Optimization and correlation of HPLC-ELSD and HPLC–MS/MS methods for identification and characterization of sophorolipids

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ABSTRACT

High-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) and HPLC with electrospray ionization tandem mass spectrometry (HPLC–ESI-MS/MS) methods were implemented and optimized to separate and identify sophorolipids (SLs) produced by *Rhodotorula bogoriensis* and *Starmerella bombicola*. SLs are carbohydrate-based amphiphilic biosurfactants with increased interest in pharmaceutical and environmental areas. *Rhodotorula bogoriensis* and *Starmerella bombicola* are mainly producers of respectively C22, and C16 and C18 SLs. Mass fragmentation patterns of SLs produced by both yeasts were investigated by HPLC–ESI-MS/MS in the positive mode for $[M+Na]^+$. Based on the established fragmentation pattern, SLs produced by both yeasts were identified and characterized. A correlation between HPLC-ELSD and HPLC- ESI-MS/MS methods was established and made possible the identification of SLs by the HPLC-ELSD technique. TLC is a common tool for the analysis of SLs mixtures. In this work, TLC scrapped bands were analysed by HPLC-ELSD and HPLC–MS allowing the correlation between R_F values and the identification of sophorolipids by this technique. Identification of monoacetylated and diacetylated C24:0 hydroxy fatty acids sophorolipids produced by *Rhodotorula bogoriensis* was for the first time accomplished with this study. Although present in lower quantity these longer chain SLs can assume special importance regarding their biological activity and surface active properties.

1. Introduction

Sophorolipids (SLs) are carbohydrate-based compounds, with a carbohydrate (hydrophilic) head and a lipid (hydrophobic) tail. A sophorose (glucose dimer) moiety is linked to a long hydroxy fatty acid tail by a glycosidic bond between the 1′-hydroxy group of the sophorose and a carbon of the fatty acid [1]. SLs can differ in saturation grade, chain length and position of hydroxylation of the hydroxy fatty acid. They can occur in the acidic or lactonic forms (carboxylic end esterified at 4″ or in some cases at 6′ or 6″ position), showing different grade of acetylation of the sugar moiety on 6′ and/or 6″ positions of sophorose (Fig. 1) [2,3].

The occurrence of different structures of SLs is dependent on producer species, substrates types and fermentation conditions. SLs are mainly produced by non-pathogenic yeasts species, including *Candida apicola* [4], *Rhodotorula bogoriensis* [5], *Candida bombicola* [6], *Wickerhamiella domericqiae* [7] and *Candida bastistae* [8].

Candida bombicola (a teleomorph of *Starmerella bombicola*) [9] produces SLs in the lactonized (Fig. 1-A₁) and acidic (Fig. 1-A₂) forms with different acetylation degree on sophorose moiety. The

hydroxy fatty acid can vary in saturation grade, hydroxylation position (terminal or sub-terminal) and chain length, mostly with 16 and 18 carbon atoms [2,10–12].

Rhodotorula bogoriensis has been described as a producer of acidic sophorolipids with longer hydroxy fatty acid chain, mainly 22 carbon atoms, where the sophorose moiety is linked to 13-hydroxydocosanoic acid (Fig. 1-B) [13].

SLs are non-ionic biosurfactant compounds. Additionally to surfactant properties, SLs also demonstrate several benefit effects on skin and hair protection and different biologic activities like: germicide (against virus, bacteria, fungi and algae), anti-cancer and anti-inflammatory [3,7,14–16]. Structure–activity relationship analysis showed that the chain length of fatty acids influenced the spermicidal activity. In fact, on the basis of dose– and time–response data [14], the SL hexyl ester with a 24-carbon chain length displayed higher spermicidal activity than the SL ethyl ester with a 20-carbon chain length. Similar phenomenon, higher spermicidal activity, has been reported for two other series of longer chains lipids [14]. These characteristics have lead to several applications in the cosmetic, food, pharmaceutical, and cleaning products industries.

The main methods applied in SLs separation include thin layer chromatography (TLC) [17] and high-performance liquid chromatography (HPLC) coupled with a light scattering detector (ELSD)

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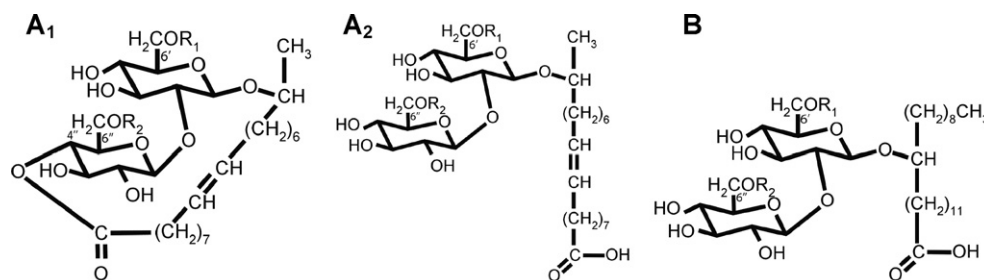E-mail address: mhribeiro@ff.ul.pt (M.H.L. Ribeiro).

Fig. 1. Examples of SLs produced by *Candida bombicola* in the lactonized form (A_1) and acid form (A_2) and acid form SLs produced by *Rhodotorula bogoriensis* (B). Different grade of acetylation on sophorose unit can occur: $R_1 = R_2 = H$; $R_1 = H$ and $R_2 = COCH_3$; $R_1 = COCH_3$ and $R_2 = H$; $R_1 = R_2 = COCH_3$.

[18–20]. Regarding SLs structure elucidation several analytical methods have been used and examples include mass spectrometry with high performance liquid chromatography (HPLC–MS) [1,10] or gas chromatography (GC–MS) [18,21,22], nuclear magnetic resonance (NMR) [23] and Fourier transform infrared spectroscopy (FTIR) [24].

TLC can be used as a preliminary method for evaluation and/or screening of SLs production. A stationary phase of silica GEL 60 or silica GEL F_{254} is frequently used in association with chloroform/methanol/water mobile phase [15,25,26]. For detection of glycolipid bands by TLC, several staining solutions in association with heat have been used and examples are anthrone [27,28], α-naphtol [15,29,30] and *p*-anisaldeheyde [26].

