Effect of Static extraction time on Extraction Efficiencies using On-line Supercritical Fluid Extraction-High Performance Liquid Chromatography for Lipoquinone Analysis in Activated Sludge

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Abstract

Analysis of microbial community is important for monitoring the performance of biological processes. One of the most simple, quantitative and high reproducible method for analysis of the microbial community is lipoquinone profile method. Lipoquinone is constituent of bacterial plasma membrane that is essential for electron transporter. Lipoquinone could be used as a biomarker to analyze the microbial community because in general one species or genus of bacteria has one dominant type of lipoquinone, thus any changes in the lipoquinone profile would reflect the changes in the microbial community. The method for lipoquinone determination in environmental sample is direct extraction using organic solvent and analysis using chromatography system. Since the method is tedious and uses a large amount of organic solvent, the on-line supercritical fluid extraction-high performance liquid chromatography (on-line SFE-HPLC) has been developed to simplify the method, and was successful determine lipoquinone compounds in activated sludges. The effect of static extraction time on extraction efficiencies of the lipoquinone was investigated in order to eliminate the water pump and methanol pump in the previous system. The CO_2 was used as an extraction solvent. The 0.1 g of freeze dried activated sludge was placed into a 1 mL stainless steel extraction vessel and methanol was spiked into the sludge as a modifier. The SFE was connected to HPLC by using trapping column as an interface for collecting lipoquinone extracted from the sludge. The static extraction time was conducted by allowed the matrix to immersed in CO_2 and methanol. When the static extraction time finished, the dynamic extraction time was carried out. The extracted and trapped lipoquinone then directly transferred to HPLC system for determination. In this study, the effect of static, dynamic extraction time and volume of spiked methanol were optimized using simplified on-line SFE-HPLC for lipoquinone analysis. The best results in terms of extraction yield were obtained at 25 MPa, 45°C, 10 min static extraction time with 500 µL methanol spiked, and 25 min dynamic extraction time with 0.9 mL min^{-1} CO_2 flow rate. It was concluded that the developed method could simplify the on-line SFE-HPLC system of lipoquinone determination which is useful for a rapid and routine analysis of microbial community in activated sludge.

Key words: microbial community, lipoquinone profile, on-line SFE-HPLC static extraction time, dynamic extraction time

Introduction

Biological processes such as wastewater treatment, anaerobic digestion, composting, and bioremediation are complex processes which involve the microbial community (Regueiroa, *et al.*, 2012). Monitoring the microbial community in biological processes is important to understand as well as ensure the process is working properly and to improve its performance. Therefore, the necessity of a simple, rapid and reliable method with a low running cost and low technical skills are basic requirements in the analysis. One of the convenient method for this purpose is lipoquinone profile method.

Lipoquinone is component of microorganisms that essential for electron transporter. Generally, a species or genus in a microbial community produces one dominant type of lipoquinone and any change in the lipoquinone profile reflects a change in the microbial community (Hiraishi, 1999; Collins, *et al.,* 1981). Lipoquinone which mainly consist of ubiquinone(UQ; associated with 1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone) and menaquinone (MK; associated with 1-isoprenyl-2-methyl-naphthoquinone), not only provided an understanding on the composition of microbial community, but also the biomass concentration of the samples (Hiraishi, 1999; Collins, *et al.*, 1981). Therefore, there is a growing interest in the analysis of microbial community based on lipoquinone profile method in environmental samples.

The conventional method for determination of lipoquinone from environmental sample consists of direct extraction using organic solvent, and analysis using high performance liquid chromatography (HPLC) (Hu, *et al.*, 1999). Since the extraction using organic solvent requires long extraction time and large volume of organic solvent, the supercritical fluid extraction (SFE) using carbon dioxide (CO_2) was introduced to be a potential alternative method to extract lipoquinone (Irvan *et al.*, 2006; Hanif *et al.*, 2012). The CO_2 under supercritical condition was used as extraction solvent to extract lipoquinone and methanol was the best modifier to adjust its polarity (Irvan *et al.*, 2006). It is to be noted that those studies were done under off-line system, where SFE and HPLC were not connected directly.

