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A novel SPE-UHPLC-DAD method for the determination of fumagillin produced by *Aspergillus fumigatus* in cell culture media

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ABSTRACT

Fumagillin is a biomolecule produced by *Aspergillus fumigatus* that is gaining relevance due to its connection with invasive aspergillosis. The determination of this molecule might help to understand the propagation of this disease and study its use as a potential biomarker. In spite of the interest of fumagillin in microbiological research, no quantitative method has been developed so far for its determination in cell culture media. In this work, the first validated method for the quantitative analysis of fumagillin in RPMI-1640 is presented. The sample treatment consists of a mixed-mode anion exchange Solid Phase Extraction that effectively removes potential interferences and offered a recovery of 83 ± 7%. The analysis was carried out by Ultra High Performance Liquid Chromatography coupled to Diode Array Detection at 336 nm. The method fulfilled the validation criteria established by EMA and FDA guidelines for bioanalysis (selectivity, carry over, linearity, accuracy, precision, dilution integrity and stability) and offers a limit of quantitation (25 $\mu g \cdot L^{-1}$) suitable for its intended use. Indeed, the method was satisfactorily applied to the quantification of the fumagillin produced by three strains of *Aspergillus fumigatus* with different toxin production capacity.

1. Introduction

Fumagillin is a complex biomolecule produced by the fungus *Aspergillus fumigatus*. Soon after its discovery in 1949 [1] it was already studied as an antiparasitic drug against *Nosema apis* in honey bees [2]. In fact, the main application of fumagillin has been as an antibiotic to prevent microsporidiosis in honey [3] and fish [4] or to deal with other kind of parasites [5]. In humans, the uses of fumagillin are scarce. Among them, it has also been applied to treat microsporidiosis in immunocompromised patients, although severe adverse effects have been reported [6]. Another field in which this biomolecule proved to be of interest is the treatment of cancer. Due to its antiangiogenic effect, fumagillin and some of its derivatives have been studied as antitumoral drugs [7], but its use is not a common practice.

According to recent studies, this mycotoxin might play an active role helping in the development of invasive aspergillosis [8–10]. Therefore, fumagillin is gaining relevance due to its potential application as a biomarker of this disease caused by *A. fumigatus*. Taking into account that this is one of the most important opportunistic fungal pathogens and that invasive aspergillosis has a mortality rate between 40% and 90% [11], the early detection of this disease is a priority challenge. For that aim, the production of fumagillin by *A. fumigatus* and the interaction of the mycotoxin with the target cells in the living organisms should be better understood. Therefore, analytical methodologies that can guarantee the reliable quantification of this biomolecule in matrices such as growth medium, biofluids or organs seem to be necessary. To the best of our knowledge, no quantitative method has been proposed yet for these matrices, apart from those that aim to carry out only a qualitative analysis of fumagillin [8,10,12].

The quantitative methods developed to date for the analysis of this mycotoxin are mainly addressed to matrices related to its use as an antibiotic, i.e. honey [13–20] and fish [21–23]. Nevertheless, some other matrices have been studied such as surface water [24] or dairy products [25]. The vast majority of these methods rely on reversed phase liquid chromatography coupled to diode array detection (DAD) [14,20–23,26] or mass spectrometry (MS) [14,15,18,19,24,25]. In spite

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of the higher sensitivity of the MS methods, photometric detection has proved to offer suitable limits of quantitation (LLOQ) for the intended use, probably due to the high absorbance of fumagillin at relatively long wavelengths (335 and 350 nm) [5]. Regarding sample treatment, solid phase extraction (SPE) is usually the technique of choice after sample dilution with water or extraction with organic solvents. Most of the SPE approaches are based on reversed phase polymeric cartridges in which the molecule is retained due to hydrophobic interactions. However, Kanda et al. [17] demonstrated that mixed-mode anion exchange sorbents could be satisfactorily used to obtain a better clean up.

Other important point to be taken into account during fumagillin analysis is its lack of stability under light exposure [27]. The molecule suffers from a fast degradation process that has been deeply studied. For instance, Assil et al. [20] observed that after one day of light exposure, only about one-third of the molecule remained in honey samples and Brackett et al. [26] estimated that only around 60% of the analyte remained after six hours of light exposure in acetonitrile solutions. The latter authors also discovered that while fumagillin is unstable when exposed to a 366 nm UV light, a 254 nm UV light did not degrade the molecule. This information is complementary to the one obtained by Garrett and Eble who observed that the photolytic degradation was minimal with light over 400 nm [27].

The aim of this work was to develop and validate an SPE-UHPLC-DAD method that would allow the reliable quantification of fumagillin produced by *A. fumigatus* in cell culture medium (RPMI-1640) for its subsequent application to study toxin release during host-fungus interaction assays using cell lines.

