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Evaluation of a method using high performance liquid chromatography with ultraviolet detection for the determination of statins in macromycetes of the genus *Pleurotus* cultivated by fermentation processes

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ABSTRACT

The applicability of high-performance liquid chromatography with ultraviolet light (HPLC-UV) for the determination of the presence of statins in macromycetes of the genus *Pleurotus* was analyzed. The fungi were obtained by liquid-state fermentation (LSF) using unconventional sources of carbon as substrates and solid-state fermentation (SSF) employing agro industrial wastes. Five statins were used as standards: lovastatin and simvastatin in the lactone form (LOVL and SIML), their corresponding hydro-acidic forms (LOVH and SIMH) and pravastatin (PRA). The following measures were evaluated: the linearity, accuracy and precision, detection limit (DL) and quantification limit (QL). The results demonstrated HPLC-UV to be an effective tool for detecting the presence of statins in extracts of LSF and SSF products. Likewise, it was hypothesized that the strains that were used for the study do not produce statins. This finding highlights the importance of continuing to evaluate other strains of the same genus by using techniques such as HPLC to first separate sufficient quantities of the compounds that were detected using the standard technique but that did not match the retention time (t_R) of any of the standards used.

1. Introduction

Statins are a class of drugs that are characterized by effectively lowering high cholesterol levels and having multiple pleiotropic effects [1–3]. These properties allow their use in the treatment of neurodegenerative disorders, non-ischemic cardiomyopathy, autoimmune diseases, such as rheumatoid arthritis, and diabetes mellitus [4,5], in the prevention of bone fractures and in reducing the incidence of some cancers [6]. Statins can be divided into two types depending on their origin: synthetic and natural. Natural statins are fungal metabolites or their fermentation products with the same basic nucleus, which differ from each other in the position of the methyl groups on the ring and the side chain and in the presence of some functional groups (Fig. 1) [3,7].

The commercially available type I statins are lovastatin, pravastatin sodium salt and simvastatin. These compounds have been isolated mainly from micromycetes *(Aspergillus, Penicillium* and *Monascus)* [8–17] and from macromycete species of the genus *Pleurotus,* which are excellent producers of these compounds [18–23], thus reaffirming the status as a functional food [24–26]. Enough research has been conducted on improvement of the production of bioactive agents from macromycetes through fermentation processes; however, research regarding the detection, identification and separation of statins from macromycetes has been lagging. The present study evaluated the applicability of using high-performance liquid chromatography with ultraviolet detection (HPLC-UV) to determine the possible presence of statins obtained through liquid-state fermentation (LSF) and solid-state fermentation (SSF). The carbon sources (CS) used for this purpose were unconventional substrates and agro industrial wastes residues for the case of SSF as applied to obtain statins from three species of the genus *Pleurotus.* The use of different types of flours as unconventional carbon sources

Abbreviations: CS, carbon source; DAD, diode array detector; DL, limit of detection; EtOAc, ethyl acetate; HCl, chlorhydric acid; HPLC, High performance liquid chromatography; KOH, potassium hydroxide; LOVH, lovastatin in hydroxy acid form LOVL, lovastatin in lactone form; LSF, liquid state fermentations; NaOH, sodium hydroxide; PRA, pravastatin; QL, limit of quantitation; RYD, fermented red rice, "Traditional Red Yeast®" Doctor's A-Z; RYN, fermented red rice "Red Yeast Rice®" Nature´s Plus; RYS, fermented red rice "Traditional Red Yeast®" Swanson Premium Brand; SIMH, simvastatin in hydroxy acid form; SIML, simvastatin in lactone form; SSF, solid state fermentations; t_R, retention time; UV, ultraviolet.

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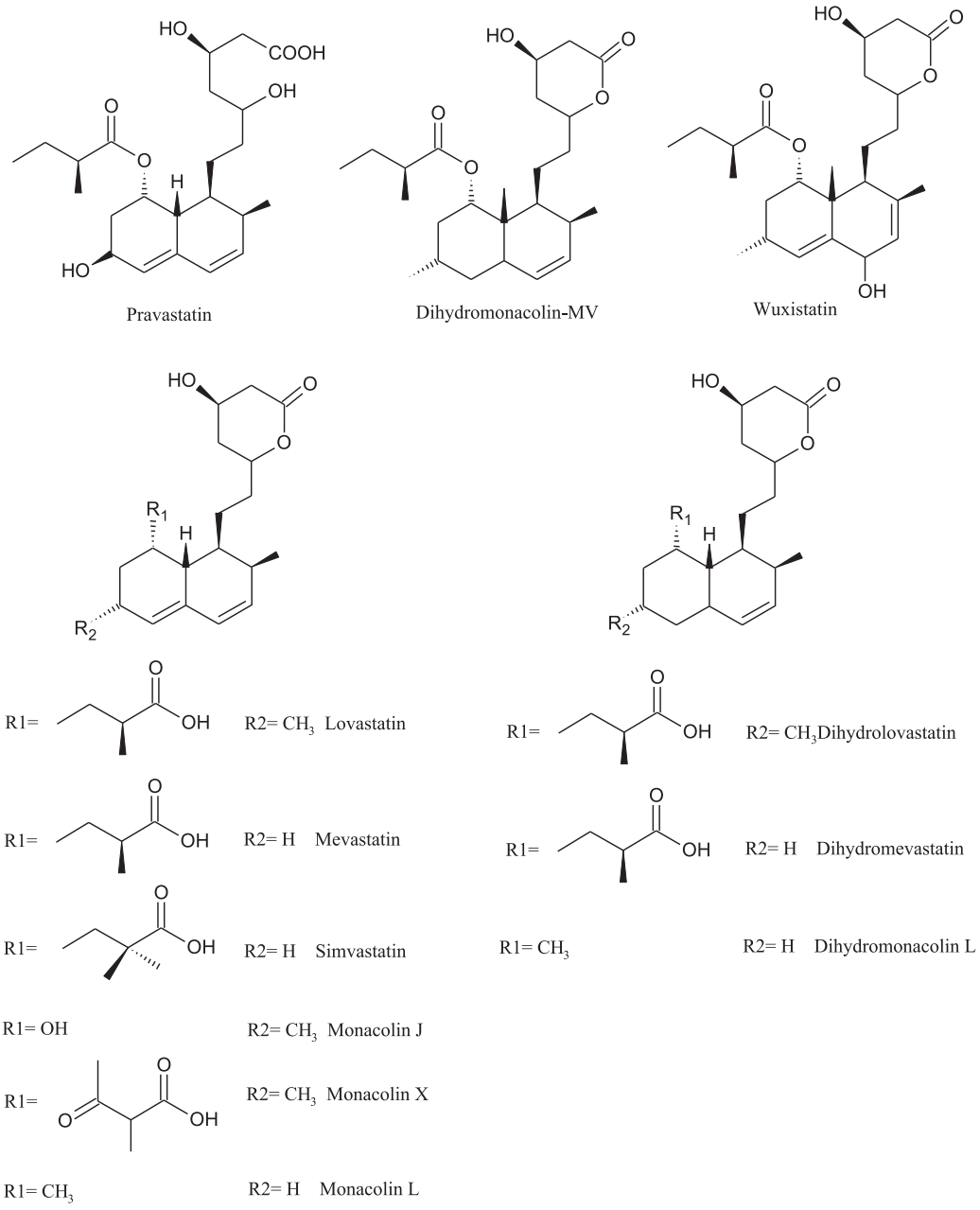

Fig. 1. Structures of type I statins.

was based on the fact that their use in LSF represents a reduction of 98% of mycelium mass-production costs and consequently a reduction of the production costs of statins [27].