The lack of chromophores and fluorophore group of sophorolipids precludes the use of ultra-violet or visible (UV–Vis) and fluorescence detectors in HPLC analysis [31]. A possible alternative could be a refractive index detector but the incompatibility with gradient elution and the lack of sensitivity makes the analysis extremely difficult [32]. These problems can be overcome, selecting an evaporative light scattering detector (ELSD). Therefore, ELSD has become popular as universal detector and sophorolipids HPLC analysis has been performed with this detector. Examples are the analysis of SLs produced by *C. bombicola* from different substrates, as referred by Bogaert et al. [20] and Davila et al. [19,33].

Moreover, as no commercial standards of SLs are available, the use of liquid chromatography coupled to mass spectrometry has increased for the characterization of these compounds in complex mixtures [1,10,23]. Most of the methods described for the characterization of SLs produced by *C. bombicola* use atmospheric pressure chemical ionization source (HPLC-APCI-MS) [2,10,30]. Electrospray ionization (ESI) technique coupled with HPLC–MS has been less used; examples are HPLC–ESI-MS analyses in Davery and Pakshirajan work [24,34] and HPLC–ESI-MS^n in Hu and Ju studies [11].

The aim of this study was the implementation, optimization and correlation of analytical methods that allow simultaneously the detection and characterization of different sophorolipids produced by *Rhodotorula bogoriensis* and *Starmerella bombicola*. Preliminary studies on sophorolipids production along time were followed by TLC. Subsequently, HPLC-ELSD and HPLC–ESI-MS/MS methods were developed respectively, for determination and identification of different sophorolipids. Furthermore a data correlation between TLC, HPLC-ELSD and HPLC–ESI-MS methods was established.

2. Materials and methods

2.1. Chemicals

The following chemicals and solvents were used: glucose, malt extract, peptone and agar from Oxoid (Hampshire, UK); yeast extract from Biokar Diagnostics (Beauvais, France); oleic acid (extra pure), methanol (pro analysis), acetic acid glacial (pro analysis), sulphuric acid 95–97% (pro analysis), formic acid (pro analysis), and

p-anisaldehyde (for synthesis) from Merck (Darmstadt, Germany); acetonitrile (HPLC grade) and methanol (HPLC grade) from Fisher Scientific (Leicestershire, UK); tetrahydrofuran (HPLC grade) from Riedel (Seelze, Germany); acetonitrile (LC–MS grade), from Scharlau (Sentmenat, Spain); ethyl acetate (pro analysis) and chloroform (pro analysis) from Carlo Erba (Rodano, Italy); water purified using a Milli-Q purification system (Millipore).

Sopholiance S, a commercially available SLs mixture produced by *Candida bombicola*, was a gift from SOLIANCE (France). It consisted in a mixture of acidic and lactonic SLs under the form of a 65% aqueous solution. Fatty acid profile of the SLs mixture include mainly C18:1 (59%), C18:2 (21%) and C18:3 (11%) and the major compound present was the 1′4″-Lactone, 6′,6″-diacetate SL. Some of the SLs in extracts obtained in *Starmerella bombicola* fermentation were similar to SLs in Sopholiance S.

2.2. Microorganisms and culture conditions

Rhodothorula bogoriensis DSM 70872 was obtained from the German Resource Centre for Biological Material, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH) and *Starmerella bombicola* CBS 6009 from Fungal Biodiversity Centre, CBS (Centraalbureau voor Schimmelcultures). *Rhodotorula bogoriensis* and *Starmerella bombicola* were maintained at 4 °C, respectively on Yeast extract–Malt extract (YM) and Glucose–Peptone–Yeast extract (GPY) agar slants, with monthly transfers.

For pre-cultivation of *Rhodotorula bogoriensis*, four colonies were used to inoculate 10 mL of YM seed medium (glucose 1% (w/v), yeast extract 0.3% (w/v), malt extract 0.3% (w/v), peptone 0.5% (w/v)) in a shake flask (50 mL) maintained for 48 h at 25 °C on a rotatory shaker (150 rpm). The same procedure was applied for *Starmerella bombicola* growth, using GPY (glucose 2% (w/v), yeast extract 0.5% (w/v), peptone 0.5% (w/v)) as seed medium.

The production medium was YM for *Rhodotorula bogoriensis* and GPY medium supplied of 2% (v/v) of oleic acid as lipidic carbon source for *S. bombicola*. The SLs production was carried out in shake flasks (150 mL) with 50 mL of medium. The production was initiated after inoculating 10% (v/v) of a seed medium volume and incubated at 25 °C on a rotatory shaker (150 rpm), for 96 h and 144 h. All assays were carried out in triplicate.

2.3. Isolation of sophorolipids

SLs mixture composition changed along fermentation process. In fact, acid SLs produced by *Rhodotorula bogoriensis* can suffer deacetylation along time, while SLs from *Starmerella bombicola* can be converted into lactones increasing the mixture in lactonic SLs. Therefore in this work, 96 and 144 h were chosen to investigate SLs production at the stationary phase.

After 96 h and 144 h of *Rhodotorula bogoriensis* culture, the medium was extracted twice with equal volume of ethyl acetate,

centrifuged at 5000 rpm for 8 min, at room temperature. After dehydration with sodium sulphate, extracts were evaporated to dryness in a rotatory evaporator, at 40 °C. *Starmerella bombicola* culture medium was submitted to the same procedure described for *Rhodotorula bogoriensis*, afterwards evaporation extracts were washed twice with 3 volumes of *n*-hexane to remove the still remaining oleic acid. Residual *n*-hexane was removed under nitrogen stream.

Therefore, after evaporation to dryness, final products (SLs) were dissolved to constant volume in ethyl acetate, one aliquot (5 μL) was used for TLC analysis and another was diluted in appropriate solvent for further HPLC-ELSD and HPLC–ESI-MS/MS analysis.

2.4. Analysis and characterization of sophorolipids

2.4.1. TLC

The sophorolipids produced by *Rhodotorula bogoriensis* and *Starmerella bombicola* were analysed using thin layer chromatography. The stationary phase selected was Merck silica Gel 60 F$_{254}$ 20 × 20 TLC plates [21,25] and the mobile phase composition was chloroform:methanol:water (65:15:2). A volume of 5 μL was applied on TLC plates. The visualization of compound bands was performed by spraying the TLC plate with *p*-anisaldehyde reagent and heated at 110 °C for 10 min [26].

SLs in Sopholiance S were extracted twice with equal volume of ethyl acetate, and after solvent evaporation a 1:10 dilution (v:v) with ethyl acetate was made. A volume of 5 μL was applied on TLC plates.

