**UNIVERSIDADE FEDERAL DE GOIÁS**
**FACULDADE DE FARMÁCIA**

RAISSA GABRIELLE LIMA FERREIRA

# FAST AND NEW MICROBIOLOGICAL METHOD FOR EVALUATING THE POTENCY OF MARBOFLOXACIN-BASED TABLETS

GOIÂNIA/GO
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UNIVERSIDADE FEDERAL DE GOIÁS
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RAISSA GABRIELLE LIMA FERREIRA

# FAST AND NEW MICROBIOLOGICAL METHOD FOR EVALUATING THE POTENCY OF MARBOFLOXACIN-BASED TABLETS

Trabalho de Conclusão de Curso apresentado ao curso de Farmácia da Universidade Federal de Goiás, como requisito parcial para a obtenção do título de Bacharel em Farmácia.

Orientadora: Profa. Dra. Ana Carolina Kogawa
Coorientador: Profa. Dra. Ieda Maria Sapateiro Torres

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2023

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# ATA DE DEFESA DE TRABALHO DE CONCLUSÃO DE CURSO

Aos sete dias do mês de dezembro do ano de 2023 iniciou-se a sessão pública de defesa do Trabalho de Conclusão de Curso (TCC) intitulado "*Fast and new microbiological method for evaluating the potency of marbofloxacin-based tablets*", de autoria de Raissa Gabrielle Lima Ferreira, do curso de Farmácia, da Faculdade de Farmácia da UFG, o qual foi publicado no *Journal of AOAC INTERNATIONAL* (FERREIRA, R. G. L.; DA SILVA JÚNIOR, J. R.; TORRES, I. M. S.; KOGAWA, A. C. *Fast and new microbiological method for evaluating the potency of marbofloxacin-based tablets*. *Journal of AOAC INTERNATIONAL* v. 106(3), p. 690-694, 2023 DOI 10.1093/jaoacint/qsac137). Os trabalhos foram instalados pela Profa. Dra. Ana Carolina Kogawa - orientadora FF/UFG com a participação dos demais membros da Banca Examinadora: Prof. Dr. Luís Antônio Dantas Silva FF/UFG e Prof. Dr. Eric de Souza Gil FF/UFG. Após a apresentação, a banca examinadora realizou a arguição do(a) estudante. Posteriormente, de forma reservada, a Banca Examinadora atribuiu a nota final de 9,5 (nove e meio), tendo sido o TCC considerado aprovada.

Proclamados os resultados, os trabalhos foram encerrados e, para constar, lavrou-se a presente ata que segue assinada pelos Membros da Banca Examinadora.

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## RESUMO

**Introdução:** O marbofloxacino (MAR) é um antimicrobiano pertencente à classe das fluoroquinolonas e é comercializado na forma de comprimidos. Não há monografia microbiológica em compêndios oficiais para avaliar a potência do produto final. Além disso, a literatura é escassa neste contexto. **Objetivo:** O objetivo é desenvolver e validar um método microbiológico por turbidimetria para avaliar a potência de comprimidos à base de MAR, baseado nos princípios da química analítica verde. **Métodos:** Água purificada-etanol (9 + 1, v/v) foi usada como diluente para preparar as soluções MAR nas concentrações de 0,25, 0,8 e 2,56 mg/mL, caldo de infusão de cérebro e coração (BHI) como meio de cultura, *E. coli* American Type Culture Collection (ATCC) 25922 a 10% e incubação em shaker a 37°C por 4 h foram utilizadas no método. **Resultados:** O método foi: linear na faixa de 0,25 a 2,56 mg/mL; seletivo contra os adjuvantes da amostra; preciso (RSD intradiário 2,26%, RSD interdia 3,49% e RSD interanalista 3,59%); exato com recuperação de 100,20%; e robusto contra alterações no volume do meio de cultura no tubo, rotação do agitador e temperatura de incubação no agitador. A potência dos comprimidos MAR foi de 96,98% utilizando o método proposto. **Conclusão:** O método turbidimétrico desenvolvido é uma opção nova, rápida e otimizada para a rotina de CQ do MAR em comprimidos, pois permite avaliar a potência do fármaco no produto final e pode ser utilizado para complementar os resultados das análises físico-químicas, além de ser uma alternativa verde e sustentável.



**Palavras-chave:** Controle de qualidade; Química analítica verde; Fluorquinolonas; Marbofloxacino; Ensaio turbidimétrico; Validação.

