ANALYSIS OF LIPOQUINONES BY ON-LINE SFE-HPLC FOR MONITORING ENVIRONMENTAL BIOLOGICAL PROCESSES

（環境生物学的プロセスのモニタリングに向けた
オンライン SFE-HPLC を用いたリポキノンの分析）

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Abstract

論文内容の要旨　(博士)

Title of Thesis 博士学位論文名	Analysis of Lipoquinones by On-line SFE – HPLC for Monitoring Environmental Biological Processes (環境生物学的プロセスのモニタリングに向けたオンライン SFE-HPLC を用いたリポキノンの分析)

Microbial community has an important role in the biological processes. Monitoring the microbial community in environmental biological processes is necessary to ensure that the process is working properly, and to improve its performance. Therefore, the necessity of a simple, rapid and reliable method with low running cost and technical skills is a basic requirement in the analysis. One of the most simple, quantitative and high reproducible method to determine the microbial community structure is lipoquinone profile method. Lipoquinone is constituent of the bacterial plasma membrane that is essential for electron transport. Lipoquinone could be used as biomarker for microbial community because lipoquinone exist in almost all bacteria, and generally, a species or genus in a microbial community contains one dominant type of lipoquinone thus any change in the lipoquinone profile reflects a change in the microbial community. Lipoquinone profile provides not only information about composition of the microbial community, but also the biomass concentration of the environmental sample.

The conventional method for lipoquinone determination is direct extraction from the matrix using organic solvent, which is usually a mixture of chloroform and methanol. Since the method requires long extraction time and large volume of organic solvent that against the principle of green chemistry, the supercritical fluid extraction (SFE) method is developed. Carbon dioxide which is used as the extraction solvent in SFE is environmentally friendly with high diffusivity, selectivity, and many other advantages.

SFE was used to extract lipoquinones from compost to monitor the microbial community dynamics during composting. The 0.3 g of dried compost was extracted using 3 mL min^{-1} of carbon dioxide (90%) and methanol (10%) at 45°C and 25 MPa for a 30 min extraction time. The extracted lipoquinones were analyzed using ultra performance liquid chromatography (UPLC) with 0.3 mL min^{-1} of methanol mobile phase for a 50 min chromatographic run time.

A comparable detected amount of lipoquinones was obtained using the SFE method and an organic solvent extraction method; being 36.06 μmol kg^{-1} and 34.54 μmol kg^{-1}, respectively. The significantly low value of dissimilarity index (D) between the two methods (0.05) indicated that the lipoquinones profile obtained by both methods was considered identical. The method was then applied to determine the maturity of the compost by monitoring the change of lipoquinones during composting. The UQ-9 and MK-7 were predominant lipoquinones in the initial stage of composting. The diversity of lipoquinones became more complex during the cooling and maturation stages. This study showed that SFE could successfully extract lipoquinones from compost which is considered to have a complex matrix of microbial community and would be useful for monitoring composting at field.

For monitoring environmental biological processes using analysis of lipoquinones, a more rapid and reliable analysis method is necessary. The on-line system between SFE and high performance liquid chromatography (HPLC) can certainly improve the performance and with several potential advantages for the qualitative and quantitative determination of lipoquinone. Therefore the main objective of this project is to develop on-line SFE-HPLC for microbial community analysis method based on lipoquinone profile.

The SFE was connected to a solid phase trapping column (Zorbax SB-C18) which is used to collect the extracted lipoquinone. This trapping column is the interface between the SFE and HPLC, and associated to six-port valve in HPLC system. Under on-line SFE-HPLC, all the extracted lipoquinone would be directly transferred to the chromatography system without tedious sample pretreatment. Therefore, the on-line SFE-HPLC does not only reduce the analysis time but also the sample preparation. In addition, since lipoquinone and their derivate are photosensitive and susceptible to oxygen, the direct transferring of extracted lipoquinone from extraction step to chromatography system can minimize the lipoquinone loss through degradation. Consequently, these can improve the reproducibility of analysis.

Optimation of the extraction and trapping column conditions were investigated using activated sludge. The reliability of the method was evaluated by comparing to the organic solvent extraction method. The optimum conditions obtained on activated sludge were as follows: 45°C; 25 MPa; 15 min; 10% methanol with flow rate of 1 mL min^{-1} and water flow of 0.04 mL min^{-1} in the trapping column. The on-line SFE-HPLC has been proved to be applicable for lipoquinone determination in various activated sludges.

