J. Braz. Chem. Soc., Vol. 21, No. 12, 2262-2271, 2010.
Printed in Brazil - ©2010 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

Article

A Method for Dextruxin Analysis by HPLC-PDA-ELSD-MS

Raquel P. Morais,[a] Simone P. Lira,[b] Mirna H. R. Seleghim[c] and Roberto G. S. Berlinck*[,a]

[a]Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, 13560-970 São Carlos-SP, Brazil

[b]Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, CP 9, 13418-900 Piracicaba-SP, Brazil

[c]Departamento de Ecologia e Biologia Evolutiva, Universidade Federal de São Carlos, CP 676, 13565-905 São Carlos-SP, Brazil

Destruxinas (Dtx) são ciclodepsipetídeos produzidos por fungos entomopatogênicos, que são utilizados como controle biológico em insetos pragas em diferentes agriculturas. A presente investigação reporta uma nova abordagem para análises de destruxinas produzidas por uma linhagem do fungo *Beauveria felina*, utilizando-se de LC-PDA-ELSD-MS. Em comparação com os métodos anteriores, a nova abordagem utiliza-se de uma limpeza prévia da amostra em cartuchos C_{18} que removem efetivamente os constituintes do meio de cultura. Além disso, o uso dos solventes MeCN/MeOH 50:50, (v/v) como eluentes mais fortes no sistema de gradiente em 0,1% de H_2O demonstrou fornecer a melhor resolução dos picos cromatográficos. Detecções simultâneas usando arranjo de fotodiodos (PDA), detector de espalhamento de luz evaporativa (ELSD) e espectrometria de massas (MS) indicaram praticamente uma resposta idêntica de todos os detectores na análise das destruxinas. Cinco amostras obtidas da cultura de *B. felina* foram analisadas, e indicaram a presença de 20 destruxinas conhecidas e de 6 ciclodepsipetídeos ainda não reportados. Considerando a redução do uso do MeCN, e a eficácia do ELSD como detector para destruxina, o método prova que pode ser de grande valia e de baixo custo operacional para controle de qualidade nas análises de destruxinas produzidas por linhagens de fungos.

Destruxins (Dtx) are cyclodepsipeptides produced by enthomopathogenic fungi, which are used in biological control of different agricultural insect plagues. The present investigation reports a new approach for analysis of destruxins produced by the fungal strain *Beauveria felina*, using LC-PDA-ELSD-MS. Compared to previous methods, the new approach uses a clean-up on C_{18} cartridges, which effectively removes growth media constituents. Moreover, the use of 50:50 (v/v) MeCN/MeOH as the strongest eluting solvent in a gradient system over 0.1% H_2O proved to give a better resolution of chromatographic peaks. Simultaneous detection using photodiode array (PDA), evaporative light scattering detector (ELSD) and mass spectrometry (MS) indicated a practically identical response of all detectors for destruxins analysis. Five samples obtained from the culture media of *B. felina* were analysed, and indicated the presence of twenty known destruxins and six yet unreported cyclodepsipeptides. Considering the reduced use of MeCN, and the effectiveness of ELSD as a detector for destruxins, the method proved to be valuable and cost-effective for quality control analysis of destruxin-producing fungal strains.

Keywords: destruxin, *Beauveria felina*, LC-PDA-ELSD-MS, light scattering, marine fungus

*e-mail: rgsberlinck@iqsc.usp.br

Introduction

Destruxins (Dtx) are cyclodepsipeptides isolated from entomopathogenic fungal strains of economic importance. Dry spores of the fungus *Metarhizium anisopliae* are currently used in biological control of agricultural plagues such as several Hemiptera and Lepidoptera species affecting sugar cane cultures and citrus plantations.[1-3] Dry spores of *Beauveria bassiana* are also used against infestations of banana plants by *Cosmopolites sordidus* (Coleoptera: Curculionidae), which causes death and a significative loss of fruit productivity.[3] Both *M. anisopliae* and *B. bassiana* produce destruxin cyclodepsipeptides and related metabolites as the active compounds against the insect plagues.[4,5] Since the cyclodepsipeptides production by these and related fungal strains is affected by growth and environmental conditions,[6-10] the biological activity of the strains used commercially can be influenced if the compounds are not produced in sufficient amount and chemical variety to maintain a high level of entomopathogenicity. Therefore, it is important to establish an analytical procedure to evaluate the composition of cyclodepsipeptides obtained from fungal cultures used in biological control on insect plagues.[11]

Although several approaches have been developed to detect and identify destruxins from different strains of *M. anisopliae*,[6,8,12-18] high cost instrumentation have been used to identify destruxins, which are unaffordable for regular use in quality control of the culture media from entomopathogenic fungi used for biological control in several developing countries.

We have recently isolated a marine-derived strain of *B. felina* fungus which is a source of new and known destruxins.[19,20] Therefore, we have been interested to develop an universal and cost effective method for destruxins analysis, in order to detect a large variety of such compounds potentially useful for insect plague biological control. Herein we present and discuss a new method using HPLC-PDA-ELSD-MS for the analysis of destruxins mixtures obtained from *B. felina*, and provide evidence that that this method is cost effective to analyse culture media of destruxin-producing fungi.

Experimental

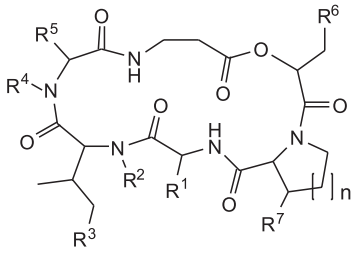
Materials and reagents

Beauveria felina fungal strain has been previously isolated from a marine alga of the genus *Caulerpa*.[19,20] A voucher specimen has been deposited in the Brazilian collection CBMAI (reference # 738 and 739). Standards used for monitoring destruxins were pseudodestruxin C, [Phe^3, *N*-Me-Val^5] destruxin B and roseotoxin B, previously isolated from *B. felina* and identified by analysis of NMR and MS data.[19] The chemical structures of the cyclodepsipeptides are shown in Figure 1. Water was purified using a double filtering system Rios/Milli-Q Gradient A 10 system (Millipore, Billerica, MA, USA). Acetonitrile, methanol and formic acid were of HPLC grade (J. T. Baker or Mallinkrodt).

Apparatus

HPLC analyses were performed using a Waters Alliance 2695 coupled online with a Waters 2996 photodiode array detector, followed by a Waters 2424 evaporative light scattering detector and a Micromass ZQ2000 mass spectrometry detector with an electrospray interface. Separations were performed on a C_{18} reversed-phase Waters X-terra (2.1 × 50 mm, 3.5 μm particle size) with a mobile phase flow rate of 0.5 mL min^{-1}. The mobile phase consisted of (A) H_2O containing 0.1% formic acid and (B) 1:1 (v/v) MeOH/MeCN containing 0.1% formic acid. A linear gradient elution program was applied as follows: 0-1.0 min hold on 10% B, 1.0-20.0 min linear gradient to 100% B, 20.0-25.0 min hold on 100% B, 25.0-30.0 min hold on 50% B for reequilibration. The total run time was of 30 min. The injection volume was of 20 μL. The pressure limits was established as follows: lowest 0 Psi, highest 5000 Psi; during elution, the highest pressure was 2200 Psi.

Determination was performed using three detectors online: a photodiode array UV detector, followed by an evaporative light scattering detector and a single quadrupole mass spectrometry detector. The photodiode array scanned the samples at λ_{max} 205 and 254 nm. The evaporative light scattering detector condition was optimized to the following conditions: drift tube temperature 75 °C, gas pressure (N_2) 50 Psi, Nebulizer 60%. The mass spectrometer detector was optimized to the following conditions: capillary voltage 3.00 kV, source block temperature 100 °C, desolvation temperature 350 °C, operating in electrospray positive mode, detection range 300-900 Da with total ion count extracting acquisition. The cone and desolvation gas flow were 50 and 350 L h^{-1}, respectively, and were obtained from a Nitrogen Peak Scientific N110DR nitrogen source. Data acquisition and processing were performed using Empower 2.0. A split-flow of the photodiode array detector effluent separated 70% of the effluent to the evaporative light scattering detector and 30% to the mass spectrometry detector.