**ABSTRACT**

**Background:** Marbofloxacin (MAR) is an antimicrobial belonging to the fluoroquinolone class and is marketed in the form of tablets. There is no microbiological monograph in official compendia to evaluate the potency of the final product. Furthermore, the literature is scarce in this context. **Objective:** The objective is to develop and validate a microbiological method by turbidimetry to evaluate the potency of MAR-based tablets, based on the principles of green analytical chemistry. **Methods:** Purified water–ethanol (9 þ 1, v/v) was used as diluent to prepare the MAR solutions at concentrations of 0.25, 0.8, and 2.56 mg/mL, brain heart infusion (BHI) broth as culture media, *E. coli* American Type Culture Collection (ATCC) 25922 at 10% and incubation in a shaker at 37C for 4 h were used in the method. Results: The method was: linear in the range of 0.25 to 2.56 mg/mL; selective against the sample adjuvants; precise (intra-day RSD 2.26%, inter-day RSD 3.49%, and inter-analyst RSD 3.59%); accurate with a recovery of 100.20%; and robust against changes in culture medium volume in the tube, shaker rotation, and incubation temperature in the shaker. The potency of MAR tablets was 96.98% using the proposed method. **Conclusion:** The turbidimetric method developed is a new, fast, and optimized option to the routine QC of MAR in tablets, since it allows the evaluation of the drug's potency in the final product and can be used to complement the results of the physicochemical analysis, in addition to being a green and sustainable alternative.

**Keywords:** Fluorquinolones; Green analytical chemistry; Marbofloxacin; Quality control; Turbidimetric test; Validation.

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MICROBIOLOGICAL METHODS

# Fast and New Microbiological Method for Evaluating the Potency of Marbofloxacin-Based Tablets

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## Abstract

**Background:** Marbofloxacin (MAR) is an antimicrobial belonging to the fluoroquinolone class and is marketed in the form of tablets. There is no microbiological monograph in official compendia to evaluate the potency of the final product. Furthermore, the literature is scarce in this context.
**Objective:** The objective is to develop and validate a microbiological method by turbidimetry to evaluate the potency of MAR-based tablets, based on the principles of green analytical chemistry.
**Methods:** Purified water–ethanol (9 + 1, v/v) was used as diluent to prepare the MAR solutions at concentrations of 0.25, 0.8, and 2.56 μg/mL, brain heart infusion (BHI) broth as culture media, *E. coli* American Type Culture Collection (ATCC) 25922 at 10% and incubation in a shaker at 37°C for 4 h were used in the method.
**Results:** The method was: linear in the range of 0.25 to 2.56 μg/mL; selective against the sample adjuvants; precise (intra-day RSD 2.26%, inter-day RSD 3.49%, and inter-analyst RSD 3.59%); accurate with a recovery of 100.20%; and robust against changes in culture medium volume in the tube, shaker rotation, and incubation temperature in the shaker. The potency of MAR tablets was 96.98% using the proposed method.
**Conclusion:** The turbidimetric method developed is a new, fast, and optimized option to the routine QC of MAR in tablets, since it allows the evaluation of the drug's potency in the final product and can be used to complement the results of the physicochemical analysis, in addition to being a green and sustainable alternative.
**Highlights:** The work shows an ecological and green alternative to the routine microbiological pharmaceutical analysis of MAR tablets.

Marbofloxacin (MAR, Figure 1) is a fluoroquinolone marketed in the form of tablets. It can be found in preparations for human use; however, the technical literature bases its use on veterinary medicine. This drug was first listed by Vetoquinol in 1995 in the United Kingdon, and later listed in the United States and Europe as an animal medicine (1, 2).

The literature shows physicochemical methods for analyzing MAR tablets by UV (3) and HPLC (4–7). However, the literature does not show microbiological methods for evaluating the potency of MAR tablets. In the official compendia (8–10) MAR does not have a monograph described. Thus, the lack of microbiological methods that are also environmentally friendly is a gap that drives scientists and researchers alike. The use of methods considered green makes the remediation of environmental impacts unnecessary.

The analytical Eco-Scale was proposed by Galuska and collaborators (11) to carry out a quantitative assessment of the green character of analytical processes, similar to the Eco-Scale

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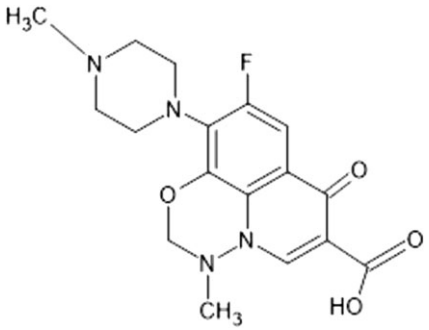


**Figure 1.** Chemical structure of marbofloxacin.

proposed by Van Aken and collaborators (12). This scale also assumes an ideal procedure, characterized by the elimination or minimization of reagents, energy consumption, and waste generation. The physical, environmental, and health risk assessment was based on the Globally Harmonized System of Classification and Labeling of Chemicals (GHS).