In order to identify the SLs (produced by *Starmerella bombicola*) in the TLC bands and correlate this technique with HPLC-ELSD and HPLC–ESI-MS/MS, TLC plates were placed under iodine vapours. The bands were marked, and after iodine evaporation they were scraped off. The collected silica gel powder was extracted with the mobile phase (chloroform:methanol:water), centrifuged, and the upper phase was evaporated, dissolved in methanol, and analysed by HPLC-ELSD and HPLC–ESI-MS/MS.

2.4.2. HPLC-ELSD

Sophorolipids were analysed by a HPLC method using a C18 reversed phase column, RP 18 Lichrospher 100 (250 mm × 4.6 mm id, 5 μm particle size) from Merck with a guard column with the same stationary phase. The liquid chromatography system comprised a Waters^{TM} Alliance 2695 HPLC Separation Module coupled to a Eurosep Instruments DDL31 evaporative light scattering detector. Nitrogen gas was generated from a nitrogen source (Parker-Domnick Hunter) and used as nebulization gas with a pressure of 1.5 bar. Mobile phase was filtered through 0.22 μm GH polypropylene membrane (Pall Corporation) before HPLC analysis. The flow rate was constant at 0.8 mL min^{-1}, the injection volume was 40 μL, and samples were maintained at 15 °C. The equipment was controlled by Waters Empower Software.

The HPLC-ELSD method described above is very useful for SLs analysis since their detection cannot be achieved with conventional UV-Vis and fluorescence detectors and when HPLC–MS equipment is not available.

2.4.3. HPLC–ESI-MS/MS

The identification of sophorolipids was performed by HPLC–ESI-MS/MS. A reversed phase Waters^{TM} Atlantis dC18 column (150 mm × 2.1 mm id, and 5 μm particle size) was used in a Waters^{TM} Alliance 2695 HPLC Separation Module connected to a Waters^{TM} Micromass Quatromicro API Tandem Quadrupole Mass Spectrometer equipped with a Waters^{TM} electrospray ionisation (ESI) source. The flow rate was constant at 0.25 mL min^{-1}, injection volume was 4 μL and samples were maintained at 10 °C. The

equipment was controlled by Waters Empower Software. The instrument was operated in the positive ion electrospray mode, ESI capillary voltage was optimized to 4 kV, cone voltage was set on 60 V. Source and desolvation temperature were adjusted to 120 °C and 350 °C respectively. Nitrogen gas was generated from a nitrogen source (Gasman G4800) and used to supply cone (50 L h^{-1}) and desolvation gas (750 L h^{-1}). For fragmentation experiments, different collision energies values (10–85 eV) were used depending on the compound analysed. Data acquisition and treatment were performed with MassLynx Software 4.1.

3. Results and discussion

This work is focused on the development and correlation of TLC, HPLC-ELSD and HPLC–ESI-MS/MS methods for detection and identification of SLs produced by *Rhodotorula bogoriensis* (mainly C22) and *Starmerella bombicola* (mainly C16 and C18). First, are reported the results on the implementation and optimization of the analytical methods used: TLC, HPLC-ELSD and HPLC–ESI-MS/MS. The HPLC–ESI-MS/MS analysis allowed characterization of fragmentation pattern of SLs. Afterwards the SLs analyses performed by the three methods were correlated, in order to identify the compounds in TLC bands and HPLC-ELSD analysis.

3.1. Analytical methods development

TLC is a common tool for the analysis of SLs mixtures, suitable for initial screening, with well described method conditions in the literature, however more specific methodologies, like HPLC-ELSD and HPLC–MS, have to be applied for identification and characterization of these compounds. Therefore, in this work optimization of HPLC-ELSD and HPLC–ESI-MS/MS methods was performed to provide the best conditions for SLs detection and identification.

Due to the lack of SLs standards available in the market, a commercially mixture of SLs, Sopholiance S, was used as standard to optimize the conditions of analysis and to evaluate repeatability at the beginning of each sample set analysis.

3.1.1. HPLC-ELSD

In order to implement the HPLC-ELSD chromatographic conditions different nebulization (NT) and evaporation (ET) temperatures were tested, respectively from 28 to 40 °C and from 40 to 60 °C. Sensitivity gain selected was 500, the nebulization temperature was 40 °C and evaporation temperature 50 °C, under nitrogen at 1.5 bar. Acetonitrile/water mobile phase was tested and following some adjustments, a suitable resolution was attained using a linear gradient for 55 min, starting with 30% of acetonitrile. To improve method resolution and peak shape, especially of SLs produced by *Rhodotorula bogoriensis*, formic acid was added at a percentage of 0.05%. The linear gradient elution with water:formic acid (99.95%:0.05%) (eluent A) and acetonitrile (eluent B) mobile phase, went from 30% to 100% of acetonitrile in 56 min. Using the above mentioned conditions an improvement on chromatographic efficiency was verified. The number of theoretical plates was calculated (USP plate count) and values between 6×10^4 and 8×10^4 were obtained for compounds detected in *Rhodotorula bogoriensis* samples.

3.1.2. HPLC–ESI-MS

The first step in the HPLC–ESI-MS analysis was the adjustment of chromatographic (mobile phase and gradient) and ionization conditions (capillary voltage, cone voltage). The chromatographic conditions were optimized and since mobile phase flow must be lower than 0.3 mL min^{-1}, a 50% starting percentage of acetonitrile in the mobile phase was selected since it did not compromise peak separation significantly. This condition resulted in earlier peak
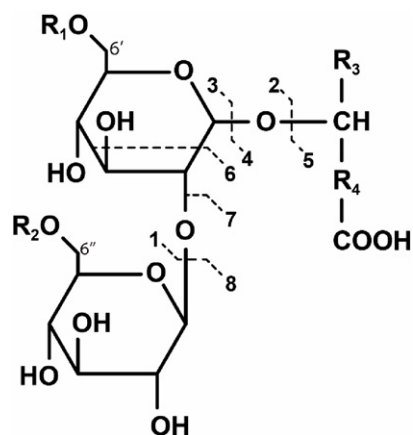
Fig. 2. MS/MS fragmentation observed for sopholipid [M+Na]$^+$ ions.

elution when compared to the HPLC-ELSD method. Mobile phase consisted of water:formic acid (99.5%:0.5%) (eluent A) and acetonitrile (eluent B). The following elution program was used: isocratic 5 min at 50% of eluent B, gradient for 20 min until 60% of eluent B, gradient for 20 min until 100% of eluent B, isocratic 10 min of 100% of eluent B and equilibrium time of 10 min. Experiments were carried out with different capillary voltage (3–4 kV) and cone voltage (30–60 V). The best conditions that allowed higher TIC signal in the chromatograms were obtained with 4 kV and 60 V, respectively.

