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Optimizing the Separation of an Antiparasitic Medication using High-Pressure Liquid Chromatography (HPLC)

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Abstract

Excess pharmaceutical waste in water is an emerging concern that can increase parasitic drug resistance, interrupt animal food chains, and threaten drinking water sources. In this work, a high-pressure liquid chromatography (HPLC) method with ultraviolet detection (210 nm) was optimized for sensitively detecting and separating antiparasitic compounds praziquantel (PZQ) and metronidazole (MET). This method has the potential to commercially monitor antiparasitic treatments administered to aquatic species, which can ultimately prevent pharmaceutical waste in water. The latest HPLC method was altered over seven experiment trials to improve resolution and Gaussian shape of chromatogram peaks. The most efficient separation of PZQ and MET was achieved on a Phenomenex™ Luna C_{18} analytical column (150 x 4.60mm, 5µm, 100A) using acetonitrile:water at alternating ratios of 20:80 v/v and 80:20 v/v as a mobile phase. This separation resulted in the shortest acquisition time with satisfactory peak shape. Aquarium facilities may ultimately use this method to understand how to safely treat parasitic fish diseases while avoiding environmental damage.

Keywords: HPLC, antiparasitics, separations, aquatic environment

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Introduction

Pharmaceutical compound waste in water sources poses a threat to environmental, animal, and human life.[1] Aquatic environments are specifically endangered as certain pharmaceutical compounds are difficult to remove in wastewater treatments.[2] Praziquantel (PZQ) is an anthelmintic compound used to treat parasitic infections in aquatic species.[3] PZQ is commonly administered to fish orally or directly dispersed in water ("bath" treatments).[4] However, there are no officially established guidelines for administering PZQ treatments.[4] As such, improper doses may indirectly harm other organisms and environments. For example, several research studies maintain anthelmintics may disturb vital food webs and lead to parasite resistance.[5] Of further concern, PZQ residue may remain in fish marketed for human consumption; there is no established limit for PZQ allowed in human foods. [4] Therefore, PZQ needs to be more effectively quantified and regulated in aquatic environments. Establishing a reliable detection method is critical for monitoring pharmaceutical environmental pollution.

This work aimed to determine a more efficient high-pressure liquid chromatography (HPLC) method for separating PZQ and related antiparasitic compound metronidazole (MET) in a powdered medication. HPLC is commonly selected for detecting and separating pharmaceuticals due to its effective trace analysis capabilities for polar compounds.[6] MET is a polar and potentially carcinogenic compound; an HPLC method can reveal how this compound is distributed and disposed of in water.[7] Many previously developed HPLC separation methods for PZQ alone are used for human antiparasitic medications.[8] While potentially useful for routine analysis, these methods are not applicable for monitoring pharmaceuticals in aquatic systems. In addition, HPLC methods used for aquaculture-based PZQ often only separate a PZQ standard rather than separating PZQ in an antiparasitic medication.[9] As PZQ may be more commonly administered with other pharmaceuticals (such as MET), it is more beneficial to explore separating PZQ from other drugs. In this work, an HPLC method was optimized for the separation of PZQ and MET in a medication. As MET is a more polar compound compared to PZQ, MET was expected to elute from the nonpolar HPLC column first. The polarity of the two compounds and mobile phase was considered to improve the separation.

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[1] *Analyst*. **2012**, *137* (17), 4037–4044.; *Chemosphere*. **2014**, *99*, 224–232.; *Anal. Bioanal. Chem*. **2010**, *398*, 1185–1194.; *Anal. Bioanal. Chem.* **2015**, *407* (30), 9085–9104.; *J. Sep. Sci*. **2014**, *37* (11), 1289–1296.; *J. Chromatogr. A.* **2012**, *1258*, 1–15.

[2] Environ. Toxicol. and Pharmacol. **2009**, *27* (2), 161–175.

[3] *Chemosphere*. **2016**, *144*, 2290–2301.; Environ. Toxicol. and Pharmacol. **2009**, *27*(2), 161–175.; Bader, C. "Use of Praziquantel."

[4] Bader, C. "Use of Praziquantel."

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[7] *Rep. Carcinog*. **2016**, *1948*, 5.