This scale takes into account the assessment of risks attributed to the reagents used, the amount used, the risk words attributed to them, the energy used (13), the occupational risks, and waste generation (Figure 2). Penalty points are assigned to these parameters and are included in the Eco-Scale calculation, according to the formula below:

$$\text{Analytical Eco–Scale} = 100 - \text{total penalty points (PP)}$$

Thus, the objective is to develop and validate a microbiological method by turbidimetry to evaluate the potency of MAR-based tablets, based on the principles of Green Analytical Chemistry (GAC).

## Experimental

### *Materials*

Marbofloxacin (MAR) reference (declared content of 99.2%, Wisdom Pharmaceuticals) and samples of MAR in tablets (declared content of 27.5 mg, CEVA) were used.

Brain heart infusion (BHI) broth (Himedia) and agar (Kasvi) were used for microorganism growth.

### *Equipment*

MAR reference and samples were weighed on an analytical balance AUW220D model (Shimadzu). Culture media were weighed on a semi-analytical balance AL-500 model (Marte). Incubation of microorganisms was performed using a shaker incubator, model TE-420 (Tecnal). A model 216/2 oven (Fabbe-Primar) and vertical autoclave, model AV-50 (Phoenix Luferco) were also used. The standardization of the inoculums and absorbance readings were performed using a SP-220 spectrophotometer (Biospectro). An ultrasound bath, USC-2800 (Unique), and water purification system, Milli Q water (Direct-Q®) were also used.

### *Preparation of MAR Solutions (Reference and Sample)*

(a) *Reference solution.*—The equivalent of 5 mg MAR reference was weighed and transferred to a 250 mL volumetric flask, and diluted to volume with purified water–ethanol (90 + 10, v/v) in order to obtain a stock solution of 20 μg/mL of MAR reference. Aliquots of this solution were transferred to 10 mL volumetric flasks and diluted with purified water–ethanol (90 + 10, v/v) to obtain concentrations of 0.25, 0.8, and 2.56 μg/mL of MAR reference (concentrations of the solutions followed a geometric progression).

(b) *Sample solution.*—Twenty tablets of MAR were crushed and homogenized (average weight 149.08 mg) and the equivalent of 5 mg was weighed and transferred to a 250 mL volumetric flask and diluted to volume with purified water–ethanol (90 + 10, v/v) ) in order to obtain a stock solution of 20 μg/mL MAR sample. Aliquots of this solution were transferred to 10 mL volumetric flasks and diluted with purified water–ethanol (90 + 10, v/v) to obtain concentrations of 0.25, 0.8, and 2.56 μg/mL MAR sample (concentrations of the solutions followed a geometric progression), which were tested against MAR reference solutions.

### *Strain and Inoculum*

(a) *Strain.*—*E.coli* American Type Culture Collection (ATCC) 25922 samples were transferred with sterile plastic handles to tubes with inclined BHI agar and incubated in an oven for 24 h at 37 ± 2°C. Subsequently, verification of the *E. coli* strain was performed using triple sugar iron agar (TM Media) and eosine methylene blue agar (Biolog), which are selective media for this microorganism (10).

(b) *Inoculum.*—A loop of *E. coli* from the prepared strain was transferred to BHI broth and incubated in an oven for 20 h at 35 ± 2°C. After this time, standardization of the broth was performed in a spectrophotometer at 580 nm for transmittance of 25 ± 2% to obtain the inoculums (10).

### *Turbidimetric Method Conditions*

A 3 × 3 parallel line assay design was used. Replicate tubes were prepared for each of the three concentrations of MAR reference and sample, one positive control tube, and one negative control tube (10).

To the tubes containing 10 mL BHI broth were added, separately, 200 μL of each MAR solution (reference and sample) at concentrations of 0.25, 0.8, and 2.56 μg/mL and 1 mL standardized inoculum. The negative control contained only 10 mL BHI broth and the positive control contained 10 mL BHI broth with 1 mL standardized inoculum. The tubes were incubated at 35 ± 2°C for 4 h. The turbidity of the culture media after this time was measured by spectrophotometry.

