Talanta 116 (2013) 743–752

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Evaluation of dispersive liquid–liquid microextraction in the stereoselective determination of cetirizine following the fungal biotransformation of hydroxyzine and analysis by capillary electrophoresis

Simone Silveira Fortes [a], Thiago Barth [b], Niege Araçari Jacometti Cardoso Furtado [c], Mônica Tallarico Pupo [c], Cristiane Masetto de Gaitani [c], Anderson Rodrigo Moraes de Oliveira [a,*]

[a] Departamento de Química, Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, Universidade de São Paulo, 14040-901 Ribeirão Preto,-SP, Brazil
[b] Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, 27930-560 Macaé, RJ, Brazil
[c] Departamento de Ciências Farmacêuticas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto, Universidade de São Paulo, 14040-903 Ribeirão Preto, SP, Brazil

ARTICLE INFO

Article history:
Received 11 April 2013
Received in revised form
25 July 2013
Accepted 25 July 2013
Available online 1 August 2013

Keywords:
Dispersive liquid–liquid microextraction
Fungal biotransformation
Cetirizine
Enantioseparation

ABSTRACT

We developed a capillary electrophoresis (CE) and dispersive liquid–liquid microextraction (DLLME) method to stereoselectively analyze hydroxyzine (HZ) and cetirizine (CTZ) in liquid culture media. The CE analyses were performed on an uncoated fused-silica capillary; 50 mmol L^{-1} sodium borate buffer (pH 9.0) containing 0.8% (w/v) S-β-CD was used as the background electrolyte. The applied voltage and temperature were +6 kV and 15 °C, respectively, and the UV detector was set to 214 nm. Chloroform (300 μL) and ethanol (400 μL) were used as the extraction and disperser solvents, respectively, for the DLLME. Following the formation of a cloudy solution, the samples were subjected to vortex agitation at 2000 rpm for 30 s and to centrifugation at 3000 rpm for 5 min. The recoveries ranged from 87.4 to 91.7%. The method was linear over a concentration range of 250–12,500 ng mL^{-1} for each HZ enantiomer ($r > 0.998$) and 125–6250 ng mL^{-1} for each CTZ enantiomer ($r > 0.998$). The limits of quantification were 125 and 250 ng mL^{-1} for CTZ and HZ, respectively. Among the six fungi studied, three species were able to convert HZ to CTZ enantioselectively, particularly the fungus *Cunninghamella elegans* ATCC 10028B, which converted 19% of (*S*)-HZ to (*S*)-CTZ with 65% enantiomeric excess.

1. Introduction

Hydroxyzine (HZ; Fig. 1) [1] is an antihistamine drug belonging to the piperazine class that acts by reversibly blocking histamine H1 receptors. HZ has a chiral center and can exist in the *R*- or *S*- form. Cytochrome P450-2D6 mediates HZ metabolism [2,3], which results in the major carboxylated metabolite (45%) cetirizine (CTZ; Fig. 1) [4]. CTZ is a more potent and long-acting agent than HZ. It is considered a second-generation histamine H1 receptor antagonist and is primarily used to treat urticaria and allergic rhinitis. Chiral CTZ can be administered as a racemic mixture; however, its pharmacological activity is primarily derived from the *R*- enantiomer, also known as levocetirizine [5–7].

Microbial models have been used to study the biotransformation of numerous drugs with the aim of producing their corresponding metabolites [8–10]. Fungi, especially those belonging to the genus *Cunninghamella*, have been extensively employed to mimic mammalian hepatic metabolism [11]. Endophytes constitute another class of fungi that have more recently been used in biotransformation procedures [12]. These microorganisms spend all or part of their life cycle colonizing the host plant healthy tissues inter- and/or intracellularly, typically causing no apparent symptoms [13].

Using fungi is advantageous because they grow quickly and easily form a multi-enzymatic system. Moreover, synthetic organic chemists now consider biotransformation an economically viable and competitive technology for identifying new production routes and obtaining fine chemicals, pharmaceuticals, and agrochemical compounds [14]. In many cases, biological transformation is enantioselective, thus allowing researchers to produce pure enantiomers from racemic mixtures [15].

Some papers have described the analysis of chiral HZ and/or CTZ in different matrices. Choi et al. [16] enantioselectively characterized CTZ

* Corresponding author. Tel.: +55 16 3602 0388; fax: +55 16 3602 4838.
E-mail addresses: armdeoliveira@gmail.com, deoliveira@usp.br (A.R.M. de Oliveira).

0039-9140/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.talanta.2013.07.062

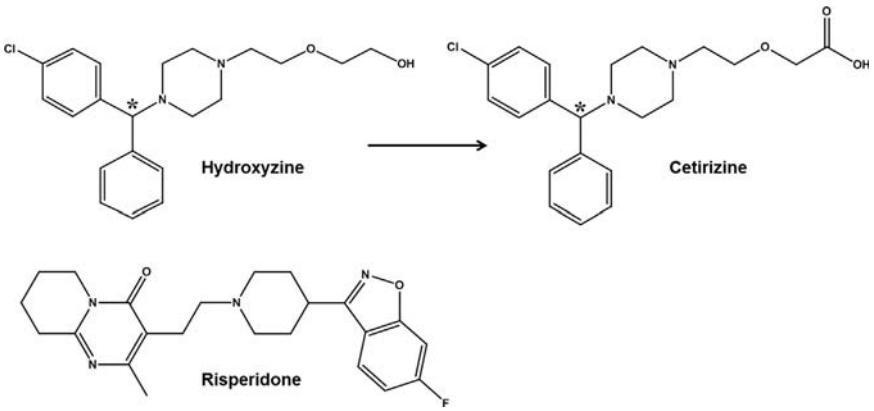

Fig. 1. Chemical structures of Hydroxyzine (HZ), Cetirizine (CTZ), and Risperidone (Risp). * Represent the chiral center.

in rat plasma using HPLC with UV detection; Gumpta et al. [17,18] enantioselectively characterized CTZ in guinea pig plasma, brain, and blood microdialysis samples using HPLC with mass spectrometric detection. Researchers have also performed chiral CTZ determination in human plasma and in a pharmaceutical formulation by capillary electrophoresis (CE) using sulfated-β-cyclodextrin (S-β-CD) as a chiral selector [5,19]. To date, only one paper has described simultaneous enantioselective HZ and CTZ characterization; in this study, CE—maltodextrin was used as a chiral selector to facilitate the separation of the analytes [1]. In summary, all these reported methods relied on the use of conventional sample preparation procedures, i.e., protein precipitation [16–18] and liquid–liquid extraction [1,5].

In 2006, Rezaee and co-workers [20] developed a novel liquid-phase microextraction technique termed dispersive liquid–liquid microextraction (DLLME). This method involves appropriately mixing the extraction and disperser solvents and injecting them into an aqueous sample containing the target analytes to form a cloudy solution. The surface areas between the extraction solvent and the sample solution are initially infinitely large; therefore, the extraction equilibrium can be rapidly reached. Following the extraction, centrifugation allows the separation of the solvent phases; a suitable analytical method determines the enriched analyte in the sedimented phase. This extraction technique is attractive because it is simple, fast, inexpensive, and efficient [21–24]. Recently, researchers have developed various methods using DLLME to extract drugs and their metabolites from different biological matrices, such as urine and plasma [25,26]. Nevertheless, the use of DLLME in fungal biotransformation studies has never been reported.

In light of the above considerations, and because a low-consumption organic solvent extraction method for enantioselectively measuring HZ and CTZ in biological matrices does not yet exist, we have developed a method to couple DLLME with CE to study enantioselective HZ fungal biotransformation.