It should be noted that the broth that contains the fungi-secreted statins constitutes a much more complex matrix compared to that of cultures in traditional culture media. This complexity increases the need for a separation and detection technique that can be applied to this matrix and that is also rapid and efficient.

2. Materials and methods

2.1. Materials

Standard statins, such as lovastatin (LOVL), simvastatin (SIML) and pravastatin (PRA), were obtained from commercial and generic medications. LOVL and SIML solutions were prepared by dissolving the solutes in acetonitrile. Given that the PRA was commercially available as a salt of a hydroxy acid, it was dissolved in a mixture of acetonitrile and 0.1% formic acid (55:45) and centrifuged to remove the remaining excipients, and the supernatant was used for the preparation of solutions. The hydroxy acid forms of lovastatin (LOVH) and simvastatin (SIMH) were obtained through a process of hydrolysis of the corresponding lactonic forms. All of the necessary dilutions were completed using the mobile phase of HPLC from a stock of 0.1 mg/ml.

The following commercial samples of red yeast rice were used in this study: "Traditional Red Yeast ®" Swanson Premium Brand (RYS), "Traditional Red Yeast ®" Doctor's AZ (RYD) and "Red Yeast Rice ®" Nature's Plus (RYN).

2.2. Optimization of the LOVL hydrolysis conditions

The method was based on a study previously reported by Yang et al. [28], introducing the use of sonication (60 min and 90 min) and reflux (90 min) during hydrolysis. This preliminary trial was

Table 1
Conditions tested to optimize the LOVL hydrolysis.

Number	Base	ACN (%)	Method	Time ($_{min}$)
1	NaOH	25	Sonication	60
2	NaOH	25	Sonication	90
3	NaOH	50	Reflux	90
4	NaOH	50	Sonication	60
5	NaOH	50	Sonication	90
6	KOH	25	Reflux	90
7	KOH	25	Sonication	60
8	KOH	25	Sonication	90
9	KOH	50	Sonication	90
10	KOH	50	Sonication	60
11	NaOH:EtOH (1:1)	NA	Reflux	20

NA: notapplicable.

performed with LOVL which contained 1 mg of the base solution (0.1 mol L^{-1} NaOH or 0.05 mol L^{-1} KOH in acetonitrile:water). The mixture was placed in a 45 °C bath for a predetermined time in each of the 10 trials (Table 1). Subsequently, the products were neutralized by reacting with a solution of 0.05 mol L^{-1} HCl. Additionally, the methodology used by Casas-Lopez [17] was adopted, analyzing in each case the hydrolysis products using HPLC-UV.

2.3. Determination of the effect of alcohol use on LOVL and SIML

The following solutions were prepared: 1 mg/mL of SIML and LOVL and mixtures of methanol with H_2O (1:1), ethanol with H_2O (1:1) and butanol with H_2O (1:1). The solutions were analyzed using HPLC-UV at 0 h and 24 h.

2.4. Determination of the interfering-components effect

The following dilutions were prepared with the aid of ultrasound, with a concentration of 1 mg/ml: triterpenoid compound mixtures, fatty acids and their esters, which are commonly reported as constituents of the genus *Pleurotus* [29] and were analyzed using HPLC-UV.

2.5. Stability of statins over 24 h

A mixture of five statins (LOVL, SIML, LOVH, SIMH and PRA) at a concentration of 12 μg/ml was prepared and injected continuously for 24 h in the HPLC-UV. The areas of each peak were integrated, and the coefficients of variation were calculated.

2.6. Analytical determination of statins

2.6.1. Equipment

The HPLC analyses were performed on a liquid chromatography column consisting of two Shimadzu LC-9A pumps, a Shimadzu CTO-to 6A column oven and a Shimadzu SIL-6B autosampler, operated by a Shimadzu SCL-6B controller. The UV detector used was an Agilent 1260 VWD (G1314B) operated at 237 nm, and the data-processing software used was the GC A5000 Workstation. The stationary phase was a Thermo Hypersil GOLD® C18 column, 150 × 4.6 mm (5 μm particles), maintained at a temperature of 30 °C. The mobile phases tested were mixtures of acetonitrile:H_2O modified with acids, such as acetic acid, phosphoric and formic acid, in different proportions, with isocratic and gradient elution modes and using the following flow rates: 0.4 mL/min, 0.6 mL/min, 0.8 mL/min and 1.0 mL/min.

The analyses by HPLC-DAD were performed on a Merck Hitachi liquid chromatography with a Merck Hitachi D-600A interface and L-6200A pump driven by a Merck Hitachi D 7000 controller. The diode-array detector used was a Merck Hitachi L-4500 operated at 237 nm.

2.6.2. Evaluation of the analytical parameters of the HPLC-UV method

Linearity was determined by preparing standard solutions of 0.05 μg/mL and 100 μg/mL that were used to create eight levels, with each performed in triplicate. The DL and QL values were estimated according to the methodology described by Martos et al. [30]. Eight replicates were prepared at concentrations of 3.12 ng/mL for PRA, 6.25 ng/mL for LOVH and SIMH and 12.5 ng/mL for LOVL and SIML. Using the response factors, the concentrations were determined for each of the integration areas, and the standard deviation of the eight tests was calculated; this value was then multiplied by 3 for DL (for seven degrees of freedom and the 99% confidence level) and by 10 for QL.

The accuracy and precision of the method was evaluated using the mycelium extracts and the broth of *Pleurotus ostreatus* that was cultured on wheat bran as a control. Three stock solutions were prepared in quadruplicate with intermediate calibration-curve concentrations of each standard (6 μg/mL, 12 μg/mL and 25 μg/mL).

2.7. Preparation of the extracts from commercial products

The red yeast-fermentation tablets were extracted as indicated by Prasad [10]. Solutions of 1 mg/mL of each extract were analyzed using HPLC-UV.