2. Material and methods

2.1. Reagents and materials

Fumagillin (≥90%), sodium diclofenac used as internal standard (IS) and RPMI-1640, Dubelcós Modified Eaglés Medium (DMEM), Haḿs F12 (HAMS) and Basal Medium Eagle (BME) cell culture media were purchased from Sigma-Aldrich (St Louis, MO, USA). All these media were supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 200 mM L-glutamine, 60 mg·L^{-1} penicillin G, 100 mg·L^{-1} streptomycin and 250 μg·L^{-1} and amphotericin B. Furthermore, lysogenic broth (LB) (Panreac), glucose minimal medium broth (GMM) (a mixture of several salts and nutrients prepared as in Shimizu et al. [28]) and yeast-glucose broth (YG) (10 g·L^{-1} glucose and 5 g·L^{-1} yeast extract) were used. The solvents for UHPLC and sample preparation (methanol and acetonitrile) were supergradient grade and were obtained from Scharlau (Barcelona, Spain). Reagents used for buffer preparation were triflouoroacetic acid (TFA) (99%, Panreac), formic acid (LC-MS quality, Fluka), ammonium acetate (≥99%, Sigma-Aldrich), ammonium hydroxide, (25%, Merck), ammonium formate (≥99%, Fluka), citric acid hydrate (99.5%, Panreac), sodium dihydrogen citrate (≥99%, Fluka), ammonium chloride (99.8%, Merck), trisodium phosphate (Merck, Pro Analysi) and sodium dihydrogen phosphate (Merck, Pro Analysi). Purified water was obtained from a Millipore (Milford, MA, USA) Milli-Q Element A10 water system. Oasis MAX cartridges (1 cc, 30 mg) were purchased from Waters (Milford, MA, USA).

2.2. Preparation of standard solutions and spiked RPMI-1640 samples

Fumagillin was dissolved in ethanol to give a 1000 mg·L^{-1} stock solution, which was stored at −20 °C in amber vial. From this solution a 20 mg·L^{-1} working solution of fumagillin in acetonitrile was prepared and kept at −20 °C. The rest of the working solutions of fumagillin were freshly prepared the day of the analysis. A 1000 mg·L^{-1} stock solution of diclofenac (IS) was prepared in acetonitrile and, by diluting it, a working solution of 20 mg·L^{-1} in acetonitrile was prepared and kept at −20 °C. Spiked RPMI-1640 samples for sample treatment optimization were prepared by spiking drug-free RMPI-1640 with the 20 mg·L^{-1} fumagillin working solution to a concentration of 200 μg·L^{-1}. Calibration standards at least at seven concentration levels ranging from 25 μg·L^{-1} to 1500 μg·L^{-1} were prepared by dilution of the fumagillin working solutions with drug-free RMPI-1640. Quality control samples (QCs) for method validation were prepared by spiking RPMI-1640 with fumagillin working solution to four levels of concentration: 25 (LLOQ), 50 (LQC), 750 (MQC), and 1200 (HQC) μg·L^{-1}. Additionally, dilution integrity was studied in a RMPI-1640 spiked at 5000 μg·L^{-1}.

2.3. Degradation of fumagillin under light exposure

Due to the fast photodegradation of fumagillin all the standards were prepared in amber vials [13]. Furthermore, the preparation of the solutions and the sample treatment was carried out under red light exposure using red filters in order to minimize the degradation of the molecule.

2.4. Chromatographic conditions

Quantitative analysis of fumagillin was carried out on an Acquity Ultra Performance Liquid Chromatography system from Waters coupled to a diode array detector. The chromatographic separation was performed on an Acquity BEH C18 column (2.1x50 mm, 1.7 μm) from Waters. In order to find the most suitable pH for the chromatographic separation, a linear gradient varying from 5% to 95% acetonitrile in 5 min was applied to a 1 mg·L^{-1} fumagillin sample (75:25, water:methanol) using different aqueous mobile phases at pH 2 (TFA, 10 mM), pH 4 (formic/formate buffer, 50 mM), pH 6 (citrate buffer, 50 mM), pH 8 (ammonium acetate solution, 50 mM) and pH 10 (ammonium/ammonia buffer, 50 mM).

The optimal chromatographic separation was achieved with a 50 mM ammonium/ammonia buffer (pH 10) as the aqueous mobile phase (A) and acetonitrile as the organic modifier (B). A flow rate of 0.40 mL/min was used with an elution gradient as follows: 0–0.5 min, 20% B; 0.5–5.5 min, linear change from 20% to 95% B; 5.5–6.5 min, 95% B; 6.5–7.0 min, from 95% to 20% B. A sample aliquot of 5 μL was injected into the column. During the chromatographic analysis, the column was thermostated at 35 °C and samples were kept at 4 °C in the autosampler. 336 and 280 nm wavelengths were employed for fumagillin and diclofenac (IS), respectively. The latter was chosen as IS due to the fact that is showed a proper chromatographic retention and a similar behavior as fumagillin during sample treatment. System control, data collection, and data processing were accomplished using Empower 2 software.

