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Distribution Coefficients of Lipopeptide Biosurfactant in Different Solvents and its Separation from a Surfactant/Polymer Mixture in Aqueous Solutions

The distribution coefficients of lipopeptide biosurfactant in four different commercially available organic solvents and water were determined by HPLC. A method with a high recovery of 92.1 % for isolation of the lipopeptide in a surfactant/polymer mixture in aqueous solutions was established by using the combined means of acid extraction, alkaline conversion and acid re-extraction. The results showed that the lipopeptide distribution coefficients range from 0.010 to 61 at different pH values in different organic solvents, which is an indicator for optimizing the process of separation of lipopeptide biosurfactant in surfactant/polymer mixtures in aqueous solutions. The proposed separation method was used to determine the lipopeptide biosurfactant concentration in production fluids of the Daqing oil field. The concentrations were 6.57 μg/L and 4.56 μg/L.

Key words: Distribution coefficient, HPLC, lipopeptide, polymer, separation method

Verteilungskoeffizienten eines Lipopeptid-Biotensids in verschiedenen Lösemitteln und seine Trennung in Tensid-Polymermischungen in wässrigen Lösungen. Die Verteilungskoeffizienten eines Lipopeptid-Biotensids in vier verschiedenen kommerziell erhältlichen organischen Lösemitteln wurden mittels HPLC bestimmt. Es wurde ein Verfahren zur Isolation von Lipopeptid mit einer hohen Rückgewinnungsrate von 92,1 % in einer wässrigen Tensid-Polymermischung entwickelt, indem man eine Kombination aus saurer Extraktion, alkalischer Konversion und saurer Rückextraktion verwendet. Die Ergebnisse machen deutlich, dass die Verteilungskoeffizienten des Lipopeptids bei verschieden pH-Werten und verschiedenen organischen Lösemitteln im Bereich von 0,001 bis 61 liegen, was ein Indikator für den Optimierungprozess der Trennung des Lipopeptid-Biotensids in wässrigen Tensid-Polymermischungen ist. Die vorgeschlagene Trennmethode wurde zur Bestimmung der Lipopetid-Biotensidkonzentration in Produktionsflüssigkeiten des Daqing-Ölfelds eingesetzt. Die Konzentrationen betrugen 6.57 μg/L und 4.56 μg/L.

Stichwörter: Verteilungskoeffizient, HPLC, Lipopeptid, Polymer, Trennverfahren

1 Introduction

A mixture of different surfactants has been widely used in many industries such as food, detergents, cosmetics, pharmaceuticals and enhanced oil recovery [1, 2]. It was reported that the mixture of the lipopeptide biosurfactant with alkylbenzene sulfonate (a synthetic surfactant) in an appropriate mixed ratio resulted in a great reduction of its the interfacial tension due to molecular interaction and synergism effect [3]. The alkyl benzene sulfonates [4, 5] and petroleum sulfonates [6–8] are petroleum-based synthetic surfactants and their mixture with the lipopeptide biosurfactant showed a great application potential in enhancement of oil recovery.

A lipopeptide is a representative member of the biosurfactants produced by microorganisms and it has attracted much attention from both the scientific and industrial communities due to its powerful interfacial and biological activities in a wide range of therapeutic applications [9]. During the last two decades, a series of lipopeptide biosurfactants covering 26 families including approximately 90 kinds of compounds [10] has been reported. Some lipopeptides seem very promising as antitumoral, antiviral, antimycoplasma and fungal phytopathogens inhibition agents [11–14]. Others have the ability to stimulate the biodegradation of hydrocarbons [15] and to remove the heavy metals in contaminated sediments and soils [16] due to their better interfacial properties and lower toxicity than chemical surfactants [17].

The reported lipopeptide is usually composed of a hydrophobic fatty acid chain and a hydrophilic peptide loop. For the structural determination of pure lipopeptide, the fatty acid chain and amino acid sequence may be determined by esterification and MS/MS respectively [18, 19]. For the quantitative analysis of pure lipopeptide, different methods including HPLC, GC-MS, ninhydrin coloration based on the equal average molar absorption coefficient of the amino acid in the peptide loop after hydrolysis [20–24] were reported.

Nevertheless, the knowledge about the quantitative determination of the lipopeptide biosurfactant in mixed surfactant solutions is still limited due to the fact of its difficulty in separation from mixtures in solutions. To this problem, the aim of this work is to propose a separation method to remove interferents and get pure lipopeptide in real samples such as oilfield production fluids, so that lipopeptide biosurfactant determination in mixed surfactant solutions will be possible and people are able to monitor easily the lipopeptide biosurfactant concentration in tertiary oil recovery.