2.8. Preparation of the fungal material

2.8.1. Liquid fermentation cultures

The fungal materials were grown in the Biotechnology and Bioprocess Laboratories of the University of Antioquia. The strains of *P. ostreatus* (BioVeg Fungi-002), *Pleurotus pulmonarius* (BioVeg

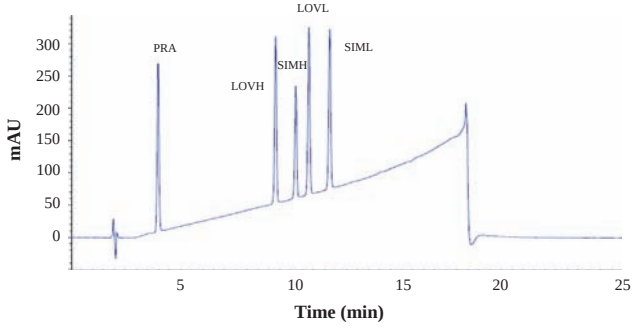

Fig. 2. HPLC-UV chromatogram of the standard mixtures (0.1 mg/mL). Gradient elution mode: ACN (B): 0.1% formic acid solution (A) transitioning from 35% to 100% B in 15 min. Flow rate: 1.0 mL/min. Thermo Hypersil GOLD® 150 × −4.6 mm C18 (5 μm) column. Retention times: PRA: pravastatin 4.02 min; LOVH: hydroxyacid lovastatin 9.37 min; SIMH: hydroxy acid simvastatin 10.20 min; LOVL: lovastatin in lactone form 10.71 min; SIML: simvastatin in lactone form 11.84 min.

Table 2
Determination of the linear relationship between the concentration and area of the studied analytes.

Analyte[a]	Linearity	
	Average response factor	% CV
PRA	25082320	4.6
LOVH	25985777	3.3
SIMH	24121549	2.3
LOVL	25980019	4.5
SIML	24148667	4.4

[a] Abbreviations used: PRA: pravastatin, LOVH:hydroxyacid lovastatin, SIMH: hydroxyacid simvastatin, LOVL: lovastatinlactoneform, SIML: simvastatin lactone form.

Fungi-001) *and Pleurotus djamor cold* (BioVeg Fungi-005) were maintained in potato dextrose agar (PDA) and were stored at 4 °C (a work strain, the first replicate of the parent strain). Subsequently, inoculates of 0.5 cm to 1 cm of mycelium and agar were transferred to Petri dishes with a medium at the following concentrations (g/L): carbon source (CS1 to CS13) 30, yeast extract 3, sucrose 5 and agar 8, with pH at 5.5 ± 0.1.

The following carbon sources were used: CS1: barley flour (*Hordeum vulgare L*), CS2: oatmeal flour (*Avena sativa L*), CS3: wheat flour (*Triticum aestivum L*), CS4: rice flour (*Oryza sativa L*), CS5: Bienestarina©, CS7: corn flour (*Zea mays L*), CS8: soy flour, CS9: wheat bran, CS10: whole-wheat flour, CS11: pinto cornmeal, CS12: seven-grain flour and CS13: yellow cornmeal.

The cultures were incubated at 26 °C in darkness for 15 days. Subsequently, flasks with 62 mL were prepared with a medium composed of the following components (concentrations expressed in units of mg/L): $NaNO_3$ 80, $MgSO_4$ 7, H_2O 20, KH_2PO_4 30, KCl 10. These components were supplemented with different carbon sources from CS1 to CS13 (except CS6). The pH of the media was adjusted to 5.6 ± 0.1, and the flasks were sterilized by autoclaving at 15 psi and 121 °C for 15 min. Disks (1 cm diameter) with agar and mycelium (1 g) were used as inoculates. The flasks were placed on an orbital agitator at 100 rpm for 9 days at 25 ± 1 °C. The method reported by Alarcón et al. [19] was used to determine the production of statins from the different strains.

2.8.2. Determination of the C/N ratio of the carbon sources

The C and N contents were determined using a Thermo FLASH 2000 Organic Elemental Analyzer. The experimental data underwent a variance analysis with the Statgraphics 5.1 software.

Table 3
Determination of the method's sensitivity.

Analyte[a]	Sensitivity	
	Detection limit (ng/mL)	Quantification limit (ng/mL)
PRA	2.3	7.8
LOVH	4.8	15.8
SIMH	7.1	23.8
LOVL	4.8	16.1
SIML	8.8	29.2

[a] Abbreviations used: PRA: pravastatin, LOVH: hydroxyacid lovastatin, SIMH: hydroxyacid simvastatin, LOVL: lovastatin lactone form, SIML: simvastatin lactone form.

Table 5
Hydrolysis percentages obtained from the conversion of LOVL to LOVH with the evaluated methods.

Treatment number[a]	LOVL (%)	LOVH (%)
1	74.3	25.7
2	28.6	71.4
3	1.5	98.5
4	89.3	10.7
5	16.2	83.8
6	94.2	5.8
7	59.6	40.4
8	2.7	97.3
9	1.7	98.3
10	47.5	52.5
11	0.0	100.0

[a] Conditions described in Section 2.2 (Table 1).

Table 4
Determination of the precision and accuracy of the developed method.

Analyte[a]	Precision[b]						Accuracy[c]					
	Mycelium extract			Broth extract			Mycelium extract			Broth extract		
	Fortification (μg/mL)			Fortification (μg/mL)			Fortification (μg/mL)			Fortification (μg/mL)		
	6	12	25	6	12	25	6	12	25	6	12	25
PRA	3.5	3.5	2.1	5.1	3.3	2.9	101	96	95	104	97	96
LOVH	4.2	3.1	1.4	5.1	3.1	1.6	100	98	98	103	101	100
SIMH	4.1	3.1	1.0	5.2	3.3	1.8	102	98	98	104	101	100
LOVL	3.6	2.9	0.9	4.7	3.3	1.6	102	99	100	104	102	102
SIML	0.9	2.9	1.4	4.3	1.6	1.2	102	96	98	112	101	103

Analit[d]	Accuracy[e]					
	Extracts from the mycelium			Extracts from the broth		
	Fortification (μg mL^{-1})			Fortification (μg mL^{-1})		
	6	12	25	6	12	25
PRA	101	96	95	104	97	96
LOVH	100	98	98	103	101	100
SIMH	102	98	98	104	101	100
LOVL	102	99	100	104	102	102
SIML	102	96	98	112	101	103

Repeatability: calculated as% of average recovery of the four replicates.

[a] Abbreviations used: PRA: pravastatin, LOVH: hydroxy acid lovastatin, SIMH: hydroxy acid simvastatin, LOVL: lactone lovastatin, SIML: lactone simvastatin.
[b] Precision calculated as the %RSD of the four replicates.
[c] Accuracy calculated as the average % recovery of the four replicates.
[d] Abbreviations used: PRA: pravastatin, LOVH: hydroxyacid lovastatin, SIMH: hydroxyacid simvastatin, LOVL: lovastatin lactone form, SIML: simvastatin lactone form.
[e] Accuracy calculated as %RSD of the four replicates.