2. Materials and methods

2.1. Chemicals and reagents

The reference substances *rac*-hydroxyzine dihydrochloride, *rac*-cetirizine dihydrochloride, and levocetirizine dihydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). Risperidone (Fig. 1), which was used as internal standard (I.S.), was acquired from Toronto Research Chemicals (North York, ON, Canada). The *rac*-hydroxyzine and *rac*-cetirizine stock solutions were prepared at a concentration of 500 μg mL^{-1}. The *rac*-hydroxyzine working solutions were prepared at concentrations of 10, 40, 80, 120, 300, 400, and 500 μg mL^{-1}, while the *rac*-cetirizine working solutions were prepared at concentrations of 5, 10, 20, 50, 100, 200, and 250 μg mL^{-1}. The I.S. solution was obtained at a concentration of 100 μg mL^{-1}. All the solutions were prepared in methanol, stored at −20 °C, and protected from light using amber glasses tubes.

The following solvents (HPLC grade) were used in DLLME and in the biotransformation procedures: acetonitrile, methanol and ethanol, purchased from JT Baker (Phillipsburg, NJ, USA); dichloromethane and chloroform, obtained from Sigma (St. Louis, MO, USA); and 1,1-dichloroethane, acquired from Fluka (Buchs, Switzerland). The reagents (analytical grade) were: sodium chloride, potassium chloride, monosodium phosphate, disodium phosphate, magnesium sulfate, and iron sulfate, all obtained from Merck (Darmstadt, Germany); sodium tetraborate decahydrate was provided by JT Baker (Phillipsburg, NJ, USA). Sodium hydroxide was purchased from Nuclear (Diadema, SP, Brazil). Potato dextrose agar (PDA), sucrose, malt extract, dextrose, tryptone soy broth, and yeast extract were obtained from Acumedia (Lansing, Michigan, USA). Sulfated-β-cyclodextrin (S-β-CD) and carboxymethyl-β-cyclodextrin (CM-β-CD) were acquired from Sigma-Aldrich (Steinheim, Germany); hydroxypropyl-β-cyclodextrin (HP-β-CD) was provided by Fluka (Buch, Switzerland).

The solutions used for the running buffer and in the CE rinse cycle procedure were filtered through a Millex-HV 0.45-μm disk filter from Millipore (Bedford, MA, USA) and degassed by ultrasound for 5 min. Water was purified with a Milli-Q plus system (Millipore, Bedford, MA, USA).

2.2. Capillary electrophoresis (CE)

The analyses were carried out using CE instrumentation from Beckman Coulter Instruments, model P/ACE MDQ (Fullerton, CA, USA). The apparatus consisted of an analyzer, an automatic sampler with temperature control, and a diode array detector (with the wavelength set to 214 nm for analyte detection). The software 32 Karat™ was used to control the instrument and to acquire data. An uncoated fused-silica capillary from Beckman Coulter Instruments (Fullerton, CA, USA) with a 75-μm id, 30-cm total length, and 20-cm effective length was used. Prior to its first use, the capillary was conditioned by rinsing with 1.0 mol L^{-1} NaOH for 10 min at 20 °C, followed by a rinse with 0.1 mol L^{-1} NaOH for 10 min at 20 °C and a final water rinse for 10 min at 20 °C. At the beginning of each working day, the capillary was rinsed with 0.1 mol L^{-1} NaOH for 10 min, followed by a water rinse for 10 min. Between consecutive analyses, the capillary was rinsed with 0.1 mol L^{-1} NaOH for 2 min, water for 2 min, and the running buffer for 2 min. After daily use, the capillary was washed with 0.1 mol L^{-1} NaOH for 10 min and then with water for 10 min. When not in use, the capillary was filled with water, and its tips were stored immersed in water. The electrophoretic separations

were carried out in 50 mmol L^{-1} sodium tetraborate buffer solution (pH adjusted to 9.0 with hydrochloric acid) containing S-β-CD 0.8% (w/v). All the experiments were conducted in the normal mode. The sample injections were performed hydrodynamically at a pressure of 0.3 psi for 5 s. The capillary and sample temperatures were set to 15 °C. A constant voltage of +6 kV was applied during the analyses.

2.3. Dispersive liquid–liquid microextraction (DLLME)

One-milliliter aliquots of Czapek liquid culture medium spiked with 50 μL of HZ and CTZ at a concentration of 200 μg mL^{-1} or samples obtained in the biotransformation process were transferred to 10-mL conical glass tubes and buffered with 1 mL of 0.25 mol L^{-1} dibasic phosphate buffer solution at pH 7.5. Subsequently, a mixture of 400 μL of ethanol (disperser solvent) and 300 μL of chloroform (extraction solvent) was rapidly injected into the sample using a 1-mL microsyringe; a cloudy solution then formed in the conical glass tube. Immediately afterward, the samples were vigorously shaken by vortex agitation at 2000 rpm for 30 s. Next, the samples were centrifuged at 3000 rpm for 5 min, and the extraction solvent was sedimented at the bottom of the conical glass tube. Following the centrifugation, 250 μL of the sedimented phase was transferred to another conical glass tube using a microsyringe. The extract was then evaporated to dryness under compressed air stream. The dried residue was reconstituted in 120 μL of water and injected into the CE system. Each extraction procedure was performed in triplicate.

2.4. Method validation

Because the entire DLLME optimization was performed in Czapek liquid culture medium and because the fungal biotransformation medium can present different characteristics due to the formation of secondary fungi metabolites, the linearity of the method was investigated in two different matrices: in Czapek liquid culture medium and in a fungi pool that was prepared in the absence of the analytes. The linearity of the method was assayed in triplicate. The results were weighted by $1/x^2$ because the residual analysis of the analytical curve exhibited heteroscedastic behavior. Analytical curves ($n=3$) were obtained by spiking aliquots of 1 mL of Czapek liquid culture medium and the fungi pool with 50 μL each of the HZ and CTZ enantiomers in the concentration range of 250–12,500 ng mL^{-1} and 125–6250 ng mL^{-1}, respectively. Linearity was determined using the correlation coefficient (r), the F test for lack-of-fit (F_{LOF}), and a p value of 0.05. The program MINITAB Release version 14.1 (State College, PA, USA) was employed for statistical analysis. In addition, the slopes of the different analytical curves that were assayed in Czapek liquid culture medium and in the fungi pool were analyzed and compared [27].

To determine the absolute recovery, aliquots of Czapek liquid culture medium (1 mL) were spiked with CTZ at concentrations of 250, 1250, and 5000 ng mL^{-1} for each enantiomer ($n=3$) and with each HZ enantiomer ($n=3$) at concentrations of 1000, 3000, and 10,000 ng mL^{-1} and submitted to the DLLME procedure. The areas obtained for these samples were compared with the areas achieved by the direct injection of pure solutions containing the same amount of each compound dissolved in water. The recovery was expressed as a percentage of the extracted amount.

The precision and accuracy of the method were assessed by the within-day ($n=5$) and between-day ($n=3$) assays using 1 mL of Czapek liquid culture medium spiked with each CTZ enantiomer at concentrations of 125, 250, 1250, and 5000 ng mL^{-1} and each HZ enantiomer at concentrations of 250, 1000, 3000, and 10,000 ng mL^{-1}. The precision results were expressed as the relative standard deviation (RSD, %); the accuracy results were expressed in terms of percentage accuracy according to the EMA guidelines [28].

The limit of quantification was defined as the lowest concentration that could be determined with accuracy and precision below 20% over five analytical runs, as recommended by the EMA [28]. The determinations were carried out using Czapek liquid culture medium (1 mL) spiked with 250 and 125 ng mL^{-1} HZ and CTZ, respectively.

The selectivity of the method was evaluated by analyzing sterile Czapek liquid culture medium and sterile Czapek liquid medium containing fungal mycelium under the previously established conditions (see Section 2.6, "HZ biotransformation study").

