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Journal of Chromatography A, 864 (1999) 211–220

JOURNAL OF CHROMATOGRAPHY A

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High-performance liquid chromatography method for the characterization of rhamnolipid mixtures produced by *Pseudomonas aeruginosa* UG2 on corn oil

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Received 30 June 1999; received in revised form 13 September 1999; accepted 13 September 1999

Abstract

A HPLC method was developed to quantify rhamnolipid species in a bacterial biosurfactant mixture. The biosurfactant mixtures containing mainly 3-[3′-(L-rhamnopyranosyl-oxy)decanoyloxy]decanoic acid ($RhC_{10}C_{10}$), 3-[3′-(2′-*O*-α-L-rhamnopyranosyl-oxy)decanoyloxy]decanoic acid ($Rh_2C_{10}C_{10}$), 3-[3′-(2′-*O*-α-L-rhamnopyranosyl-oxy)decanoyloxy]dodecanoic acid ($Rh_2C_{10}C_{12}$), and a dehydrogenated variety of the latter, 3-[3′-(2′-*O*-α-L-rhamnopyranosyl-oxy)decanoyloxy]dodecenoic acid ($Rh_2C_{10}C_{12}-H_2$), were isolated from *Pseudomonas aeruginosa* UG2 cultures grown on corn oil as sole carbon. The rhamnolipid species were identified and quantified after their derivatization to the corresponding phenacyl esters. To confirm the reliability of the HPLC method, the biosurfactant mixtures and the HPLC isolated species were further analyzed. Mass spectroscopy (electrospray ionization and atmospheric pressure chemical ionization techniques) was used to confirm their molecular mass, gas chromatography to verify their fatty acid content, and a colorimetric assay to quantify the rhamnose content.

Keywords: *Pseudomonas aeruginosa*; Rhamnolipids; Lipids

1. Introduction

Biosurfactants are amphipatic molecules produced by a wide variety of bacteria, yeast and filamentous fungi. They include peptides, glycolipids, lipopeptides, fatty acids and phospholipids [1]. Up to now, the most commonly isolated and best studied group of biosurfactants are the glycolipids and phospholipids [2]. Although the exact mechanism is not clear, it is known that biosurfactants enhance the cellular uptake and use of substrates with low degrees of aqueous solubility [1]. When associated with the cell, they promote transport across the membrane, and as extracellular compounds they help to solubilize the substrate. Some microorganisms such as *Rhodoccocus* and *Corynebacterium* produce biosurfactants only during growth on hydrophobic substrates, while others like *Pseudomona aeruginosa* and *Torulopsis bombicola* seem to produce them to some degree regardless of the hydrophobicity of the substrate. The latter microorganisms are the most adapted for growing and producing biosurfactants in complex matrices such as soil systems and wastes [1].

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00979-6

Rhamnolipids produced by *P. aeruginosa* strains are among the most effective surfactants when applied for the removal of hydrophobic compounds from contaminated soils. They posses low average minimum surface tension (30–32 mN/m), high average emulsifying activity (10.4–15.5 U/ml filtrate), low critical micelle concentration (CMC) (5–65 mg/l), and high affinity for hydrophobic organic molecules [3,4]. Those properties confer upon rhamnolipids optimal characteristics as potential carriers of pollutants in soil systems. Being microbially produced, rhamnolipids' high biodegradability provides them additional advantage over synthetic surfactants when performing soil washing and bioremediation processes. Other advantages of rhamnolipids over other biosurfactants are their ease of isolation from the culture (they are exo-biosurfactants), and the fact that they can be produced using relatively cheap hydrophobic or hydrophilic substrates such as carbohydrates, hydrocarbons, vegetable oils, or even wastes from the food industry [1,3–9].

Rhamnolipids produced by *Pseudomonas* strains grown with different carbon sources have been traditionally reported as mixtures of the species RL-1 ($RhC_{10}C_{10}$), RL-2 (RhC_{10}), RL-3 ($Rh_2C_{10}C_{10}$), and RL-4 (Rh_2C_{10}) as classified by Syldatk and Wagner [10]. Several authors reported rhamnolipid mixtures produced by *P. aeruginosa* where the monorhamnolipid $RhC_{10}C_{10}$ was the predominant component [5,8,11,12]. Champion et al. [13] and Zhang and Miller [8] reported the presence of other monorhamnolipids with different fatty acid chains (C_{18}, C_{22} and C_{24}) as minor components of the monorhamnolipid mixture, but their percentages were not quantified.

While an extra rhamnose ring confers more hydrophilicity to rhamnolipids (monorhamnolipids vs. dirhamnolipids), additional carbons in the fatty acid chains can increase their hydrophobicity. These properties can affect the stability of rhamnolipids in the aqueous phase (as monomers or micellar conglomerates), their capability to solubilize hydrophobic organic compounds, and the bioavailability of such compounds [14]. More hydrophilic rhamnolipids like RhC_{10} or Rh_2C_{10} yielded CMC values as high as 200 mg/l [10], whereas lower values of 5–60 mg/l have been reported for mixtures containing mainly $RhC_{10}C_{10}$ monorhamnolipid [3,10,15]. The dirhamnolipid $Rh_2C_{10}C_{10}$ shows intermediate CMC values of 40–65 mg/l [9,10]. The $RhC_{10}C_{10}$ monorhamnolipid observed higher micellar solubilization capacity than $Rh_2C_{10}C_{10}$ with phenanthrene, but proved to reduce the extent of its bioavailability while $Rh_2C_{10}C_{10}$ enhanced it [12].