In the present work, the distribution coefficients of lipopeptide biosurfactant in different solvents were determined and a method was established to remove the coexistent substances and to separate lipopeptide from the complex surfactant/polymer mixture.

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2 Experimental

2.1 Instrumentation

A WuFeng HPLC with UV-detector and fluorescence detector, and the chromatographic column (C_{18} (5μm) 4.6 × 250 mm, Dalian Elite Analytical Instruments Co. Ltd) were used for determination of lipopeptide contents. Redistilled water (A) and acetonitrile (B) were chosen as the mobile phase. Gradient elution was set as follows: 0 min: 10% A, 2 min: 10% A, 22 min: 60% A. The column temperature and flow velocity are set at 30 °C and 1 mL min^{-1}, respectively. The samples to be detected were filtered by a pinhole membrane filter (φ = 0.22 μm, d = 13 mm) before HPLC determination. The peak area signals were recorded.

2.2 Reagents and solutions

Commercial available reagents of analytical grade were used in our experiment, including ethyl ether ($CH_3CH_2OCH_2CH_3$), dichloromethane (CH_2Cl_2), *n*-hexane (C_6H_{14}), *n*-pentane (C_5H_{12}), tetrachloromethane (CCl_4), 1-fluoro-2,4-dinitrobenzene (FDNB, $C_6H_4N_2O_4F$), acetonitrile (C_3H_6O), ammonium chloride (NH_4Cl) and dansyl chloride ($C_{12}H_{12}ClNO_2S$). The FDNB working solution was prepared by diluting FDNB with acetonitrile to a concentration of 1% (*v/v*) and kept in a refrigerator at 4 °C. The dansyl chloride working solution was prepared by dissolving dansyl chloride into acetone to a concentration of 0.05% (*w/w*) and kept also in a refrigerator at 4 °C. The pH values were adjusted by addition of HCl (6 M) or NaOH (0.1 M) solutions.

2.3 Sample preparation

2.3.1 Samples for distribution coefficient determination

Lipopeptide samples were prepared from the cell-free culture broth of *Bacillus subtilis* HSO121 in our laboratory [25]. The lipopeptide biosurfactant was identified by ESI-MS (supporting information, see Fig. S1, appendix). The samples were prepared by adding HCl (6 M) or NaOH (0.1 M) solution into 10.0 mL lipopeptide broth to adjust their pH to 1, 7 and 13, respectively, and the solutions were diluted with deionized water to 15.0 mL, followed by addition of 15.0 mL of organic solvent, i. e. ethyl ether, dichloromethane, *n*-hexane, *n*-pentane, or tetrachloromethane and by vibrating the mixed solution vigorously to blend them sufficiently. The mixed solutions were placed in quiescence to achieve a phase separation and the equilibrium distribution of lipopeptide in the two phases. Then 12.0 mL of the equilibrated organic phase was taken and the lipopeptide in the organic phase sample was obtained by removing organic solvents. 12.0 mL of the aqueous phase was also shifted out and the pH was adjusted to pH = 1 by adding 6 M HCl. The lipopeptide in the aqueous phase was extracted with of 3.0 mL, 2.0 mL, 2.0 mL, 2.0 mL ethyl ether for 4 times. Finally, the ethyl ether solutions were combined and the lipopeptide in the aqueous solutions was obtained by removing ethyl ether.

2.3.2 Preparation of mixed samples

The mixed samples were prepared according to the formula of industrial applied composition. The polyacrylamide solutions were prepared by adding 0.72 g polyacrylamide into 500 mL deionized water in batches and stirred at room temperature for 12–24 h until the polyacrylamide was completely dissolved. The surfactant/polymer mixture samples were prepared by adding 2.40 mL lipopeptide broth, 1.20 g petroleum sulfonate and 3.60 g Na_2CO_3 successively to the polyacrylamide solutions, and were finally diluted with deionized water to 600 mL. After sufficient mixing, a 100 mL solution was taken for separation and determination in parallel for five times.

2.4 Separation of lipopeptide from mixed samples

For the lipopeptide/polymer/petroleum sulfonates system, the separation process was operated as follows: On acid aqueous solution, lipopeptide and oil-soluble interference were firstly extracted by CH_2Cl_2 or ethyl ether with higher distribution coefficients, and the water-soluble impurities were removed. The lipopeptide was then transferred into alkaline aqueous solutions to remove the oil-soluble interferences with lower distribution coefficient by *n*-hexane. Finally, in acid aqueous solution, the lipopeptide was extracted by ethyl ether with higher distribution coefficient.