Compound	Name	MW (DA)	R^1	R^2	R^3	R^4	R^5	R^6	R^7	n
1	Dtx E diol	611	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHOHCH_2OH$	H	1
2	Dtx Ed_1	625	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHOHCH_2OH$	H	2
3	Chlorohydrin Dtx A_4	643	$CH(Me)CH_2Me$	Me	Me	Me	Me	$CHOHCH_2Cl$	H	1
4	Dtx A_1	591	$CH(Me)CH_2Me$	Me	H	Me	Me	$CH=CH_2$	H	2
5	Dtx A_4	591	$CH(Me)CH_2Me$	Me	Me	Me	Me	$CH=CH_2$	H	1
6	Roseotoxin B	591	$CH(Me)CH_2Me$	Me	H	Me	Me	$CH=CH_2$	Me	1
7	Dtx B_1	607	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHMe_2$	H	2
8	Dtx E_1	607	$CH(Me)CH_2Me$	Me	H	Me	Me	oxirane	H	2
9	HomoDtx B	607	$CH(Me)CH_2Me$	Me	Me	Me	Me	$CHMe_2$	H	1
10	Roseotoxin A	607	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHMe_2$	Me	1
11	[Phe^3, *N*-Me-Val^5] Dtx B	655	CH_2Ph	Me	H	Me	$CHMe_2$	$CHMe_2$	H	1
12	PseudoDtx B	669	CH_2Ph	Me	H	Me	CH_2CHMe_2	$CHMe_2$	H	1
13	PseudoDtx C	669	CH_2Ph	Me	H	Me	$CHMe_2$	$CHMe_2$	Me	1
14	Dtx C_2	595	$CHMe_2$	Me	H	Me	Me	$CHMeCH_2OH$	H	1
15	DesmethylDtx C	595	$CH(Me)CH_2Me$	H	H	Me	Me	$CHMeCH_2OH$	H	1
16	Dtx F	595	$CH(Me)CH_2Me$	Me	H	Me	Me	CHOHMe	H	1
17	HydroxyDtx B	609	$CH(Me)CH_2Me$	Me	H	Me	Me	CMeMeOH	H	1
18	Dtx C	609	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHMeCH_2OH$	H	1
19	Dtx D_2	609	$CHMe_2$	Me	H	Me	Me	$CHMeCO_2H$	H	1
20	Dtx D_1	637	$CH(Me)CH_2Me$	Me	H	Me	Me	$CHMeCO_2H$	H	2

Figure 1. Structures of destruxins (Dtx).

Preparation of standard solutions

Accurately weighed samples of each cyclodepsipeptide standard were dissolved in MeOH to prepare stock solutions of 1.0 mg mL^{-1}. The stock solutions were kept closed in appropriate vials, at 4 °C until needed. The PDA, ELSD and MS chromatograms of the three cyclodepsipeptide standards are shown in Figures 2-5.

Sample preparation

B. felina was grown in 500 mL erlenmeyer flasks containing 250 mL of MF broth as a culture medium. After 14 days of incubation at 28 °C, the culture medium and mycelia suspension were filtered through a 0.7 µm membrane. The filtered liquid medium was adsorbed into a solid-phase extraction (SPE) C_{18} Waters SepPak cartridge (5 g). Dessorption was performed using: 75:25 (fraction 1), 50:50 (fraction 2), 25:75 (fraction 3) H_2O/MeOH and 100% MeOH (fraction 4). All fractions were dried in a Speedvac Savant high vacuum centrifuge. Dried samples were accurately weighed and solutions were prepared at a concentration of 1.0 mg mL^{-1}.

Peak identification

Isobaric cyclodepsipeptides were identified by analysis of MS spectra and comparison with retention times of standards and literature data.[5]

Method validation

The validation protocol was performed following literature procedures.[21,22] Acceptable values were defined for the following parameters: selectivity, precision (repeatability and intermediate precision) and stability.

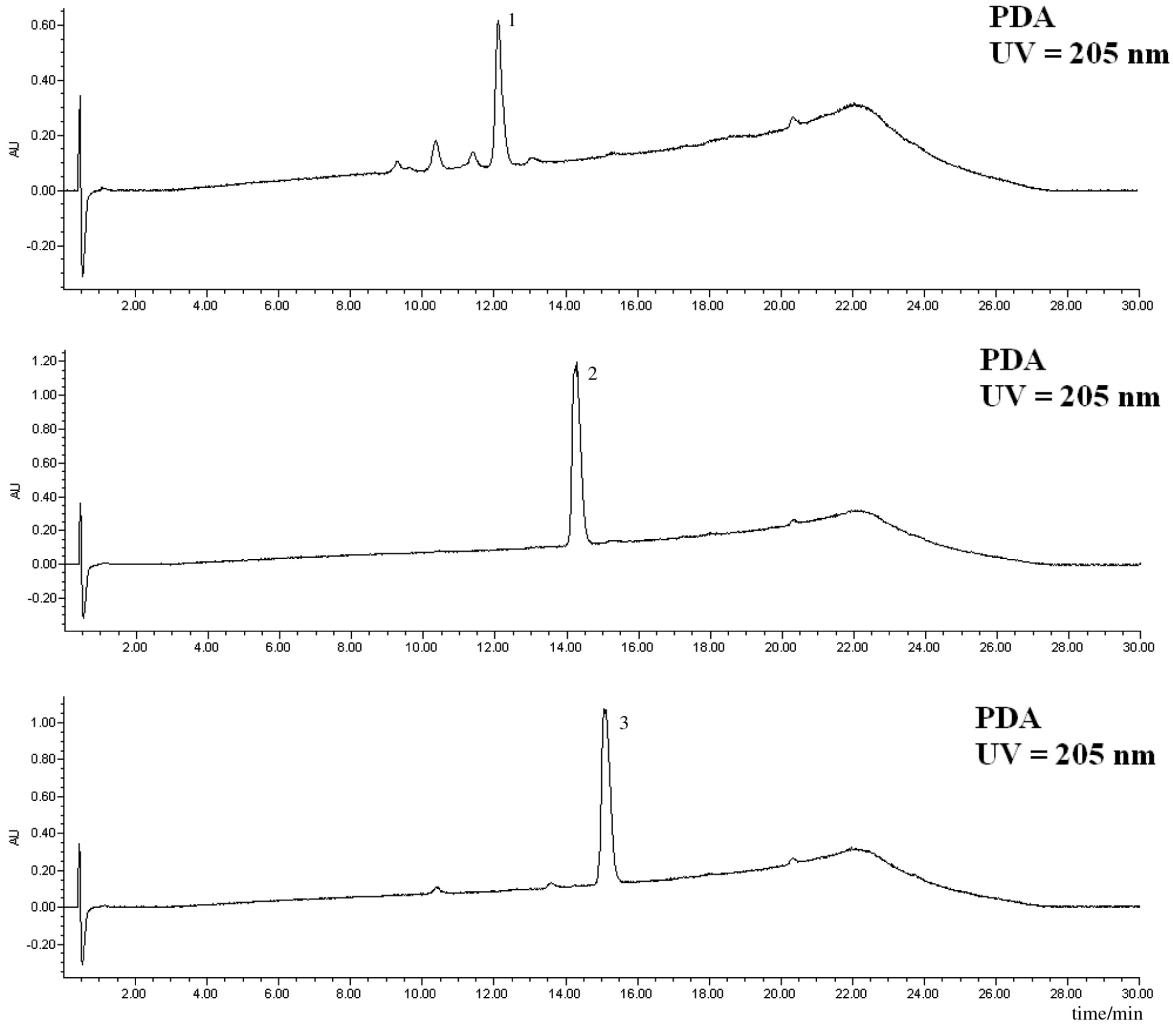


Figure 2. Photodiode array (λ_{max} 205 nm) chromatogram of the three Dtx standards and linear gradient elutions of water containing 0.1% formic acid. Experimental condition: Phenomenex C_{18} column was used; the concentrations of the three Dtx were 1.0 mg mL^{-1}; injection volume, 20 µL. The linear gradient elution profiles are given, along with other details, in Experimental section. Peaks: 1 = roseotoxin B; 2 = [Phe^3, *N*-Me-Val^5] Dtx B; 3 = pseudoDtx C.

Selectivity: The peak purity of the [Phe^3, *N*-Me-Val^5] Dtx B (**11**), and of each chromatographic peak of samples C2-fr3-MF, C2-fr4-MF, C3-fr3-MF, C3-fr4-MF and C4-fr4-MF, was evaluated by comparison of the MS and UV spectra obtained at three points of each peak, using the MassLinx-Empower software (Waters Co.). Peaks were considered pure when their UV spectra similarity (230 to 400 nm) and mass spectra similarity was greater than 95%.