2.5. SPE conditions

For the sample treatment of RPMI-1640, a SPE treatment previously optimized by the research group was applied. In brief, the most adequate sorbent for SPE was chosen among several reversed phase cartridges (Oasis HLB, Waters; Strata-X, Phenomenex) and mixed mode cartridges (BondElute NH2, Agilent; Oasis MAX, Waters). In a latter step, the SPE procedure with Oasis MAX (Mixed-mode strong anion-exchange) cartridges was further optimized by experimental design.

The optimal sample treatment conditions were established as follows: 500 μL of RMPI-1640 sample was spiked with 37.5 μL of 20 mg·L^{-1} IS solution in acetonitrile and 465 μL of phosphate buffer (100 mM, pH = 12). After vortex mixing the solution was transferred to Oasis MAX cartridges. The cartridges had been previously activated with 1 mL methanol and conditioned with 1 mL phosphate buffer (100 mM, pH = 12). After sample loading, the cartridges were washed with 1 mL phosphate buffer (100 mM, pH = 12):methanol (55:45). Then the cartridges were dried for 5 min at high vacuum and 1 mL of 3.5% formic acid solution in methanol was used for eluting the analyte. 500 μL of aqueous mobile phase were added to 500 μL of the eluate and after centrifugation, the solution was transferred to autosampler vials and subsequently injected into the UHPLC system for analysis.

In order to calculate the recovery of the sample treatment, five replicates spiked at 200 μg·L^{-1} and a blank RMPI-1640 sample spiked after the SPE were analyzed.

2.6. Validation of the analytical method

Method validation was based on the European Medicine Agency (EMA) [29] and the Food and Drug Administration (FDA) [30] guidelines and was performed to evaluate the method in terms of selectivity, carryover, linearity, accuracy, precision, dilution integrity and stability of fumagillin in several conditions.

The selectivity of the method was aimed to check the interferences at the retention time of the analyte and the IS by comparing the response of blank samples with a sample spiked with the IS and fumagillin at LLOQ. For acceptance, the signal obtained in the blank matrices must be lower than 20% the response of fumagillin at the LLOQ (5% for the IS). The regulatory agencies ask to study the selectivity at six different sources of the matrix in a scenario in which matrices with a high variability such as blood or urine are usually analyzed. The authors did not consider that analyzing six different sources of RPMI-1640 medium was necessary, and decided to apply the analytical method to other growth media instead. For his aim, we used three common cell culture media (DMEM, HAMS F12 and BME) as well as three standard microbiological broths (LB, GMM and YG). Furthermore, in order to study a potential interference the antimicrobial agents added to the cell culture media, standard solutions of 15 mg·L^{-1} penicillin G, 25 mg·L^{-1} streptomycin and 72.5 μg·L^{-1} amphotericin B in methanol:aqueous mobile phase (1:1) were chromatographically analyzed.

Carryover was tested by the injection of a RMPI-1640 blank sample directly after the injection of a high concentration standard at a concentration of 5000 μg·L^{-1}. The response in the blank sample was compared with the response at the LLOQ, and was considered acceptable when the signal obtained at the fumagillin retention time was below 20% of the signal at the LLOQ (5% for the IS).

Calibration curves consisted of a blank RPMI-1640, a zero calibration standard and at least seven non-zero calibration standards spiked with fumagillin from 25 to 1500 μg·L^{-1} in order to cover the expected range for the production of fumagillin by *A. fumigatus*. The calibration curves were built by plotting the corrected peak area of fumagillin against its nominal concentration. The acceptance criterion for the calibration curve was that the calculated concentration of at least six calibration standards should be ±15% of the nominal value (±20% for LLOQ). The LLOQ was defined as the lowest amount of analyte which can be quantified reliably complying with the criteria for accuracy and precision, and had been previously estimated from signal to noise ratio (S/N) and the analysis of several replicates in the 15–90 μg·L^{-1} range.

In order to evaluate the accuracy and precision, five replicates were prepared by spiking RMPI-1640 at four concentration levels of fumagillin: 25 (LLOQ), 50 (LQC), 750 (MQC) and 1200 (HQC) μg·L^{-1}. For intra-day accuracy and precision five replicates of each concentration were analyzed the same day. These experiments were also repeated in three different days for the evaluation of inter-day accuracy and precision. The concentration value of the spiked samples was obtained from interpolation of the resulting corrected area in the regression equation. Accuracy was expressed as relative error (%RE), and precision was evaluated as relative standard deviation (%RSD). The acceptance criterion for accuracy was that the mean value should be within ± 15% of the nominal value (20% for the LLOQ) and for precision was that %RSD must be ≤ 15% (20% for LLOQ).