The freeze–thaw cycle stability, short-term room temperature stability, and stability under the biotransformation conditions were determined. To perform the freeze–thaw cycle stability studies, three aliquots ($n=3$) of the samples prepared in Czapek liquid culture medium at concentrations of 250 and 5000 ng mL^{-1} of each CTZ enantiomer and 1000 and 10,000 ng mL^{-1} of each HZ enantiomer were stored at −20 °C for 24 h, followed by thawing at room temperature. Once completely thawed, the samples were refrozen for 12 h under the same conditions. The freeze–thaw cycle was repeated twice, and the samples were analyzed in the third cycle. To determine the short-term room temperature stability, aliquots of the samples prepared in liquid culture medium at the concentrations specified above were kept at room temperature (22 ± 2 °C) for 12 h and then analyzed. To determine the stability under the biotransformation conditions, an aliquot of 5 mg of HZ (free-base form) dissolved in 1 mL of sterile water was added to an Erlenmeyer flask containing 100 mL of Czapek medium (25 μg mL^{-1} of each enantiomer) and submitted to the same conditions used in the biotransformation procedure (see Section 2.6, "HZ biotransformation study"). During the biotransformation period (15 days), aliquots of 1 mL ($n=3$) were analyzed. The stability of the samples placed in the CE auto-sampler was evaluated. This procedure was performed using three aliquots ($n=3$) of samples prepared in Czapek liquid culture medium at CTZ enantiomer concentrations of 250 and 5000 ng mL^{-1} and HZ enantiomer concentrations of 1000 and 10,000 ng mL^{-1}. The samples were extracted and placed in the CE auto-sampler at room temperature and analyzed after 24 h. The samples submitted to the stability studies were considered stable if the relative error (RE%) from the nominal concentration was within ± 15% and the RSD was below 15%.

To investigate whether the resolution of the enantiomers would change in the presence of different fungi, the relative standard deviation (RSD%) and the resolution of the enantiomers were determined separately in the presence of the following fungi: *Penicillium crustosum* (VR4), *Mucor rouxii* NRRL 1894, *Cunninghamella echinulata* var. *elegans* ATCC 8688A and *Cunninghamella elegans* ATCC 10028B ($n=3$). The resolution values were determined by analyzing the samples from the biotransformation study.

2.5. Fungi

The fungus *Mucor rouxii* NRRL 1894 was kindly provided by Dr. C.W. Hesseltine (Northern Utilization Research and Development Division, ARS, USDA, Peoria, IL, USA) and belonged to a collection of fungal cultures of the Departamento de Biologia da Faculdade de Filosofia, Ciências e Letras de Ribeirão Preto, University of São Paulo (FFCLRP/USP). The microorganism was stored as a conidial suspension on silica gel (6–12 mesh, grade 40, desiccant activated) at 4 °C and on slants of solid oatmeal baby food consisting of 0.4% (w/v) oatmeal and 1.8% (w/v) agar.

The fungi *Cunninghamella echinulata* var. *elegans* ATCC 8688A and *Cunninghamella elegans* ATCC 10028B were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

The endophytic fungi *Nigrospora sphaerica* (Sacc.) E.W. Mason (SS67) and *Fusarium oxysporum* (SS50) were previously isolated as endophytes from the plant *Smallanthus sonchifolius* [29]; the fungus *Penicillium crustosum* (VR4) was isolated from the plant *Viguiera robusta*. The endophytic fungi and *Cunninghamella* strains were maintained as potato dextrose agar plugs in 80% glycerol (*v*/*v*) and stored at −20 °C in the Laboratório de Química de Micro-organismos, FCFRP/USP, Brazil.

2.6. HZ biotransformation study

The biotransformation study was performed as previously described by our group [30,31]. Three discs with 0.5-cm diameters containing the fungal mycelia were aseptically transferred to 9.0-cm diameter Petri dishes containing potato dextrose agar medium and allowed them to grow for 7 days at 30 °C. Subsequently, three uniform discs of 0.5-cm diameter of the fungus mycelia were cut with a transfer tube (Fisher Scientific, Pittsburgh, PA, USA) and inoculated in 50-mL Falcon tubes containing 20 mL of pre-fermentative medium (10 g of malt extract, 10 g of dextrose, 5 g of triptone, and 3 g of yeast extract and deionized water to 1 L; the pH was adjusted to 6.2 with a solution of 0.5 mol L^{-1} HCl). The Falcon tubes were incubated for 7 days (168 h) at 30 °C on a rotatory shaker (Cientec, CT712RN, SP, Brazil) operating at 125 rpm. Finally, the mycelium was completely transferred to a 250-mL Erlenmeyer flask containing 100 mL of Czapek liquid culture medium (25.0 g of sucrose, 2.0 g of $NaNO_3$, 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 0.01 g of $FeSO_4 \cdot 7H_2O$, and deionized water to 1.0 L; pH adjusted to 5 with a solution of 1 mol L^{-1} HCl). At this point, HZ (5 mg) was dissolved in 1 mL of sterile water and added to the Czapek liquid culture medium. The cultures were incubated for 360 h at 30 °C with shaking at 125 rpm. The three control flasks consisted of (1) sterile Czapek culture medium without HZ and the fungus, (2) sterile culture medium with HZ and without the fungus, and (3) culture medium with the fungal mycelium of the studied fungi and without HZ.

The CTZ enantiomeric excess (*ee*) following biotransformation was determined by the equation $ee=(A-B/A+B) \times 100$, where *A* is the peak area of the enantiomer present in higher concentration and *B* is the peak area of the enantiomer present in lower concentration [32]. The biotransformation kinetic studies were presented as the concentration versus the collection interval (hours). To assess the efficiency of the biotransformation process, the amount of CTZ in the culture medium was quantified and correlated with the initial amount of HZ (time 0). The biotransformation procedure was performed in triplicate ($n=3$).

3. Results and discussion

3.1. Selection of the separation conditions

To achieve enantiomeric separation, we considered the physicochemical properties of hydroxyzine (HZ, basic compound; pKa's 2.13 and 7.13) and cetirizine (CTZ, zwitterionic compound; pKa's 2.19, 2.93, and 8.0). Theoretically, CTZ can be considered a dication at pH < 2.19, a cation at a pH range from 2.19 to 2.93, a zwitterionic compound at a pH range from 2.93 to 8.0, and an anion at pH > 8.0. In turn, HZ can be considered a dication at pH < 2.3, a cation at a pH range from 2.13 to 7.13, and a neutral compound at pH 7.13 [1].

We evaluated how pH affects the migration and separation of the enantiomers at pH values of 8, 9, 10, 11, and 12 using a 50 mmol L^{-1} sodium tetraborate solution containing S-β-CD (0.5% w/v). In this pH range, HZ is in the neutral form, whereas CTZ and S-β-CD are negatively charged. In this situation, electroosmotic flow induces the movement of the analytes toward the cathode. We achieved the best resolution and suitable migration times at pH 9.0, which was the pH value we selected for further experiments.

We assessed the effect of the sodium tetraborate buffer (pH 9.0) concentration in the range of 5–100 mmol L^{-1} in the presence of S-β-CD (0.5% w/v). The detection was optimal at buffer concentrations of 50 and 100 mmol L^{-1}, but 50 mmol L^{-1} provided superior resolution coupled with acceptable current levels, optimal peak shape, and the lowest migration times. Hence, we employed 50 mmol L^{-1} in further experiments.