Precision: The precision was calculated by the evaluation of repeatability and intermediate precision at one concentration level (1.0 mg mL^{-1}) for each analysis. In order to measure the repeatability (intra-day precision), samples were analyzed in triplicate during the same day. For intermediate precision (inter-day precision), samples were analyzed in triplicate in three different days. The precision was expressed as the relative standard deviation (RSD) of the concentration of [Phe^3, *N*-Me-Val^5] Dtx B (**11**).

Stability: For the internal and external standard methods, the standard [Phe^3, *N*-Me-Val^5] Dtx B (**11**) stability test was performed at one concentration level (1.0 mg mL^{-1}), in triplicate, at regular intervals of 0, 24 and 48 h. The solution was stored at 20 °C during this period. The results are reported in the Supplementary Information section.

Results and Discussion

Analysis of crude fractions obtained from microbial culture media can be complicated in the presence of culture media constituents. Therefore, it is highly desirable to perform the sample clean-up before analysis by HPLC. Previous methods of sample preparation for destruxin

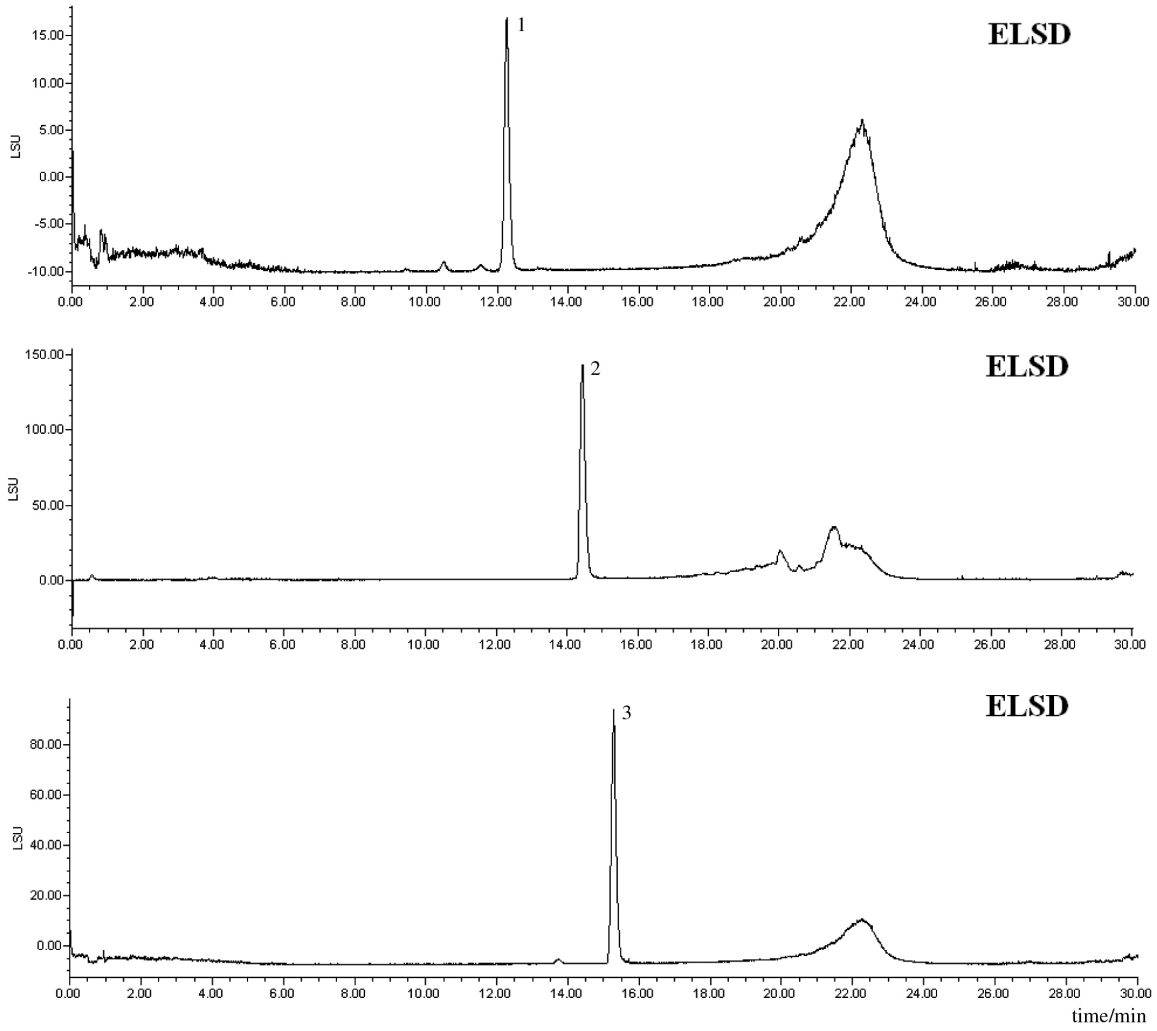


Figure 3. Evaporative light scattering detector chromatogram of the three Dtx standards and linear gradient elutions of water containing 0.1% formic acid. Experimental condition: Phenomenex C_{18} column was used; the concentrations of the three Dtx were 1.0 mg mL^{-1}; injection volume, 20 µL. The linear gradient elution profiles are given, along with other details, in Experimental section. Peaks: 1 = roseotoxin B; 2 = [Phe^3, *N*-Me-Val^5] Dtx B; 3 = pseudoDtx C.

analysis reported the direct HPLC analysis of the crude culture media,[6,7,18] HPLC analysis after extraction of the culture media with organic solvents such as CH_2Cl_2/EtOAc,[8,18] or with CH_2Cl_2 followed by pre-purification by silica-gel column chromatography,[14,17] or by purification by ion-exchange and silica-gel chromatography,[16] culture media centrifugation and filtration through a size-exclusion membrane,[12] or culture media adsorption in XAD-16 followed by desorption and chromatography on Sephadex LH-20.[15] We selected a pre-purification method to remove most of culture media contaminants and avoid the use of environmentally unfriendly organic solvents. Indeed, after solid-phase extraction of *B. felina* culture media on C_{18} reversed-phase cartridges, we observed that only apolar fractions 3 and 4 (see Experimental section) presented Dtx, but the most polar fractions 1 and 2 were devoid of cyclodepsipeptides. Additionally, fractions 3 and 4 presented only destruxins and possibly lipids eluting later, but no media components such as sugars or amino acids were detected. Therefore, the clean-up procedure proved to be effective to remove culture media contaminants.

Mobile phases tested for the optimization of HPLC analyses were: MeOH/H_2O (0.1% formic acid) (1), MeCN/H_2O (0.1% formic acid) (2) and MeOH/MeCN/H_2O (0.1% formic acid) (3). Mobile phase 1 was too weak to elute Dtx, required 40 min run time, and did provide a poor separation, since many cyclodepsipeptides appeared to be eluting with very close retention times. A similar, but inverted resulted, was obtained with mobile phase 2, which was too strong, provided a poor separation resolution and all Dtx eluted in less than 10 min. The use of mobile phase 3, in which the