In SLs, for the same hexose acetylation grade, retention time increased as unsaturation grade of the hydroxy fatty acid moiety decreased (*c.f.* 3.3.2). The following elution order was verified: Cn:2 fatty acid chain < Cn:1 fatty acid chain < Cn:0 fatty acid chain.

3.2. Sophorolipids pattern of fragmentation

Initially full scan MS data over the *m/z* range of 60–2000 were obtained in order to have information about the molecular ion of each compound separated in the chromatogram. In order to study the fragmentation pattern of the SLs, MS/MS experiments were performed using different collision energy values (10–85 eV).

The majority of the SLs produced by *Rhodotorula bogoriensis* and *Starmerella bombicola* showed a common fragmentation pattern regardless of the molecule. The spectrum of SLs showed a base peak corresponding to an adduct of sodium with the molecular ion [M+Na]$^+$. Generally the protonated molecule [M+H]$^+$ was not found and although [M+K]$^+$ was also present [M+Na]$^+$ was the more intense peak. MS/MS spectra are presented in Fig. 2 and product ions can be assigned to hexose moiety fragments (2, 3, 6, 7, 8), fatty acid portion fragments (5, 4) and sophorolipid fragments after losing one hexose molecule (1). Only some of these fragments (1, 3, 6, 8) were previously described [23], using FAB-MS/MS equipment for the analysis of [M+Na]$^+$ ions of SLs produced by *S. bombicola*.

Common fragments that occurred in all MS/MS spectra of acidic SLs were the sodium adduct of the disaccharide generating peaks at *m/z* 365, *m/z* 407 or *m/z* 449, depending on the acetylation grade (2) (Fig. 2). Furthermore, the hydroxy fatty acid (4) and hydroxy fatty acid followed by the loss of one water molecule (5) fragments were also present as sodium adducts in our study. A not so frequent fragment, especially in SLs produced by *S. bombicola*, was the fragment (7) corresponding to the sodium adduct of the terminal hexose [C$_i$+H+Na]$^+$ (e.g. *m/z* 203, *m/z* 245), originated from the cleavage of the glycosidic bond between hexoses. This fragment, as well as fragment (2), have been pointed out by Damon and Costelo [35] as a carbohydrate fragment (C$_i$) present in fast atom bombardment (FAB)-MS and FAB-MS/MS spectra in positive mode of glycoconjugates appearing as [C$_i$+2H]$^+$. Fragment 2 (C$_2$) and

fragment 7 (C$_1$) correspond to sodium adducts of a sophorose unit and a glucose unit respectively, with different grade of acetylation. The presence of these SLs fragments is for the first time identified on HPLC–MS or HPLC–MS/MS sophorolipids studies. Other fragments that occurred are represented as 3 and 8 and involve the loss of water when comparing to the corresponding sodium cationized fragment 2 and 7, respectively. According to Damon and Costello [35], fragment named as ^{1,3}A$_2$ represents cross-ring fragmentations (6) that occurred as sodium adducts (e.g. *m/z* 287) and were present in almost all acidic SLs MS/MS spectra. This fragment obtained from a double cleavage across the hexose ring (A ions) tend to occur under high-energy collisions from sodium adducts [36].

The fragments obtained are in accordance with Cancilla et al. [37], who referred that the three main processes in the fragmentation of alkali metal ion coordinated oligosaccharide are the loss of the metal ion, glycosidic bond cleavage and cross-ring cleavage.

Fragmentation pattern of sophorolipids (from *S. bombicola*) in the lactonized form, obtained with collision induced dissociation (CID) of [M+Na]$^+$, showed less fragments than SLs in the acidic form. In fact, in the lactonic form the disaccharide moiety fragments (2, 3) were not present and neither was fragment 6 (Fig. 2). Nevertheless, lactonic SLs identification was attained as fragments resulting by the loss of a hexose moiety (and subsequent loss of water molecule), also hydroxy fatty acid moiety fragment (4 and 5, in Fig. 2) and hexose moiety fragment (and subsequent loss of water molecule) were present. In this study, we were able to interpret the MS/MS spectra of sodium cationized lactones, [M+Na]$^+$, contrary to what was mentioned by Koster et al. [23].

Some lactonic SLs produced by *Starmerella bombicola* were also analysed by MS/MS using [M+H]$^+$ as precursor ion. The pattern of fragmentation of [M+H]$^+$ was similar to [M+Na]$^+$ but some differences were observed. The spectra of the product ion of [M+H]$^+$ (spectrum not shown) of lactonic C18:1 and C18:0 hydroxy fatty acid SLs, revealed fragments originated by consecutive loss of water molecules [M−nH$_2$O+H]$^+$ and [M−C$_6$H$_9$O$_5$R−nH$_2$O+H]$^+$ (with *n* = 1, 2, 3). This fact had already been pointed out by Koster et al. [23]. Product ion of [M+H]$^+$ spectra also showed the loss of the hydroxy fatty acid by originating disaccharide fragments at *m/z* 409, 391 and 373 that were not present on [M+Na]$^+$ spectra.

3.3. Chromatographic analysis of sophorolipids and methods correlation

3.3.1. SLs produced by Rhodotorula bogoriensis

It is known that *Rhodotorula bogoriensis* produces mainly, four different 13-hydroxydocosanoic acid sophorolipids [13] and as cultures become older deacetylation of these compounds is more evident [38,39].

Preliminary analyses of SLs produced by *Rhodotorula bogoriensis* were carried out by TLC. The 4 bands observed in Fig. 3-I correspond to the main SLs produced by *Rhodotorula bogoriensis*, where the sophorose moiety is linked to 13-hydroxydocosanoic acid. Those compounds showed *R$_F$* value of 0.18 for the deacetylated SL (A), 0.27 and 0.3 for isomers of monoacetylated SLs (B and C) and 0.41 for the diacetylated SL (D). The same SLs were detected on the HPLC-ELSD chromatogram (Fig. 3-II) showing a retention time (RT) of 26.2 (A), 30.5 (B), 31.7 (C) and 36.7 min (D).