We investigated the efficiency of various chiral selectors using CM-β-CD, S-β-CD, and HP-β-CD. S-β-CD was previously reported to provide enantioselective resolution for CTZ enantiomers [5,19]; in our experiments, S-β-CD was the only CD able to separate the CTZ and HZ enantiomers. We examined the effects of S-β-CD concentration on the enantioseparation using a 50 mmol L^{-1} sodium tetraborate buffer solution (pH 9.0) over a concentration range of 0.5–1.0% (w/v) S-β-CD at an applied voltage of +10 kV. At the highest S-β-CD concentration, we observed diminished and improved HZ and CTZ enantiomer resolutions, respectively. The S-β-CD concentration that afforded the best resolution for all the analytes was 0.8% (w/v), which provided resolutions of 1.21 and 1.39 for the HZ and CTZ enantiomers, respectively.

We also optimized the capillary temperature and length. The capillary temperature did not significantly alter the CTZ or HZ enantiomer resolution. With respect to the capillary length, the analysis time decreased from 22 to 9 min by replacing a 40-cm effective length with a 20-cm effective length capillary.

Therefore, the optimized conditions constituted a 50 mmol L^{-1} sodium tetraborate buffer solution (pH 9.0) containing 0.8% (w/v) S-β-CD and a voltage and temperature of +6 kV and 15 °C, respectively. Under these conditions, we achieved suitable resolutions (HZ enantiomers, Rs = 1.76; CTZ enantiomers, Rs = 1.52) in 9 min with an acceptable current level ($\approx$ 75 μA). Nojavan and Fakhari [1] previously reported a method to enantioselectively characterize HZ and CTZ by CE using maltodextrin as the chiral selector and a 75 mmol L^{-1} sodium phosphate solution (pH 2) as the running buffer; under these conditions, the authors were able to separate all the enantiomers in 33-min analytical runs. Deng et al. [19] developed a method to analyze CTZ enantiomers and to control the enantiomeric purity and the quality of (*R*)-CTZ in pharmaceutical formulations. These authors also used S-β-CD as the chiral selector and achieved a resolution of 3.1 with a migration time of 7 min. Despite the high resolution values achieved by the aforementioned authors, we had difficulty separating the HZ and CTZ enantiomers in a single run with a suitable resolution and migration time. Increasing the S-β-CD concentration led to improved CTZ enantiomer resolution; however, the HZ enantiomer resolution decreased dramatically. Thus, we found 0.8% (w/v) S-β-CD to be the optimal chiral selector.

We determined the CTZ enantiomer migration order by injecting a levocetirizine (*R*-CTZ) solution using the developed and validated method. This analysis confirmed that the second enantiomer to migrate was (*R*)-CTZ, indicating that the first enantiomer corresponded to (*S*)-CTZ. Because we did not establish the HZ enantiomer migration order, we simply designated the first and second HZ enantiomers to migrate as HZ (E1) and HZ (E2), respectively.

3.2. Development of the DLLME procedure

Various factors influence the extraction efficiency of the analytes by DLLME, such as the disperser and extraction solvent type and volume, the extraction time, the centrifugation time, and the extraction rate. Other frequently optimized parameters are the salting-out effect and the pH of the sample [24,33]. However,

we did not evaluate the two latter parameters because we had previous knowledge about the neutral pH range for both analytes [1] and considered salt addition unnecessary due to the large amount of salt present in the matrix.

3.2.1. Effect of the disperser and extraction solvent type

An appropriate extraction solvent is the most important parameter when optimizing DLLME [23] because the organic solvents used as the DLLME extraction solvent must be denser than water. This density difference enables the separation of the extraction solvent from the aqueous phase (matrix) by centrifugation. We used chloroform, dichloromethane, and 1,1-dichloroethane to investigate HZ and CTZ extraction from Czapek liquid culture medium. In these experiments, we tested 200 μL of each extraction solvent and a fixed volume of 500 μL of acetone (disperser solvent used in this stage). According to Fig. 2A, dichloromethane provided larger peak area responses for the HZ enantiomers, whereas chloroform produced the highest peak area responses for the CTZ enantiomers. The latter solvent was selected for further experiments.

The solubility of the disperser solvent in the extraction solvent and in the aqueous phase determines the choice of disperser, which influences the generation of fine droplets of the extraction solvent in the aqueous phase [34]. Therefore, we evaluated acetone, acetonitrile, methanol, and ethanol as disperser solvents. We performed the experiments using 500 μL of each disperser solvent and 200 μL of the optimized extraction solvent (chloroform). Fig. 2B reveals that acetonitrile furnished higher peak area responses for the HZ enantiomers, while methanol and ethanol produced better responses for the CTZ enantiomers. Ultimately, ethanol was selected as the disperser solvent due to its lower toxicity.

3.2.2. Effect of the extractor and disperser solvent volumes

To evaluate the effect of the extraction solvent volume on the extraction efficiency, we subjected a constant ethanol volume (400 μL) containing different volumes of chloroform (100–500 μL) to the same DLLME procedure. Fig. 2C reveals higher peak areas for the CTZ and HZ enantiomers when 300 μL of chloroform was employed, which was the extraction solvent volume selected for subsequent experiments. The disperser solvent volume affects the formation of the cloudy state. To investigate this effect, we evaluated the disperser solvent volume in the range of 300–700 μL (Fig. 2D) using 300 μL of chloroform (extraction solvent). At low ethanol volumes, we observed low extraction recoveries, most likely because the cloudy solution was not well-formed. At high ethanol volumes, the solubility of the analytes in the aqueous phase increased, decreasing the extraction efficiency. We selected an ethanol volume of 400 μL, which furnished the highest recoveries.

3.2.3. Effect of extraction time

The extraction time is defined as the interval between the injection of the disperser solvent and the extraction solvent mixture and centrifugation [20]. According to several studies [23,33], the extraction time affects the extraction efficiency in DLLME to a small extent. This effect occurs because the surface area between the extraction solvent and the aqueous phase (sample) is infinitely large. Hence, the transfer of the analytes from the aqueous phase to the extraction phase is rapid (a few seconds) [20]. Furthermore, the use of ultrasound (US) and vortex (VX) improves the extraction efficiencies in DLLME [35,36]. Thus, we evaluated assisted DLLME by comparing ultrasound-assisted DLLME for 5 min, vortex-assisted DLLME for 30 s, and the combination of these two forms of assisted DLLME extraction. The cloudy solution (CS) in combination with vortex agitation was able to more efficiently extract HZ and CTZ than the ultrasound-assisted method, most likely because the mechanical and thermal effects of the ultrasound treatment resulted in the loss of the volatile extraction solvent [35]. Moreover, fast vortex agitation most likely broke the chloroform into fine droplets, which more