In our recent study, the on-line SFE-HPLC has been developed and was successful determine the lipoquinone in various activated sludges. In the system, SFE was connected to the solid phase trapping column (Zorbax SB-C18) as an interface between the SFE and HPLC system. The trapping column, as a collector of extracted lipoquinone, was associated to six-port valve in HPLC system. Therefore, using on-line SFE-HPLC, all the extracted lipoquinone could be directly transferred to the HPLC system without tedious sample pretreatment which can reduced the total analytical time and improve the reproducibility of analysis. In addition, since lipoquinone and their derivate are photosensitive and susceptible to oxygen (Collins, *et al.*, 1981), the direct transferring of extracted lipoquinone from extraction step to chromatography system can minimize the lipoquinone loss through degradation. The direct transferring of extracted lipoquinone also could reduce the amount of sample requirement.

In the previous system, two pumps were used in the extraction mode, it is for delivering CO_2 and methanol. In addition, to increase the trapping efficiency of extracted lipoquinone due to the flowing of high amount of methanol under on-line SFE-HPLC, the water flow was introduced to adjust the solubility of extracted lipoquinone in extraction fluid. This led to the usage of one more pump, which eventually could cause higher running cost and maintenance. Therefore, the static extraction time was proposed to combine with the dynamic extraction time and methanol spiked directly into the sample to eliminated the pump used for flowing methanol and water. In this study, the optimum conditions for static and dynamic extraction time were investigated using simplified on-line SFE-HPLC. In

addition, the volume of methanol spiked was also investigated. The aim is to establish a more simplified method for lipoquinone analysis which is essential for rapid monitoring of biological processes.

Materials and Methods

Chemicals and reagents

The solvents used were HPLC grade. Standards for ubiquinone-10 (UQ-10) and Menaquinone-7 (MK-7), diisopropyl ether, methanol and acetone were obtained from Wako Co. (Osaka, Japan). The standard solutions were prepared to the desired concentration with acetone. The CO_2 (99.9%) from Taiyo Nippon Sanso Corporation (Japan) was used in all of the on-line SFE experiment.

Sample Preparation

The activated sludge that was obtained from the aeration tank of the domestic wastewater treatment plant at Toyohashi University of Technology (TUT), with the capacity of 3.8×10^2 m^3 day^{-1} was used as a sample in this study. The sample was dried in a vacuum freeze dryer for 24 hours and then homogenized by crushing and sieving to collect particles smaller than 500 µm. Freeze-dried sample was stored at -30°C until analysis.

Apparatus

The schematic diagram of on-line SFE-HPLC system is shown in Figure 1. It consists of cooler (Scinics CH-201, Tokyo, Japan), CO_2 pump (Jasco PU-1580 HPLC, Tokyo, Japan) that equipped with valve to change the static or dynamic mode, preheated column (0.25 mm id × 1.59 mm od × 2 m), oven (GC-353B, GL Sciences, Tokyo, Japan), and backpressure regulator (BPG 880-81, Jasco). The extraction was accomplished using a 1 mL inner volume stainless steel extraction vessel (SUS316, Jasco). The guard-column (Zorbax SB C18, Agilent, Santa Clara, CA; 4.6 mm id × 12.5 mm, particle size 5 m) was used as a trapping column to collect the extracted lipoquinone, which was connected to six-port valve (Rheodyne®, Cotati, CA) in HPLC system. The six-port valve provided two switching modes: extraction mode and analysis mode. This valve was connected to the injection valve which is part of HPLC system. Separation and detection of the lipoquinone were performed by HPLC that equipped with degasser (DGU-14A, Shimadzu, Kyoto, Japan), two high-pressure pumps (LC-10AD VP, Shimadzu), an oven (CTO-10AS VP, Shimadzu), a UV-Vis detector (SPP 10A VP, Shimadzu) and a photo diode array (PDA) detector (SPD M10A VP, Shimadzu). The analytical column (CD-C18, Cadenza, Imtakt Technologies, Portland, OR; 4.6 mm id × 250 mm, particle size 3 m) was applied for separation of lipoquinone.