### *Method Validation*

Microbiological method validation was carried out according to the literature recommendation (8–10, 14–26) for linearity, selectivity, precision, accuracy, and robustness parameters.

(a) *Linearity and selectivity.*—Linearity and selectivity parameters were evaluated by construction of three calibration curves, for reference and sample, performed on three different days in triplicate. The concentrations used were 0.25, 0.8, and 2.56 μg/mL. The results were analyzed by the least-squares method and the linearity deviation and parallelism were evaluated by analysis of variance (ANOVA).

(b) *Precision.*—The precision parameter was evaluated at three levels, intra-day, inter-day, and inter-analyst. The intra-day level was carried using six analyses of MAR reference solution at 0.8 μg/mL on the same day, with the same analyst using the same working conditions. The RSD (%) of the six absorbances were calculated. The inter-day level was

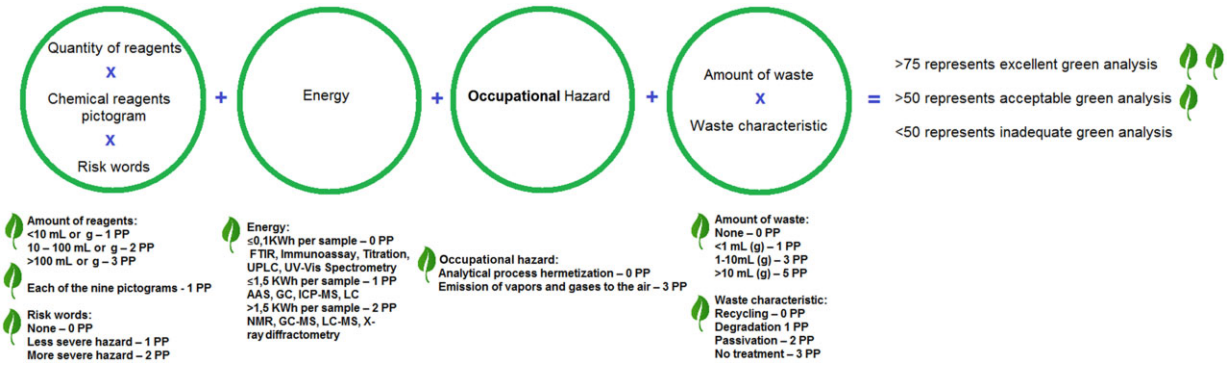


**Figure 2.** Example of the calculation for classifying an analysis according to its greenness: excellent green analysis, acceptable green analysis, or inadequate green analysis.

carried out using 12 analyses of MAR reference solution at 0.8 μg/mL, six analyses each day, with the same analyst using the same working conditions. The RSD (%) of the 12 absorbances were calculated. The inter-analyst level was carried out using 12 analyses of MAR reference solution at 0.8 μg/mL, six analyses by each analyst, using the same working conditions. The RSD (%) of the 12 absorbances were calculated.

(c) *Accuracy*.—The accuracy parameter was proved by the recovery test, to which was added a known amount of reference in a known quantity of sample. The method was performed at three different levels, 80% (R1), 100% (R2), and 120% (R3), in triplicate on three different days, as shown in Table 1.

(d) *Robustness*.—The robustness parameter was evaluated by modifying some conditions of the method and later evaluating the results with the results of an analysis with normal conditions (without modifications) using an F-test (Snedecor) and *t*-test (Student). The modified conditions were: culture medium volume in tube: 9.5 (modified) and 10 mL (normal); shaker rotation: 50 (modified) and 52 revolutions per minute (normal); and incubation temperature in the shaker: 36 ± 2°C (modified) and 37 ± 2°C (normal).

## Results and Discussion

### *Development*

In developing the turbidimetric method for determining the potency of MAR to establish appropriate conditions, different concentrations of MAR, inoculum and different culture media and microorganisms were tested in order to find a satisfactory combination.

Based on the response of absorbance values, RSDs, least-squares and ANOVA of the conditions tested, the best combination was: microorganism: *E. coli* ATCC 25922; MAR concentrations: 0.25, 0.8, and 2.56 μg/mL; culture medium: BHI broth; and inoculum volume: 1 mL.

### *Linearity and Selectivity*

In the validation of microbiological methods for evaluating the potency of antimicrobials, analytical curves for the sample and for the reference are made simultaneously and linearity is evaluated at a confidence level of 95% generally.