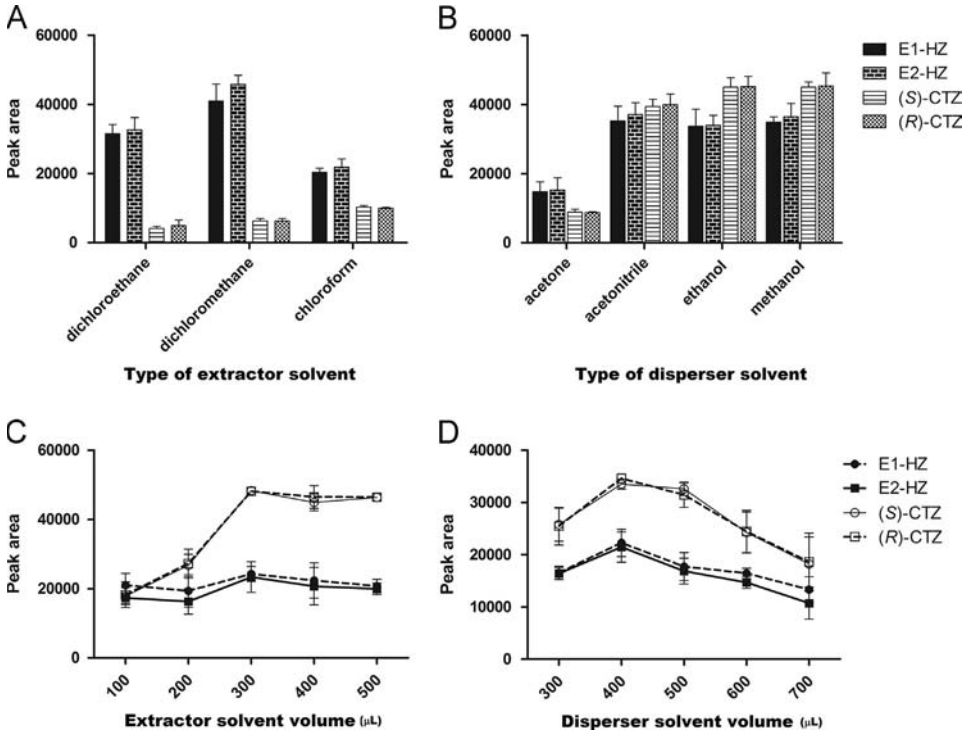

Fig. 2. DLLME optimization. Selection of extraction solvent type (A), disperser solvent type (B), extraction solvent volume (C) and disperser solvent volume (D) on the extraction of HZ and CTZ enantiomers from Czapek culture medium. Extraction efficiencies represented by peak areas. Sample pH 7.5 and 5 min of centrifugation at 3000 rpm. The bars denote the standard deviation of replicate ($n=3$).

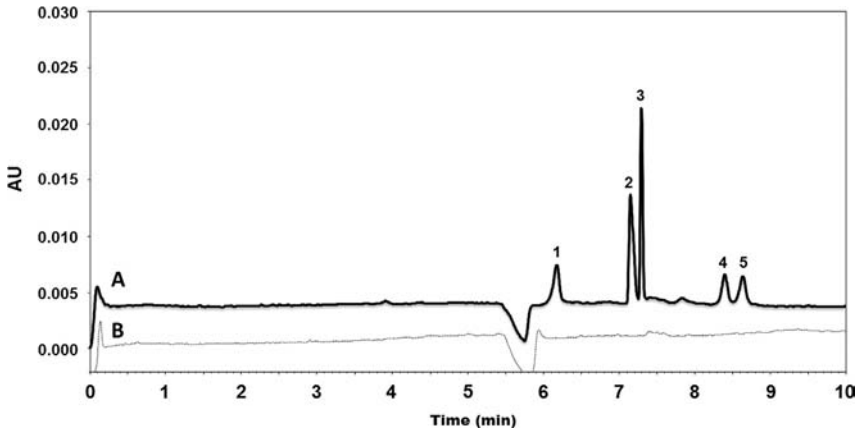

Fig. 3. Representative electropherograms of the enantioselective analysis of HZ and CTZ in Czapek culture medium after DLLME extraction. (A) Czapek culture medium spiked with (1) internal standard, (2) E1-HZ and (3) E2-HZ (7500 ng mL^{-1}), (4) (*S*)-CTZ and (5) (R)-CTZ (2500 ng mL^{-1}). (B) Drug-free Czapek culture medium. Electrophoretic and DLLME conditions are described in Sections 2.2 and 2.3, respectively.

Table 1
Linearity of the method for analysis of the analytes in Czapek liquid culture medium and in a fungi pool.

Analytes	Type of matrix	Linear equation[a]	*r*[b]	Ratio of slopes	ANOVA lack-of-fit	
					F-value	*p*-value
(1)-HZ	Fungi pool	$y=0.000157x+0.0042$	0.998	0.93	1.17	0.371
(1)-HZ	Czapek medium	$y=0.000169x+0.0101$	0.998		0.77	0.585
(2)-HZ	Fungi pool	$y=0.000154x+0.0013$	0.999	0.90	1.32	0.312
(2)-HZ	Czapek medium	$y=0.000171x+0.0124$	0.998		1.39	0.288
(S)-CTZ	Fungi pool	$y=0.000243x+0.0035$	0.999	1.00	2.86	0.055
(S)-CTZ	Czapek medium	$y=0.000242x+0.0024$	0.998		2.66	0.068
(R)-CTZ	Fungi pool	$y=0.000243x+0.0030$	0.997	1.05	1.78	0.181
(R)-CTZ	Czapek medium	$y=0.000232x+0.0045$	0.998		1.90	0.159

[a] Three replicates ($n=3$) for each concentration. Range: HZ=250–12,500 ng mL^{-1}; CTZ=125–6250 ng mL^{-1}.
[b] Correlation coefficient.

efficiently dispersed the droplets within the aqueous solution [36,37]. As a result, vortexing provided enhanced mass transfer and extraction efficiency.

3.2.4. Centrifugation parameters

Following vortex agitation, it was necessary to centrifuge the cloudy solution to guarantee complete separation of the organic and aqueous phases. We evaluated centrifugation times from 5 to 15 min at a fixed rotation and temperature of 3000 rpm and 10 °C, respectively. We selected a 5 min centrifugation time for subsequent experiments because no appreciable improvement in the recovery was observed with longer centrifugation periods. Fig. 3 displays the electropherograms following the DLLME optimization. The final conditions for the DLLME consisted of ethanol (400 μL) as the disperser solvent and chloroform (300 μL) as the extraction solvent, followed by vortex agitation at 2000 rpm for 30 s. The samples were then centrifuged at 3000 rpm for 5 min, and the extraction solvent was sedimented to the bottom of the conical glass tube for further analysis.

3.2.5. Method validation

We validated the CE method according to the EMA guidelines [28]. We performed regression analyses by plotting the peak area ratio of the analytes and I.S. versus the theoretical analyte concentration. The method proved to be linear (Table 1) over the concentration range of 125–6250 ng mL^{-1} for each CTZ enantiomer and 250–12,500 ng mL^{-1} for each HZ enantiomer; the correlation coefficient was above 0.998, and the relative error for each point of the analytical curves was below 15%. The slopes of the analytical curves prepared in different matrices differed by less than 10%, which indicated that the interferents present in the fungi pool did not significantly affect the DLLME procedure [27,28,38]. In addition, we submitted the constructed analytical curves to the ANOVA lack of fit test and demonstrated their validity (Table 1). The matrix did not influence the analyte recovery; hence, we conducted the entire method validation in Czapek liquid culture medium, which does not produce any biological residues.

Table 2
Limit of quantification and recovery values of the method.

Analyte	Nominal concentration (ng mL^{-1})	Obtained concentration (ng mL^{-1})	Accuracy	Precision	Recovery	
			%	RSD (%)[a]	%	RSD (%)[a]
(1)-HZ	250	231.7	92.6	11	90	7
(2)-HZ	250	241.4	96.5	10	91	5
(S)-CTZ	125	137.2	109.7	7	87	4
(R)-CTZ	125	136.7	109.3	7	87	6

[a] Relative standard deviation in percentage.

The mean (E1)-HZ, (E2)-HZ, (*S*)-CTZ, and (*R*)-CTZ recoveries were 90, 92, 87, and 87, respectively, with RSDs below 7%. The lowest concentrations that the validated method was able to quantify were

Table 3
Precision and accuracy of the method.