The static extraction then was proposed to combine with the dynamic extraction and methanol spiked directly into the sample to simplified the system. The effect of static extraction on extraction efficiencies of the lipoquinone was then investigated in order to eliminate the water pump and methanol pump in the previous system. The best results in terms of extraction yield were obtained at 25 MPa, 45°C, 10 min static extraction with 500 μL methanol spiked, and 30 min dynamic extraction with 0.9 mL min^{-1} CO_2 flow rate. The development of on-line SFE-HPLC method offers simplification for a rapid and routine analysis of lipoquinone to monitor the performance of environmental biological processes based on lipoquinone profile.

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1.1 Background and Motivation

Microbial communities are abundant in the environment and encompass the vast majority of species on the earth. It play the most important aspects in the regulation of ecosystem processes, such as anaerobic digestion, wastewater treatment, and composting. In those processes, the decomposition of organic matters as well as generation of renewable energy through the conversion of organic feedstock into energy-rich compounds was occurred [1-7]. Since the process are entirely depends upon the concerted and syntrophic activity of microorganisms, those the changes of the microbial communities, would influence the whole ecosystem process through metabolic feedback [3]. Study of the microbial community in the environment would not only give insight into the process of the ecosystem, but also provide information that could be used to improve the performance of the process. Due to its vital contribution to the ecosystem, it is a great interest to study the microbial community structure.

Within the natural population of microbial community, large amounts of information could be revealed which are needed to be discovered. On the other hand, the existing tools remain inadequate for elaborating the complexity of microbial community. Even though the recent methods support the exploration of microbial community analysis with the innovative processes, new tools with wider throughput, higher quantitative, and better cost effectiveness are needed to help revealing structures and functions in parallel [1].

Since only a minority of microbial communities is culturable, a number of microbial community analysis methods that extract the components of microorganisms directly from the sample without cultivation were developed [8-12]. One of the most commonly used methods is lipoquinone profile analysis. The lipoquinone analysis, which corresponds to the mole fraction of each lipoquinone type in a mixed culture, has gained recognition as a highly

quantitative and repeatable method [13]. It provides the composition of the microbial community and their biomass concentration [14]. The analysis of lipoquinone has been utilized for the determination of microbial community structure in various biological processes, such as waste water treatment [13,15], composting [16-18], bioremediation and marine sediments [19].

The lipoquinone profile analysis is conducted by using organic solvent extraction which consists of a mixture of chloroform and methanol, and followed by re-extraction using hexane and water. The fractionation of lipoquinone then conducted using Sep-Pak Plus Silica cartridges [20]. Determination of extracted lipoquinone was then performed by using high performance liquid chromatography (HPLC) [20, 21]. However, the procedures are considered to be tedious and almost all the steps have to be performed with extra caution as lipoquinone and their derivate are photosensitive and susceptible to oxygen [14]. Moreover, this conventional method uses a large volume of expensive and hazardous organic solvents. From the view of practical application, these disadvantages would be vital, as study of environmental biological process usually requires routine sampling and analysis. To effectively study the microbial community based on lipoquinone profile, an alternative analysis method which would only need short time yet comes with high sensitivity would be ideal. Therefore, there is an urgent need to seek alternative approaches on current analysis of lipoquinone. On the other hand, increased awareness in the use of environmentally safety and cost effective solvent, has stirred interest in the concept of green chemistry.

One of green chemistry method for analysis of lipoquinone is the supercritical fluid extraction (SFE) using carbon dioxide (CO_2) as an extraction solvent. The supercritical carbon dioxide ($scCO_2$) has the potential to replace the organic solvent extraction, mainly for its rapid extraction and low volume usage of solvent [22, 23]. The achievement of SFE as an effective, rapid and quantitative extraction technique in many areas has been approved due to its success to determine the lipoquinone from environmental biological samples [23-25].

Nevertheless, the reported achievements on analysis of lipoquinone using SFE still required pretreatments, which included re-extraction using hexane and fractionation using Sep-Pak Plus Silica before injecting into HPLC. As mentioned previously, monitoring environmental biological process is a routine and continuous work that results into a large of number of samples needed to be analyzed. Therefore, the concept to connect the extraction and analysis technique directly, in which the pretreatments stage is to be omitted, could simplify the analysis of lipoquinone. This connecting concept, which could called as on-line method, creates a straightforward approach in both preparation and analysis steps. On-line method can improve the performance and cost-effectiveness of analysis. Besides, the on-line method is valuable for unstable analytes. The analytes which is limited in amount was also beneficial to analyze using the on-line method since all the extracted analytes are transferred directly to the chromatographic system without further expose to the environment. Since lipoquinone and their derivatives are photosensitive and susceptible to oxygen, the on-line method has a high potential to minimize lipoquinone loss through volatilization or degradation.