The conditions of analyses in HPLC-ELSD and HPLC–ESI-MS methods are similar, therefore the SLs are eluted in the same order (Fig. 3-II and III). The confirmation of the C22:0 hydroxy fatty acid SLs was accomplished by the fragmentation experiments and the results are presented in Table 1. Respectively, [M+Na]$^+$ peaks detected at *m/z* 703 with a RT of 13.7 min (A), *m/z* 745 with a RT of 20.7 min (B), 22.8 min (C) and *m/z* 787 with a RT of 31.6 min (D), correspond to SLs with different acetylation grade (Fig. 3-III and
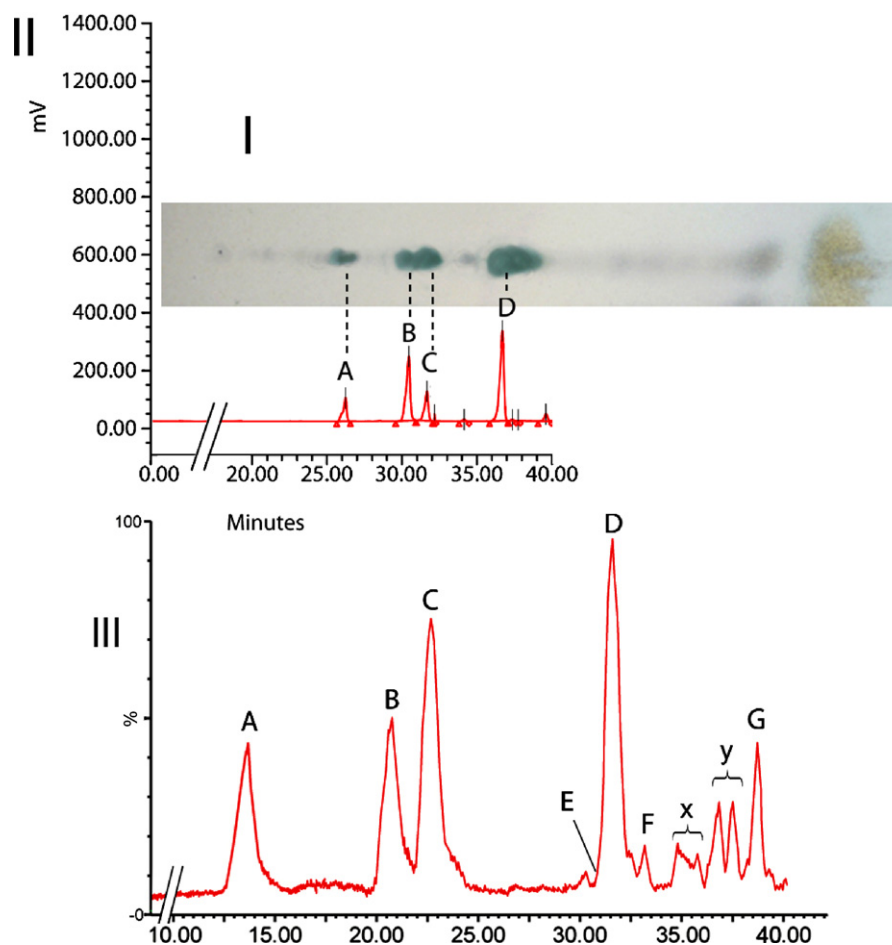
Fig. 3. SLs produced by *Rhodotorula bogoriensis.* I – TLC separation; II – HPLC-ELSD chromatogram; III – HPLC–ESI-MS TIC chromatogram. Same compounds are marked with the same letter in the chromatograms. TIC chromatogram was obtained from unpurified extract (non scraped) SLs mixture. TLC bands were correlated with HPLC-ELSD peaks and LC–ESI-MS TIC peaks via scrapping.

Table 1). These compounds were previously identified by Nuñez et al. [13].

3.3.1.1. *New compound identification.* In the present work, C24:0 hydroxy fatty acid sophorolipids (E, F and G – Fig. 3-III) were identified for the first time. Fragments identified in all MS/MS spectra are included in Table 1.

New SLs were detected in samples corresponding to adducts of molecular ions [M+Na]$^+$ at *m/z* 773 with a retention time of 31.2 and 33.2 min, and at *m/z* 815 with a retention time of 38.5 min. Spectra in Fig. 4 and Table 1 presents ions corresponding to the free acidic form of a monoacetylated and diacetylated sophorolipid with a 24 carbon saturated hydroxy fatty acid. Nuñez et al. [13] identified C22:0 hydroxy fatty acid sophorolipids by APCI-MS and mentioned that after hydrolysis traces of a hydroxy fatty acid with

Table 1
Product ions obtained in MS/MS spectra of [M+Na]$^+$ ions (product ion scan) of SLs produced by *Rhodotorula bogoriensis*.

		C22:0				C24:0		
		deAc	6″-Ac	6′-Ac	6′,6″-Ac	6″-Ac	6′-Ac	6′,6″-Ac
[M+Na]$^+$		703 (A)	745 (B)	745 (C)	787 (D)	773 (E)	773 (F)	815 (G)
(1) M + Na−C$_6$H$_9$O$_5$R	R = H	541	–	583	–	–	611	–
	R = COCH$_3$	–	541	–	583	569	–	611
(2) RC$_{12}$H$_{20}$O$_{11}$R + Na		365	407	407	449	407	407	449
(3) RC$_{12}$H$_{20}$O$_{11}$R + Na−H$_2$O		347	389	389	431	389	389	431
(4) FA(OH) + Na		379	–	–	–	–	–	–
(5) FA(OH) + Na−H$_2$O		361	361	361	361	–	–	–
(6) C$_6$H$_9$O$_5$R + C$_2$H$_3$O$_2$ + Na	R = H	245	–	245	–	–	245	–
	R = COCH$_3$	–	287	–	287	287	–	287
(7) C$_6$H$_{11}$O$_6$R + Na	R = H	203	–	–	–	–	–	–
	R = COCH$_3$	–	–	–	245	245	–	245
(8)	R = H	185	–	185	–	–	185	–
C$_6$H$_{11}$O$_6$R + Na−H$_2$O	R = COCH$_3$	–	227	–	227	227	–	227