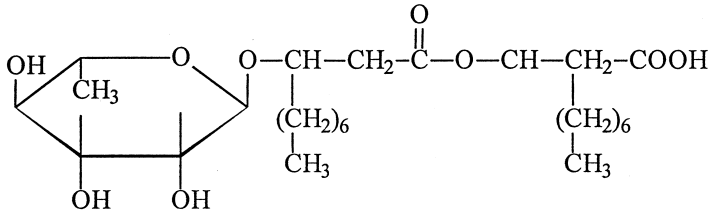
As supported by the facts previously stated, there is a need to develop accurate analysis of rhamnolipid mixtures, prior to their application on environmental processes. The composition of a rhamnolipid mixture will greatly influence its performance as a carrier of contaminants and its capability to enhance contaminants bioavailability [14]. Traditional analysis of rhamnolipids such as turbidity assays [4] or colorimetric determination of total rhamnose content [16], do not provide information on the composition of the mixture. High-performance liquid chromatography (HPLC) methods developed for more precise determination of rhamnolipid mixtures are limited to the quantification of the $C_{10}C_{10}$ species [5,11,15]. As opposed to results by other authors, rhamnolipid mixtures reported in this present work are mainly composed by dirhamnolipids where not only the $C_{10}C_{10}$, but also the $C_{10}C_{12}$ species constitute important percentages of the total amount (see Fig. 1). The present work describes the development of an HPLC method that separates and quantifies the major components of a rhamnolipid mixture (after derivatization to their phenacyl esters) obtained from *P. aeruginosa* UG2 grown on corn oil as a sole carbon source.

2. Experimental

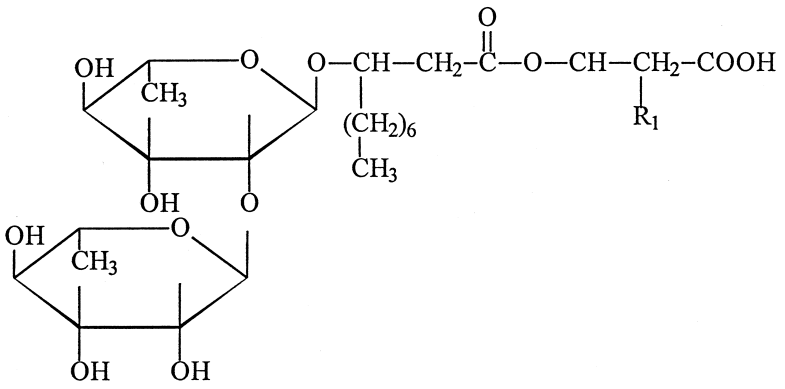
2.1. Microorganism and cultivation conditions

P. aeruginosa UG2 was provided by the Department of Environmental Biology at the University of Guelph, Ontario, Canada. Microorganisms were maintained on L-agar (Gibco) plates throughout the experiments.

For growth of *P. aeruginosa* UG2 in liquid culture, the minimal media of Hylemon and Phibbs Jr. [17] as modified by Tomasek and Karns [18] was used. Additional $(NH_4)_2SO_4$ and trace metals were delivered to the culture on identical concentrations



Monorhamnolipid ($RhC_{10}C_{10}$)



Dirhamnolipids ($Rh_2C_{10}C_n$)
n = 8, 10, 12

Fig. 1. Structures of rhamnolipids produced by *P. aeruginosa* UG2. R1=C_5H_{11}, C_7H_{15}, C_9H_{19}, or C_9H_{17} (dehydro species).

every two days up to the tenth day of cultivation. A total concentration of 12.75 g/l (15 ml/l) of corn oil (Mazola 100% corn oil CPC International) was added to the media as carbon source. Preliminary experiments (not shown) proved that this substrate concentration was optimal for obtaining high product yield combined with a minimum of impurities (mainly non-metabolized fatty acids). A pH of 6.25 was kept as recommended by Guerra-Santos et al. [6]. All the cultivation experiments were performed in 2.8 Fernbach flasks with a total broth volume of 1 l. Starter cultures (100 ml in 250-ml Tunaire flasks) were inoculated from L-agar plates. After 24 h, 50 ml was used for the inoculation of Fernbach flasks. Once inoculated, all flasks were incubated at 37°C on a gyratory shaker at 220 rpm (New Brunswick Scientific series G25) for 16 days.