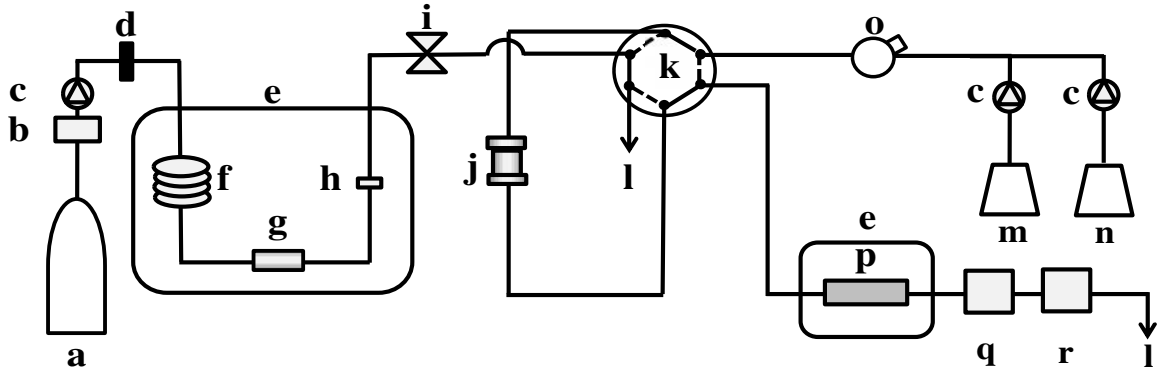

Figure 1. Diagram of static and dynamic on-line SFE-HPLC for Quinone. (a) CO2; (b) Cooler; (c) Pump; (d) CO2 Valve; (e) Oven; (f) Preheated Column; (g) Extraction Vessel; (h) Filter; (i) Back Pressure Regulator; (j) Trapping Column; (k) Six-port Valve; (l) Waste; (m) Methanol; (n) Diisopropyl Ether; (o) Injector; (p) Separation Column; (q) UV-Vis Detector; (r) PDA Detector

Analytical Method

The SFE was performed at the pressure of 25 MPa and the extraction temperature of 45°C with the CO_2 flow rate of 0.9 mL min^{-1}. The 0.1 g of freeze dried activated sludge was placed into a 1 mL stainless steel extraction vessel, and methanol was spiked directly into the sludge as a modifier. The extraction vessel then set in the SFE system. After reaching the corresponding supercritical fluid conditions, the static extraction time was conducted by closing the CO_2 valve. In the end of the static extraction time, the dynamic extraction time with the constant flow rate of CO_2 was carried out by opening the CO_2 valve, and the extracted lipoquinone was then carried through the restrictor, and collected in the trapping column. The temperature of the trapping column was 24°C (room temperature). When the extraction was completed, extraction mode was switched to the analysis mode to connect the trapping column into the HPLC system.

An isocratic flow mixture of 80% methanol and 20% diisopropyl ether with a flow rate of 1 mL min^{-1} was used for the separation. The temperature of the column oven was maintained at 30°C. The wavelength was set at 270 nm and the analysis time was set for 75 min. The UQ and MK species were identified based on the retention time and the spectrum. The identification of the lipoquinone species were conducted by understanding the correlation between the logarithm of retention time of lipoquinone and equivalent number of isoprenoid unit (ENIU) (Collins, *et al.*, 1981). The amounts of lipoquinone then calculated from the peak area based on the mole absorption coefficient (UQ: 14.4 mM^{-1} cm^{-1}; MK: 17.4 mM^{-1} cm^{-1}) (Kroger, 1978). Triplicate experiments were carried out to determine the precision of all experiments.

Results and Discussion

Effect of static extraction time on the extraction efficiency using simplified on-line SFE-HPLC

The static extraction time was optimized in the range of 0 to 25 min and followed by dynamic extraction time. As shown in Figure 2, the comparable detected amounts of lipoquinone were obtained at different static extraction times, therefore, the static extraction time is not so important.