Dilution integrity was performed to ensure the reliable quantification of samples greater than the upper limit of quantitation (ULOQ) after being diluted with blank matrix. For that aim RPMI-1640 spiked with fumagillin at a concentration of 5000 μg·L^{-1} was diluted ten times with blank RPMI-1640 and analyzed. It was considered acceptable when both the %RE and %RSD were ≤15%.

Stability assays of fumagillin under different conditions were carried out in spiked RPMI-1640 samples at low (50 μg·L^{-1}) and high (1200 μg·L^{-1}) levels of concentration, using three replicates for each assay. Stability of spiked RPMI-1640 samples was tested following three freeze/thaw cycles, consisting each cycle on a 24 h period at −20 °C before thawing; after 24 h in the autosampler at 4 °C; and after 2 months storage at −20 °C. Furthermore, bench-top stability was assessed at two different conditions: four hours under red light exposure and four hours under regular laboratory light exposure. In this case, the same experiment was carried out in a sample without matrix (i.e. water) in order to better understand the photosensitivity of the analyte. The concentrations obtained under the different conditions were compared to the concentration of the same samples freshly prepared. The mean concentration at each level should be within ±15% of the nominal concentration to be considered stable.

2.7. Application of the method to real samples

The *A. fumigatus* Cea10 and Δ*akuB*^{KU80} reference strains were used in this study; furthermore, we included an environmental *A. fumigatus* strain (PB2021). The fungus was maintained on glucose minimal medium agar (GMM) for seven days at 37 °C as previously described [31]. The conidia of the strains were obtained by harvesting and cleaning twice with saline-Tween solution (SS-T) (0.9% NaCl and 0.02% Tween 20).

To study the *A. fumigatus* fumagillin secretion ability 5 × 10^6 conidia·mL^{-1} of each strain were seeded in 2 mL of RPMI-1640 using 6-well plates (ThermoFisher, Waltham, MA, USA). The plates were incubated at 37 °C, 5% CO_2 and 95% of humidity. After 48 h incubation period, 1 mL of the cultures was centrifuged at 14.000 rpm during 5 min to obtain the supernatant that was transferred to a light safe microtube and kept in ice until analysis.

3. Results and discussion

3.1. Chromatographic conditions

Fumagillin determination is usually performed in reversed-phase columns at acidic pH values in which the carboxylic group of the molecule is protonated, favoring the retention in the chromatographic column of the neutral species [5]. Nevertheless, we observed that the best results in terms of peak shape and efficiency were obtained at higher pH values as can be observed in Fig. 1. Other authors have worked before at pH values in which fumagillin acidic group is in its anionic form [14]. Therefore, pH 10 was chosen as optimal value for the aqueous mobile phase and the gradient was fine-tuned in order to obtain a good retention factor for both the fumagillin and the IS.

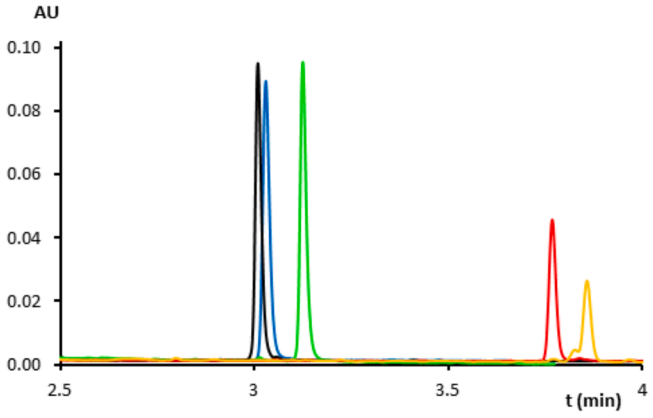

Fig. 1. Chromatograms obtained for 1 mg·L^{-1} fumagillin solution using aqueous mobile phases at pH 2 (orange), pH 4 (red), pH 6 (green), pH 8 (blue) and pH 10 (black) under conditions explained in 2.4.

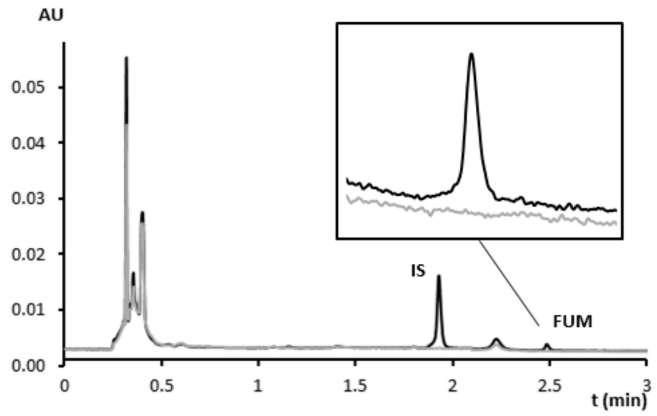

Fig. 2. Chromatograms of a blank RPMI-1640 (grey) and RMPI-1640 spiked at 25 $\mu g \cdot L^{-1}$ (LLOQ) (black). The enlarged area shows the chromatographic signal for fumagillin.