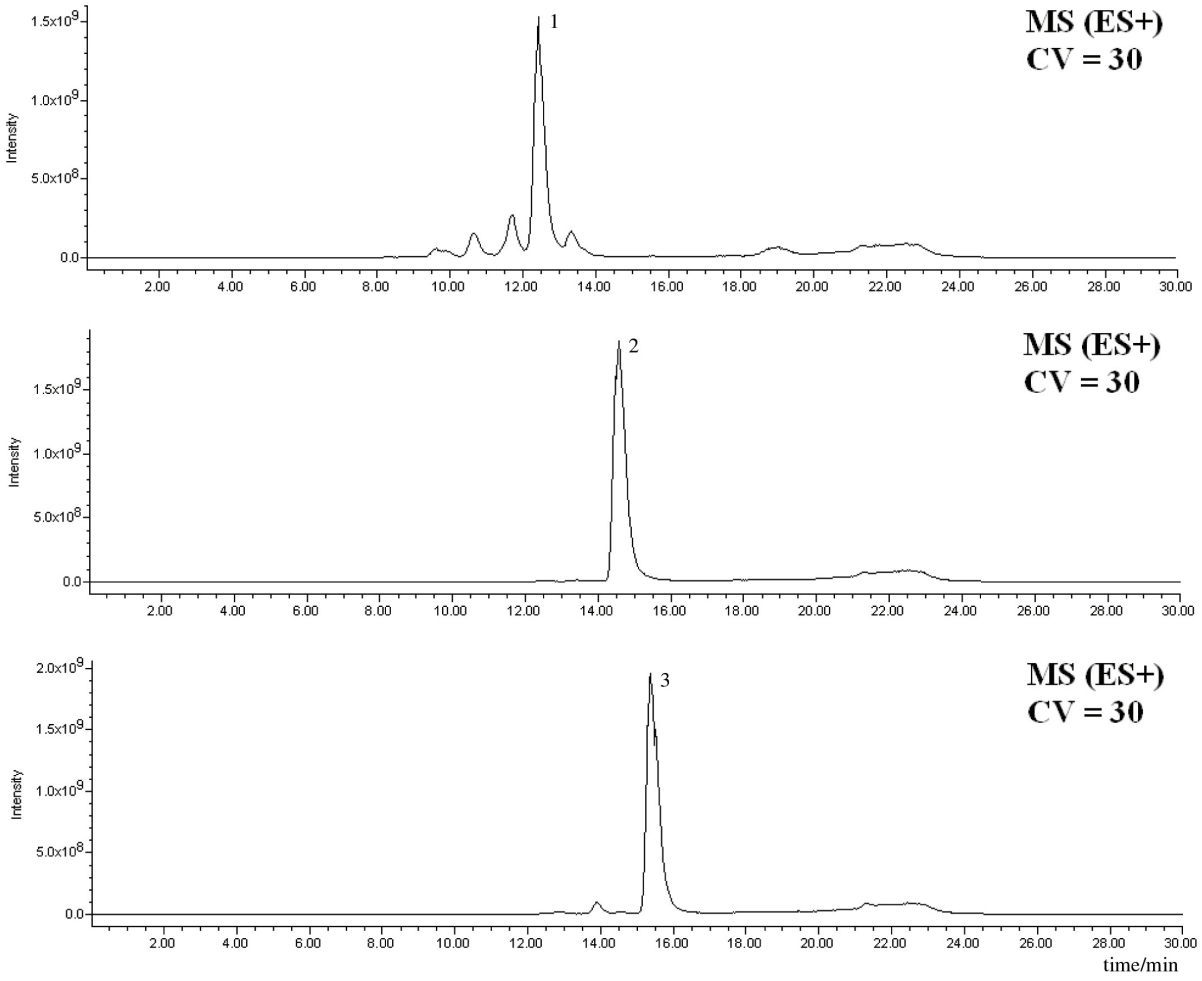


Figure 4. Mass spectrometry detector chromatogram of the three Dtx standards and linear gradient elutions of water containing 0.1% formic acid. Experimental condition: Phenomenex C_{18} column was used; the concentrations of the three Dtx were 1.0 mg mL^{-1}; injection volume, 20 µL. The linear gradient elution profiles are given, along with other details, in Experimental section. Peaks: 1 = roseotoxin B; 2 = [Phe[3], *N*-Me-Val[5]] Dtx B; 3 = pseudoDtx C.

proportion between MeOH and MeCN remained constant (50:50, v/v) in increased amounts related to H_2O (linear gradient), provided the best resolution for peaks separation. All previous HPLC methods for Dtx analysis consisted of MeCN/H_2O as mobile phases.[6,8,12-19] However, the number of Dtx derivatives detected using these previous methods were much smaller than in the present investigation: one,[7] two,[6,16] three,[8,12] four,[14] and seven.[18] Only one previous study detected 24 Dtx derivatives, but with a poor separation resolution.[17] Our method enabled us to detect up to twelve Dtxs derivatives in a single run (Figure 5). Furthermore, due to the current high cost of acetonitrile, the amount of MeCN required in the present method is reduced by the half with a best separation resolution. Moreover, in using a short (2.1 × 50 mm) HPLC column and a smaller flow rate (0.5 mL min^{-1}), the analysis cost can be significantly reduced, and very suitable for dried spores quality control of *M. anisopliae*, *Beauveria* spp. or other Dtx producing fungal strains.

Previous methods for Dtx analysis used detectors such as UV,[6,7,16] UV-MS,[8] PDA-MS,[12,14] MS,[17-18] and MS/MS.[15] Interest in using an evaporative light scattering detector (ELSD) is based on the fact that in optimized and standardized conditions the signal intensity can be directly related to analyte amount, by plotting the peak area versus sample size in double logarithmic.[23] Since the response of light scattering is a function of the solute particle diameter, and that the structure of Dtx are closely related one to another (Figure 1), the signal intensity of each peak can be directly related to the amount of each Dtx in the sample under analysis. The same is not true for the UV detection, since some Dtx present a benzyl chromophore.[19] Although the ELSD detection limit is lower than UV (one to two orders of magnitude),[23-25] such is not the case for

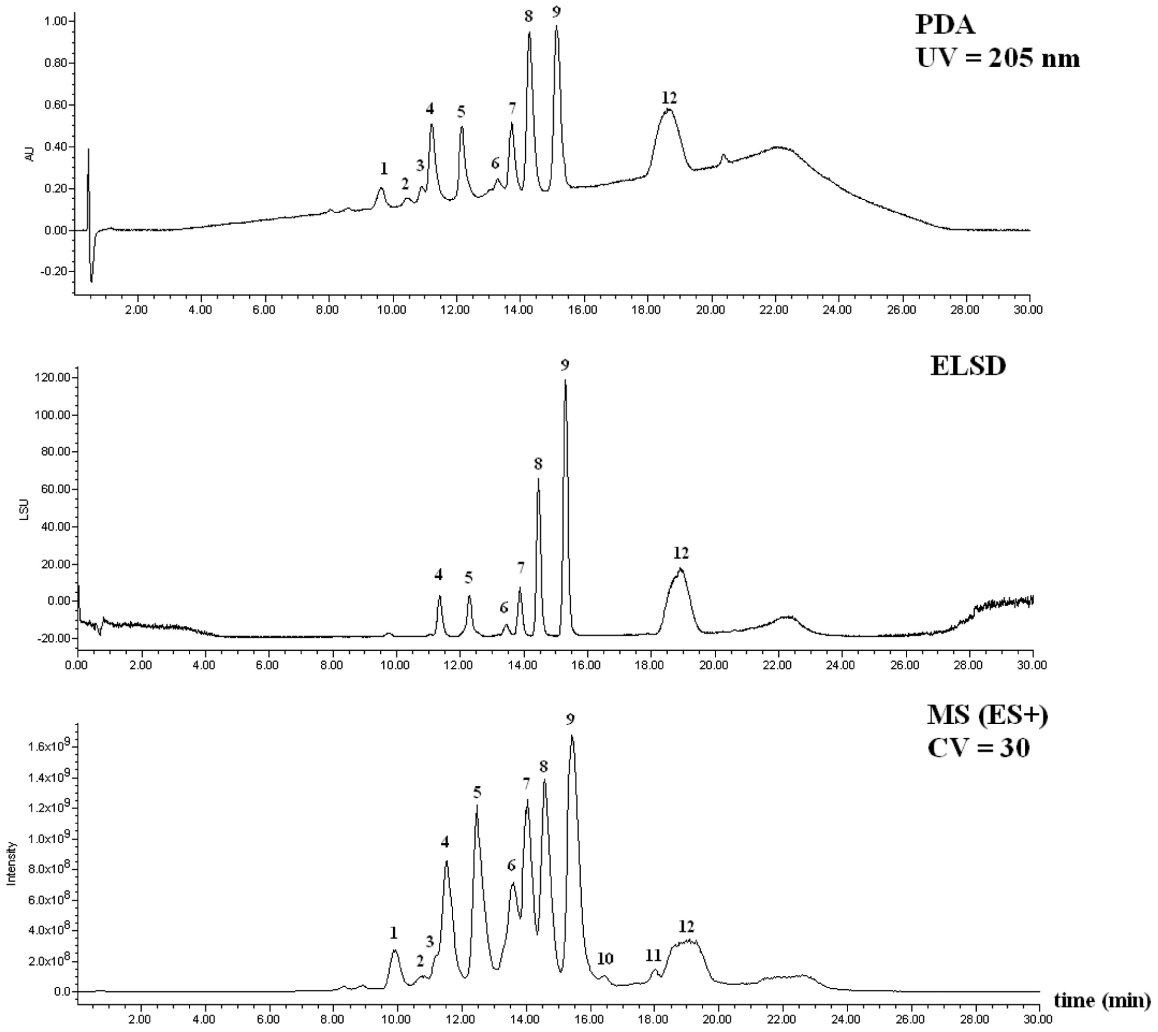


Figure 5. Typical PDA-ELSD-MS chromatograms of Dtx detected in fractions obtained from *B. felina*. Chromatograms with PDA, ELSD and MS detection of fraction C2-fr4-MF. For experimental conditions see Experimental section. For peaks assignments, see Table 1.

analytes with a non-conjugated chromophore for which the UV response is much weaker. Since the large majority of Dtx present UV absorption at very low wavelengths (below λ_{max} 210 nm), due to the absence of conjugated chromophores, the use of UV detection implies mobile phase absorption and a compromised detection. Moreover, the detection in such a high energy wavelength implies in a considerable reduction of expensive UV lamps lifetime. The laser LED of a light scattering detector is not only cheaper than deuterium UV lamps, but also has a considerably longer lifetime.[26] More important, in the case of Dtx analyses we observed an excellent relatedness of signal intensity for the three detectors used simultaneously, PDA-ELSD-MS. Therefore, it is possible to use either PDA-MS or ELSD-MS detection for Dtx analysis, without detection compromise. The parameters to be optimized for ELSD detection (drift tube temperature, gas pressure and nebulizer temperature) are of easy operation and the detector can be easily cleaned as well. Therefore, Dtxs analysis using ELSD gave reliable results, with a cheapest and easy-to-operate detector.