In this study, the on-line SFE-HPLC was developed to introduce an alternative analysis method for microbial community analysis based on lipoquinone profile. Development of on-line SFE-HPLC requires optimization of several parameters. Not only the extraction conditions have to be optimized but also the trapping column which serves as an interface of the on-line SFE-HPLC. At the end, the on-line SFE-HPLC was simplified by combining the static and dynamic extraction with the methanol spiked directly into the extraction vessel. The developed method could be an essential method for routine analysis when come with large number of samples.

1.2 Objectives and Structure of Thesis

The main objective of this thesis is to develop fast, sensitive, high reproducibility method for high-throughput analysis of microbial community in the environmental biological processes such as wastewater treatment, composting, and anaerobic digestion, based on lipoquinone profile. Since the

possibilities of the connecting SFE and HPLC are reasonably well known, also with regard to the potential of automation, our major focus is on developing the on-line SFE-HPLC and simplified on-line SFE-HPLC system. This doctoral thesis is divided into 6 chapters. The brief description and objectives of each chapter are as follows:

Chapter 1:

This chapter states the background, the motivation, the objectives and the structure of this thesis. In addition, this chapter reviews the importance of the microbial community structure in the environmental biological process with the common techniques that are used to analysis the microbial community. The new approach of microbial community analysis that appropriated with the concept of green chemistry was also discussed. Finally, the introduction of on-line SFE-HPLC system was described.

Chapter 2:

This chapter described about the chemicals and reagents used, sample pretreatments, apparatus, and analytical procedures of all methods that used in this study.

Chapter 3:

In this chapter, the expand application of SFE to analyze microbial community from compost based on lipoquinone profile has done. As compost has a more complex matrix compare to the activated sludge, the amount and diversity of microbial community also higher. Therefore, optimization of the conditions for extracting lipoquinone from compost using SFE is required. In this study, the extraction temperature and extraction time, were optimized. The lipoquinone profile obtained using this method was then compared with those obtained using organic solvent extraction method. The SFE method was then apply to extract the lipoquinone for monitoring the composting.

Chapter 4:

In this chapter, the on-line SFE-HPLC was developed to analyze the microbial community structure based on lipoquinone profile. Activated sludge taken from

Toyohashi University of Technology (TUT) was used as samples in optimization of the conditions for the developed method. Effects of water flow rate and temperature on the efficiency of the trapping column as an interface to collect the lipoquinone extract were examined. Furthermore, the comparison between this developed method and the conventional organic solvent extraction method was also discussed. The optimum conditions that obtained using this method were then applied to study the microbial community structure in various activated sludge samples.

Chapter 5:

This chapter described the further simplified on-line SFE-HPLC for microbial community analysis based on lipoquinone profile. 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, 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 combination of static extraction and dynamic extraction was proposed with the methanol spiked directly into the sample to eliminate the pump used.

Chapter 6:

Some general conclusions and future perspectives of this study are described in this chapter.

1.3 Importance of Microbial Community Analysis

Microbial community is the basic unit of the ecosystem, which addresses a variety of issues that range from the function of a single population to the interactions of complex communities [26]. The changes in microbial communities are often a precursor to understand the performance of the process [27]. Therefore, the analysis of microbial community is one of the ways to understand the composition and the function of microbial community in the environment.

Research in the microbial community field has been developed since the increasing awareness of the environmental issues. The activity of microbial community plays the central role in minimizing the environmental pollution and monitoring the biological process such as wastewater treatment, anaerobic digestion, composting and bioremediation [4-7, 28]. Nevertheless, the microbial community structure still remains as a black box for the scientist. Their function and their mechanisms throughout the whole process is still unknown. Therefore, analysis of the microbial community structure in the environment has been always a continuous work which needs a huge amount of information based on proven data.

There are many methods used for microbial community analysis [8-12]. The main methods for microbial community analysis are shown in **Figure 1.1.** The methods mostly involve the resolution of genetic and phenotypic. The microbial enrichment and isolation method are useful tools for studying the culturable microorganisms. Since only a minority of microbial communities is culturable [29], the suitable methods that extract the components of microorganisms directly from the sample without cultivation are considered as the better approach.

There are several non-cultivated-based techniques for microbial community analysis. For example, direct extraction of DNA/RNA sequence from environmental samples which followed by subsequent analysis using denaturing gradient gel electrophoresis (DGGE), temperature gradient gel electrophoresis (TGGE) or terminal-restriction fragment length polymorphism analysis (T-RFLP). These DNA-based methods made the studies of microbial communities become easier and have a great potential to further explore the black box of the microbial community structure. However, these methods have a huge deviation in the copy number of DNA in each species of microorganisms and need long time of analysis [30]. Another method, fluorescence in situ hybridization (FISH), is a method for detection of the whole-bacterial cells and the chromosomes with fluorescence probes. Although this method gives quantitative information on microbial community structure, it is time consuming and less sensitive [29].