The method was linear in the range between 0.25 and 2.56 μg/mL, as shown in Figure 3. The representative equation and correlation coefficients (*r*) for reference and sample were, respectively, $y = -0.3198 \ln(x) + 0.5271$, $r = 0.9996$ and $y = -0.3188 \ln(x) + 0.5332$, $r = 0.9992$. ANOVA of data from the curves is shown in Table 2.

**Table 1.** Preparation of the MAR solutions for the recovery test of the turbidimetric method

| | 20 μg/mL MAR sample, μL | 20 μg/mL MAR reference, μL | Final theoretical concentration, μg/mL[a,b] |
|---|---|---|---|
| Sample | 125 | —[c] | 0.25 |
| R1 (80 %) | 125 | 725 | 1.70 |
| R2 (100 %) | 125 | 937.5 | 2.125 |
| R3 (120 %) | 125 | 1125 | 2.50 |
| Reference | — | 125 | 0.25 |

[a] Volumetric flask of 10 mL.
[b] Concentration levels prepared in triplicate.
[c] — = Not applicable.

ANOVA results indicate non-linearity deviation, with Fcal < Ftab (cal = calculated; tab = tabulated) in the quadratic ($0.07 < 4.96$) and quadratic difference ($1.96 < 4.96$). Furthermore, the preparation and between-tube (same dose) sources was also non-significant ($0.18 < 4.96$, $3.52 < 4.10$, respectively) and the regression and between-dose sources were significant ($1919.11 > 4.96$, $384.27 > 3.33$, respectively). Therefore, the method developed was considered linear.

### *Precision*

Absorbance and RSD (%) values for the MAR reference solution obtained for the precision parameter are shown in Table 3. The results show RSDs for intra-day, inter-day, and inter-analyst precision levels less than 5%, with a maximum limit allowed of 15% (14), which proves the precision of the method.

### *Accuracy*

The values obtained through the standard recovery test are described in Table 4. The average recovery was 100.20%, which confirms the ability of the method to accurately determine the MAR content (16, 17).

### *Robustness*

The results of the method's robustness are presented in Table 5. They were statistically evaluated by the F-test and the *t*-test and

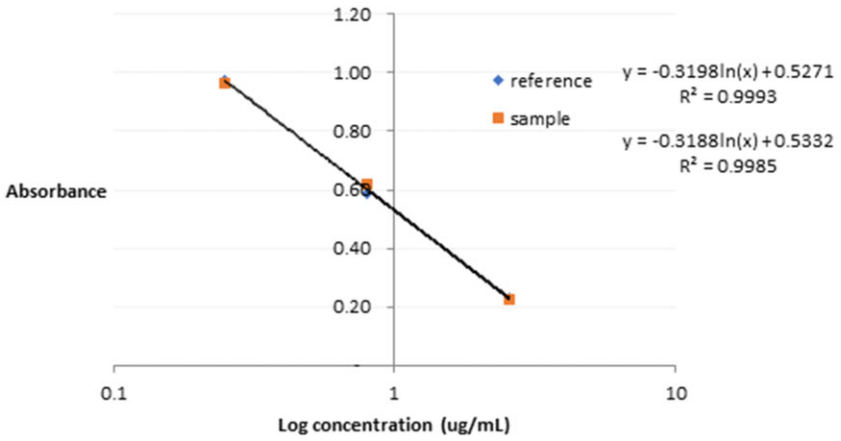


**Figure 3.** Analytical curve of reference and sample at concentrations of 0.25, 0.8, and 2.56 μg/mL, obtained by the turbidimetric method.

**Table 2.** ANOVA of absorbance values obtained from the analytical curve of MAR by the turbidimetric method

| Variation sources | Degrees of freedom | Sum of quadratic | Mean quadratic | $F_{cal}$ | $F_{tab}$ |
|---|---|---|---|---|---|
| Preparation | 1 | 0.0002 | 0.0002 | 0.18 | 4.96 |
| Regression | 1 | 1.6554 | 1.6554 | **1919.11**[a] | 4.96 |
| Parallelism | 1 | 0.0000 | 0.0000 | 0.00 | 4.96 |
| Quadratic | 1 | 0.0001 | 0.0001 | 0.07 | 4.96 |
| Quadratic difference | 1 | 0.0017 | 0.0017 | 1.96 | 4.96 |
| Between-doses | 5 | 1.66 | 0.33 | **384.27**[a] | 3.33 |
| Between-tubes | 2 | 0.01 | 0.00 | 3.52 | 4.10 |
| Within (error) | 10 | 0.01 | 0.00 | —[b] | — |
| Total | 17 | 1.67 | — | — | — |

[a] Significant for $P < 5\%$.
[b] — = Not applicable.
Bold values indicates the significant values.