Five fractions obtained from two distinct growth experiments of *B. felina* have been obtained and analyzed by HPLC-PDA-ELSD-MS: C2-fr3-MF, C2-fr4-MF, C3-fr3-MF, C3-fr4-MF and C4-fr4-MF. Analysis of all five fractions indicated the presence of several Dtx. A typical analysis outset is shown in Figure 5, in which peaks are simultaneously detected by UV/photodiode array, evaporative light scattering and mass spectrometry detectors. The chromatograms obtained using all three detectors showed an excellent agreement of signal intensity in each analysis (Figures 2-5). Since the optimized ionization conditions favored the observation of both $[M+H]^+$ and $[M+Na]^+$ quasi-molecular ion peaks,

the molecular mass assignment relied on the detection of both peaks for a single Dtx or isobaric Dtxs. Peak assignments are included in Table 1, while the structure of Dtxs detected in fractions obtained from *B. felina* are represented in Figure 1. Fraction C2-fr3-MF showed the presence of ten peaks, seven of which could be assigned to known and two to unknown destruxin. The mass spectra recorded for the remaining peak in this fraction (P10) did not provide information to assign it to any Dtx. Fraction C2-fr4-MF showed the presence of twelve peaks (Figure 5), eight of which could be assigned for Dtxs, two of which are unknown. The mass spectra recorded for the remaining peak in this fraction (P2) did not provide information to assign it to any Dtx. Fraction C3-fr3-MF showed the presence of ten peaks, six of which could be assigned for Dtxs, but three are yet unknown. The mass spectra recorded for the remaining peak in this fraction (P1) did not provide information to assign it to any Dtx. Fraction C3-fr3-MF showed the presence of ten peaks, nine of which could be assigned for Dtxs, but three are yet unknown. Fraction C3-fr4-MF presented seven Dtxs, two of which are unknown, while fraction C4fr4-MF presented eight Dtxs, three of which are unknown. Overall, twenty six distinct Dtx cyclohexadepsipeptides have been detected in fractions obtained from *B. felina*, six of which have not yet been reported in the literature. The $[M+H]^+$/$[M+Na]^+$ values recorded for the unknown Dtxs (630.0/652.0; 568.0/590.0; 601.6/624.6; 557.1/579.9; 628.1/650.0; 688.0/710.0) did not allow us to suggest possible structures for these compounds. Further investigations on semi-preparative scale production of Dtxs are currently underway in order to isolate and identify the unknown cyclodepsipeptides produced by *B. felina*.

As shown in Table 1, use of HPLC-PDA-ELSD-MS could be applied for the analysis of four fractions obtained from distinct growth experiments of *B. felina*, in order to detect and assign Dtxs. The separation conditions indicated

Table 1. Dtxs in fractions obtained from growth experiments of *B. felina*

Fraction/peak assignments	t_R	*m/z* $[M+H]^+$	*m/z* $[M+Na]^+$
C2-fr3-MF			
P1: **1**	9.160	612.0	634.0
P2: **2**	9.958	626.0	648.0
P3: unknown	10.832	630.0	652.0
P4: **3**	11.569	644.0	666.0
P5: **4** or **5** or **6**	12.509	592.0	614.0
P6: unknown	13.649	568.0	590.0
P7: **7** or **8** or **9** or **10**	14.074	608.0	630.0
P8: **11**	14.636	656.2	678.1
P9: **14** or **15** or **16**	15.390	596.0	618.0
C2-fr4-MF			
P1: **2**	9.923	626.0	648.0
P3: **3**	11.494	644.0	666.0
P4: **4** or **5** or **6**	12.446	592.0	614.0
P5: unknown	13.624	568.0	590.0
P6: **7** or **8** or **9** or **10**	14.028	608.1	630.0
P7: **11**	14.584	656.0	678.1
P8: **12** or **13** and **14** or **15** or **16**	15.443	670.1 and 596.0	692.1 and 618.0
P9: **17** or **18** or **19**	16.474	610.1	632.0
P10: **20**	18.060	638.1	660.1
P11: unknown	19.153	601.6	624.6
C3-fr3-MF			
P2: **1**	9.331	612.0	634.0
P3: **2**	9.905	626.0	648.0
C3-fr3-MF			
P4: unknown	10.841	630.0	651.9
P5: **3**	11.512	644.0	666.0
P6: **4** or **5** or **6**	12.460	592.0	614.0
P7: unknown	13.627	568.1	590.0
P8: **7** or **8** or **9** or **10**	14.012	608.1	630.0
P9: **11**	14.606	656.1	678.1
P10: unknown	15.973	557.1 and 628.1	579.0 and 650.0
C3-fr4-MF			
P1: unknown	13.608	568.1	590.0
P2: **7** or **8** or **9** or **10**	14.001	608.0	630.0
P3: **11**	14.516	656.1	678.1
P4: **12** or **13**	15.461	670.1	692.1
P5: **17** or **18** or **19**	16.501	609.9	632.1
P6: **20**	18.065	638.1	660.1
P7: unknown	19.218	601.5	624.5
C4-fr4-MF			
P1: **4** or **5** or **6**	12.492	592.0	614.0
P2: unknown	13.162	688.0	710.0
P3: unknown	13.572	568.0	590.0
P4: **7** or **8** or **9** or **10**	14.010	608.1	630.0
P5: **11**	14.575	656.1	678.1
P6: **12** or **13** and **14** or **15** or **16**	15.428	670.1 and 596.0	692.0 and 618.1
P7: **20**	18.078	638.1	660.1
P8: unknown	19.040	601.5	624.5

that all peaks in the chromatograms of the fractions analyzed were well separated, with exception of only three peaks, P8 of fraction C2-fr4-MF, P10 of fraction C3-fr3-MF and P6 of fraction C4-fr4-MF. These peaks presented two different Dtxs each one. Therefore, the separation conditions using 50:50 (v/v) MeOH/MeCN as the organic solvent in the gradient proved to be useful for the optimal separation of Dtx on a short reversed-phase C_{18} column. Moreover, as shown in Figure 2, the response of PDA, ELSD and MS detectors to Dtxs proved to be almost identical. Therefore, it is possible to use a cheapest HPLC-ELSD analysis for Dtxs detection and even quantification, provided that calibration curves are constructed using different Dtx standards. Doubtless, the present method can be used for quality control of destruxin-producing fungal strains, whose dried mycelia is used in biological control of insect plagues.

As for the method validation, the selectivity was evaluated by measuring the peak purity of [Phe^3, *N*-Me-Val^5] Dtx B (**11**) in fractions C2-fr3-MF, C2-fr4-MF, C3-fr3-MF, C3-fr4-MF and C4-fr4-MF. Selectivity was checked by carefully analyzing the UV spectra of all chromatographic peaks in three different regions of its chromatographic peak (smaller rt, center and longer rt), and no interference was observed. As for the precision, the relative standard deviation (RSD) to repeatability did not exceed 5.68% (UV detector), at the concentration level of 1.0 mg mL^{-1} for [Phe^3, *N*-Me-Val^5] Dtx B (**11**). As for the intermediate precision, the RSD did not exceed 5.66% (UV detector) when it was calculated for measurements realized during three consecutive days (n = 9), at the concentration level of 1.0 mg mL^{-1} for [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as well. These precision values are slightly above the accepted values of high standard quality methods (ANVISA, RSD at 5%).[22] In the case of the external standard method, the RSD of repeatability (n = 3) did not exceed 5.57 % (MS detector), for **11** at 1.0 mg mL^{-1}. Finally, the samples C2-fr3-MF, C2-fr4-MF, C3-fr3-MF, C3-fr4-MF and C4-fr4-MF stability tests performed at one concentration level (1.0 mg mL^{-1}) showed no significant differences in the concentration values of [Phe^3, *N*-Me-Val^5] Dtx B (**11**) after 24 and 48 h of samples preparation, using either the internal standard method or no standard. The overall results obtained showed very good agreement of selectivity, precision and stability, for samples analyzed with photodiode array, evaporative light scattering and mass spectrometry detectors. Therefore, our method could be conveniently validated.