[8] *Marmara Pharm. J*. **2015**, *19* (1), 27–35; *Arab. J. Chem.* **2017**, *10*, S35–S41; *Int. J. Pharm. Pharm. Sci*. **2019**, *11* (5), 62–67.

[9] "Praziquantel Determination in Aquarium Water."

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Experimental Methods

An ultraviolet-visible (UV-Vis) light spectrum was taken of a 22.6 ppm PZQ standard (Sigma-Aldrich, St. Louis, MO) with a LAMBDA™-365 UV-Vis spectrophotometer (PerkinElmer, Waltham, MA) to determine an optimal wavelength for the HPLC-UV detector. The standard was prepared in 50:50 v/v acetonitrile:water (ACN:H_2O) based on previous literature methods.[10]

A PerkinElmer Flexar™ FX-20 HPLC-UV (Waltham, MA) was used to detect the PZQ standard and separate PZQ and MET in the powdered antiparasitic medication (API®, Chalfont, PA) with a Phenomenex™ Aqua C_{18} analytical column (150 x 4.60mm, 5µm, 100A) (Torrance, CA; later changed to a Luna C_{18} analytical column).

The PZQ and MET structures and chemical details are shown in Figure 1 and Table 1, respectively. Table 2 describes the injected samples. The samples were run in triplicate; blank samples were injected before and after each triplicate run.

Results and Discussion

In this work, an HPLC method was optimized for separating PZQ and related compound MET in an antiparasitic medication. The HPLC instrument was coupled to a UV detector, an appropriate choice as the detector is compatible with a gradient mobile phase.[11] The selected wavelength of a UV detector is crucial for determining the absorption properties of an injected sample.[12] The optimal wavelength was determined as 210 nm based on the most relevant shoulder peak displayed in Figure 2.

[10] Praziquantel Determination in Aquarium Water; *J. Braz. Chem. Soc.* **2015**, *26* (4), 729–735.
[11] LaCourse, M. E.; LaCourse, W. R. *General Instrumentation in HPLC.*
[12] Snyder et al. *Introduction to Modern Liquid Chromatography.*

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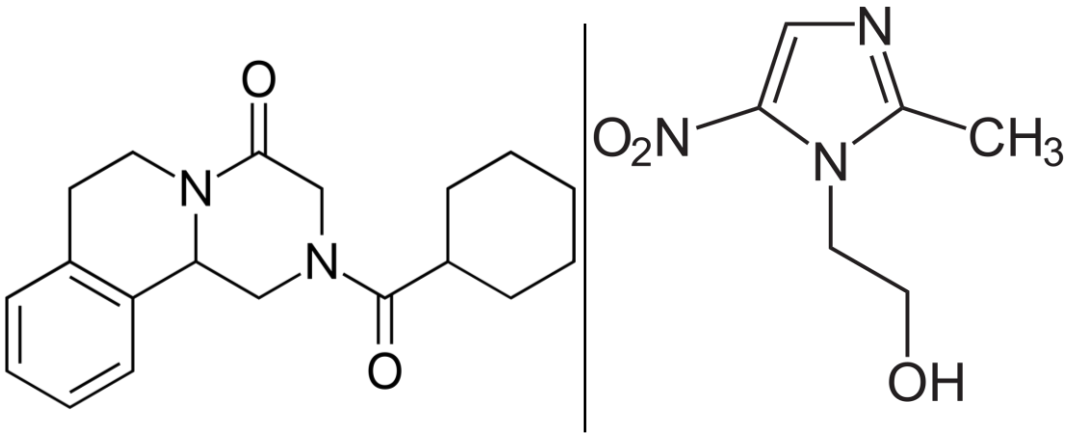

Figure 1. Praziquantel (PZQ) (left) and metronidazole (MET) (right) 2D structures. The compounds were ultimately separated with an optimized HPLC method.

Table 1. Relevant chemical properties of PZQ and MET. Higher hydrophobicity indicates higher affinity for a nonpolar HPLC column.

	Praziquantel (PZQ)	**Metronidazole (MET)**
Molecular Weight ($gmol^{-1}$)	312.4	171.2
Water Solubility at 25˚C (mgL^{-1})	400	11,000
logKow (Hydrophobicity Constant)	2.42	-0.02

Table 2. Summary of injected samples for HPLC trials. The PZQ standard was used to confirm the compound's identity in an HPLC method. The treatment powder was the sample used for separating PZQ and MET. ACN = acetonitrile.