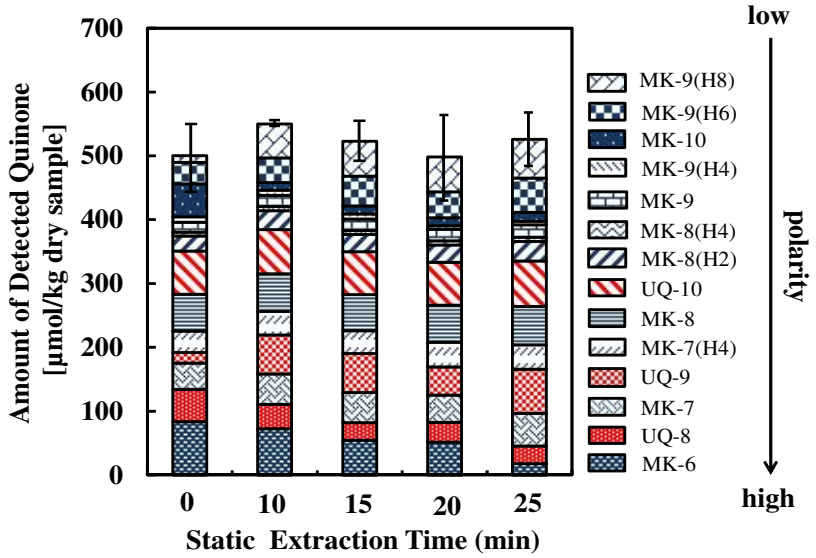

Figure 2. Effect of static extraction time on the amount of detected lipoquinone using simplified on-line SFE- HPLC. Sample: 0.1 g dried activated sludge. SFE conditions: 25 MPa; 45°C; 500 µL methanol spiked; 15 min dynamic extraction time with 0.9 mL min^{-1} CO_2 flow rate. Trapping conditions: Zorbax SB-C18 (4.6 mm id × 12.5 mm, 5 m) at room temperature (24°C).

The 10 min was chosen as static extraction time because of the highest detected amount of lipoquinone with the lowest error bar. The increasing error bar without increasing in detected amount of lipoquinone were obtained when the static extraction time was longer than 10 min; it might due to matrix swelling, longer time in high extraction temperature (45°C) that could cause decomposition of lipoquinone, or lipoquinone lost by leaking from the system.

Effect of dynamic extraction time on the extraction efficiency using simplified on-line SFE-HPLC

With the 10 min of static extraction time, the effect of dynamic extraction time on the extraction efficiency was studied in the range of 10 to 30 min. The results were shown in Figure 3. The comparable detected amount of lipoquinone was obtained at 20 min and 25 min of dynamic extraction time; however, the lowest error bar was obtained at 25 min of dynamic extraction time. Hence, 25 min was chosen as the optimum dynamic extraction time. The longer dynamic extraction time could increase the amount of CO_2 to be eluted from the trapping column which could lead to the higher lipoquinone lost. Therefore, the increase of dynamic extraction time caused decrease in detected amount of lipoquinone.