3.2. Recovery of the sample treatment

Once the optimal parameters for SPE extraction were fixed, five RMPI-1640 samples spiked at 200 $\mu g \cdot L^{-1}$ with fumagillin were analyzed and the response compared with the results obtained for a RPMI-1640 blank sample spiked after extraction with 50 $\mu g \cdot L^{-1}$. In this way, the obtained recovery was 83 ± 7%.

3.3. Validation of the analytical method

3.3.1. Selectivity

In order to assess the selectivity of the method the chromatograms obtained for a RMPI-1640 blank and RPMI-1640 spiked at the LLOQ were studied. No interference were observed at the retention time of the analyte or the IS as shown for fumagillin in Fig. 2.

Furthermore, another six media with different components were analyzed and, in spite of the complexity of some of them, none showed any significant interference at the retention time of the fumagillin or the IS (Fig. 3). It is especially remarkable the case of LB medium, which includes yeast extract, a fact that might explain the complex chromatogram obtained for this medium. Nevertheless, the analytical method proved to be selective enough also for this matrix thanks to the combination of the SPE extraction, the UHPLC separation and the choice of a proper detection wavelength.

Finally, another important source of interferences in media analysis could be the antimicrobials employed for the cell culture. In order to study a potential interference, a standard solution of these compounds was injected and compared with a RMPI-1640 blank sample containing the antimicrobials that was subjected to the SPE treatment. As expected, streptomycin standard was not detected in the chromatographic analysis since it is not a suitable analyte for reversed-phase analysis coupled to photometric detection [32]. Regarding penicillin G and amphotericin B standards, their chromatographic response were observed at 0.9 and 2.1 min, respectively (Fig. 4). Although in the RPMI-1640 sample