2.2. Extraction of rhamnolipid mixture from culture

After 16 days, the pH of the culture was adjusted to 8.0 (using 10 *M* NaOH) and biomass was removed by centrifugation for 20 min at 10 000 *g*. The supernatant pH was adjusted to 2 (using 3 *M* H_2SO_4) and an equal volume of chloroform–methanol (2:1) was added. The mixture was shaken for 10 min. Centrifugation was performed for 10 min at 10 000 *g*, and the organic phase removed. The extraction operation was repeated once more. The rhamnolipid product was concentrated from the pooled organic phases using a rotary evaporator (Yamato Hi Tec RE-51, Water Bath BM-51). The thick yellowish product was dissolved in methanol, filtered (Sterivex-GV 0.22 μm, Millipore, Bedford, MA,

USA), and concentrated again using the rotary evaporator.

2.3. Isolation of rhamnolipid species

Rhamnolipid species were isolated from the mixture using an HPLC instrument equipped with Waters Model 712 WISP autosampler, two Waters Model 510 pumps, and a Waters Model 996 photodiode array detector. A Novapak C_{18} 100 mm×8 mm column (8NVC18, 4 μm Waters Assoc., Milford, MA, USA), and a gradient method using acetonitrile–H_3PO_4 (3.3 m*M*) (2:98, v/v, during 1 min; from 2:98 to 60:40, v/v, for 49 min; from 60:40 to 100:0, v/v, for 15 min), at a flow-rate of 1.0 ml/min was used to separate the rhamnolipid species. Fractions were collected every 3 min, and the presence of rhamnolipids species in individual fractions (from 39 to 51 min) was determined using a colorimetric assay (orcinol reaction) for rhamnose identification [16]. Briefly, samples (taken from fractions) were air dried and re-dissolved in same volume of water (pH adjusted to 7.5). To 0.1 ml of sample, 0.9 ml of a solution containing 0.19% orcinol (in 53% H_2SO_4) was added. After heating for 30 min at 80°C, the samples were cooled for 15 min at room temperature and the absorbance at 421 nm was measured with a Beckman DU-7 spectrophotometer. The concentration of rhamnolipids was calculated in terms of rhamnose content by comparing the data with those of rhamnose (Sigma) standards.

Fatty acid analyses were conducted on HPLC fractions and rhamnolipid mixtures. Samples containing rhamnolipids were dried and then saponified in 1.0 ml of 3.75 *M* NaOH solution (water–MeOH, 1:1, v/v) at 100°C for 30 min, followed by methylation in 2.0 ml of 3.0 *M* HCl–CH_3OH (1.18:1, v/v), at 80°C for 10 min. Samples were then extracted once with 1.25 ml of hexane–methyl-*tert*.-butyl ether (MTBE) (1:1, v/v). Fatty acid analysis was performed on a Hewlett-Packard 5890A GC instrument, equipped with an HP Ultra 2 crosslinked 5% Ph Me Silicone column, 25 m×0.2 mm I.D. Injector and flame ionization detection (FID) system temperatures were 250 and 300°C, respectively. Oven temperature was increased from 170 to 260°C at 5°C/min for 18 min, from 260 to 310°C at 40°C/min for 0.8 min, and held at 310°C for 1.5 min. Standards of 3-hydroxydecanoic acid (3-OHC_{10}), and 3-hydroxydodecanoic acid (3-OHC_{12}) (Sigma) were processed in an identical way to the samples.

2.4. HPLC method for derivatized rhamnolipids

Derivatization of rhamnolipid samples was performed using a modification of the technique described by Schenk et al. [11]. Briefly, samples were air dried completely and dissolved in 1 ml of CH_3CN containing 2-bromoacetophenone and triethylamine (Et_3N) in molar ratios of approximately 1:4:2 (glycolipid–2-bromoacetophenone–Et_3N). Reactions were carried out at 80°C for 1 h. Products were filtered (0.22 μm Millex-HV Millipore Products) prior to HPLC analysis.

Derivatized rhamnolipid phenacyl esters were separated by HPLC using a gradient of CH_3CN–3.3 m*M* H_3PO_4 (50:50, v/v, for 3 min; from 50:50 to 100:0, v/v, for 19 min; 100:0, v/v, for 5 min; from 100:0 to 50:50, v/v, for 3 min; 50:50, v/v, for 10 min), at a flow-rate of 1.0 ml/min on the same Novapak C_{18} (8NVC18, 4 μm) column previously used for the isolation of rhamnolipid fractions. Absorbance of derivatized rhamnolipids was measured at a wavelength of 244 nm. Isolated rhamnolipid species in fractions (fully characterized through GC, mass spectroscopy and orcinol reaction) were initially used as standards, and hydroxyatrazine (Ciba Geigy) was added as an internal standard. The compound was chosen because its UV absorbance spectrum resembles that of the rhamnolipid phenacyl esters.

2.5. Mass spectroscopy

Samples were air dried and dissolved in methanol. Underivatized rhamnolipid species in fractions were identified by mass spectroscopy in a Finnigan LCQ instrument using ESI (electrospray ionization) technique with N_2 as the primary ionizing source. Derivatized rhamnolipid species were detected with the same instrument by APCI (atmospheric pressure chemical ionization) using a mobile phase of CH_3CN–water–CH_3COOH (49.5:49.5:1) and a flow-rate of 2 ml/min. While ESI generated [M−

$H]^-$ negatively charged rhamnolipid ions, APCI generated positive $[M+Na]^+$ adducts.