Injected Samples		
Type	**Conc. of PZQ (ppm)**	**Matrix**
Praziquantel Standard	22.6	50:50 ACN:H_2O
API® Parasitic Fish Disease Treatment Powder	21.2	80:20 ACN:H_2O, then 50:50 H_2O dilution

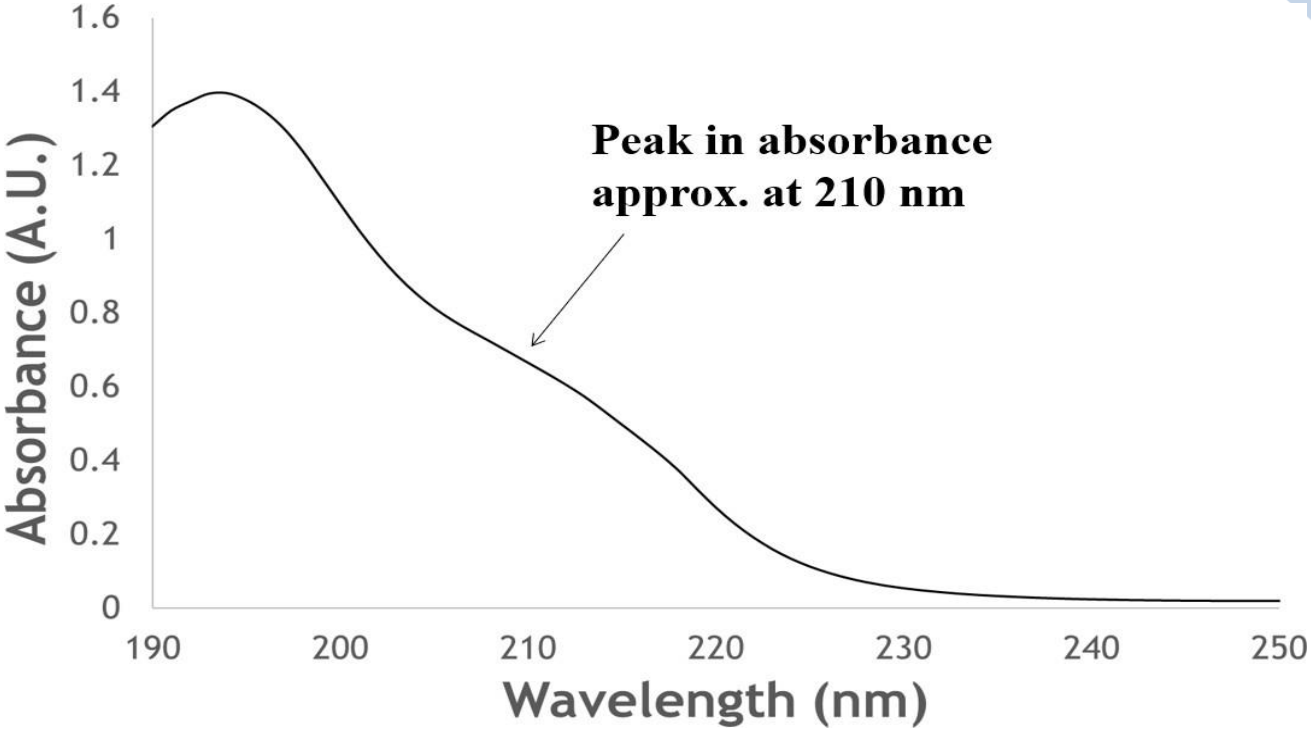

Figure 2. UV-Vis spectrum recorded between 190 and 250 nm for PZQ standard in 50:50 ACN:H_2O. 210 nm was selected as the wavelength for the UV detector in the HPLC instrument.

