phosphate without β-cyclodextrin (+, −) and D, 30 mM phosphate with 10 mM β-cyclodextrin (−, +)  
Software: Design-Expert 12
Fig. 5 Electropherogram (EG) of virginiamycin extraction in wastewater by MEKC. Bottom EG is from a blank sample and top EG is a spike added after extraction. Software: Karat 32
Fig. 6 Electropherogram (EG) of virginiamycin (1) extraction in wastewater by MEKC. Bottom EG is at 50 µg/L and top EG is at 500 µg/L. Software: Karat 32
Ethical Approval

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author C.Díaz-Quiroz, upon reasonable request.

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**Statements and Declarations**

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6.2 Competing Interests
All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.
6.3 Authors Contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Juan Francisco Hernández-Chávez and Jesús Fernando Robles-Castro. The first draft of the manuscript was written by Carlos Abraham Díaz-Quiroz. Gabriela Ulloa-Mercado and Carlos Díaz contributed to original concept, funding, supplied reagent and manuscript revisions and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.