2.8.3. Solid-state fermentation cultures

The three species of *Pleurotus* were cultivated in bags containing 400 g of sugar cane bagasse, 10% CS1 and 4% $CaCO_3$. These bags were then inoculated with 40 g of spawn.

The sporocarps were collected in the first harvest, with pileus sizes of 5 cm in diameter. The lamellae were removed from the carpophores.

2.9. Fungal sample preparation

The products of the LSF were filtered to separate the mycelium from the broth. The mycelium was washed with a 0.05 mol L^{-1} HCl solution to assure the reincorporation of all of the hydroxyacid statins that could be in the broth or accumulated within the pellets. The lyophilized broth, mycelium, lamellae and fruiting bodies were extracted with AcOEt. The extracts were desiccated with anhydrous Na_2SO_4 and dried in a rotary evaporator. For the HPLC analysis, solutions of 1 mg/mL were prepared and sonicated before injection.

3. Results and discussion

3.1. Determination of the optimal chromatography parameters

To determine the applicability of the HPLC-UV chromatography method, different variables must be evaluated, such as the type of stationary phase, composition of the mobile phase, elution mode, flow rate and temperature of the oven, among others.

Despite the various existing reports discussing the analysis of statins using this method, the majority of such studies focus on a single statin [31–36], with the exception of the study published by Yang et al. [28]. This research group applied this technique to determine the statin content of Pu-Erh tea. In addition, there have been no previous studies to determine the statin contents of the substrates evaluated in this investigation. This limitation means that the results of the present work are pioneering regarding the determination of these five statins in the LSF and SSF products of *Pleurotus*.

The effect of the aforementioned variables on the chromatography parameters indicated that a gradient with acetonitrile and formic acid mixture gave the optimum values of k', R_s and peaks with a perfect Gaussian distribution for the analytes (Fig. 2). The ramp was as follows: 0.1% formic acid solution (A) in a linear gradient from 35% to 100% of (B) in 15 min, with a flow rate of 1.0 mL/min and an oven temperature of 35 °C.

This method has an additional advantage compared to that published by Yang et al. [28]. With the gradient used in the present method, the PRA has a greater interaction with the stationary phase, which allows the separation of polar interferences that could be present in the evaluated extracts, thereby facilitating the detection of PRA or other structurally similar derivatives with a similar polarity.

3.2. Evaluation of the parameters of the chromatography method

Based on the tests conducted to determine the reliability of the method, the relationship between the concentration and the area of each of the analytes fit a perfectly straight line, with similar response factors and variation coefficients between triplicate samples of less than 5% (Table 2).

The DL and QL values were at the bottom limit of the ranges published in the literature (1–100 ng/mL and 0.5–125 ng/mL, respectively) [31,32,37] (Table 3).

Because the HPLC-UV profile of the 72 matrices did not vary significantly, as will be explained below, the accuracy and precision were evaluated by selecting a single strain and a culture medium to simplify the process. The extracts used were those of *P. ostreatus* mycelium and broth cultured in wheat bran. The gas chromatography and mass spectroscopy analyses (not included in this paper) demonstrate the presence of various important triterpenoid compounds. From a bio-synthetic point of view, these results suggest the absence of statins because if statins were present, they would inhibit HMG-CoA and thus drive the concentration of triterpenoids to an undetectable level. This characteristic

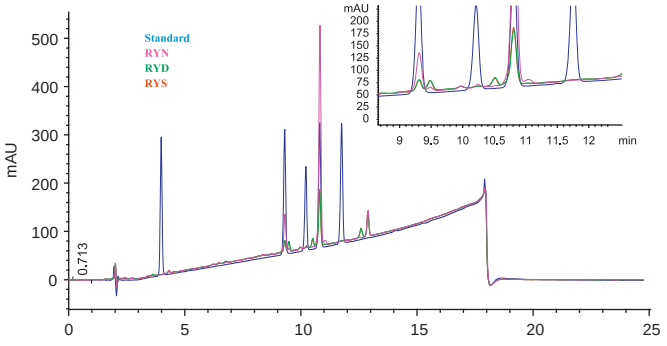

Fig. 3. HPLC-UV chromatogram of the extract in AcOEt from samples of fermented commercial red yeast rice.**RYS**: "Traditional Red Yeast ®" Swanson Premium Brand, **RYD:** "Traditional Red Yeast ®" Doctor's AZ, **RYN:** "Red Yeast Rice ®" Nature's Plus.

Table 6
C/N ratio of the carbon sources used for the LSF of the *Pleurotus* species.

Carbon source	C (%)	N (%)	C/N
CS1	40.2804 ± 0.0069 c	1.8427 ± 0.0163 b	21.8555 ± 0.1996 f
CS2	42.3532 ± 0.0132 i	2.5853 ± 0.0298 d,e	16.3823 ± 0.1951 d
CS3	39.7866 ± 0.0096 b	2.2063 ± 0.0325 c	18.0332 ± 0.2550 d,e
CS4	39.4205 ± 0.0077 a	1.3733 ± 0.0004 a	28.7049 ± 0.0093 h
CS5	40.4564 ± 0.0086 d	3.4513 ± 0.1068 f	11.7221 ± 0.3400 b
CS7	40.2361 ± 0.0172 c	1.616 ± 0.0174 b	24.8986 ± 0.2519 g
CS8	46.6606 ± 0.0167 k	5.2838 ± 0.0166 g	8.8309 ± 0.0298 a
CS9	41.5307 ± 0.0194 h	2.7823 ± 0.0376 e	14.9268 ± 0.2108 c
CS10	40.8197 ± 0.0033 f	2.2241 ± 0.0293 c	18.3534 ± 0.2359 e
CS11	41.2568 ± 0.0090 g	1.7484 ± 0.0140 b	23.5969 ± 0.1973 g
CS12	42.6171 ± 0.0167 j	2.4293 ± 0.0131 c.d	17.5430 ± 0.0905 d.e
CS13	40.6038 ± 0.0039 e	1.5768 ± 0.0412 b	25.7508 ± 0.6358 g

The results are given after multiple comparisons, based on significant differences: the mean values obtained from three repetitions are the indicated letters, from letter a to letter k. Two mean values accompanied by the same letter indicates that these means were not significantly different from each other; otherwise, different letters indicate significant differences.