2.6. Surface tension

Surface tension measurements to evaluate CMC of rhamnolipid mixtures were performed at 24–25°C with a C.S.C. Scientific Co. Model 70545 Du Nouy ring tensiometer. All rhamnolipid solutions were buffered at pH 7.6 with 15 m*M* $NaHCO_3$ and NaCl was added to a concentration of 10 m*M* to keep a constant ionic strength. Multiple testing of each surfactant solution was performed. The CMC was determined from the inflection point of a plot of the surface tension (mN/m) against the logarithm of the surfactant concentration.

3. Results and discussion

3.1. Characterization of rhamnolipids from HPLC isolated fractions

The underivatized rhamnolipid fraction isolated at 42–45 min yielded an ESI mass spectrum with a most intense signal at m/z 649 $[M-H]^-$ that corresponds to the $Rh_2C_{10}C_{10}^-$ negative ion (Fig. 2A). The fraction isolated from 45–48 min showed the most intense signal at m/z 503 $[RhC_{10}C_{10}-H]^-$, and minor ones at m/z 677.5 $[Rh_2C_{10}C_{12}-H]^-$ and m/z 675.6 $[Rh_2C_{10}C_{12}-H_2-H]^-$ (Fig. 2B), whereas these same two signals at m/z 677.5 and m/z 675.6 were the most intense in the fraction isolated between 48–51 min (see Fig. 2C). Analysis of fatty acids released from fractions after saponification confirmed a major presence of 3-hydroxydecanoic acid (3-OHC_{10}) in fractions isolated from 42-45 and 45-48 min. In the fraction 48-51 min 3-hydroxydodecanoic acid (3-OHC_{12}) and a dehydrogenated variant (3-$OHC_{12}-H_2$) predominated (Fig. 3A). A further comparison was made between total content of rhamnose in all fractions and a theoretical estimation of rhamnose using GC results and assuming the presence of the rhamnolipid species identified by mass spectrometry. Differences of less than 20% between theoretical and observed rhamnose contents in the fractions confirmed the identification of the rhamnolipid species (see Fig. 3B).

3.2. HPLC method

A chromatogram of the derivatized rhamnolipids from a culture of *P. aeruginosa* UG2 is shown in Fig. 4. Isolated rhamnolipid fractions were derivatized and used as standards to identify the species represented by each peak on the chromatogram. To confirm the identity of the rhamnolipid species, the peaks were collected and their content analyzed through APCI-MS (Fig. 5). The minor peak at 18–18.5 min gave an intense signal at m/z 763.4

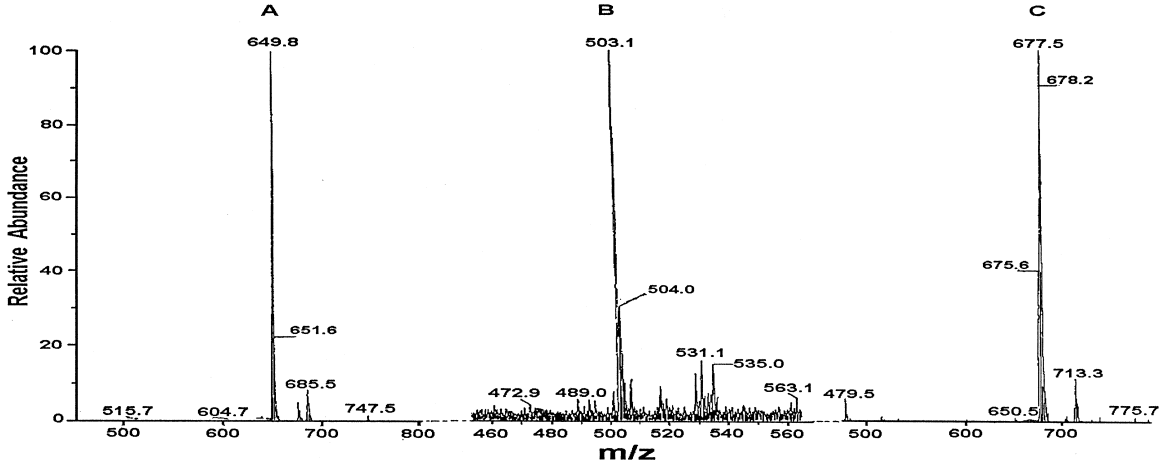


Fig. 2. ESI mass spectra of HPLC fractions from underivatized rhamnolipid mixture: (A) 42–45 min m/z 649.8 $[Rh_2C_{10}C_{10}-H]^-$; (B) 45–48 min m/z 503.1 $[RhC_{10}C_{10}-H]^-$; (C) 48–51 min m/z 677.5 $[Rh_2C_{10}C_{12}-H]^-$, m/z 675.6 $[Rh_2C_{10}C_{12}-H_2-H]^-$.