Conclusions

HPLC-PDA-ELSD-MS analysis of Dtxs mixtures showed that HPLC-ELSD can be used for qualitative analysis of such compounds. The use of a 50:50 (v/v) MeOH/MeCN mixture as the organic eluent showed a better resolution for Dtx separation than only MeOH or MeCN. The use of a short HPLC column coupled to a smaller flow rate without resolution compromise could significantly reduce solvent consumption for the HPLC analyses. Sample clean-up using C_{18} SPE showed excellent results to obtain fractions largely enriched in Dtxs. Compared to methods previously reported, the present method proved to be among the best, including the advantage of using HPLC-ELSD as a cost effective alternative for destruxin analysis.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br, as PDF file.

Acknowledgments

The authors thank to Dr. Álvaro Esteves Migotto, director of the Centro de Biologia Marinha of the Universidade de São Paulo, for providing facilities for *B. felina* collection and to Aline Maria de Vita-Marques for the isolation and preservation of *B. felina*. Financial support provided by FAPESP BIOTA/BIOprospecTA research program (05/60175-2) is gratefully acknowledged. R. P. Morais also thanks FAPESP for a PhD scholarship (06/61694-6).

References

1. Fernandez, F. B.; *Int. Sugar J.* **2000**, *102,* 482.
2. Acevedo, J. P. M.; Samuels, R. I.; Machado, I. R.; Dolinski, C.; *J. Invertebr. Pathol.* **2007**, *96,*187.
3. Dolinski, C.; Lacey, L. A.; *Neotropic. Entomol.* **2007**, *36*, 161.
4. Jegorov, A.; Hajduch, M.; Sulc, M.; Havlicek, V.; *J. Mass Spectrom.* **2006**, *41,* 563.
5. Pedras, M. S. C.; Zaharia, L. I.; Ward, D. E.; *Phytochemistry* **2002**, *59,* 579.
6. Hu, Q. B.; Ren, S. X.; Wu, J. H.; Chang, J. M.; Musa, P. D.; *Toxicon* **2006**, *48,* 491.
7. Feng, K. C.; Rou, T. M.; Liu, B. L.; Tzeng,Y. M.; Chang, Y. N.; *Enzyme Microb. Technol.* **2004**, *34*, 22.
8. Wang, C.; Skrobek, A.; Butt, T. M.; *J. Invertebr. Pathol.* **2004**, *85*, 168.
9. Amiri-Besheli, B.; Khambay, B.; Cameron, S.; Deadman, M. L.; Butt, T. M.; *Mycol. Res.* **2000**, *104*, 447.
10. Liu, B. L.; Chen, J. W.; Tzeng, Y. M.; *Biotechnol. Prog.* **2000**, *16*, 993.
11. Strasser, H.; Vey, A.; Butt, T. M.; *Biocont. Sci. Technol.* **2000**, *10*, 717.

12. Seger, C.; Sturm, S.; Stuppner, H.; Butt, T. M.; Strasser, H.; *J. Cromatogr., A* **2004**, *1061*, 35.
13. Liu, C. M.; Huang, S. S.; Tzeng, Y. M.; *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 1013.
14. Hsiao,Y. M.; Ko, J. L.; *Toxicon* **2001**, *39*, 837.
15. Potterat, O.; Wagner, K.; Haag, H.; *J. Chromatogr., A* **2000**, *872*, 85.
16. Chen, J.-W.; Liu, B.-L.; Tzeng, Y.-M.; *J. Chromatogr., A* **1999**, *830*, 115.
17. Jegorov, A.; Havlicek, V.; Sedmera, P.; *J. Mass Spectrom.* **1998**, *33*, 274.
18. Loutelier, C.; Cherton, J. C.; Lange, C.; Traris, M.; Vey, A.; *J. Chromatogr., A* **1996**, *738*, 181.
19. Lira, S. P.; Vita-Marques, A. M.; Seleghim, M. H. R.;Bugni, T. S.; La Barbera, D.; Sette, L. D.; Sponchiado, S. R. P.; Irleland, C. M.; Berlinck, R. G. S.; *J. Antibiot.* **2006**, *59*, 553.
20. Vita-Marques, A. M.; Lira, S. P.; Berlinck, R. G. S.; Seleghim, M. H. R.; Sponchiado, S. R. P.; Tauk-Tornisielo, S. M.; Barata, M.; Pessoa, C.; Moraes, M. O.; Coelho, B.; Nascimento, G. G. F.; Souza, A. O.; Minarini, P. R. R.; Silva, C. L.; Silva, M.; Pimenta, E. F.; Thiemann, O.; Passarini, M. R. Z.; Sette, L. D.; *Quim. Nova* **2008**, *31*, 1099.
21. Bandeira, K. F.; Cavalheiro, A. J.; *Chromatographia*, **2009**, *70*, 1455.
22. National Agency of Sanitary Vigilance of Brazil-ANVISA. Resolução RE no. 889 de 29 de maio de 2003. Available at: http://www.anvisa.gov.br/legis/resol/2003/re/899_03re.htm, accessed in May 2010.
23. Kong, L.; Li, X.; Zou, H.; Wang, H.; Mao, X.; Zhang, Q.; Ni, J.; *J. Chromatogr., A* **2001**, *936*, 111.
24. Megoulas, N. C.; Koupparis, M. A.; *Crit. Rev. Anal. Chem.* **2005**, *35*, 301.
25. Ganzera, M.; Stuppner, H.; *Curr. Pharm. Anal.* **2005**, *1*, 135.
26. Seller representatives cost/lifetime estimation for a UV lamp is approximately US$ 1.00/h (continuously working at λ_{max} 200-210 nm), while for a ELSD LED is approximately US$ 0.32/h.

Submitted: December 2, 2009
Published online: September 8, 2010

FAPESP has sponsored the publication of this article.

J. Braz. Chem. Soc., Vol. 21, No. 12, S1-S9, 2010.
Printed in Brazil - ©2010 Sociedade Brasileira de Química
0103 - 5053 $6.00+0.00

A Method for Dextruxin Analysis by HPLC-PDA-ELSD-MS

Raquel P. Morais,[a] Simone P. Lira,[b] Mirna H. R. Seleghim[c] and Roberto G. S. Berlinck*[,a]

[a]Instituto de Química de São Carlos, Universidade de São Paulo, CP 780, 13560-970 São Carlos-SP, Brazil

[b]Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, CP 9, 13418-900 Piracicaba-SP, Brazil

[c]Departamento de Ecologia e Biologia Evolutiva, Universidade Federal de São Carlos, CP 676, 13565-905 São Carlos-SP, Brazil

Table S1. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr3-MF using UV; ELSD and MS HPLC detectors; peaks areas were measured relative to [Phe[3], *N*-Me-Val[5]] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
1	-	-	-	-	-	-	3.09	1.57	2.72
2	3.92	5.09	1.51	-	-	-	3.79	1.43	5.25
unknown	2.73	4.50	1.85	-	-	-	1.17	3.74	3.90
3	3.62	3.13	0.43	-	-	-	3.20	2.27	1.19
4, 5, 6	2.48	1.17	2.40	-	-	-	3.44	1.06	4.18
unknown	-	-	-	-	-	-	0.97	2.42	5.31
7, 8, 9, 10	2.99	3.18	2.65	-	-	-	0.84	2.09	3.13
11	0.00	0.00	0.00	-	-	-	0.00	0.00	0.00
14, 15, 16	2.43	2.01	2.05	-	-	-	2.34	2.93	3.26
no destruxin	-	-	-	-	-	-	0.20	3.66	2.77