On the other hand, the lipid biomarker analysis has a high correlation to the biomass, but inferior to molecular methods for taxonomic resolution. For practical monitoring environmental biological processes based on microbial community structures, the rapid, quantitative and reliable method are a substantial focus rather than taxonomic resolution. The lipid biomarker method based on analysis of lipoquinone is one of the simplest methods that provides both compositions and the biomass concentration of the microorganisms.

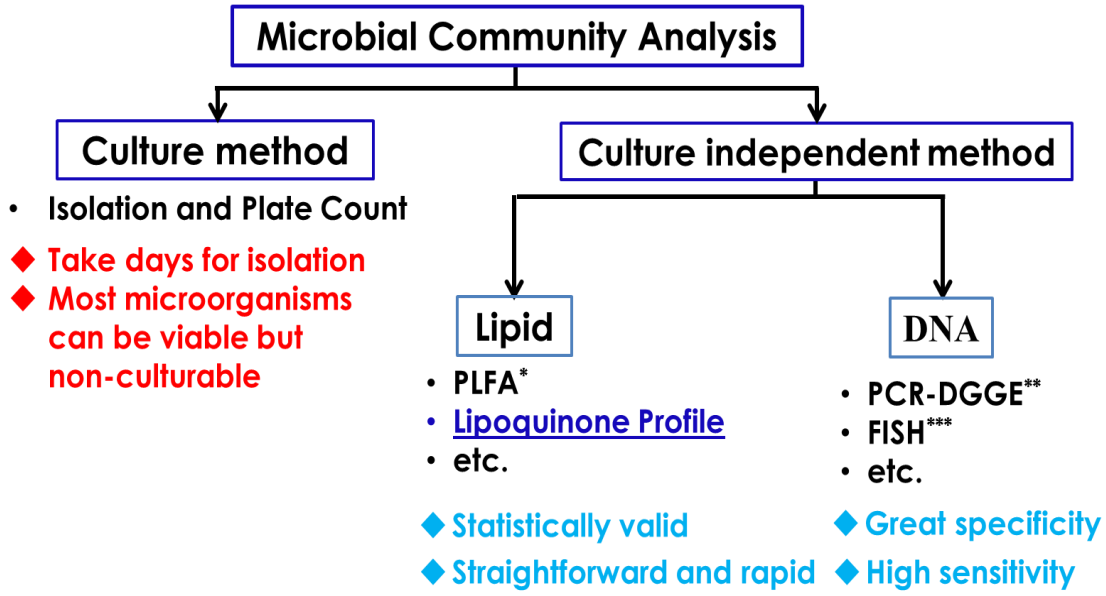

Figure 1.1

Schematic overview of various methods used in microbial community analysis [31].

It is useful to understand on how the methods providing information in the taxonomic resolution as well as the complexity of the analysis method before deciding the suitable method to characterize the microbial community for the different purposes. It is necessary to characterize correctly and effectively the microbial community in different samples, under different conditions, and for different purposes. Therefore, the selection of an appropriate method to characterize the microbial community is clearly depending on the requirements and constraints for information collection, costs, and acquisition time for the

efficient analysis. The taxonomic resolution of some microbial techniques are shown in **Figure 1.2.**