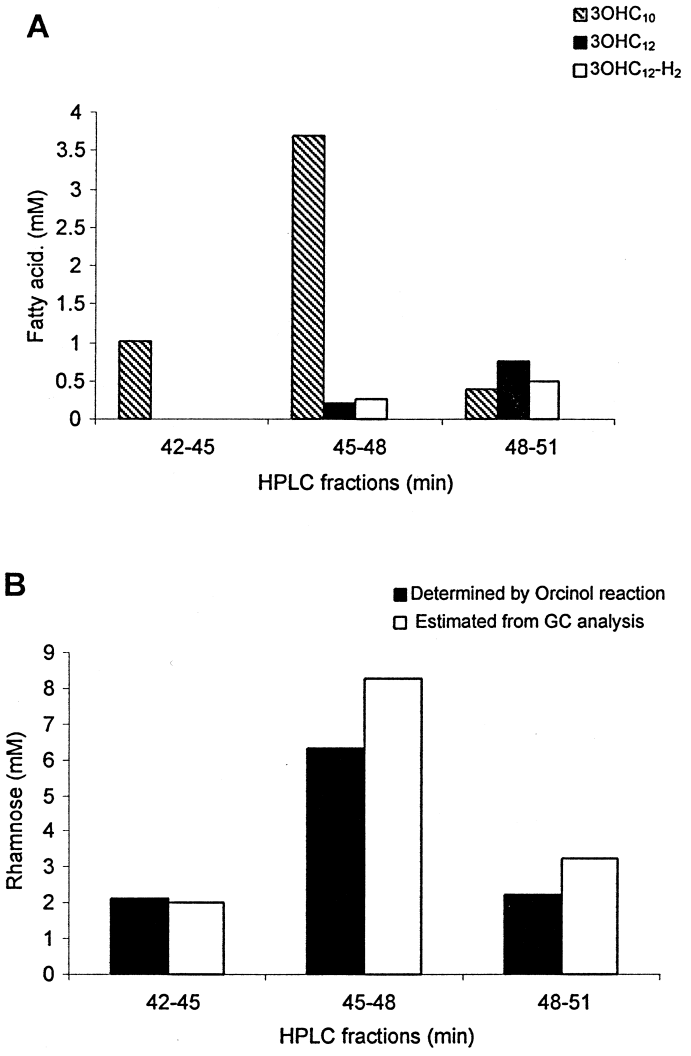


Fig. 3. (A) Fatty acid content of rhamnolipid fractions. (B) Comparison between total rhamnose content of HPLC fractions determined experimentally, and indirectly from calculation of the theoretical rhamnose content of the parent rhamnolipid species that released the fatty acids analyzed by GC.

corresponding to the sodium adduct $[M+Na^+]$ of the $Rh_2C_{10}C_8$-phenacyl ester, while the peak at 19–19.5 min showed a strong signal at m/z 791.6 corresponding to the $Rh_2C_{10}C_{10}$-phenacyl ester$+Na^+$ adduct. A signal at m/z 817.5 indicated that the 20.5–21 min peak represented the $Rh_2C_{10}C_{12}-H_2$-phenacyl ester$+Na^+$ adduct. A signal at m/z 645.5 corresponding to the $RhC_{10}C_{10}$-phenacyl ester$+Na^+$ adduct was yielded by the content of the peak at 21.5–22 min, and a signal at m/z 819.5 indicated that the $Rh_2C_{10}C_{12}$-phenacyl ester$+Na^+$ adduct comprised the peak at 22.5–23 min.

Validation of the HPLC method was done by quantifying the content of rhamnolipid species in four mixtures extracted from different *P. aeruginosa* UG2 cultures grown under the conditions previously described. Samples of the four mixtures were also analyzed through GC to quantify the amount of fatty acids released by the different rhamnolipid species after their hydrolysis under alkaline conditions.