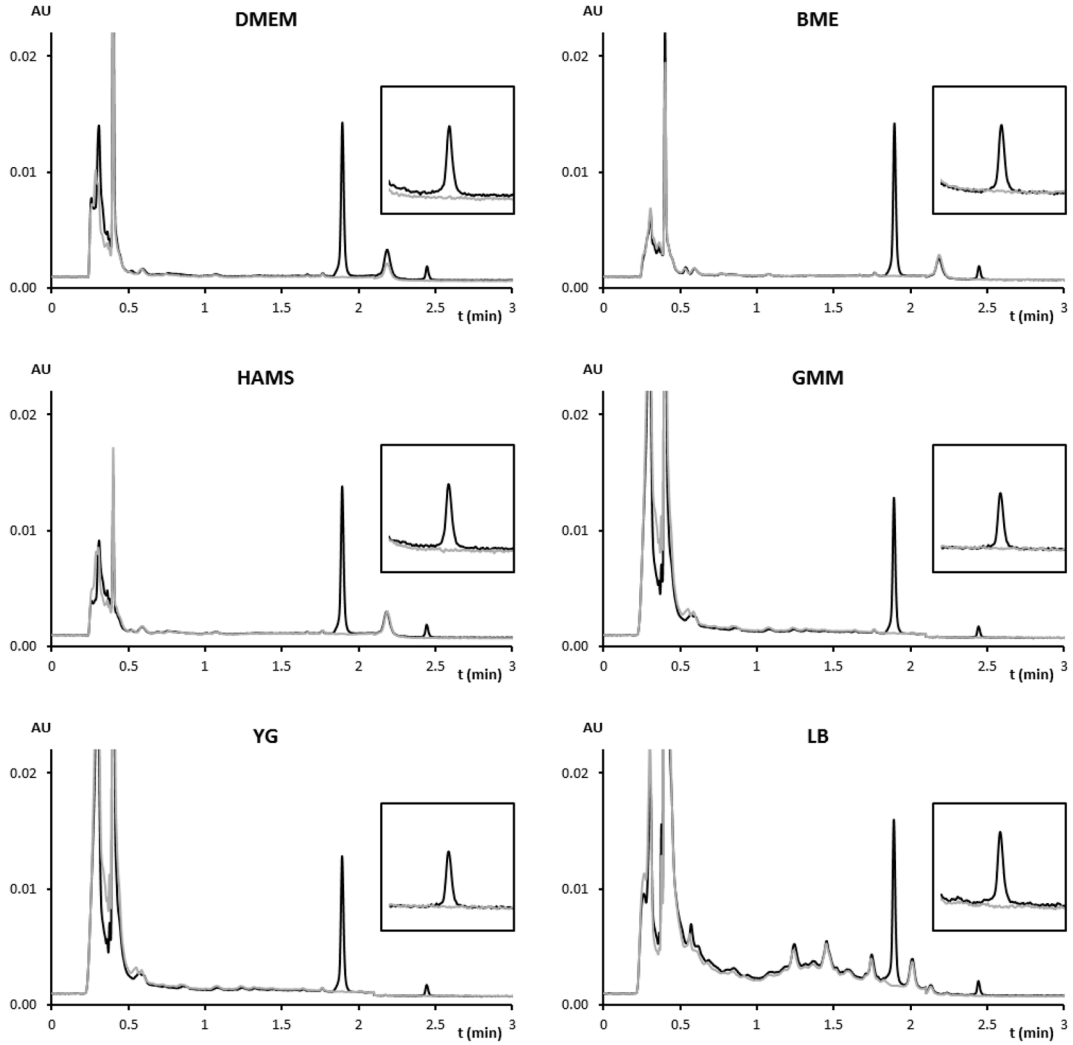

Fig. 3. Chromatograms obtained for the different growth media. The enlarged areas show the chromatographic signal for fumagillin.

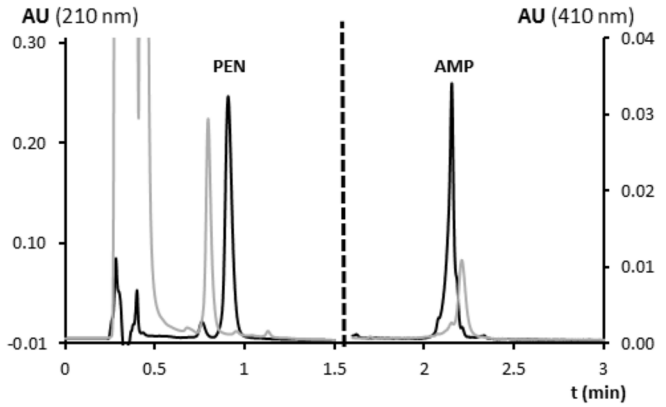

Fig. 4. Chromatogram obtained for RMPI-1640 blank (supplemented with the antimicrobial agents) after sample treatment (grey) together with the chromatograms of a penicillin G (PEN) and amphotericin B (AMP) standard solutions (black). The chromatograms have been processed at maximum absorption wavelengths for those compounds: 210 nm (0–1.55 min) and 410 nm (1.55–3.0 min), respectively.

chromatogram some compounds elute close to those retention times, the UV–Vis spectra clearly differ from the ones obtained for the antimicrobials. Therefore, those compounds are successfully removed during the sample treatment. Moreover, in the event of a potential retention of these antimicrobials in the SPE cartridge, the chromatographic method would allow to separate them from diclofenac (1.9 min retention time) and fumagillin (2.5 min retention time).

3.3.2. Carryover

No significant chromatographic peak at the retention time of fumagillin was observed in the blank analyzed after injecting RPMI-1640 spiked at 5000 μg·L^{-1} with fumagillin.

3.3.3. Linearity

Calibration curves were obtained plotting the corrected area (ratio fumagillin area/IS area) for each concentration level versus the nominal concentration levels corresponding to each calibration solution. The calibration curves generated were fitted to a regression line by applying the lineal regression model based on the least square method. All of them met the criteria established for linearity from 25 to 1500 μg·L^{-1} with correlation coefficients ranging from 0.994 to 0.998. At least, six concentration levels with a %RE lower than 15% were used in each calibration curve and a heterogeneous distribution of the residuals was observed.

Table 1
Intra- and inter-day accuracy and precision results (n = 3 days, 5 replicates) in terms of %RE and %RSD, respectively, at four concentration levels. For intra-day assays, apart from the mean value of the three days, the minimum (min) and maximum (max) values obtained are shown.

Concentration (μg·L^{-1})	Intra-day		Inter-day	
	Accuracy (% RE) Mean (min.-max.)	Precision (% RSD) Mean (min.-max.)	Accuracy (%RE)	Precision (% RSD)
25 (LLOQ)	11.0 (1.5–17.3)	10.9 (7.2–15.3)	0.5	16.8
50 (LQC)	4.1 (0.1–7.2)	11.5 (7.5–14.8)	4.0	11.6
750 (MQC)	8.2 (7.3–9.6)	9.1 (4.0–11.8)	8.1	9.3
1200 (HQC)	5.4 (0.9–10.1)	4.0 (1.7–5.6)	5.7	5.3

3.3.4. Accuracy and precision

The results obtained for accuracy and precision are shown in Table 1. The %RE and %RSD of the QC samples was lower than 15% at all concentration levels (both intra-day and inter-day).

3.3.5. Dilution integrity

From the replicates of the samples spiked at 5000 μg·L^{-1} fumagillin concentration and then diluted and analyzed, a %RSD of 14.3% and a % RE of 13.0% were obtained, fulfilling the validation criteria for dilution integrity.