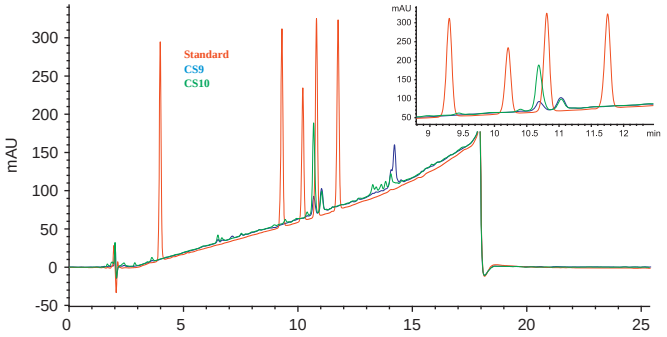

Fig. 4. HPLC-UV chromatogram of the *P. djamor* extract inEtOAc. The mycelium was cultured in wheat bran (CS9) and whole wheat flour (CS10) via SSF.

makes the chosen extracts ideal control samples to evaluate accuracy and precision [38].

Both the accuracy and precision in all the cases were within the expected values, with variation coefficients under 5% and recovery percentages close to 100% (Table 4).

All of the evidence indicates that the developed method effectively detects and quantifies PRA, LOVL, SIML, LOVH and SIMH.

3.3. Optimization of the hydrolysis conditions of LOVL

In previous studies, it was determined that statins obtained via fermentation can be found in their lactonic forms in the mycelia and fruiting bodies and in their hydroxyl acidic form in the broth [14,39–41]. Therefore, it is important to determine the optimal conditions to extract both the lactonic and hydroxyl acidic statin forms. This extraction will allow for their identification and quantification in the products of LSF and SSF with *Pleurotus*.

Table 5 contains the results of the lactonic and hydroxyl acidic percentages extracted from the 11 tests. The method selected was that reported by Casas et al. [17], which allows the complete hydrolysis of the lactonic form in a short period of time (20 min). This hydrolysis differs from the results reported by Yang et al. [28], who used an alcohol in the hydrolysis reaction and observed peaks attributed to the formation of the corresponding esters.

Similar results were obtained after using the same conditions with SIML. For this reason, during this study, the conversion from the lactonic forms to the hydroxyl acidic forms was obtained with a reflux for 20 min of a mixture of 0.1 mol L^{-1} NaOH and ethanol (1:1 v/v) at 50 °C and subsequent neutralization with 0.05 mol L^{-1} HCl.

3.4. Effect of alcohol use on the extraction, extract interferents and time associated with the determination of statins in Pleurotus

The factors evaluated were the use of alcohols for the extraction processes, the presence of other compounds, such as fatty acids and triterpenoid compounds, and the time from the sample preparation to the injection into HPLC-UV. For the first variable, it was found that use of alcohol was not adequate because the observed peaks of different polarities did not correlate with the t_R of any of the standards, which can be attributed to the formation of esters with the alcohol used. This result was previously reported by authors conducting similar studies [28].

With respect to interfering substances, there were no observed peaks that could alter the determination of the statins. None of the compounds used absorb at the wavelength of detection of the statins. These compounds include those typically found in

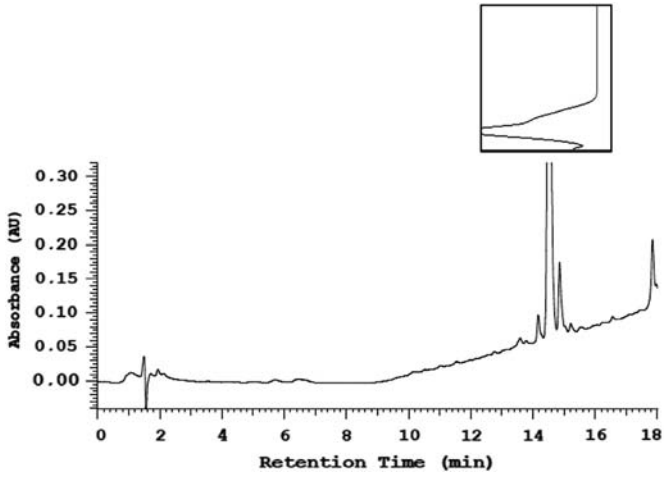

Fig. 5. HPLC-DAD chromatogram of the *P. djamor* extract in AcOEt. The mycelium was cultured in whole wheat flour (CS10) via SSF.

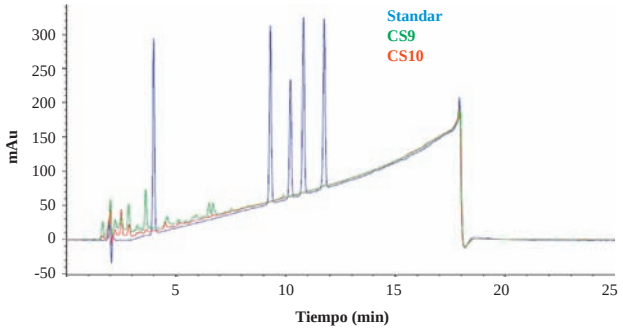

Fig. 7. HPLC-UV chromatograph of the extracts from the broth of *Pleurotus djamor* cultured in wheat bran (CS9) and whole wheat flour (CS10).

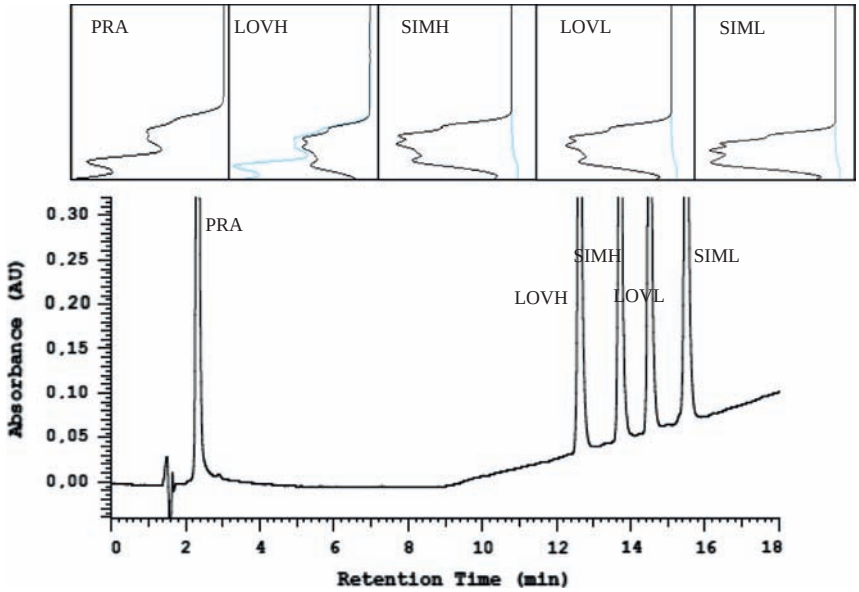

Fig. 6. HPLC-DAD chromatogram of the standard mixture (0.1 mg/mL). **PRA**: pravastatin, **LOVH**: hydroxyacid lovastatin, **SIMH**: hydroxyacid simvastatin, **LOVL**: lactonic lovastatin, **SIML**: lactonic simvastatin.