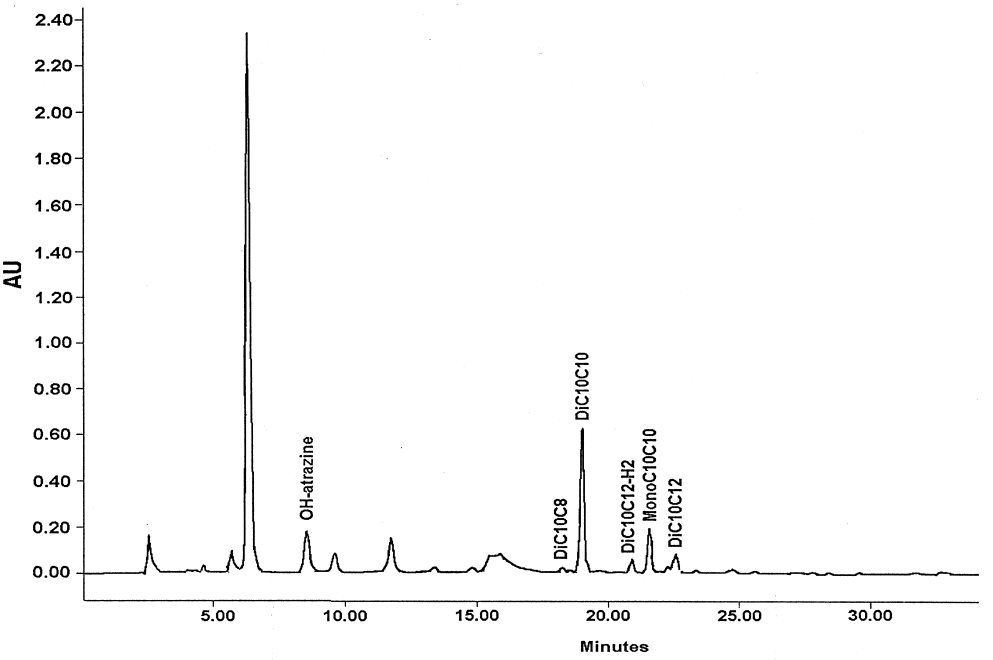


Fig. 4. HPLC chromatogram of derivatized rhamnolipid species from a culture medium of *P. aeruginosa* UG2, and hydroxyatrazine (internal standard). The amount injected was 15 μl of a 1.52 m*M* (total rhamnolipids) derivatized rhamnolipid solution.

Moles of fatty acids could be co-related to moles of rhamnolipid species, since a molecule of fatty acid is released from each hydrolyzed rhamnolipid molecule. The results of rhamnolipid direct quantification by HPLC in the four mixtures, and those indirectly obtained by quantifying the fatty acids analysis are shown in Fig. 6. An average difference of 9.6% between the HPLC and the GC results was obtained

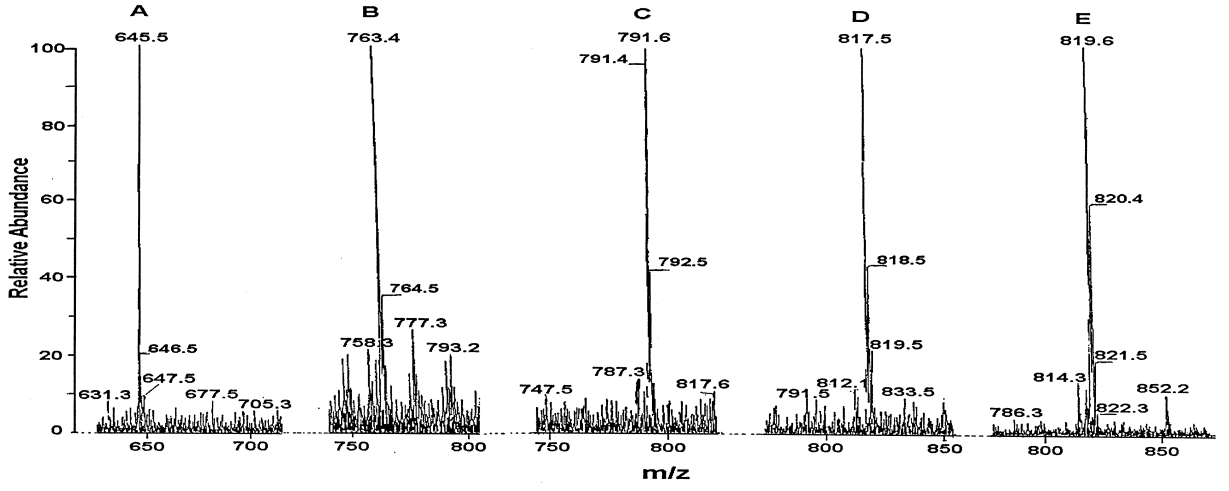


Fig. 5. APCI mass spectra of rhamnolipid derivatives from HPLC peaks: (A) 21.5–22 min *m/z* 645.5 [$RhC_{10}C_{10}$-phenacyl ester+Na^+]; (B) 18–18.5 min *m/z* 763.4 [$Rh_2C_{10}C_8$-phenacyl ester+Na^+]; (C) 19–19.5 min *m/z* 791.6 [$Rh_2C_{10}C_{10}$-phenacyl ester+Na^+]; (D) 20.5–21 min *m/z* 817.5 [$Rh_2C_{10}C_{12}-H_2$-phenacyl ester+Na^+], 21.5–22 min *m/z* 819.5 [$Rh_2C_{10}C_{12}$-phenacyl ester+Na^+].