**Table 3.** Results of the evaluation of the precision parameter by the turbidimetric method

| | Absorbance | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Precision level | 1 | 2 | 3 | 4 | 5 | 6 | Average | RSD, % |
| Intra-day | 0.537 | 0.550 | 0.543 | 0.530 | 0.522 | 0.519 | 0.534 | 2.26 |
| Inter-day | 0.537 | 0.550 | 0.543 | 0.530 | 0.522 | 0.519 | 0.545 | 3.49 |
| | 0.537 | 0.571 | 0.561 | 0.536 | 0.582 | 0.546 | | |
| Inter-analyst | 0.581 | 0.520 | 0.531 | 0.547 | 0.521 | 0.564 | 0.539 | 3.59 |
| | 0.537 | 0.571 | 0.561 | 0.536 | 0.582 | 0.546 | | |

**Table 4.** Results of the evaluation of the accuracy parameter by the turbidimetric method

| Level | MAR reference added, μg/mL | MAR reference found[a], μg/mL | Recovery, % | RSD, % | Average recovery, % |
|---|---|---|---|---|---|
| R1 | 1.45 | 1.46 | 101.03 | 0.26 | |
| R2 | 1.875 | 1.864 | 99.41 | 0.72 | 100.20 |
| R3 | 2.25 | 2.25 | 100.17 | 0.35 | |

[a] Average of three determinations.

the changes in culture medium volume in the tube ($t_{cal}$ 0.54 < 2.78 $t_{crit}$ (crit = critical)), shaker rotation ($t_{cal}$ 0.50 < 2.78 $t_{crit}$), and incubation temperature in the shaker ($t_{cal}$ 0.22 < 4.30 $t_{crit}$) do not interfere with the method, which is robust in the face of these changes.

### *Potency of MAR in Tablets*

The potency of MAR in tablets, 96.98%, was determined using the developed and validated method. This value was compared with other trials for evaluating MAR in tablets (ref. 3, Table 6). The comparison was not ideal, since the literature does not show

**Table 5.** Results of the evaluation of the robustness parameter by turbidimetric method

| Parameters | Normal condition | Modified condition[a] | F-Test | *t*-Test | Robustness |
|---|---|---|---|---|---|
| Culture medium volume | 10 mL | 9.5 mL | $F_{cal}$ 4.38 < 19.0 $F_{crit}$ | $t_{cal}$ 0.54 < 2.78 $t_{crit}$ | Robust |
| Shaker rotation | 52 rpm | 50 rpm | $F_{cal}$ 15.71 < 19.0 $F_{crit}$ | $t_{cal}$ 0.50 < 2.78 $t_{crit}$ | Robust |
| Incubation temperature in the shaker (±2°C) | 37°C | 36°C | $F_{cal}$ 56.29 < 19.0 $F_{crit}$ | $t_{cal}$ 0.22 < 4.30 $t_{crit}$ | Robust |

[a] Each modification was analyzed in triplicate; $F_{cal} = F_{calculated}$; $F_{crit} = F_{critical}$; $t_{cal} = t_{calculated}$; $t_{crit} = t_{critical}$.

**Table 6.** Results of MAR potency in tablets and comparison with another method in the literature

| Method | Potency, % | | | Average, % | RSD, % |
|---|---|---|---|---|---|
| Present[a] | 98.97 | 96.60 | 95.37 | 96.98 | 4.41 |
| | Content, % | | | | |
| (3)[b] | 104.43 | 103.67 | 101.59 | 103.67 | |

[a] Microbiological method.
[b] Physicochemical method.

microbiological methods for evaluating the potency of MAR tablets for the proposed method to be compared (which shows the relevance and importance of the developed method). Thus, the comparison was made with a physicochemical method and the results between them showed non-significant RSD (%).

According to Eco-Scale, the proposed method is considered an excellent green analysis, since the greenness of the analytical process was 91 (>75 represents excellent green analysis, Figure 2; 11–13, 27).

## Conclusions

The developed and validated turbidimetric method is able to assess the potency of MAR tablets reliably, safely, quickly, and within the principles of GAC. It is an excellent alternative, as the official compendia do not provide the option of evaluating the potency of the final product for MAR.

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## Conflict of Interest

The authors declare that there is no conflict of interest.

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