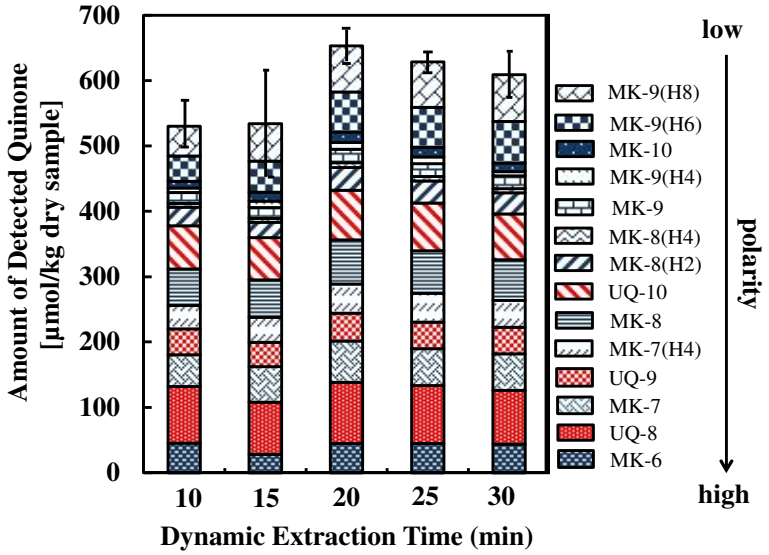

Figure 3. Effect of dynamic extraction time on the amount of detected lipoquinone using simplified on-line SFE-HPLC. Sample: 0.1 g dried activated sludge. SFE conditions: 25 MPa; 45°C; 500 µL methanol spiked; 10 min static extraction time; 0.9 mL min^{-1} CO_2 flow rate (in dynamic extraction time). Trapping conditions: Zorbax SB-C18 (4.6 mm id x 12.5 mm, 5 m) at room temperature (24°C).

Effect of methanol spiked volume on the extraction efficiency using simplified on-line SFE-HPLC

Volume of methanol spiked is one of the essential parameters in this study. If the methanol spiked was not enough, the adjustment of polarity for the extraction solvent would not be sufficient and would affect to the extraction efficiency. Volume of methanol spiked from 100 µL to 500 µL was examined at the optimized SFE conditions. For the 1 mL inner volume of extraction vessel with 0.1 g sample, the maximum methanol spiked was considered to be 500 µL. The results were shown in Figure 4. The detected amount of lipoquinone with the methanol spiked volume 400 µL and 500 µL were almost same. However, the 500 µL methanol spiked volume has the lowest error bar, therefore, 500 µL was used as the optimum volume for methanol spiked in simplified on-line SFE-HPLC.

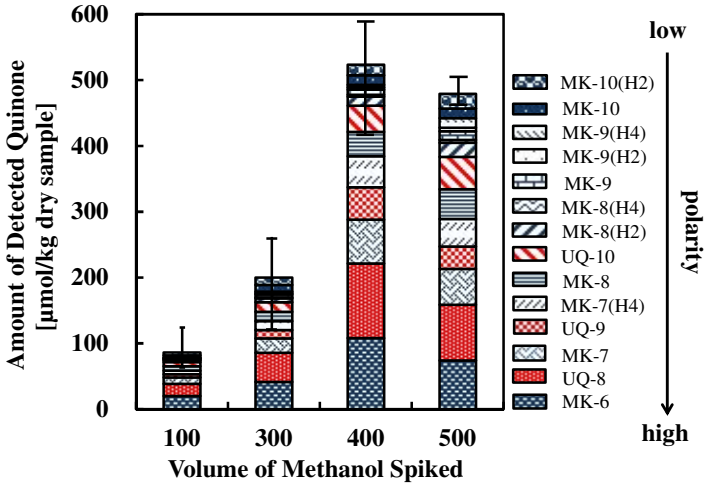

Figure 4. Effect of methanol spiked volume on the amount of detected lipoquinone using simplified on-line SFE-HPLC. Sample: 0.1 g dried activated sludge. SFE conditions: 25 MPa; 45°C; 10 min static extraction time; 25 min dynamic extraction time with 0.9 mL min^{-1} CO_2 flow rate. Trapping conditions: Zorbax SB-C18 (4.6 mm id × 12.5 mm, 5 m) at room temperature (24°C).

Conclusion

The combination of 10 min static extraction time and 25 min dynamic extraction time with 500 µL methanol spiked has successfully determined lipoquinone from activated sludge by the simplified on-line SFE-HPLC developed in this study. The two pumps needed in the previous study were eliminated for extraction and trapping of lipoquinone in this simplified on-line SFE-HPLC. With the advantage of this study compared to previous studies, the lower cost for running and maintenance of the pump could be obtained. This simplified on-line SFE-HPLC system offered a more effective method for lipoquinone determination thus is expected to be a breakthrough technology for routine analysis in monitoring environmental biological processes.

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