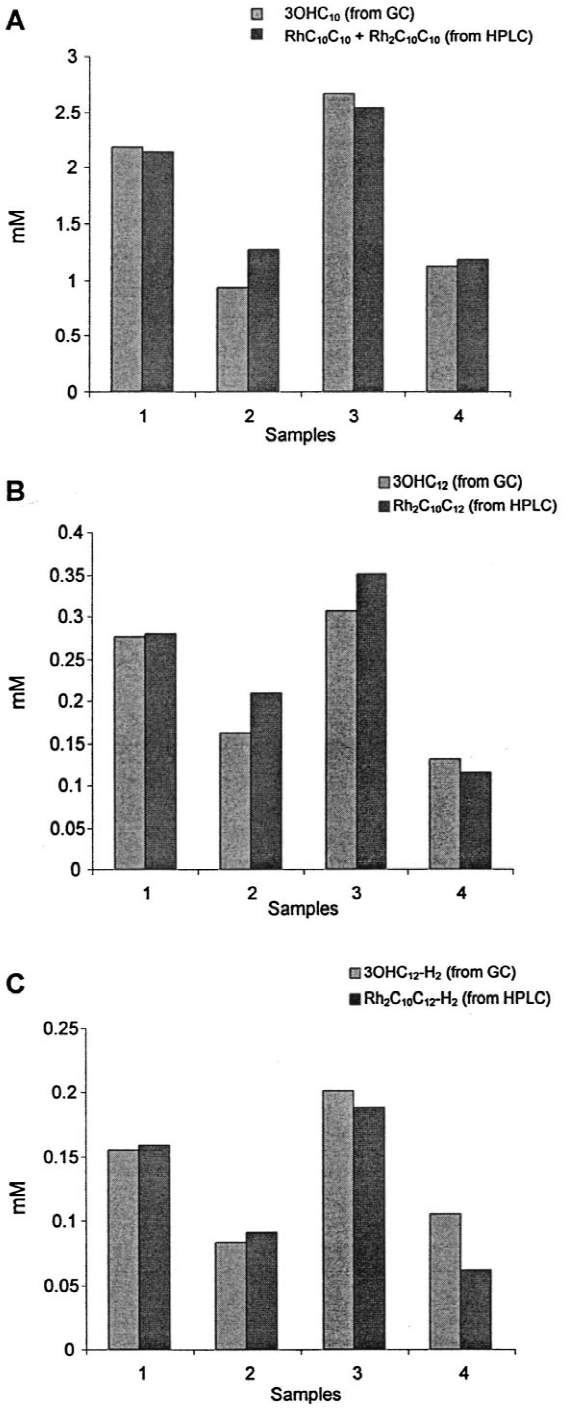


Fig. 6. Comparison of four rhamnolipid mixtures between the molar content of rhamnolipid species (HPLC method) and the molar amounts of fatty acids released after their hydrolysis (GC analysis).

after their comparison. This proves the accuracy and reliability of the proposed HPLC method. Further rhamnolipid quantification on HPLC using hydroxyatrazine as internal standard (results not shown) gave similar average difference in the results when compared to GC analysis.

3.3. Interactions of rhamnolipid mixtures with the aqueous phase

Minimal surface tension and CMC were measured for two different rhamnolipid mixtures, one with a high content of $Rh_2C_{10}C_{12}$ (41%) and $Rh_2C_{10}C_{12}-H_2$ (38%) species, and the other a typical rhamnolipid mixture produced by *P. aeruginosa* UG2 using corn oil as carbon source under normal conditions [$Rh_2C_{10}C_{10}$ (61%), $RhC_{10}C_{10}$ (20%), $Rh_2C_{10}C_{12}$ (about 11%), and $Rh_2C_{10}C_{12}-H_2$ (about 8%)] (Fig. 7). The mixture rich in $Rh_2C_{10}C_{12}$ and $Rh_2C_{10}C_{12}-H_2$ species was obtained from an aged culture (30 days). In old cultures of *P. aeruginosa* UG2, a reduction of $Rh_2C_{10}C_{10}$ and $RhC_{10}C_{10}$ species was observed whereas the concentration of $Rh_2C_{10}C_{12}$ and $Rh_2C_{10}C_{12}-H_2$ species remained constant [19]. Once the carbon source (corn oil) was completely depleted, microorganisms could have preferentially degraded $Rh_2C_{10}C_{10}$ and $RhC_{10}C_{10}$ species, contributing to an eventual enrichment of $Rh_2C_{10}C_{12}$ and $Rh_2C_{10}C_{12}-H_2$ species in rhamnolipid mixtures extracted from aged cultures.

The mixture rich in $Rh_2C_{10}C_{12}$ and $Rh_2C_{10}C_{12}-H_2$ species showed a lower CMC of 37 mg/l and a higher average minimum surface tension (AMST) of 36 mN/m, compared to the CMC of 53 mg/l and AMST of 31 mN/m for the one with high content of $Rh_2C_{10}C_{10}$ (Fig. 8). $Rh_2C_{10}C_{12}$ and $Rh_2C_{10}C_{12}-H_2$ species contain larger fatty acid chains that confer higher hydrophobicity to the molecules, and so they tend to aggregate as micelles at lower concentrations than the $Rh_2C_{10}C_{10}$ species.