2.5 Hydrolysis of lipopeptide

In this procedure, the dried samples extracted from the lipopeptide broth and separated from mixed samples were transferred into the ampoule bottles. 1.0 mL 6 M HCl solution was added into the bottles and later the bottles were put into a hermetic reactor. For hydrolysis the reactor was placed in a 90 °C oven for 24 h [26]. At the end of the reaction, the ampoule bottles were taken out for natural cooling.

2.6 Labeling and HPLC determination of lipopeptide

After hydrolysis, the water mist and HCl were removed with an air pump at 50 °C. For the determination of the distribution coefficient, 1.0 mL deionized water, 2 drops of triethylamine and 1.0 mL 1% FDNB-acetonitrile were added into the residue in ampoule bottles. Then the bottles were heated in water bath to 60 °C for 30 min one by one, and finally the destination product DNP-Leu was detected immediately by HPLC UV-detector at 360 nm [27].

As to separate the lipopeptide samples from the mixed samples, 200 μL $Na_2CO_3/NaHCO_3$ buffer (pH = 9.5) and 200 μL dansyl chloride-acetone solution were added into the residue, and the mixture was placed in a water bath at 60 °C for 30 min one by one. Finally the product Dansyl-Leu was detected immediately by HPLC fluorescence detector at an excitation of 340 nm and emission of 510 nm.

2.7 Determination of distribution coefficients

The relative content of lipopeptide was determined by HPLC and represented by the peak area of a labeled amino acid, since the lipopeptide content is directly proportional to that of amino acids or the peak area of labeled amino acids. Thus, the lipopeptide distribution coefficients, the ratios of the lipopeptide content in organic phase to that in aqueous phase, were calculated through the peak areas of labeled leucine in organic solvent dividing by that in aqueous solutions.

3 Results and discussion

3.1 Distribution coefficients of lipopeptide in common solvents

The amino acids from the peptides of lipopeptides after hydrolysis in different organic solvents and aqueous solutions were labeled by FDNB, respectively. The peptide loop of lipopeptide contains 4 leucines, 1 glutamic acid, 1 valine and 1 as-

partic acid, but in order to acquire better signal response, the most abundant amount of amino acid leucine was chosen to represent lipopeptide content. The distribution coefficients of lipopeptides in the solvents were obtained by using the ratios of the peak areas of organic phase samples and aqueous phase samples determined by HPLC. The distribution coefficients of lipopeptide between aqueous solution and ethyl ether, dichloromethane, tetrachloromethane, *n*-hexane, *n*-pentane at different pH values are listed in Table 1.

It is shown in Table 1 that the lipopeptide distribution coefficients generally decreased with the increase of pH values. On acid conditions, the lipopeptide tended to dissolve in ethyl ether and dichloromethane, while on alkaline conditions, the lipopeptide showed a high solubility in aqueous solution, especially in *n*-hexane or *n*-pentane, since the carboxyl groups in the peptide loop would change to carboxyl ions on alkaline condition and this resulted in an easier dissolution of the lipopeptide in water.

The HPLC spectra of the blank control groups, leucine labelled by FDNB and the hydrolized lipopeptide sample labelled by FDNB were shown in Fig. 1. Compared with that of the control group, the peak 1 with the elution time of 10.79 min indicated the DNP-Leu. It implied that the lipopeptide distribution coefficients provided a basis for a solvent selection in the lipopeptide separation from mixed samples. General, the lipopeptide could be separated by multiple extraction approaches with ethyl- or dichloromethane on acid conditions, if it coexisted with hydrosoluble interferents in a mixed sample. But in the case of lipopeptide coexisted with oil-soluble interferents, organic solvents, such as ethyl ether, were used to extract the lipopeptide and oil-soluble interferents on acid conditions, and the solvents with lower distribution coefficients, such as *n*-hexane, were then used on alkaline condition to remove the oil-soluble impurities.