Analyte	Nominal concentration (ng mL^{-1})	Within-day (n=5)[a]			Between-day (n=3)[b]		
		Concentration (ng mL^{-1})	RSD [c] (%)	accuracy (%)	Concentration (ng mL^{-1})	RSD[c] (%)	accuracy (%)
(1)-HZ	250	255.6	2	102.2	245.5	5	98.2
	1000	1092.2	3	109.2	1043.8	7	104.3
	3000	3144.8	2	104.8	3231.6	2	107.7
	10,000	9847.6	4	98.4	9724.1	6	97.2
(2)-HZ	250	258.2	6	103.2	248.3	5	99.32
	1000	996.7	5	99.6	1008.9	8	100.8
	3000	3101.6	1	103.3	3163.5	2	105.4
	10,000	9759.3	2	97.5	9292.1	4	92.9
(S)-CTZ	125	120.5	4	96.4	125.9	8	100.7
	250	249.4	5	99.7	255.0	5	102
	1250	1200.3	3	96.0	1211.5	7	96.9
	5000	4940.6	2	98.8	5065.4	6	101.3
(R)-CTZ	125	127.1	5	101.6	129.8	5	103.8
	250	233.3	5	93.3	238.0	6	95.2
	1250	1215.5	3	97.2	1215.1	5	97.2
	5000	4904.8	2	98.0	5031.7	9	100.6

[a] Number of replicates.
[b] Number of days.
[c] Relative standard deviation in percentage.

Table 4
Freeze-thaw and short-term room temperature stability of HZ and CTZ enantiomers in Czapek culture medium.

Analyte	(1)-HZ		(2)-HZ		(S)-CTZ		(R)-CTZ	
Stability								
Freeze-thaw cycles (n=3)								
Concentration (ng mL^{-1})	243.2	8914.0	236.0	9073.4	225.2	4386.8	232.7	4471.2
Precision (RSD)[a]	1	7	1	6	4	6	2	7
Accuracy (RE, %)[b]	−2.7	−10.8	−5.5	−9.2	−9.8	−12.2	−6.8	−10.5
Short-term (n=3)								
Concentration (ng mL^{-1})	242.2	8714.3	233.1	8706.2	238.8	4719.7	234.5	4603.5
Precision (RSD)[a]	1	8	3	7	3	5	2	3
Accuracy (RE, %)[b]	−3.1	−12.8	−6.7	−12.9	−4.4	−5.6	−6.1	−7.9

n=number of determinations.
[a] Expressed as relative standard deviation, RSD (%).
[b] Expressed as relative error, RE (%).

250 and 125 ng mL^{-1} for each HZ and CTZ enantiomer, respectively (Table 2). We assessed the precision and accuracy of the method for both within-day (five spiked Czapek culture medium samples for each concentration on the same day) and between-day (five spiked Czapek culture medium samples for each concentration for three consecutive days) determinations (Table 3). The RSDs were under 15%, and the accuracy values were within 15% of the nominal values.

The HZ and CTZ freeze-thaw and short-term room temperature stability in Czapek culture medium revealed RSDs of less than 15%, and the RE% from the nominal concentrations fell within ± 15% (Table 4). CTZ and HZ were stable in the auto sampler for 24 h and for 360 h under the biotransformation conditions (RSD < 15% and RE % ± 15%); we verified that no CTZ or HZ degradation occurred during this period (data not shown). The selectivity of the separation was high, as the studied fungus did not produce any secondary metabolites with migration times close to those of the HZ and CTZ enantiomers (Fig. 4). Moreover, the Czapek liquid culture medium did not present any interfering peaks (data not shown).

The investigation of the resolution values in different fungal biotransformation media and in a pure Czapek medium revealed that the type of fungus can influence the resolution value of the enantiomers (Table 5). This effect can be attributed to the difference in the ionic strengths of the culture media, which can lead to an anti-stacking effect [39] that affects the resolution of the enantiomers. Although the composition of the culture medium was the same for all the evaluated fungi (Czapek medium), the consumption of salts by the fungi can differ during the biotransformation period, leading to differences in the culture medium composition.

3.3. HZ biotransformation study

The monitored HZ biotransformation involves a carboxylation reaction that yields the active metabolite CTZ. We selected fungi belonging to the genus *Cunninghamella* because they had been previously shown to perform carboxylation reactions on various drugs, including celecoxib [40], ebastine [41], and muraglitazar [42]. Our group also verified other classes of oxidation reaction for these fungi; using the fungi *M. rouxii*, *N. sphaerica*, *F. oxysporum*, and *P. crustosum*, we observed a sulfoxidation reaction for the drug albendazole [9,30]. The fungus *P. crustosum* also performed a sulfoxidation of the drug thioridazine [15]; furthermore, it was able to biotransform the drugs propranolol [43] and ibuprofen [44] *via* a hydroxylation reaction. We also reported that the fungus *M. rouxii* performed hydroxylation during risperidone biotransformation [14].

We followed the HZ biotransformation for 15 days (360 h). Aliquots were collected every 48 h until day 10 and then again on day 15. We analyzed the collected samples by CE using a simultaneously

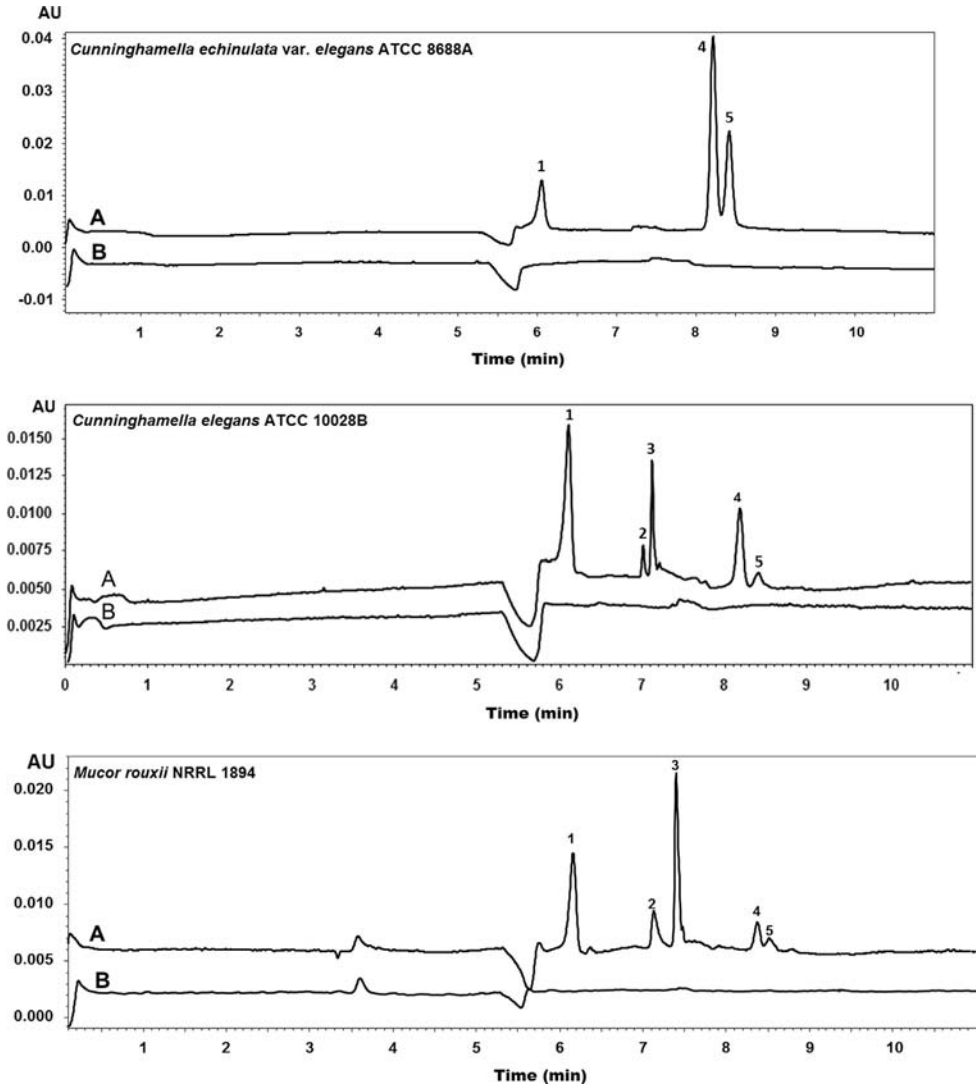

Fig. 4. (A) Representative electropherograms for 360-h HZ incubation with the studied fungi (B) Czapek culture medium incubated with the studied fungi (fungus control) showing that these fungi did not produce any secondary metabolite in the migration time of the analytes. (1) internal standard, (2) E1-HZ, (3) E2-HZ, (4) (*S*)-CTZ and (5) (*R*)-CTZ. Electrophoretic and DLLME conditions are described in Sections 2.2 and 2.3, respectively.