Table S2. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr3-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
1	-	-	-	-	-	-	2.99	1.65	1.67
2	2.05	1.52	1.25	1.88	1.32	1.81	4.01	2.89	3.82
unknown	0.34	2.28	2.59	-	-	-	1.09	4.78	2.33
3	1.63	2.36	0.56	0.74	1.17	2.00	3.46	1.57	2.05
4, 5, 6	0.81	0.04	1.56	0.64	2.35	1.10	3.24	1.43	3.55
unknown	-	-	-	-	-	-	1.23	0.78	3.14
7, 8, 9, 10	1.76	0.55	1.81	2.52	1.27	2.89	0.76	1.40	1.23
11	3.01	1.79	0.84	-	-	-	0.27	2.95	2.24
14, 15, 16	1.20	2.25	1.63	2.34	2.91	0.64	2.36	1.98	1.36
no destruxin	-	-	-	-	-	-	0.17	3.98	2.60

*e-mail: rgsberlinck@iqsc.usp.br

Table S3. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr3-MF using UV; ELSD and MS HPLC detectors. Retention times were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
1	-	-	-	-	-	-	1.24	0.51	0.12
2	0.24	0.22	0.06	-	-	-	1.52	0.49	0.04
unknown	0.22	0.17	0.23	-	-	-	1.46	0.42	0.18
3	0.16	0.19	0.03	-	-	-	1.34	0.30	0.05
4, 5, 6	0.09	0.74	0.06	-	-	-	1.28	0.14	0.03
unknown	-	-	-	-	-	-	1.23	0.20	0.05
7, 8, 9, 10	0.01	0.05	0.01	-	-	-	1.19	0.11	0.07
11	0.00	0.00	0.00	-	-	-	0	0	0
14, 15, 16	0.09	0.04	0.06	-	-	-	1.11	0.12	0.12
no destruxin	-	-	-	-	-	-	1.06	0.12	0.04

Table S4. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr3-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
1	-	-	-	-	-	-	0.18	1.07	0.14
2	0.43	0.36	0.06	0.48	0.39	0.14	0.46	0.42	0.04
unknown	0.24	0.38	0.16	-	-	-	0.41	0.35	0.14
3	0.35	0.26	0.08	0.27	0.27	0.09	0.28	0.36	0.04
4, 5, 6	0.26	0.54	0.03	0.27	0.30	0.18	0.22	0.21	0.09
unknown	-	-	-	-	-	-	0.17	0.17	0.02
7, 8, 9, 10	0.19	0.14	0.08	0.11	0.10	0.16	0.13	0.15	0.09
11	0.20	0.13	0.07	-	-	-	1.06	0.16	0.07
14, 15, 16	0.12	0.10	0.12	0.11	0.11	0.08	0.16	0.08	0.16
no destruxin	-	-	-	-	-	-	0.04	0.18	0.05

Table S5. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr4-MF using UV; ELSD and MS HPLC detectors. Peaks areas were measured relative to [Phe[3], *N*-Me-Val[5]] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
2	2.72	3.55	2.78	3.30	1.22	4.29	3.81	1.86	1.34
no destruxin	0.70	4.71	0.74	-	-	-	0.71	0.87	2.16
3	2.34	1.58	3.85	1.17	2.03	4.09	3.57	1.38	2.41
4, 5, 6	2.29	2.33	1.49	2.88	2.44	4.95	2.31	1.01	0.50
unknown	2.91	5.66	2.63	2.69	3.80	1.95	1.08	1.51	3.58
7, 8, 9, 10	0.06	1.68	1.86	2.59	0.90	4.29	0.60	4.30	1.65
11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
12, 13 and **14, 15, 16**	0.20	3.58	3.40	1.20	1.82	3.11	1.02	1.57	2.00
17, 18, 19	-	-	-	-	-	-	1.12	3.37	1.66
20	-	-	-	-	-	-	1.13	2.70	1.75
unknown	1.11	3.31	2.36	2.44	0.80	2.57	0.46	0.10	1.42

Table S6. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr4-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
2	2.27	0.37	1.24	2.14	0.60	1.65	4.04	1.42	0.62
no destruxin	3.20	1.37	2.33	-	-	-	1.65	2.51	2.27
3	2.49	1.94	1.60	0.30	0.53	0.57	3.17	1.35	3.74
4, 5, 6	0.26	1.66	1.34	2.73	0.40	3.64	3.09	2.24	1.10
unknown	1.14	1.01	0.88	2.01	1.72	1.66	1.11	1.37	3.67
7, 8, 9, 10	2.50	3.64	4.66	1.26	2.08	0.82	1.58	0.99	0.12
11	2.52	1.56	2.81	1.35	1.53	3.48	0.98	1.42	1.58
12, 13 and **14, 15, 16**	2.47	1.03	0.97	2.42	1.24	2.66	1.37	1.50	0.87
17, 18, 19	-	-	-	-	-	-	0.35	0.63	1.48
20	-	-	-	-	-	-	0.17	2.42	2.27
unknown	1.54	0.75	0.70	3.75	1.83	0.98	0.52	0.41	0.81

Table S7. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr4-MF using UV; ELSD and MS HPLC detectors. Retention times were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
2	0.02	0.03	0.09	0.37	0.12	0.15	0.19	0.15	0.11
no destruxin	0.08	0.02	0.14	-	-	-	0.30	0.19	0.18
3	0.09	0.02	0.09	0.13	0.21	0.35	0.13	0.08	0.10
4, 5, 6	0.05	0.01	0.08	0.05	0.18	0.21	0.06	0.23	0.10
unknown	0.02	0.01	0.02	0.10	0.09	0.25	0.19	0.06	0.15
7, 8, 9, 10	0.05	0.03	0.07	0.09	0.12	0.15	1.21	0.12	0.07
11	0	0	0	0	0	0	0	0	0
12, 13 and **14, 15, 16**	0.05	0.03	0.04	0.07	0.06	0.13	0.13	0.05	0.10
17, 18, 19	-	-	-	-	-	-	0.05	0.12	0.11
20	-	-	-	-	-	-	0.04	0.06	0.07
unknown	0.02	0.08	0.18	0.15	0.20	0.16	0.38	0.75	0.90

Table S8. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C2-fr4-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
2	0.03	0.09	0.14	0.34	0.07	0.07	0.13	0.04	0.06
no destruxin	0.04	0.11	0.13	-	-	-	0.29	0.30	0.17
3	0.08	0.10	0.10	0.15	0.05	0.20	0.05	0.04	0.07
4, 5, 6	0.03	0.11	0.12	0.04	0.07	0.03	0.06	0.21	0.19
unknown	0.03	0.11	0.11	0.08	0.08	0.09	0.13	0.07	0.02
7, 8, 9, 10	0.05	0.10	0.11	0.11	0.06	0.05	1.17	0.06	0.11
11	0.04	0.12	0.13	0.03	0.17	0.19	0.09	0.12	0.16
12, 13 and **14, 15, 16**	0.06	0.09	0.13	0.06	0.12	0.05	0.07	0.08	0.09
17, 18, 19	-	-	-	-	-	-	0.05	0.01	0.06
20	-	-	-	-	-	-	0.05	0.06	0.10
unknown	0.05	0.05	0.19	0.13	0.08	0.08	0.47	0.74	0.84

Table S9. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr3-MF using UV; ELSD and MS HPLC detectors. Peaks areas were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
no destruxin	1.78	1.58	3.50	-	-	-	0.44	0.51	1.19
1	1.77	2.79	4.29	-	-	-	1.76	0.58	0.89
2	0.66	3.42	4.67	3.34	3.30	1.88	1.12	0.66	3.12
unknown	2.55	5.65	2.98	-	-	-	3.21	0.60	4.45
3	1.03	2.73	2.61	3.97	1.48	3.18	2.91	0.92	2.19
4, 5, 6	0.23	1.08	1.77	4.97	1.88	2.41	1.43	0.29	2.77
unknown	3.93	3.65	5.32	-	-	-	4.26	2.18	2.01
7, 8, 9, 10	2.05	2.81	3.34	3.94	0.43	0.99	0.43	1.58	2.07
11	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
unknown	1.78	1.58	3.50	3.38	2.54	4.64	1.31	1.82	1.01

Table S10. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr3-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
no destruxin	2.78	1.42	4.38	-	-	-	0.74	1.7	2.45
1	0.17	1.02	2.61	-	-	-	2.15	1.93	1.2
2	2.32	0.76	2.28	1.36	1.45	0.79	0.82	0.33	1.2
unknown	1.05	2.68	0.34	-	-	-	3.89	1.42	4.78
3	2.42	1.5	5.29	0.57	0.15	0.9	2.21	0.7	0.25
4, 5, 6	2.06	1.13	3.03	1.24	1.18	0.57	0.79	0.67	1.35
unknown	2.71	3.58	2.08	-	-	-	3.8	4.35	0.13
7, 8, 9, 10	1.12	4.87	1.46	1.14	3.34	1.86	0.88	1.38	1.71
11	1.84	0.47	3.34	3.66	1.67	2.69	0.69	0.39	1.93
unknown	2.78	1.42	4.38	3.52	2.22	2.96	1.23	0.71	2.81