3.2 Separation of lipopeptide from mixed samples

The determination results of five parallel samples separated from mixed samples are summarized in Table 2. The amino acids hydrolyzed from the peptides of lipopeptide in mixed samples were labeled by dansyl chloride since dansyl chloride has the advantage of higher sensitivity than FDNB and the lipopeptide contents in mixed samples were in trace range. As shown in Table 2 the standard deviation (S_D) and relative deviation (r_D) of the five parallel samples were 0.47% and 6.63%, respectively, which demonstrated that the separation procedure was reproducible and reliable. Good recovery of 92.1% lipopeptide in the surfactant/polymer mixture indicated that this separation procedure was effective in removing the coexistent interferents and in separating the pure lipopeptide in a mixture.

If 100 mL mixed sample contain m g lipopeptide, from the first extraction with 15.0 mL CH_2Cl_2, m_1 g of lipopeptide could be obtained based on the distribution coefficient:

$$m_1 = m \times \frac{34 \times 15}{34 \times 15 + 100} = 0.84\ m \qquad (1)$$

From the second extraction with 10.0 mL CH_2Cl_2, m_2 g lipopeptide could be obtained:

$$m_2 = (m - m_1) \times \frac{34 \times 10}{34 \times 10 + 100} = 0.12\ m \qquad (2)$$

The calculation showed that the residual amount of lipopeptide which remained in the complex system was less than 0.05% after five extractions. Lipopeptide biosurfactant usually coexisted with petroleum sulfonates which may have a negative effect to lipopeptide determination. Petroleum sulfonates are mixtures which are obtained by treating high-boiling petroleum fractions with oleum, sulfur trioxide, or sulfuric acid followed by neutralization [28, 29], usually they are composed of alkylaromatic sulfonates that have different polarities [30]. Thus, petroleum sulfonates can be grouped into two categories, water-soluble and oil-soluble.

The separation process of lipopeptide from mixed samples is summarized in Scheme 1. In addition to the five parallel samples, two control groups were set: (i) mixed sample

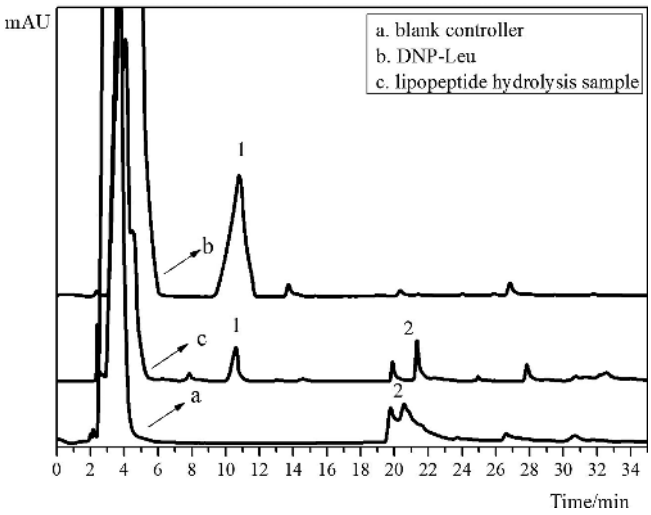


Figure 1 HPLC spectrum of DNP-Leu; The curves a, b and c represent the spectrum of the blank controller, leucine labeled by FDNB and lipopeptide hydrolysis sample labeled by FDNB, respectively; peak 1 stands for DNP-Leu

Parallel samples	n = 5
Mean value	7.05
^1S_D	0.47
^2r_D (%)	6.63
Working curve	Y = 12.15 c
Recovery (%)	92.1

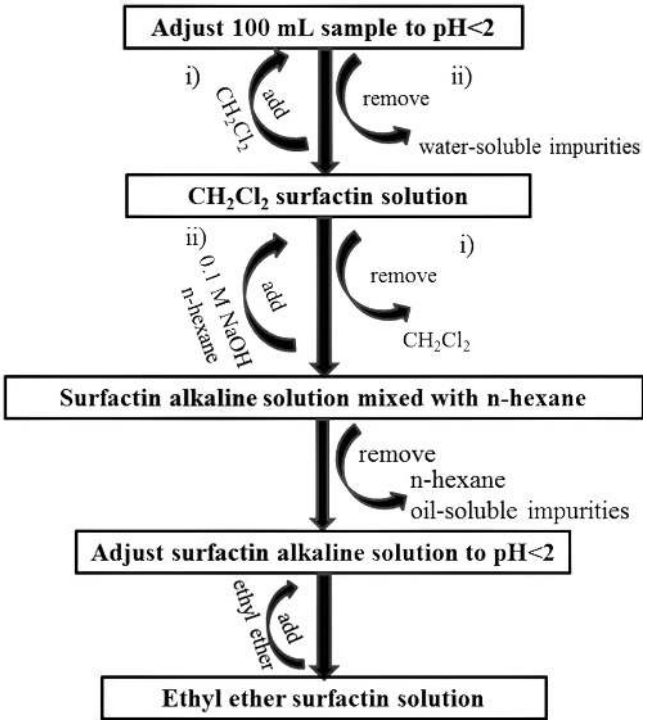
^1S_D = Standard Deviation; ^2r_D = Relative Deviation