3.3.6. Stability

Fumagillin proved to be stable under all the studied conditions except for regular laboratory light exposure (Table 2). After 4 h under those conditions, an analyte loss of 31.0% (HQC) and 41.2% (LQC) was observed, far above the acceptable levels (15%). Thereby, the sample processing under red light exposure is strongly advisable in order to guarantee a more reliable quantitation.

The degradation of fumagillin can be clearly observed in the chromatograms shown if Fig. 5. In those samples exposed to regular laboratory light, fumagillin response decreases giving rise to at least two degradation products. These products are slightly more non-polar and showed a similar UV–Vis spectra as fumagillin. Photolytic degradation of fumagillin under similar conditions has been previously reported and associated to the appearance of new chromophores called neofumagillin (s). For instance, Brackett *et al.* [26] observed that around 40% of a 20 mg·L^{-1} fumagillin had been degraded after 6 h in acetonitrile and Kochansky and Nassr that 62% of a 42 mg·L^{-1} fumagillin solution in 50% ethanol had been degraded after 5.5 h [34]. On the contrary, Dmitrovic and Durden [19] only observed a degradation of 3% in a 10 mg·L^{-1} fumagillin solution in acetonitrile after 3 h. The same authors reported a complete degradation of the analyte under sunlight exposure. Thus, this difference might be explained by different conditions in laboratory lighting.

It is also noteworthy that the degradation in RPMI-1640 under fluorescence illumination was lower than the one observed on samples without matrix (i.e. in water): 68.7% and 56.6% for LQC and HQC, respectively (Fig. 5). Therefore, the presence of RPMI slows down the degradation of the analyte to some extent.

3.4. Application of the method to real samples

The analytical method was applied to study the ability to secrete fumagillin of three different strains of *A. fumigatus*. For that aim, the method was applied to the RMPI-1640 in which the strains had been cultivated for 48 h. As can be observed in Fig. 6, these strains produced different levels of fumagillin that could be successfully quantified: 150 ± 16 μg·L^{-1} (Cea 10), 604 ± 52 μg·L^{-1} (Δ*akuB*^{KU80}) and 221 ± 23 μg·L^{-1} (PB2021).

The variability in the production of the toxin by the different strains tested is significant and it is especially remarkable the high production of fumagillin by the Δ*akuB*^{KU80} mutant strain. This indicates that the mutation made in the nonspecific recombination system (ku70/ku80) of this fungus may have deregulated the secondary metabolism of this

Table 2
Concentration variation percentage of the stability samples compared with a freshly prepared sample. The samples that show a significant statistic difference ($p < 0.05$) are noted (*).

	50 μg·L^{-1} (LQC)	1200 μg·L^{-1} (HQC)
Autosampler stability	+7.3	−0.6
Freeze-thaw stability	−6.7	+2.9
Long-term stability	−11.6	−6.3
Bench-top stability (regular light)	−41.2*	−31.0*
Bench-top stability (red light)	−3.3	−0.5

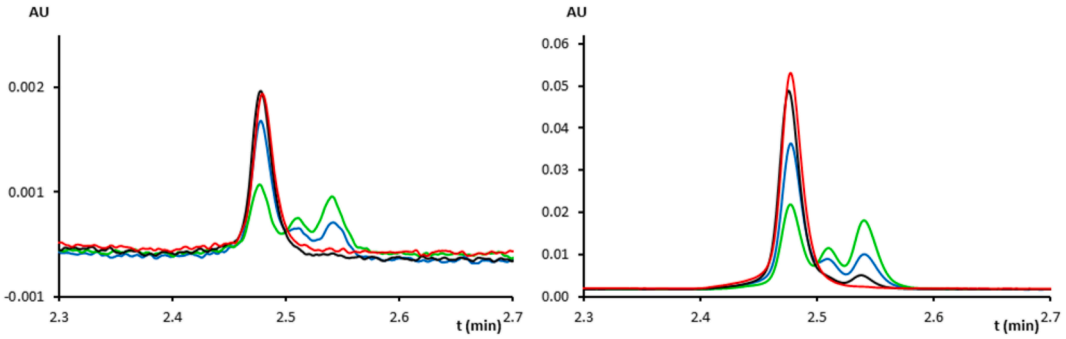

Fig. 5. Chromatograms of 50 μg·L^{-1} (left) and 1200 μg·L^{-1} (right) fumagillin samples after 4 h of: (i) red light exposure in RMPI (red), red light exposure in water (black), regular fluorescence exposure in RMPI (blue) and regular light exposure in water (green). RPMI samples followed a SPE extraction and water samples a 1:4 dilution to injection conditions.