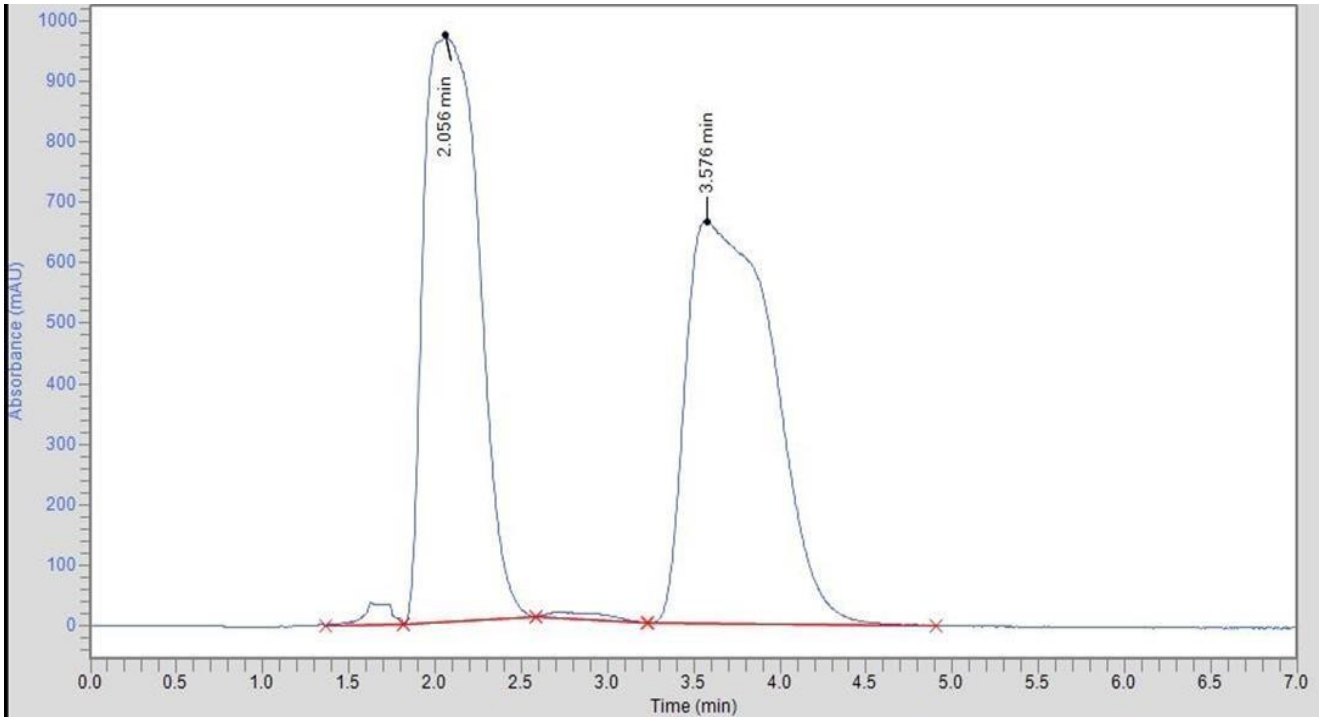

Figure 3. Separation of 50µL sample injection with 50:50 ACN:H_2O as the mobile phase (based on literature parameters).[9] The separation was insufficient based on non-Gaussian peak shape.

Because a shorter wavelength will detect more extraneous compounds in a solution, the prominent peak at approximately 195 nm was not considered. The 210 nm wavelength was satisfactory for HPLC-UV experiments because PZQ typically absorbs light between 200-220 nm and UV detection is most sensitive at wavelengths lower than 220 nm.[13] Figure 3 shows the results of the first attempted HPLC method prior to optimization adjustments. The 50:50 ACN:H_2O v/v mobile phase was selected to retain the UV-Vis matrix conditions and verify the method proposed by Herzig and Gilde.[9] The Aqua C_{18} analytical column was the most readily available nonpolar stationary phase when initially conducting experiments. While the compound peaks in Figure 3 are generally separated, they do not have an ideal Gaussian distribution for properly quantifying the compounds. This indicates the method was not efficient enough. Because the polarity of the mobile phase strongly affects the resolution of peaks, the ratio of organic and aqueous solvent in the mobile phase was modified over six following experiment trials. In addition, the stationary phase was upgraded to a newer nonpolar column, a Phenomenex™ Luna C_{18} analytical column (150 x 4.60mm, 5µm, 100A), in between trials to improve resolution quality. Tables 3A and 3B show trial parameters and advantages and disadvantages of each trial modification. The method was officially finalized with the parameters described in the final row of Table 3A.