Table 5
Resolution values for different fungal biotransformation media.

Medium	HZ resolution (RSD%)	CTZ resolution (RSD%)
Czapek liquid medium	1.76 (1.5)	1.52 (0.3)
Fungus 10028B	1.55 (5.9)	1.52 (3.1)
Fungus VR4	1.78 (3.9)	1.70 (7.3)
Fungus 8688A	1.77 (5.4)	1.50 (4.0)
Fungus *Mucor rouxii*	2.52 (0.79)	1.47 (0.8)

RSD, relative standard deviation in percentage ($n=3$).

obtained calibration curve. Following the extraction and analysis, we determined the analyte concentrations and plotted the concentration as a function of the incubation time. The fungi SS50 and SS67 did not biotransform HZ under the evaluated conditions. The fungus VR4 did not afford a good biotransformation yield; CTZ enantiomers were only detected after 360 h of incubation (data not shown). The fungus *Cunninghamella elegans* ATCC 8688 A produced the highest (*S*)-CTZ yield: at 96 h of incubation, only (*S*)-CTZ was present in the culture medium (Fig. 4), and at 144 h, (*R*)-CTZ was also present. At 360 h, the maximum (*S*)-CTZ and (*R*)-CTZ concentrations in the culture medium were 12,270 and 6277 ng mL^{-1}, respectively (57 and 26% biotransformation, respectively, Fig. 5A), corresponding to a (*S*)-CTZ *ee* of 32%. HZ biotransformation by the fungus *C. elegans* ATCC 10028B resulted in predominantly (*S*)-CTZ formation (Fig. 4). The CTZ enantiomer production began after 96 h of incubation. At 360 h, the maximal CTZ enantiomer concentrations in the culture medium were 2693 and 579 ng mL^{-1} (19 and 4% biotransformation, respectively) for (*S*)-CTZ and (*R*)-CTZ, respectively (Fig. 5B), which corresponded to a (*S*)-CTZ *ee* of 65%.

The biotransformation of HZ by the soil fungus *Mucor rouxii* NRRL 1894 resulted in the predominant formation of the *S*- enantiomer of CTZ (Fig. 4). After 360 h of incubation, the maximal (*S*)-CTZ and (*R*)-CTZ concentrations in the culture medium were 1957 and 495 ng mL^{-1}, respectively (7 and 2% of biotransformation, respectively, Fig. 5C), which corresponded to a (*S*)-CTZ *ee* of 60%.

4. Conclusion

This study demonstrated the successful use of DLLME–CE to enantioselectively determine HZ and CTZ in a complex matrix. The assessed validation parameters met the literature requirements [28]. This method constitutes the first report of simultaneous HZ and CTZ extraction by DLLME, which is a powerful tool for studying HZ fungal biotransformation. In comparison with other

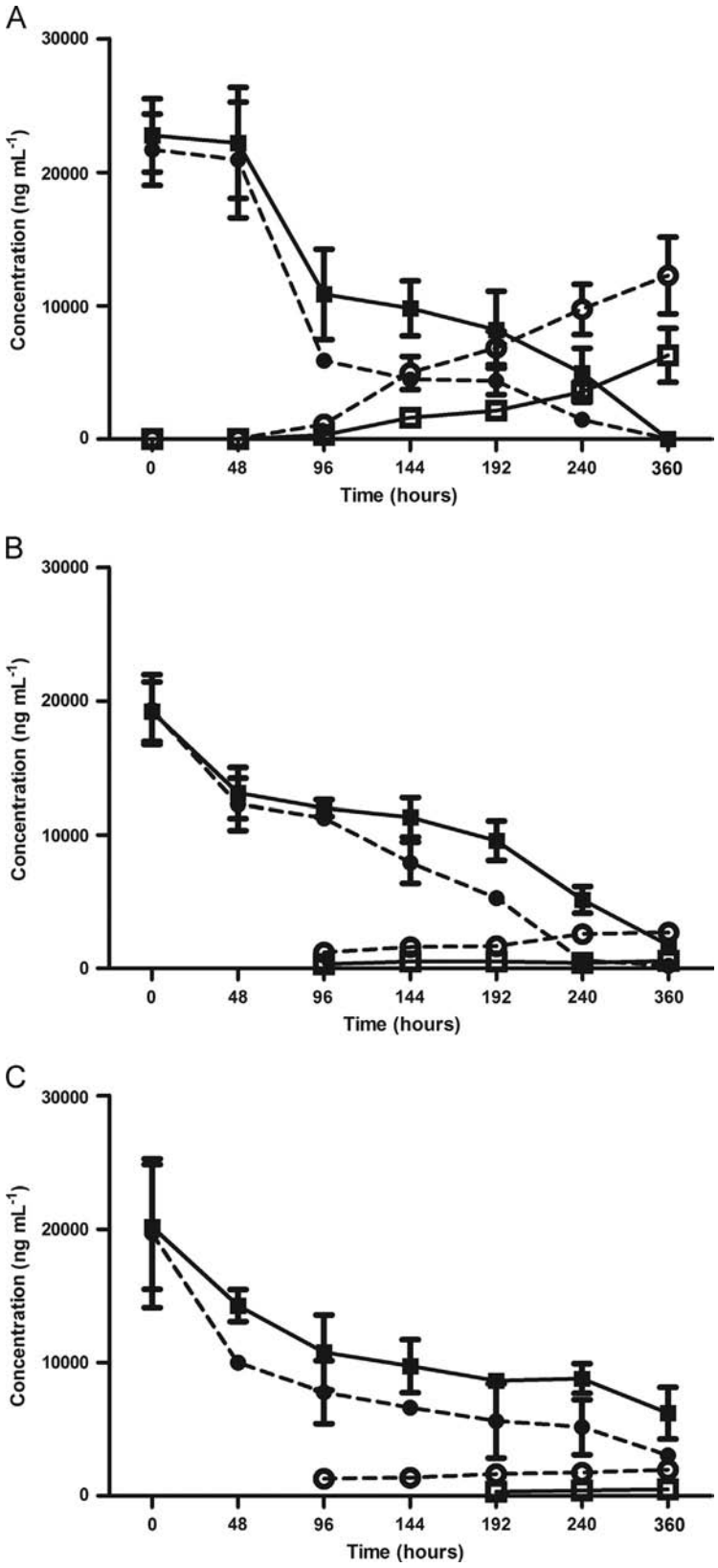

Fig. 5. Concentration-time profiles for the biotransformation of HZ by the fungi (A) *Cunninghamella echinulata* var. *elegans* ATCC 8688A, (B) *Cunninghamella elegans* ATCC 10028B, and (C) *Mucor rouxii* NRRL 1894. The bars denote the standard deviation of replicate ($n=3$). (●) E1-HZ, (■) E2-HZ, (○)-CTZ and (□) (*R*)-CTZ.

literature methods, DLLME-CE offers the following advantages: (i) shorter analysis times compared with the procedure described by Nojavan et al. [1] and (ii) a simpler extraction procedure as well as lower solvent consumption in comparison with the previously described protein precipitation [16–18] and LLE [1,5] procedures. Moreover, the recovery was significantly improved in our DLLME

procedure through the addition of a vortex-assisted step relative to the cloudy solution only. In addition, this is the first report of the enantioselective HZ biotransformation to CTZ with predominant (*S*)-CTZ formation.