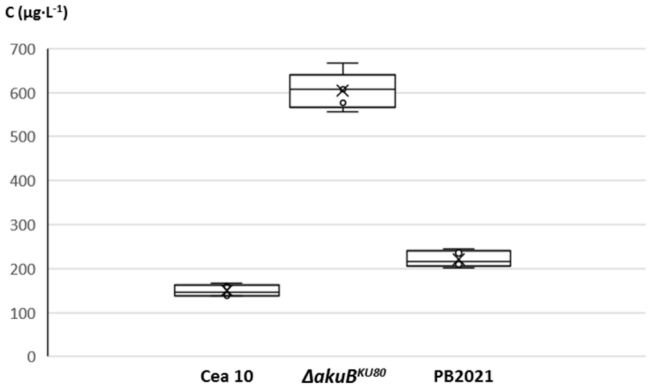

Fig. 6. Box plot of the three strains analyzed.

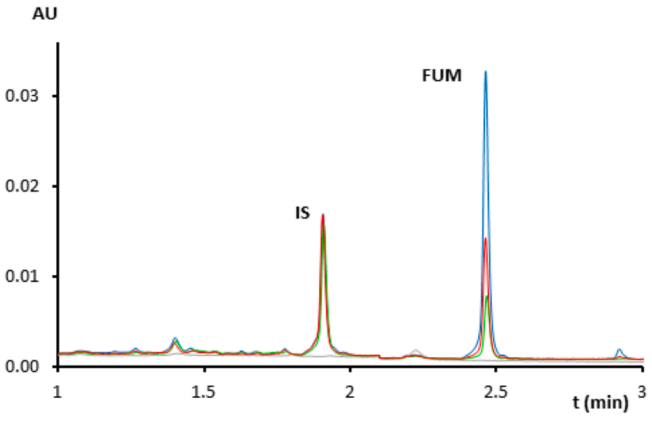

Fig. 7. Chromatograms obtained for a RPMI-1640 blank (grey) and three strains of *A. fumigatus* producing different amounts of fumagillin (FUM): Cea 10 (green), Δ*akuB*^{KU80} (blue) and PB2021 (red).

fungus. Since this strain is used in genetic studies for the generation of gene deletion mutants, this possible deregulation should be confirmed by the possibility of affecting nonspecifically the results of these genetic studies. In any case, it must be highlighted that the differences observed in the production of the toxin do not affect the reliability of the method as can be observed in Fig. 7.

4. Conclusions

Fumagillin is a mycotoxin that is gaining interest as a potential biomarker for invasive aspergillosis. In this scenario, quantitative methods as the one we propose in this research are necessary to carry out toxin release and fungal diagnosis studies. Even if several analytical methods have been developed for the determination of fumagillin in matrices such as honey or fish, this is, to our knowledge, the first validated quantitative method applied to cell cultures. The mixed mode anion exchange SPE allows a suitable cleaning-up of the matrix with a high recovery, which in combination with the UHPLC separation and the DAD detection provides a reliable method. In this way, the method has demonstrated to fulfill the requirements of the regulatory guidelines in terms of selectivity, carry over, linearity, accuracy, precision, dilution integrity and stability. Regarding the latter, one of the most important points to be taken into account during fumagillin analysis is minimizing light exposure to avoid photodegradation. In this aspect, the use of red light during sample treatment has demonstrated to be an appropriate measurement.

The LLOQ values reported in literature for the analysis of fumagillin vary depending on the matrix and the detection method. Evidently, the methods based on mass spectrometry offer lower values ranging between 0.1 and 10 μg·Kg^{-1}. Regarding photometric detection, Fekete et al. obtained a 100 μg·Kg^{-1} LLOQ in fish that could be improved to 5 μg·Kg^{-1} when a enrichment process is applied [21]. With DAD detection Nozal et al. obtained a LLOQ ranging from 95 to 150 μg·Kg^{-1} depending on the botanical origin of the honey [14]. Therefore, the developed method offers a LLOQ (25 μg·L^{-1}) comparable or even better than other methods with photometric detection and, most importantly, shows to be enough for the intended use, as it has been satisfactorily applied to the analysis of fumagillin obtained from strains of *A. fumigatus*. Nevertheless, if a lower concentration of the analyte was to be determined, an evaporation and preconcentration step could be applied after SPE elution.

The capacity to produce fumagillin has been related to the virulence capacity of the fungus as overexpression of the fumagillin production gene cluster has been detected during experimental animal infections [8]. Therefore, the determination of the production capacity of strains causing human infections is of high interest. In this work, we proved that the analytical method was applicable to strains producing very different amounts of the toxin.

Regarding the results obtained from the analysis of different strains of *A. fumigatus*, a significant difference in the production of the toxin was observed.

In conclusion, the method we present in this publication is an important step forward for the development of microbiological studies involving fumagillin and could help to better understand the role of this molecule in the propagation of invasive aspergillosis. It must be highlighted that, although the method has been optimized for its application in cell culture medium, it could be easily transferred to other matrices of interest such as plasma, urine or tissues.

Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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