[13] "Praziquantel Determination"; *J. Braz. Chem. Soc.* **2015**, *26* (4), 729–735; Snyder et al. *Introduction to Modern Liquid Chromatography*.

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Table 3A. HPLC experimental trials showed how changes in the mobile phase and method acquisition time affected compound separation. Note acquisition time does not include the pre-equilibrium time of the total method (five minutes). ACN = acetonitrile.

Trial	Mobile Phase	Stationary Phase	Matrix	Injection Volume (µL)	Acquisition Time (min.)
1	50:50 ACN:H_2O	Phenomenex ™ Aqua C18	Prepared in mobile phase; diluted 50:50 in H_2O	50	7
2	50:50 ACN:H_2O	Aqua	Prepared in mobile phase; diluted 50:50 in H_2O	5	7
3	50:50; 45:55; 40:60; 35:65; 30:70 ACN:H_2O (non-gradient; in consecutive injections)	Phenomenex ™ Luna C18	Prepared in 50:50 ACN:H_2O	5	7
4	30:70 ACN:H_2O; immediate switch to 80:20 ACN:H_2O	Luna	Prepared in 80:20 ACN:H_2O; diluted 50:50 in H_2O	5	35
5	20:80 ACN:H_2O; immediate switch to 80:20 ACN:H_2O	Luna	Prepared in 80:20 ACN:H_2O; diluted 50:50 in H_2O	5	13; 12 (consecutive trials)
6	20:80 ACN:H_2O; immediate switch to 80:20 ACN:H_2O	Luna	Prepared in 80:20 ACN:H_2O; diluted 50:50 in H_2O	5	20
7	20:80 ACN:H_2O; immediate switch to 80:20 ACN:H_2O	Luna	Prepared in 80:20 ACN:H_2O; diluted 50:50 in H_2O	5	10

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Table 3B. Observations and modifications after each HPLC experimental trial. The final optimized trial resulted in the shortest acquisition time with satisfactory peak shape and separation.

Trial	Primary Observation(s)	Proposed Modification(s)
1	• Non-Gaussian peak shapes • Noisy baselines	• Lower injection volume
2	• Non-Gaussian peak shapes • Noisy baselines	• Change ratio of organic modifier in mobile phase in separate injections • Use only mobile phase as sample matrix • Upgrade stationary phase
3	• Improved peak shapes; significant baseline drift as organic modifier decreases (noticed with 30:70 ACN:H_2O) • Overlapping injections (diminishes Gaussian peak shape)	• Use mix of higher polarity (30:70 ACN:H_2O) and lower polarity (80:20 ACN:H_2O) mobile phase ratios to improve separation • Increase acquisition time for equilibrium stabilization
4	• Improved Gaussian peak shapes • Residues in first injection	• Increase step time between gradient switches to achieve equilibrium • Decrease total acquisition time • Decrease organic solvent use
5	• Overlapping injections	• Increase acquisition time of 80:20 ACN:H_2O method portion
6	• Improved Gaussian peak shapes	• Decrease total acquisition time
7	• Maintained Gaussian peak shapes	