Acknowledgments

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for financial support and research fellowships.

References

[1] S. Nojavan, A.R. Fakhari, Electrophoresis 32 (2011) 764–771.
[2] K. Tekes, H. Kalász, M.Y. Hasan, E. Adeghate, F. Darvas, N. Ram, A. Adem, Curr. Med. Chem. 18 (2011) 4885–4900.
[3] H. Yu-Hsiang, W. Hsin-Lung, W. Shou-Mei, C. Su-Hwei, K. Hwang-Shang, Anal. Bioanal. Chem. 376 (2003) 859–863.
[4] A.M. Beltagi, O.M. Abdallah, M.M. Ghoneim, Talanta 74 (2008) 851–859.
[5] Y.W. Chou, W.S. Huang, C.C. Ko, S.W. Chen, J. Sep. Sci. 31 (2008) 845–852.
[6] P. Mikus, I. Valásková, E. Havránek, J. Sep. Sci. 28 (2005) 1278–1284.
[7] A. Van Eeckhaut, Y. Michotte, Electrophoresis 27 (2006) 2376–2385.
[8] T. Barth, M.T. Pupo, K.B. Borges, L.T. Okano, P.S. Bonato, Electrophoresis 31 (2010) 1521–1528.
[9] D.B. Carrão, K.B. Borges, T. Barth, M.T. Pupo, P.S. Bonato, A.R.M. de Oliveira, Electrophoresis 32 (2011) 2746–2756.
[10] K.B. Borges, W.S. Borges, R. Durán-Patrón, M.T. Pupo, P.S Bonato, I.G. Collado, Tetrahedron Asymmetry 20 (2009) 385–397.
[11] S. Asha, M. Vidyavathi, Biotechnol. Adv. 27 (2009) 16–29.
[12] W.S. Borges, K.B. Borges, P.S. Bonato, S. Said, M.T. Pupo, Curr. Org. Chem. 13 (2009) 1137–1163.
[13] R.X. Tan, W.X Zou, Nat. Prod. Rep. 18 (2001) 448–459.
[14] L.I. Jesus, N.C.P. Albuquerque, K.B. Borges, R.A. Simões, L.A. Calixto, N.A.J. C. Furtado, C.M. Gaitani, M.T. Pupo, A.R.M. de Oliveira, Electrophoresis 32 (2011) 2765–2775.
[15] K.B. Borges, W.S. Borges, M.T. Pupo, P.S. Bonato, Appl. Microbiol. Biotechnol. 77 (2007) 669–674.
[16] S.O. Choi, S.H. Lee, H.S. Kong, E.J. Kim, H.Y. Choo, J. Chromatogr. B 744 (2000) 201–206.
[17] A. Gupta, P. Chatelain, R. Massingham, E.N. Jonsson, M. Hammarlund-Udenaes, Drug Metab. Dispos. 34 (2006) 318–323.
[18] A. Gupta, B. Jansson, P. Chatelain, R. Massingham, M. Hammarlund-Udenaes, Rapid Commun. Mass Spectrom. 19 (2005) 1749–1757.
[19] X. DengB. de CockR. VervoortD. PamperinE. AdamsA.V. Schepdael, Chirality 24 (2012) 276–282.
[20] M. Rezaee, Y. Assaddi, M.R.M. Hosseini, E. Aghaee, F. Ahmadi, S. Berijani, J. Chromatogr. A 1119 (2006) 1–9.
[21] M. Rezaee, Y. Yamini, M. Faraji, J. Chromatogr. A 1217 (2010) 2342–2357.
[22] A. Zgoła-Grzeskowiak, T. Grzeskowiak, Anal. Chem. 30 (2011) 1382–1399.
[23] L. Meng, B. Wang, F. Luo, G. Shen, Z. Wang, M. Guo, Forensic Sci. Int. 209 (2011) 42–47.
[24] Z. Xiao-Huan, W. Qiu-Hua, Z. Mei-Yue, X. Guo-Hong, W. Zhi, Chinese J. Anal. Chem. 37 (2009) 161–168.
[25] H.A. Mashayekhi, P. Abroomand-Azar, M. Saber-Tehrani, S.W. Husain, Chromatographia 71 (2010) 517–521.
[26] H. Ghambari, M. Hadjmohammadi, J. Chromatogr. B 899 (2012) 66–71.
[27] B.K. Matuszewski, J. Chromatogr. B 830 (2006) 293–300.
[28] Guideline on bioanalytical method validation, ⟨http:/www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf⟩.
[29] M.B. Gallo, F.O Chagas, M.O. Almeida, C.C. Macedo, B.C. Cavalcanti, F.W. Barros, M.O. De Moraes, L.V. Costa-Lotufo, C. Pessoa, J.K. Bastos, M.T Pupo, J. Basic Microbiol. 49 (2009) 142–151.
[30] V.C. Hilário, D.B. Carrão, T. Barth, K.B. Borges, N.A.J.C. Furtado, M.T. Pupo, A.R. M. de Oliveira, J. Pharm. Biomed. Anal. 61 (2012) 100–107.
[31] M.Z. Bocato, R.A Simões, L.A. Calixto, C.M. Gaitani, M.T. Pupo, A.R.M. de Oliveira, Anal. Chim. Acta 742 (2012) 80–89.
[32] K.D. Altria, in: H. Shintani, J. Polonský (Eds.), Handbook of Capillary Electrophoresis Applications, Blackie Academic and Professional, London1996, pp. 334–344.
[33] C.L. Ye, Q.L. Liu, Z.K. Wang, J. Fan, Intern. J. Environ. Anal. Chem 20 (2012) 1176–1186.
[34] X. Wen, Q. Yang, Z. Yan, Q. Deng, Microchem. J. 97 (2011) 249–254.
[35] A.M. Carro, S. Fernández, I. Racamonde, D. García-Rodríguez, P. González, R.A. Lorenzo, J. Chromatogr. A 1253 (2012) 134–143.
[36] J.L. Darias, M.G. Hernández, V. Pino, A.M. Afonso, Talanta 80 (2010) 1611–1618.
[37] Z.G. Shi, K. Lee, Anal. Chem. 82 (2010) 1540–1545.
[38] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Anal. Chem. 75 (2003) 3019–3030.
[39] B. Perlatti, E. Carrilho, F.A. Aguiar, in: C.D. García, K.Y. Chumbimuni-Torres, E. Carrilho (Eds.), Capillary Electrophoresis and Microchip Capillary Electrophoresis, John Wiley & Sons, Inc., New Jersey2013, pp. 23–39.
[40] K. Srisailam, C. Veeresham, Appl. Biochem. Biotechnol. 160 (2010) 2075–2089.
[41] H. Schwartz, A. Liebig-Weber, H. Hochstätter, H. Böttcher, Appl. Microbiol. Biotechnol. 44 (1996) 731–735.
[42] D. Zhang, H. Zhang, N. Aranibar, R. Hanson, Y. Huang, P.T. Cheng, S. Wu, S. Bonacorsi, M. Zhu, A. Swaminathan, W.G. Humphreys, Drug Metab. Dispos. 34 (2006) 267–280.
[43] K.B. Borges, M.T. Pupo, P.S. Bonato, Electrophoresis 30 (2009) 3910–3917.
[44] K.B. Borges, A.R.M. de Oliveira, T. Barth, V.A.P. Jabor, M.T. Pupo, P.S. Bonato, Anal. Bioanal. Chem. 399 (2011) 915–925.