Ac, COCH$_3$; 6′ and 6″, acetylation position in hexose rings; FA(OH), hydroxylated fatty acid.
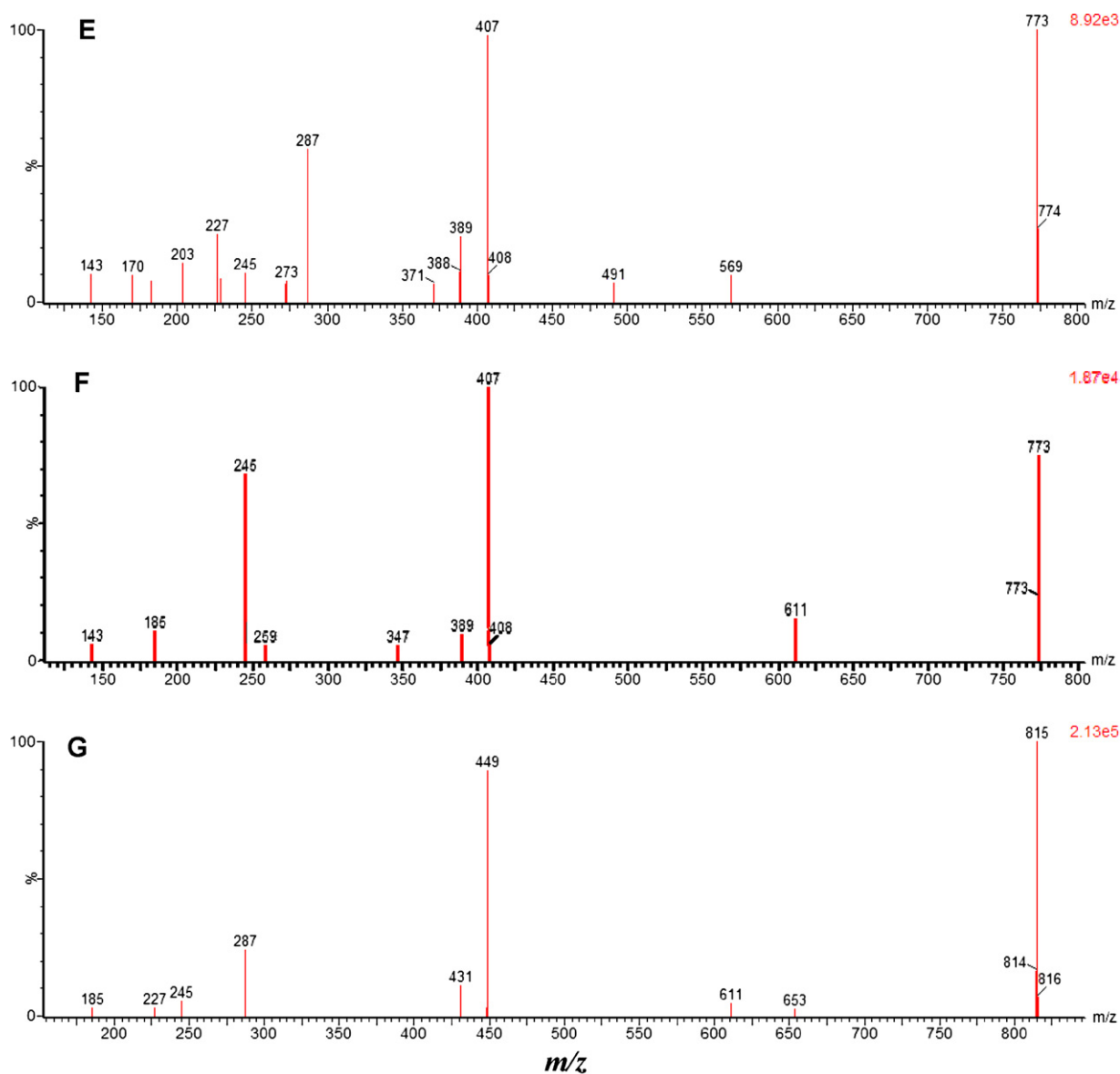
Fig. 4. MS/MS spectra of [M+Na]$^+$ ions of SLs produced by *Rhodotorula bogoriensis*. E – *m/z* 773 (RT = 31.2 min); F – *m/z* 773 (RT = 33.2 min) (F); G – *m/z* 815 (RT = 38.5 min).

24 carbon chain SL was present, but they were not able to identify these compounds.

The *m/z* 773 ions are isomers differing in the acetylation position (6′ or 6″) of sophorose moiety. In the first eluted compound (E) a fragment at *m/z* 569 corresponds to the loss of an acetylated hexose (204 Da) indicating 6″ acetylation while for compound in peak (F) the fragment at *m/z* 611 represents the loss of a deacetylated hexose (162 Da) indicating 6′ acetylation. The loss of the fatty acid moiety (366 Da) originates the monoacetylated disaccharide fragment at *m/z* 407 and peaks at *m/z* 389 and *m/z* 371 that stand for the loss of one or two H$_2$O molecules from *m/z* at 407. Different fragments could also be observed on the MS/MS spectra of the isomers. The peak with retention time 31.2 min gave peaks at *m/z* 287 (264 Da) and *m/z* 227 (204 Da) while the peak with the retention time 33.2 gave peaks at *m/z* 245 (222 Da) and *m/z* 185 (162 Da). According to the nomenclature suggested by Domon and Costelo [35] the sodium adducts at *m/z* 287 and 245 stand for $^{1,3}A_2$ hexose cross ring fragment, derived from the cleavage of two bonds in the glycosidic ring, including a acetylated and deacetylated terminal

hexose, respectively. Furthermore, when SL had a terminal acetylated hexose, the fragment at *m/z* 287, showed large intensity, but when the terminal hexose was deacetylated appeared the fragment at *m/z* 245 (Fig. 4-E, F).

The peak at 38.5 min corresponding to [M+Na]$^+$ at *m/z* 815 presents analogous fragments (Fig. 4-G and Table 1). The peak at *m/z* 611 corresponded to the loss of an acetylated hexose (204 Da) and a diacetylated disaccharide fragment at *m/z* 449 (426 Da) can also be observed, which after losing one water molecule appeared as *m/z* 431.

Moreover a base peak at *m/z* 717 corresponding to a sodium adduct with a C20:0 hydroxy fatty acid monoacetylated SL in the acidic form [M+Na]$^+$ was also found in the samples analysed. In MS/MS spectra (data not shown) the loss of an acetylated hexose ring was observed at *m/z* 513, the presence of a monoacetylated disaccharide was shown by peaks at *m/z* 407 and 389 and a cross ring fragment at *m/z* 287 was also present.