Table S11. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr3-MF using UV; ELSD and MS HPLC detectors. Retention times were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
no destruxin	0.02	0.18	0.02	-	-	-	0.05	0.12	0.08
1	0.06	0.04	0.14	-	-	-	0.04	0.08	0.17
2	0.12	0.05	0.06	0.33	0.25	0.3	0.05	0.3	0.24
unknown	0.03	0.1	0.08	-	-	-	0.07	0.13	0.1
3	0.05	0.04	0.02	0.28	0.03	0	0.04	0.05	0.03
4, 5, 6	0.05	0.02	0.02	0.49	0.15	0.3	0.03	0.11	0.02
unknown	0.16	0.08	0.42	-	-	-	0.08	0.21	0.31
7, 8, 9, 10	0.07	0.03	0.03	0.33	0.28	0.15	0.12	0.15	0.15
11	0	0	0	0	0	0	0	0	0
unknown	-	-	-	0.3	0.25	0.16	0.02	0.12	0.1

Table S12. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr3-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
no destruxin	0.01	0.14	0.07	-	-	-	0.03	0.12	0.11
1	0.04	0.03	0.06	-	-	-	0.07	0.01	0.15
2	0.12	0.11	0.1	0.11	0.21	0.24	0.06	0.23	0.13
unknown	0.03	0.04	0.04	-	-	-	0.09	0.05	0.16
3	0.04	0.05	0.09	0.08	0.05	0.08	0.06	0.05	0.11
4, 5, 6	0.04	0.06	0.09	0.24	0.17	0.09	0.06	0.06	0.11
unknown	0.15	0.12	0.39	-	-	-	0.07	0.15	0.18
7, 8, 9, 10	0.06	0.07	0.11	0.2	0.32	0.18	0.13	0.08	0.07
11	0.02	0.06	0.08	0.29	0.04	0.08	0.03	0.08	0.13
unknown	-	-	-	0.07	0.28	0.12	0.03	0.04	0.11

Table S13. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr4-MF using UV; ELSD and MS HPLC detectors. Peaks areas were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
unknown	-	-	-	-	-	-	0.88	1.76	1.03
7, 8, 9, 10	2.01	2.05	5.32	3.54	2.46	3.48	0.99	1.35	4.47
11	0	0	0	0	0	0	0	0	0
12, 13	2.83	1.11	0.83	1.82	1.21	1.38	1.35	2.91	3.1
17, 18, 19	-	-	-	-	-	-	1.23	1.98	1.05
20	-	-	-	-	-	-	1.78	1.77	1.52
unknown	3.34	3.84	5.36	2.48	1.73	1.81	1.86	3.77	2.46

Table S14. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr4-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
unknown	-	-	-	-	-	-	1.2	3.41	3.33
7, 8, 9, 10	0.72	2.84	2.76	1.19	2.48	2.93	1.5	0.76	1.72
11	1.29	0.35	3.76	2.34	1.71	1.97	0.56	0.64	2.93
12, 13	1.91	0.51	3.23	3.58	1.75	0.65	1.83	2.11	2.78
17, 18, 19	-	-	-	-	-	-	1.69	3.27	2.37
20	-	-	-	-	-	-	1.36	2.64	1.53
unknown	2.36	1.51	2.45	0.3	0.69	0.28	1.84	2.08	0.56

Table S15. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr4-MF using UV; ELSD and MS HPLC detectors. Retention times were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
unknown	-	-	-	-	-	-	0.29	0.08	0.28
7, 8, 9, 10	0.03	0.16	0.26	0.06	0.08	0.11	0.2	0.05	0.19
11	0	0	0	0	0	0	0	0	0
12, 13	0.14	0.29	0.27	0.07	0.09	0.05	0.27	0.06	0.25
17, 18, 19	-	-	-	-	-	-	0.2	0.11	0.34
20	-	-	-	-	-	-	0.39	0.06	0.24
unknown	0.23	0.05	0.22	0.14	0.19	0.13	0.32	0.24	0.51

Table S16. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C3-fr4-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
unknown	-	-	-	-	-	-	0.08	0.09	0.1
7, 8, 9, 10	0.05	0.2	0.12	0.01	0.22	0.2	0.02	0.11	0.13
11	0.07	0.3	0.13	0.07	0.14	0.1	0.22	0.11	0.32
12, 13	0.2	0.22	0.15	0.13	0.05	0.05	0.13	0.08	0.09
17, 18, 19	-	-	-	-	-	-	0.08	0.07	0.03
20	-	-	-	-	-	-	0.19	0.12	0.1
unknown	0.18	0.27	0.14	0.2	0.16	0.19	0.12	0.13	0.54

Table S17. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C4-fr4-MF using UV; ELSD and MS HPLC detectors. Peaks areas were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
4, 5, 6	1.89	1.22	3.52	-	-	-	0.56	4.58	1.11
unknown	2.22	2.04	3.85	-	-	-	2.59	3.89	2.41
unknown	1.36	3.92	2.03	0.66	3.47	1.38	2.53	4.65	4.32
7, 8, 9, 10	2.78	1.68	5.31	1.44	1.09	2.9	1.54	4.72	2.45
11	0	0	0	0	0	0	0	0	0
12, 13 and **14, 15, 16**	5.68	2.45	5.18	0.38	0.55	0.85	2.29	4.61	3.98
20	1.89	1.22	3.52	0.66	3.47	1.38	2.31	2.44	4.25
unknown	2.22	2.04	3.85	1.44	1.09	2.9	1.57	3.24	0.77

Table S18. Peak areas relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C4-fr4-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
4, 5, 6	0.22	1.35	1.13	-	-	-	1.71	5.57	0.61
unknown	0.68	2.8	2.11	-	-	-	3.58	4.75	2.55
unknown	0.77	1.91	1.31	0.56	1.65	2.05	0.63	2.34	4.7
7, 8, 9, 10	2.03	3.75	5.04	1.02	3.43	0.59	1.13	2.04	1.75
11	1.66	1.14	2.71	0.48	1.69	3.44	2.16	2.52	0.79
12, 13 and **14, 15, 16**	4.34	1.08	2.75	0.67	1.67	3.52	0.57	0.43	4.71
20	0.22	1.35	1.13	0.56	1.65	2.05	3.44	0.79	4.95
unknown	0.68	2.8	2.11	1.02	3.43	0.59	0.74	0.14	0.17

Table S19. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C4-fr4-MF using UV; ELSD and MS HPLC detectors. Retention times were measured relative to [Phe^3, *N*-Me-Val^5] Dtx B (**11**) as internal standard

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
4, 5, 6	0.28	0.04	0.06	-	-	-	0.16	0.27	0.15
unknown	0.05	0.09	0.17	-	-	-	0.45	0.05	0.74
unknown	0.05	0.12	0.19	0.22	0.05	0.14	0.23	0.25	0.09
7, 8, 9, 10	0.07	0.09	0.13	0.03	0.06	0.09	0.14	0.32	0.07
11	0	0	0	0	0	0	0	0	0
12, 13 and **14, 15, 16**	0.07	0.12	0.1	0.01	0.08	0.13	0.39	0.14	0.09
20	-	-	-	-	-	-	0.23	0.22	0.12
unknown	-	-	-	-	-	-	0.25	1.01	0.08

Table S20. Retention times relative standard deviation (%) of repetibility (RP). Intermediate precision (IP) and stability (S) measured for peaks observed in fraction C4-fr4-MF using UV; ELSD and MS HPLC detectors. No internal standard was used

	UV			ELSD			MS		
Compound	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S	RP (n = 3)	IP (n = 9)	S
4, 5, 6	0.41	0.09	0.05	-	-	-	0.07	0.15	0.19
unknown	0.1	0.09	0.13	-	-	-	0.61	0.11	0.76
unknown	0.11	0.03	0.08	0.15	0.05	0.23	0.05	0.09	0.11
7, 8, 9, 10	0.08	0.04	0.09	0.04	0.05	0.11	0.05	0.16	0.1
11	0.15	0.13	0.11	0.07	0.01	0.13	0.19	0.16	0.04
12, 13 and **14, 15, 16**	0.13	0.06	0.05	0.06	0.07	0	0.2	0.18	0.1
20	-	-	-	-	-	-	0.06	0.06	0.16
unknown	-	-	-	-	-	-	0.15	0.85	0.11