Table 2 Determination results of lipopeptide separation from mixed samples

	Ethylether	Dichloromethane	Tetrachloromethane	*n*-Hexane	*n*-Pentane
pH = 1	61	34	6.2	5.0	3.5
pH = 7	17	11	2.0	0.44	0.74
pH = 13	0.075	0.16	0.013	0.010	0.023

Table 1 Distribution coefficients of the lipopeptide in common solvents

only containing 0.12% polyacrylamide, 0.6% Na_2CO_3, 0.2% petroleum sulfonates, and (ii) the mixed sample of the same industrial applied composition just extracted by ether ester for five times. The HPLC spectra of experimental group and control groups are shown in Fig. 2, in which peak 3 stands for Dansy-NH_4^+ and the NH_4^+ may come from three sources: i) experimental environment, ii) product of amino acid hydrolysis, and iii) NH_4^+ remained in the broth (see supporting information Fig. S2 in the appendix). A comparison of the control groups with the experimental group showed that the existence of petroleum sulfonates made great influence in determination of lipopeptide in mixed samples.



Scheme 1 Separation process of lipopeptide from mixed samples

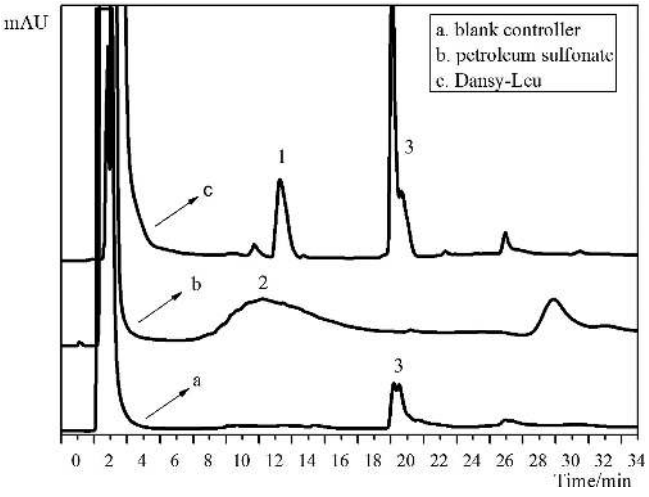


Figure 2 HPLC spectrum of Dansy-Leu; the curves a, b and c represent the spectrum of the blank controller, petroleum sulfonates and complex hydrolysis sample labeled by Dansy-Cl, respectively; peak 1 stands for Dansy-Leu, peak 2 for petroleum sulfonates, peak 3 for Dansy-NH_4^+

3.3 Application to real samples

The two production fluids were sampled from different wells of Daqing oilfield. For lipopeptide separation and determination 700 mL production fluids were taken out. The separation and determination process were operated as above (see supporting information Fig. S3 in the appendix). By HPLC determination, we measured the lipopeptide concentration in the two production fluids which were 6.57 μg/L and 4.56 μg/L, respectively. The results provide a basis for people to estimate how much lipopeptide biosurfactant needed to be re-added to the wells to meet the practical production requirements.

4 Conclusions

The distribution coefficients of lipopeptide biosurfactant in different solvents and at different pH values were determined. A method for separation of lipopeptide with a recovery of 92.1% from the mixture containing petroleum sulfonates and polyacrylamide in aqueous solutions was accordingly established. It suggested that the lipopeptide can be separated from a surfactant/polymer mixture by multiple extraction approaches with diethyl- or dichloromethane on acid condition, if it coexisted with hydrosoluble interferents in a mixed sample; while in the case of lipopeptide coexists with oil-soluble interferents, organic solvents with higher distribution coefficients should be used to extract lipopeptides and oil-soluble interferents on acid condition, and solvents with lower distribution coefficients should then be used on alkaline condition to remove the oil-soluble impurities. The proposed separation method had been successfully applied to the determination of lipopeptide biosurfactant concentration in oilfield production fluids.