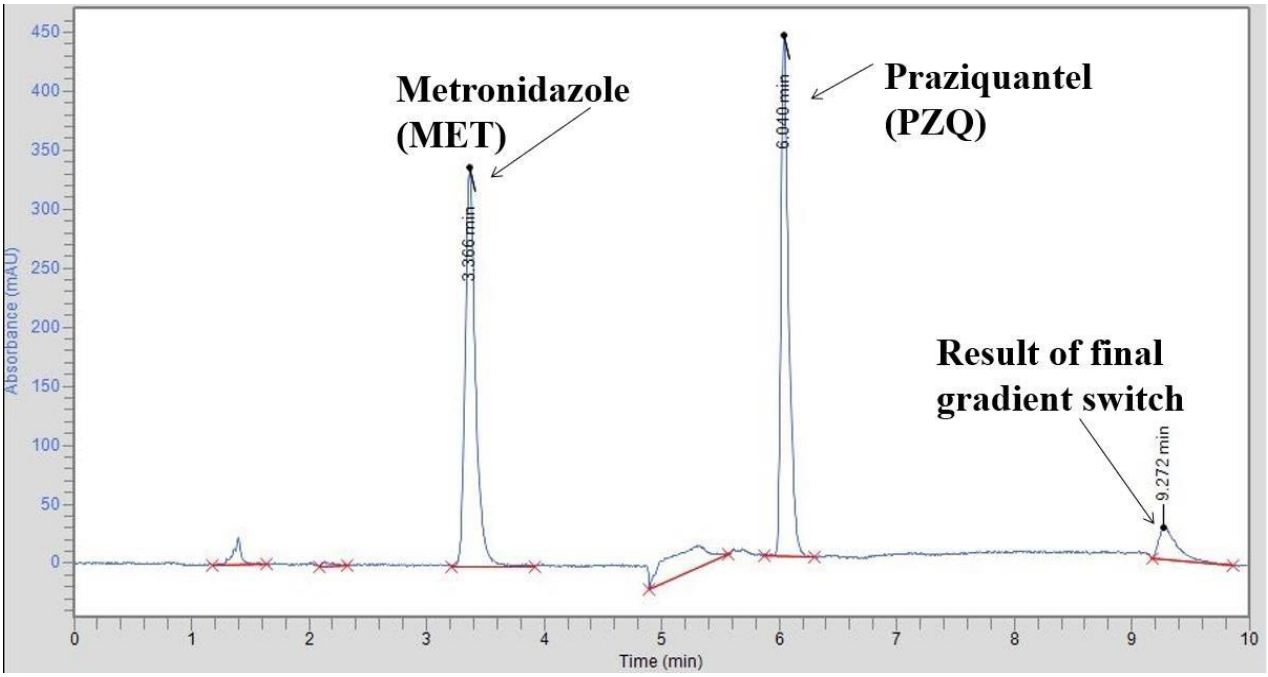

Figure 4. Optimized separation of PZQ and MET based on the final optimized method. The resolution of both peaks was significantly improved with the method. Based on literature review, the second peak was identified as PZQ; thus, MET was identified as the first peak. The 9.272 min peak resulted from switching the mobile phase composition from 80:20 ACN:H_2O to 20:80 ACN:H_2O.

Table 4. The final optimized HPLC method and parameters for separating PZQ and MET in an antiparasitic medication.

Final Optimized HPLC Method	
Parameter	**Value**
Equilibrium Time (min.)	5
Optimized Mobile Phase (for separation)	0 to 3 min. - 20:80 ACN:H_2O 3.5 to 7.5 min. - 80:20 ACN:H_2O 8 to 10 min. - 20:80 ACN:H_2O
Flow Rate ($mLmin^{-1}$)	1
Injection Volume (µL)	5
Column	Phenomenex™ Luna C_{18} (150 x 4.60mm, 5µm, 100A)
HPLC Detector	UV at 210 nm
Column Oven Temperature (°C)	30

Figure 4 shows the chromatogram produced with this method. Compared to Figure 3, the Figure 4 chromatogram displays more ideal Gaussian separation results. The compounds were identified based on separation results from Rajesh and James.[14] The peaks achieved a more optimal resolution and will be sufficient for future quantitative analysis. The compound elution order correlates with the hydrophobicity of PZQ. As predicted, because PZQ is the more nonpolar compound, it was retained in the C_{18} column longer than MET. The peak intensity relates to the detected compound concentration in the injected sample. Though MET was the major compound in the original antiparasitic medication, PZQ was more concentrated in the injected sample. This indicates that PZQ quantification may be more accurate. The final optimized method is described in detail in Table 4.

Future work will be done to quantify the compounds with analytical figures of merit (AFMs) (i.e., linearity, limit of detection, relative standard deviation). Calculating AFMs will validate the separation for the two compounds in a water matrix. The method can then be tested with the compounds in a seawater matrix. Results will indicate if the method can be proposed to aquarium facilities for commercial use.

Conclusion

In this work, antiparasitic compounds PZQ and MET were successfully separated in an optimized HPLC method. Future work includes reporting AFMs for method validation and improving the method for separating compounds in seawater. Aquarium facilities may use a developed method to understand how to safely treat parasitic fish diseases while avoiding environmental damage.

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