Pleurotus, such as palmitic, stearic, oleic and linoleic acids together with their corresponding esters and triterpenoids [29,42]. This pattern allows for the conclusion that there is no interference from such compounds that complicate the detection of statins from *Pleurotus* fermentations.

A mixture of 5 standards was injected over the course of 24 h to determine the possibility of converting or degrading the statins over time. During this time, the coefficient of variance of the computed areas was calculated for each standard and in all cases was below 5%. This result provides evidence that there was no significant conversion or degradation during the 24 h at room temperature for the five standards in solution.

3.5. Application to the extracts from commercial products

With the goal of determining the efficiency of this methodology for the determination of statins in commercial products, the same extraction and post-HPLC-UV analysis was conducted. The test specimens were three different brands of red yeast rice fermented with *Monascus purpureus* that reportedly contain statins.

Fig. 3 contains the chromatographs for the three cases in which peaks that correspond to LOVL and LOVH can be observed in all of the extracts. Thus, it can be concluded that the method is applicable to the study of statins in this matrix.

3.6. C/N ratio of the carbon sources

Because the C/N ratio is an important parameter in the LSF production of fungal metabolites, it is necessary to calculate the ratio from the utilized carbon sources. Previous reports have established that this ratio directly affects the production of statins by both micromycetes and macromycetes [17,19].

The obtained results (Table 6) demonstrate that there are important differences between carbon sources that can influence the production of statins. Alarcón et al. reported that the production of lovastatin by *P. ostreatus* cultured via LSF increases as a function of increasing substrate content of C and N. The maximum production is achieved with a C/N ratio of 6.07 [19]. For this reason, in this study, one would expect that the CS8 culture would be favored in the production of these metabolites.

3.7. Application of HPLC-UV to Pleurotus extracts

The extracts of the analyzed specimens were prepared in AcOEt based on previous solubility studies of the metabolites [43,44] and based on the results obtained by Yang et al., which reported greater extraction efficiency when using AcOEt to eliminate the possibility of creating statin esters when including alcohols. All of the extracts obtained from the broth and the mycelia, cultured with different carbon sources were analyzed with HPLC-UV. There

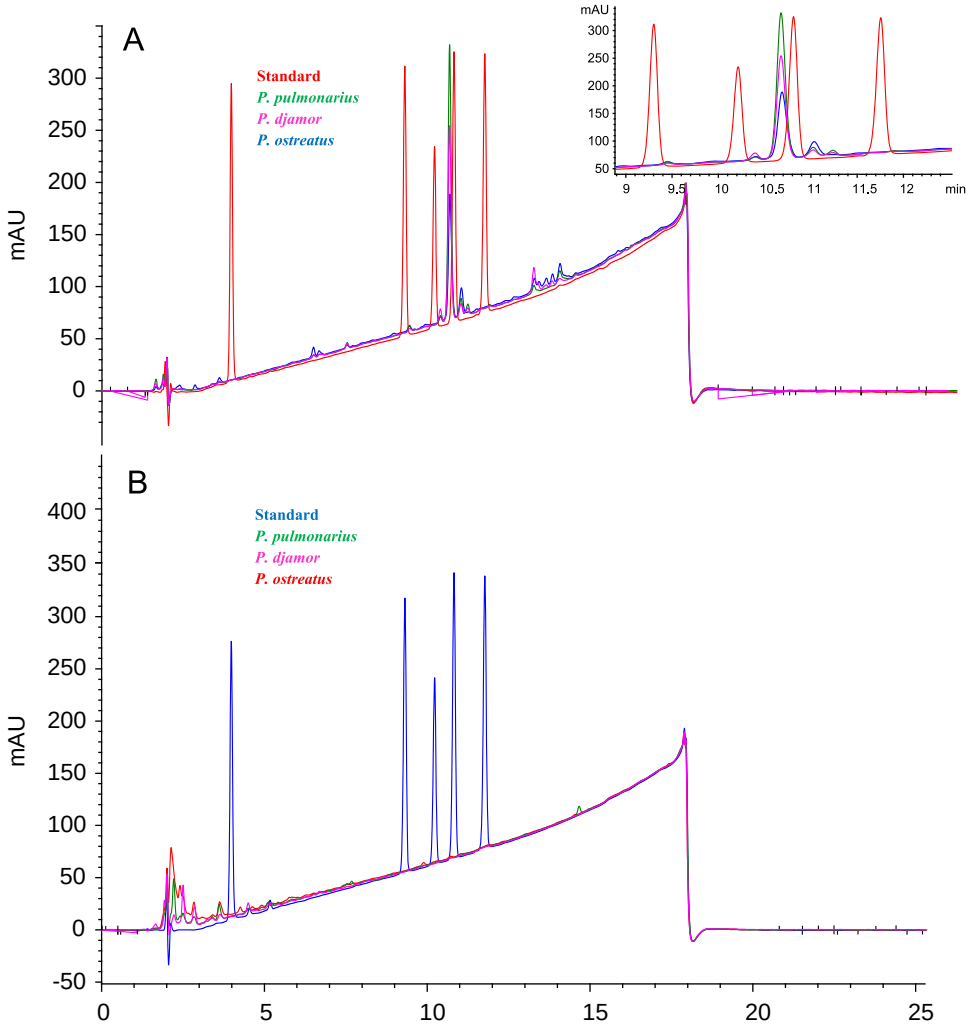

Fig. 8. HPLC-UV chromatogram of the mycelium extract (A) and broth (B) of the three strains of *Pleurotus* cultured under the conditions specified by Alarcón et al. [19].

were a total of 72 matrices resulting from three *Pleurotus* species (*ostreatus, djamor* and *pulmonarius*) produced through LSF and 12 carbon sources. Analysis of the different mycelia revealed that the results were independent of both the carbon source and the species used. No sample had a detected peak that correlated to the t_R of the standards used. However, there were peaks identified that corresponded to t_R values between those of the selected standards; these peaks varied in intensity among both the carbon source and the analyzed species. Fig. 4 contains the chromatogram for the extract of *P. djamor* cultured in CS9 and CS10, which shows a common peak at a t_R value equal to 14.07 min with a higher concentration in CS10. All of the detected compounds had different t_R values than those of the standards.

This result makes it necessary to explore other techniques that would allow confirmation of the presence of the metabolites of interest.

Analyzing via HPLC-DAD the extract of *P. djamor* cultured in CS10 (Fig. 5) demonstrated that the UV spectrum was not consistent with those of the desired statins. The spectrum should contain a major peak at 237 nm and two minor peaks at 230 nm and 248 nm, which would be consistent with the spectra of the standards used (Fig. 6).

Similarly to the case of the mycelium extracts, the analysis of the broth extracts demonstrates that the results are independent of the carbon source and species used. Fig. 7 contains the chromatograms of the broth of *P. djamor* cultured in CS9 and CS10. The results suggest that there was no secretion of the hydroxyacid statins or if there were, the quantities were below the DL of this method. This result contrasts with other studies concerning the use of *Pleurotus* that reported the presence of statins in the culture broths [20,22,45].