In the total ion current (TIC) chromatogram, at 35.1 and 35.7 min, were detected two compounds (marked as *x* in Fig. 3-III)
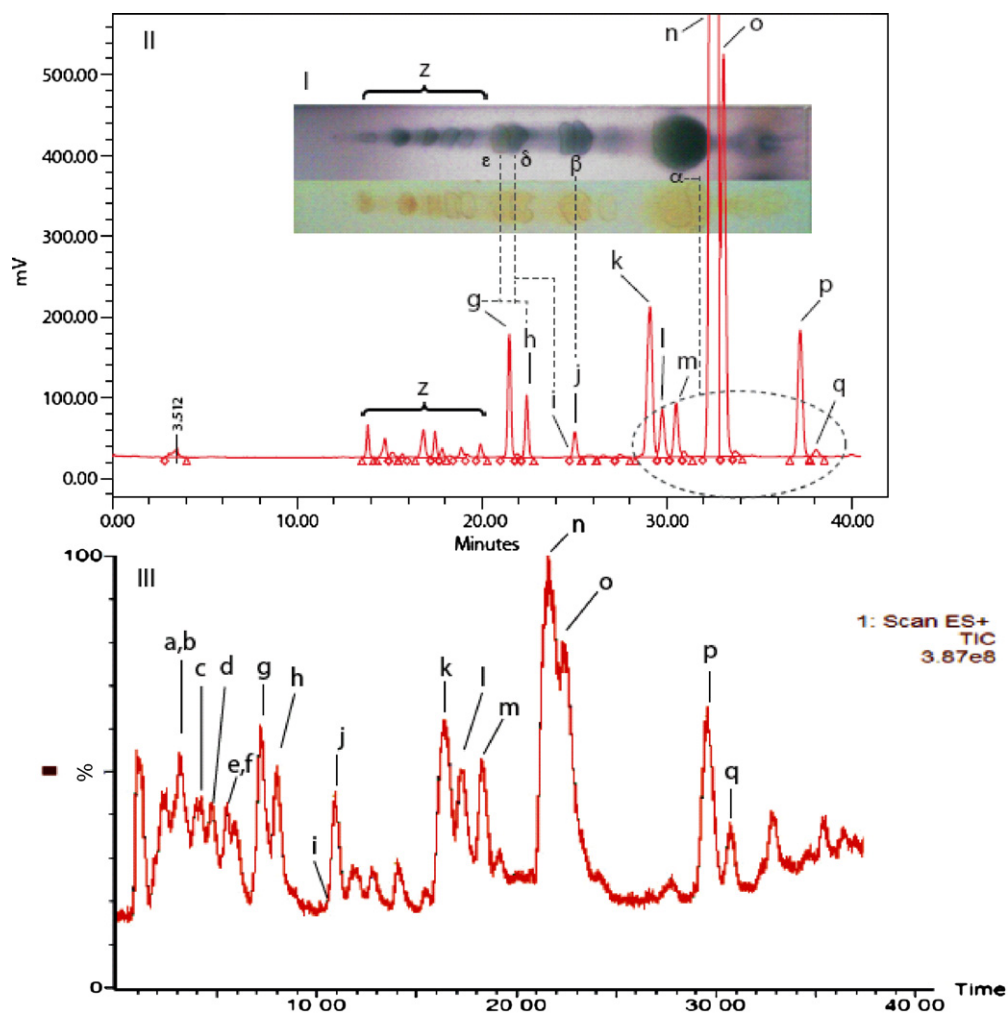
Fig. 5. SLs produced by *Starmerella bombicola* when supplied of glucose and oleic acid. I – TLC separation with *p*-anisaldehyde and iodine vapours; II – HPLC-ELSD chromatogram; III – HPLC/MS TIC. Same compounds are marked with the same letter in the chromatograms. TIC chromatogram was obtained from unpurified extract (non scraped) SLs mixture. TLC bands were correlated with HPLC-ELSD peaks and LC–ESI-MS TIC peaks via scrapping.

with the base peak at m/z 801. The MS/MS spectra revealed characteristic fragments of sophorolipids, but further work must be performed to identify those compounds.

The fragmentation spectra of the peaks detected at 36.8 and 37.5 min (marked as y in Fig. 3-III) showed a base peak at m/z 583 corresponding to a C22:0 hydroxy fatty acid linked to one acetylated hexose (Table 1). The MS/MS experiments revealed hydroxy fatty acid fragments at m/z 379 and m/z 361 (after the loss of one water molecule) and also acetylated hexose fragments at m/z 245 and m/z 227 (Table 1).

3.3.2. SLs produced by S. bombicola

The methodology used for the analysis of SLs from *R. borgoriensis* was applied to the analysis of SLs produced, in GPY medium supplied with oleic acid, by *S. bombicola*.

In Fig. 5-I are presented the TLC bands (using *p*-anisaldehyde and iodine staining solutions) corresponding to SLs mixtures. From the analysis of these bands (*c.f.* 2.4), by HPLC–MS, was possible to conclude that SLs first eluted by TLC are all lactonic diacetylated, identified as: lactonic C18:2 diacetylated SL (k), lactonic C16:0 diacetylated SL (l, m), lactonic C18:1 diacetylated SL (n, o) and lactonic C18:0 diacetylated SL (p, q) (Fig. 5 and Table 2). These compounds were present in α bands (R_F 0.71, and R_F 0.77), but compounds p and q were mainly present in the R_F 0.77 band. Asmer et al.

[17] identified lactonic C18:1 and C18:0 SLs in the first TLC band, but our results showed that others SLs are eluted, simultaneously. Also Davila et al. [18] referred that the first eluted band corresponded to 6′ and 6″ diacetylated SLs supporting our results. Another compound identified, was the lactonic C18:1 monoacetylated SL with an R_F of 0.52 (β in Fig. 5-I) corresponding to j (Fig. 5 and Table 2), also identified by Davila et al. [18]. Moreover acidic SLs were identified on band δ (R_F 0.37) and ε (R_F 0.34) as C18:1 diacetylated SLs, while C18:0 diacetylated SLs was present on band δ (Fig. 5-I).