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Appendix

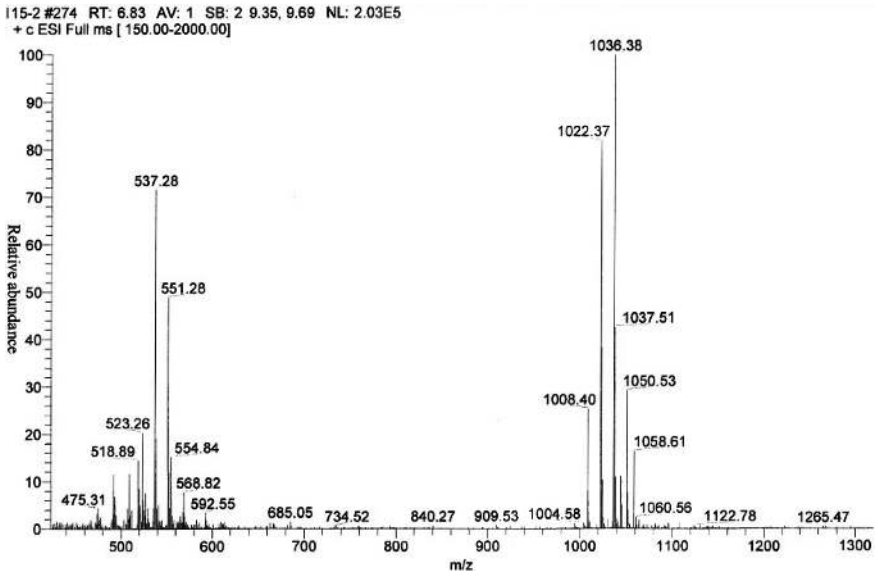

Figure S1 ESI-MS of lipopeptide biosurfactant (formula weights: 1008.40, 1022.37, 1036.38 and 1050.50 stand for surfactin-C13, surfactin-C14, surfactin-C15, and surfactin-C16, respectively)

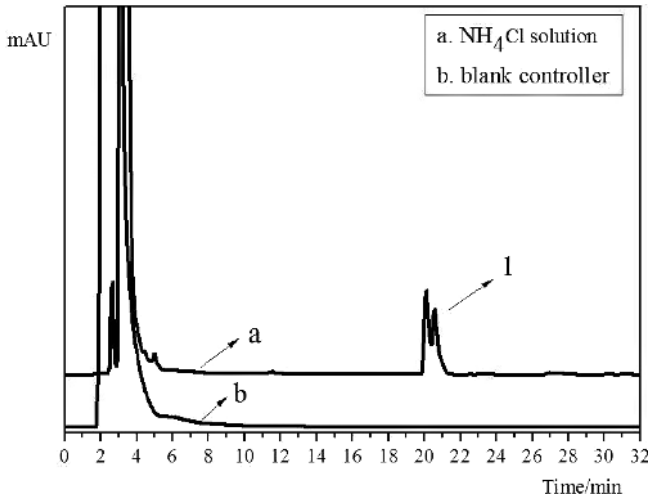

Figure S2 Identification of NH_4^+ by HPLC; the curve a stands for the experimental group NH_4Cl solution labeled by Dansy-Cl, the curve b stands for blank controller, peak 1 stands for Dansy-NH_4^+)

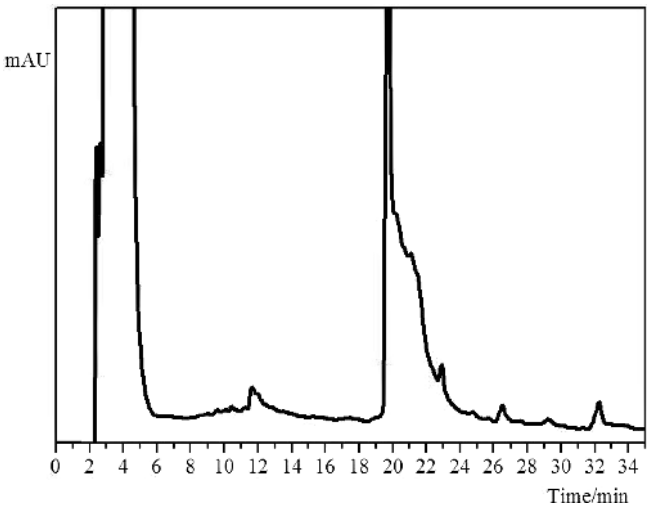

Figure S3 HPLC of Daqing oilfield production fluid