The results above allow for the formulation of different hypotheses: (1) The CS used are not an adequate substrate for the biosynthesis of statins; (2) there is no statin production during the mycelium growth phase; (3) the working strain stocks do not produce the compounds of interest.

To evaluate the first hypothesis, the three strains of *Pleurotus* used in this study were cultured in the conditions reported by Alarcón et al. [20]. Fig. 8 shows the chromatogram for the mycelia and broth extracts, whose analysis reveals that the substrate did not inhibit the biosynthesis of statins. If this inhibition were the case, these statins would have been produced when using the media reported by Alarcón. The answer to this hypothesis becomes clearer when noting the absence of statins in CS9 (wheat bran), which has been reported to be one of the most efficient substrates for statin production during SSF of micromycetes and *Pleurotus* [20,46–48].

SSF tests were performed, and both the carpophores and lamellae were analyzed to evaluate the effect of maturity on the production of the metabolites. The lamellae were chosen based on the work of Gunde-Cimerman, who determined that these structures and the sporocarps with a diameter of 5 cm contained the highest lovastatin contents [21].

The results of the extracts of both the carpophores and lamellae mimicked those of the mycelium (Fig. 9). This result discards the hypothesis that the growth phase of the mycelium strains avoids the synthesis of statins.

Combining the aforementioned results infers that the strains of *Pleurotus* used in this study are not statin producers. This deduction is agreed with literature reports by Inga Schneider et al. and Alicia Gil-Ramirez et al., who found that two *Pleurotus* strains, *ostreatus* and *ostreatus* (*Jacq. Ex Fr.*), do not produce this type of secondary metabolite; however, these strains present a cholesterol-reducing

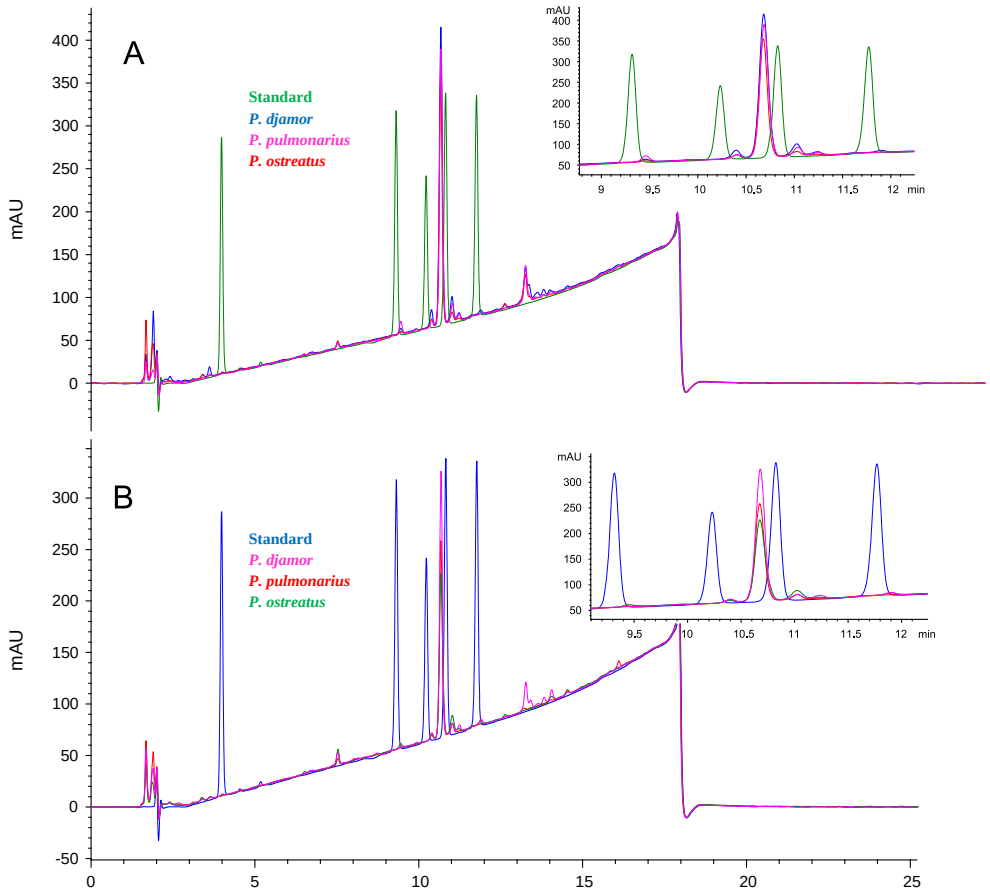

Fig. 9. HPLC-UV chromatogram of the extracts from the fruiting bodies with 5 cm diameters (A) and the lamellae (B) of the three strains of *Pleurotus* cultured via SSF.

effect that is attributed to the presence of sterols and/or linoleic acid, which are compounds that reduce lipid content.

Gil-Ramírez et al. mentioned that although a statin was not responsible, there was a change in the synthesis of endogenous cholesterol due to the inhibition of HMG-CoA reductase, which is a pathway used by statins [18,49].

Another possible explanation of the presence of peaks similar to those of the standards is that the compounds detected using HPLC-UV are structural derivatives of statins or their intermediates. If this is the case, the hexahydro-naphthalene ring must contain substitute functional groups that alter the UV profiles by maintaining the maximum peak at 237 nm but eliminating the absorption of the other two wavelengths.

A technique such as HPLC-MS is a tool that would allow the confirmation of whether the strains in this study are statin producers. However, this technique was not considered appropriate given that the non-production of these metabolites by the *Pleurotus* genus was not exceptional.

4. Conclusions

In conclusion, the results of this investigation showed that a very efficient method was developed for the detection and quantification of LOVL, LOVH, SIML, SIMH and PRA in the products obtained from the LSF of *Pleurotus* strains. The strains utilized in this study do not produce any of the five statins used as standards. However, a peak with a t_R close to LOVL was detected in the results of all the cultures at different concentrations, depending on the utilized carbon source. Currently, other HPLC-UV studies are being conducted regarding the extracts of mycelia and sporocarps of different strains to confirm whether the specific strains of this study do not produce the bio-active compounds of interest, result would agree with other studies in the literature, which have confirmed that not all of the *Pleurotus* strains studied are statin producers [18,49].

To determine the structure of said metabolites, similar work is being conducted to use a preparative HPLC-UV technique to separate sufficient quantities of the major compounds that were detected analytically.