HPLC-ELSD and HPLC–ESI-MS simultaneous analysis of TLC scraped bands allowed comparison between the different chromatographic methodologies. This information is summarized in Fig. 5 and Table 2 and was achieved for lactonic and some acid SLs. The correlation of compounds marked as z in TLC plates and HPLC-ELSD chromatograms (Fig. 5-I and II) was not accomplished by HPLC–MS analysis since the corresponding scraped bands extracts was not achieved. However, they probably correspond to the peaks labeled as a–f (Fig. 5-III and Table 2) identified on unpurified extract analyzed by HPLC–MS/MS.

The SLs produced by *Starmerella bombicola* were identified (on SLs mixture extract) performing MS/MS experiments and showed to be 18 and 16 carbon hydroxy fatty acid SLs. The compounds identified by mass spectrometry showed the same pattern of fragmentation referred in Fig. 2 and are assigned in Table 2 where acidic

Table 2

SLs produced by *Starmerella bombicola* identified (by product ion scan) in CID spectra of [M+Na]+ ions and in some cases also [M+H]+, that also allowed TLC and HPLC-ELSD SLs identification.

Peak	Detection			MW	SLs	References
	TLC	HPLC ELSD	HPLC MS			
a			√	620	A – C18:2 deacetylated	–
b			√	622	A – C18:1 deacetylated	[23]
c			√	706?	A – C18:1 diacetylated[c]	–
d			√	664	A – C18:1 monoacetylated	[23]
e; f			√	704	A – C18:2 diacetylated[b]	–
g; h	√	√	√	706	A – C18:1 diacetylated[b]	[23]
i	√	√	√	708	A – C18:0 diacetylated	–
j	√	√	√	646	L – C18:1 monoacetylated	[23]
k	√	√	√	686	L – C18:2 diacetylated	[10]
l; m	√	√	√	662	L – C16:0 diacetylated[a]	[10]
n; o	√	√	√	688	L – C18:1 diacetylated[a]	[1,10,23]
p; q	√	√	√	690	L – C18:0 diacetylated[a]	[10]

√ – identification accomplished with mentioned methodology; A – free acid form; L – lactonic form.

[a] Isomers: the 1′-hydroxy group of the sophorose sugar is linked to the $\omega - 1$ or ω carbon of the fatty acid corresponds to the first and second SL eluted respectively [1,10].

[b] Likely to be $\omega - 1$ or ω isomers.

[c] C18:1 diacetylated SLs adduct?

SLs correspond to compounds represented from *a* to *i* and lactonic SLs from *j* to *q*.

Acidic C18:2 diacetylated SLs, *e* and *f* (Fig. 5-III), were identified and those isomers are likely to include, respectively, a sub-terminal ($\omega - 1$) and terminal (ω) hydroxylated fatty acid. Moreover, C18:1 diacetylated acidic SLs were also identified in peak *g* and *h* (Fig. 5-III) and the same assumption was made. This could be also verified with the lactonic C16:0 diacetylated SL (*l*, *m*), lactonic C18:1 diacetylated SLs (*n*, *o*) and lactonic C18:0 diacetylated SLs (*p*, *q*). Similar elution order was verified for SLs isomers by Ahsby et al. [1] and Nuñez et al. [10].

In the present study correlation of SLs elution order was established by simultaneous TLC, HPLC-ELSD and HPLC–MS/MS analysis. The SLs mixture was produced by *Starmerella bombicola* when glucose and oleic acid were supplied. *Starmerella bombicola* produces a vast number of different SLs that are difficult to identify when using TLC and HPLC-ELSD methods. With the HPLC–MS/MS equipment, identification of compounds present in TLC bands and HPLC-ELSD chromatogram was much effortless and less time consuming than other methodologies used in the past, like SLs hydrogenation and hydrolytic reactions, followed by determination of the hydroxy fatty acid moiety by GC–MS [18].

3.3.2.1. New compound identification. Our study showed that C18:2 deacetylated acidic SL was produced in the medium with glucose supplied of oleic acid and this compound had not been reported so far.

In this work, the C18:2 diacetylated and C18:0 diacetylated acidic SLs were also produced with the oleic acid addition to the medium with glucose and had only been mentioned when *Candida bombicola* growth medium was supplied of glucose and soybean oil as carbon sources [10,11].

At 3.9 min the chromatographic peak presented a base at *m/z* 1137. The MS/MS spectra of this peak (data not shown) revealed the loss of a diacetylated hexose dimer (408 Da) originating a fragment at *m/z* 729. This fragment (*m/z* at 729) most likely represents a sodium adduct of a C18:1 diacetylated acidic SL, since other fragments present in CID spectra equal the ones obtained for peak *g* and *h* (Fig. 5-III and Table 2). The compound with a base peak at *m/z* 1137 seems to include a C18:1 diacetylated acidic SL structure linked to a diacetylated hexose dimer. It is represented as compound *c* in Table 2 considering that it is an adduct of a different acidic C18:1 hydroxy fatty acid SL (MW 706).

4. Conclusions

The analytical methods described in this work are for the first time the correlation of HPLC-ELSD and HPLC–ESI-MS/MS methods for separation and identification of sophorolipids produced by *Rhodotorula bogoriensis* and *S. bombicola*. The method optimization was crucial to provide the best conditions for SLs detection and identification.

Simultaneous analyses with both techniques, HPLC-ELSD and HPLC–MS, of the TLC scrapped bands also allowed establishing a correlation between R_F values and identification of sophorolipids.

As common knowledge the HPLC–MS/MS technique is a powerful tool in the identification and characterization of compounds and samples. The developed method of HPLC–ESI-MS gave contribute to the existing methods for SLs analysis since most of HPLC–MS methods published used an APCI probe. HPLC–ESI-MS/MS analysis allowed identification and guarantee that sophorolipids were present in samples, confirming the reliability of HPLC-ELSD analysis.

A [M+Na]+ fragmentation pattern of SLs produced by *Rhodotorula bogoriensis* and *Starmerella bombicola* was established. Fragmentation pattern of lactonic SLs differed of acid ones by lack of internal hexose fragments and hexose dimers fragments, and the respective identification was also accomplished.

The HPLC–ESI-MS/MS allowed for the first time the identification of C24:0 monoacetylated and diacetylated SLs produced by *Rhodotorula bogoriensis*.

This work is an important contribution for the use of HPLC-ESLD method in SLs analysis. The correlation established between the methods (HPLC-ELSD and HPLC–MS) allowed identification of compounds on the HPLC-ELSD chromatogram.

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