From an environmental standpoint, low values of CMC, AMST, and interfacial tension are among the optimal characteristics that surfactants should have in order to promote remediation of contaminated subsurface environments [2–4,12,15,20–22]. It is clear that such parameters are intimately related with the rhamnolipid composition of a biosurfactant, and that different *P. aeruginosa* strains are able to produce

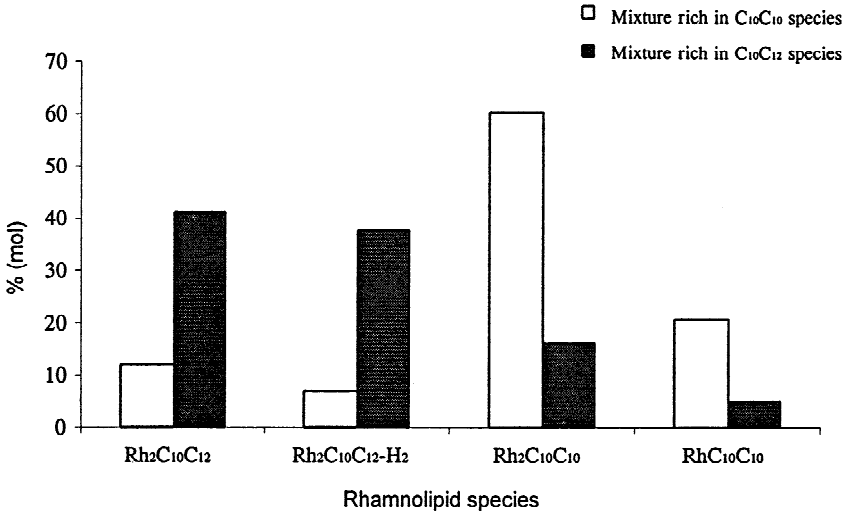


Fig. 7. Composition of two rhamnolipid mixtures, one rich in $C_{10}C_{12}$ dirhamnolipids (79%) and the other rich in $C_{10}C_{10}$ mono- and dirhamnolipids (81%).

several varieties of rhamnolipid mixtures. Wide ranges of CMC values, from 5 to 200 mg/l, have been reported for rhamnolipids. Compositions with different CMCs will have different potential as carriers of contaminants and promoters of pollutant bioavailability in subsurface environments. Thus there is a need to report an accurate biosurfactant composition together with their performance as contaminant carriers [4,5,8,11,12,14,15].

A rapid and precise determination of rhamnolipid

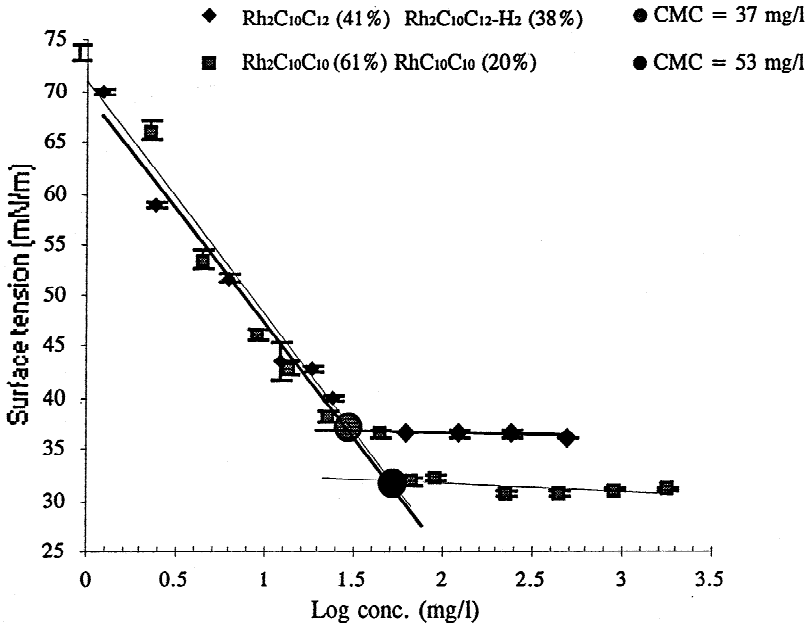


Fig. 8. Surface tension curves and critical micelle concentration (CMC) of two rhamnolipid mixtures, one with $Rh_2C_{10}C_{12}$ and $Rh_2C_{10}C_{12}-H_2$ dirhamnolipids as major components (79%), and the other rich in $RhC_{10}C_{10}$ and $Rh_2C_{10}C_{10}$ species (81%).

species assessed by the proposed HPLC method can help in getting a better knowledge of the processes involved in the production and composition of biosurfactants by other *P. aeruginosa* strains. In addition, future use of the HPLC method will permit a more complete insight of the interactions and stability that rhamnolipid species can individually have when involved in sorption–desorption and/or biodegradation processes in subsurface environments.

Acknowledgements

The authors want to thank Dr. J. Trevor from the Department of Environmental Biology at the University of Guelph, for supplying the *P. aeruginosa* UG2 cultures. The authors acknowledge the constructive comments on mass spectroscopy provided by Dr. Judd Nelson from the Entomology Department at University of Maryland. We also want to thank Sunita Prasad for helping in generating part of the experimental results presented in this work. Our acknowledgements to the U.N.A.M. (Universidad Nacional Autónoma de México), CONACYT (Consejo Naconal de Ciencia y Tecnología), and The Fulbright Comission for their economical support on the development of this work. The authors want to acknowledge the National Science Foundation Environmental Engineering Program (Grant BES-9702603) and the US Department of Agriculture for funding part of this research.

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