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References

[1] K. Almuti, R. Rimawi, D. Spevack, R.J. Ostfeld, Int. J. Cardiol. 109 (2006) 7.
[2] N.R Veillard, F. Mach, Cell. Mol. Life Sci. 59 (2002) 1771.
[3] C. Chegwin-Angarita, I.J. Nieto-Ramírez, L. Atehortúa, L. Sepúlveda, Rev. Colombiana Biotecnol. 14 (2012) 157.
[4] M. Endres, Atheroscler. Suppl. 7 (2006) 31.
[5] O. Stüve, S. Youssef, S. Dunn, A.J. Slavin, L. Steinman, S.S. Zamvil, Cell. Mol. Life Sci. 60 (2003) 2483.
[6] A. Sassano, L.C. Platanias, Cancer Lett. 260 (2008) 11.
[7] E. Istvan, Atheroscler. Suppl. 4 (2003) 3.
[8] J. Barrios-González, R.U. Miranda, Appl. Biochem. Biotechnol. 85 (2010) 869.
[9] B.P. Panda, S. Javed, M. Ali, Biotechnol. Bioprocess Eng. 14 (2009) 123.
[10] B.P. Panda, S. Javed, M. Ali, Food Bioprocess Technol. 3 (2010) 373.
[11] K. Gupta, P.K. Mishra, P. Srivastava, Biotechnol. Bioprocess Eng. 14 (2009) 207.
[12] S.A. Sayyad, B.P. Panda, S. Javed, M. Ali, Appl. Microbiol. Biotechnol. 73 (2007) 1054.
[13] M.A. Dhale, S. Divakar, S.U. Kumar, G. Vijayalakshmi, Appl. Microbiol. Biotechnol. 73 (2007) 1197.
[14] M. Bizukojc, S. Ledakowicz, J. Biotechnol. 130 (2007) 422–435.
[15] E. Rodríguez, J.L. Casas, M.A. Vilches, J.A. Sánchez, J.L. García, Y. Chisti, Biochem. Eng. J. 29 (2006) 1.
[16] B.-J. Xu, Q.-J. Wang, X.-Q. Jia, C.-K. Sung, Bioprocess Biosyst. Eng. 10 (2005) 78.
[17] J.L. Casas, J.A. Sánchez, J.M. Fernández, F.G. Acién, E. Molina, Y. Chisti, Enzyme Microb. Technol. 33 (2003) 270.
[18] I. Schneider, G. Kressel, A. Meyer, U. Krings, R.G. Berger, A. Hahn, J. Funct. Foods 3 (2011) 17.
[19] J. Alarcón, S. Águila, Z. Naturforsch. C: J. Biosci. 61c (2006) 95.
[20] J. Alarcón, S. Águila, P. Arancibia-Avila, O. Fuentes, E. Zamorano-Ponce, M. Hernández, Z. Naturforsch. C: J. Biosci. 58c (2003) 62.
[21] N. Gunde-Cimerman, A. Cimerman, Exp. Mycol. 19 (1995) 1.
[22] N. Gunde-Cimerman, A. Plemenitas, A. Cimerman, FEMS Microbiol. Lett. 113 (1993) 333.
[23] N. Gunde-Cimerman, J. Friedrich, A. Cimerman, N. Benicki, FEMS Microbiol. Lett. 111 (1993) 203.
[24] E. Wisbeck, A.P. Robert, S.A. Furlan, Rev. Saúde e Ambiente 3 (2002) 7.
[25] V.P. Gerasimenya, O.V. Efremenkova, O.V. Kamzolkina, T.A. Bogush, T. A. Bogush, V.A. Zenkova, Int. J. Med. Mushrooms 4 (2004) 106.
[26] R. Cohen, L. Persky, Y. Hadar, Appl. Microbiol. Biotechnol. 58 (2002) 582.
[27] P. Zapata, D. Rojas, L. Atehortúa, Int. J. Med. Mushrooms 14 (2012) 197.
[28] D.-J. Yang, L.S. Hwang, J. Chromatogr. A 1119 (2006) 277.
[29] I.J. Nieto R, C. Chegwin A, J. Chil. Chem. Soc. 53 (2008).
[30] P.A. Martos, W. Thompson, G.J. Diaz, World Mycotoxin J. 3 (2010) 205.
[31] L. Novakova, D. Satınsky, P. Solich, TRAC—Trends Anal. Chem. 27 (2008) 352.
[32] S. Bauer, J. Mwinyi, A. Stoeckle, T. Gerloff, I. Roots, J. Chromatogr. B 818 (2005) 257.
[33] A. Álvarez-Lueje, J. Pastine, J.A. Squella, L.J. Nuñez-Vergara, J. Chil. Chem. Soc. 50 (2005) 639.
[34] S.J. Rajh, S. Kreft, B. Strukelj, F. Vrecer, Croat. Chem. Acta 76 (2003) 263.
[35] S. Erturk, A. Onal, S.M. Cetin, J. Chromatogr. B 793 (2003) 193.
[36] L.Y. Ye, P.S. Firby, M.J. Moore, Ther. Drug Monit. 22 (2000) 737.
[37] A. Kocijan, R. Grahek, A. Bastarda, L.Z. Kralj, J. Chromatogr. B 822 (2005) 311.
[38] H.J. Kim, I.-S. Lee, S.S. Kang, Stud. Natl. Prod. Chem. 33 (2006) 751.
[39] M. Bizukojc, B. Pawlowska, S. Ledakowicz, J. Biotechnol. 127 (2007) 258.
[40] M. Manzoni, S. Bergomi, M. Rollini, V. Cavazzoni, Biotechnol. Lett. 21 (1999) 253.
[41] M. Manzoni, M. Rollini, S. Bergomi, V. Cavazzoni, Biotechnol. Tech. 12 (1998) 529.
[42] I.J. Nieto R., C. Chegwin A, J. Chil. Chem. Soc. 58 (2013) 1580.
[43] T. Eksangsri, H. Habaki, J. Kawasaki, Chem. Eng. Process. 43 (2004) 1203.
[44] J. Nti-Gyabaah, Y.C. Chiew, J. Chem. Eng. Data 53 (2008) 2060.
[45] A.R. El-Shami, E.R. Hamed, Egypt. J. Biotechnol. 25 (2007) 102.
[46] R.C. Pansuriya, R.S. Singhal, Braz. J. Microbiol. 41 (2010) 164.
[47] H.R. Valera, J. Gomes, R. Lakshmi, S. Gururaj, D. Suryanarayan, Kumarc, Enzyme Microb. Technol. 37 (2005) 521.
[48] P.-L. Wei, Z.-N. Xu, P.-L. Cen, J. Zhejiang Univ.—Sci. A 8 (2007) 1521.
[49] A. Gil-Ramirez, C. Clavijo, M. Palanisamy, C. Soler-Rivas, A. Ruiz-Rodriguez, F.R. Marín, G. Reglero, M. Pérez, in: Proceedings of the 7th International Conference on Mushroom Biology and Mushroom Products, 2011, pp. 110–119.