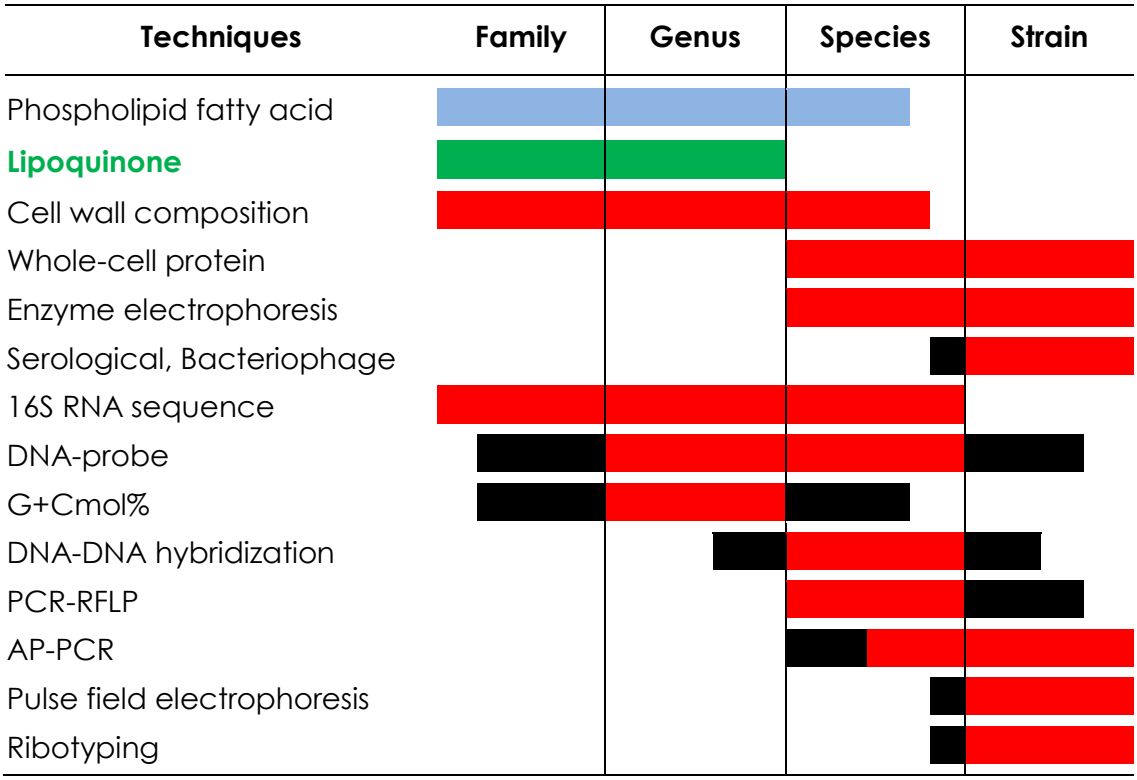

Figure 1.2

Taxonomic resolution of some of the microbial techniques [31].

1.4 Lipoquinone Analysis

Recently, characterization and identification of microbial community using chemotaxonomic approached are in great interest. The lipoquinone profile is one of the most promising methods for this purpose [32]. This technique has been considered to be a useful tool for the analysis of changes in microbial population dynamics in mixed cultures [14].

Lipoquinone, which is constituents of the bacterial plasma membrane that essential for electron transport, is divided into two main types: ubiquinone (associated with 1-methyl-2-isoprenyl-3,4-dimethoxyparabenzoquinone) and menaquinones (associated with 1-isoprenyl-2-methyl-naphthoquinone). The nomenclature of quinone is as follows: the abbreviation of quinone type

(ubiquinone: UQ, menaquinone: MK) followed by a dash and number of isoprenoid units in its side chain. For example, UQ-8 indicates a ubiquinone with 8 isoprene units in its side chain, and MK-8(H2) shows a menaquinone with 8 isoprene units in its side chain and one of the double bonds in the side chain is saturated with 2 hydrogen atoms [14, 21, 33]. Generally, a species or genus in a microbial community produces one dominant type of lipoquinone and any changes in the lipoquinone profile could reflect the changes in the microbial community [13-15].

Lipoquinone are lipid-soluble substances that existed in almost all species of organisms. In prokaryotic cells, lipoquinone located exclusively in their cytoplasmic membrane. In eukaryotic cells, most of the lipoquinone are associated with the inner membrane of mitochondria [13]. **Figure 1.3** shows the quinone in the cell membrane of the microorganism.

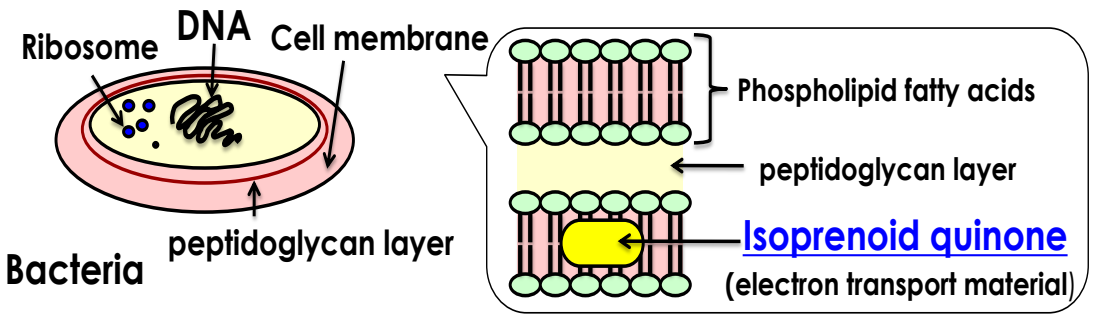

Figure 1.3
Quinone in cell membrane of microorganism.

The use of lipoquinone profile is convenient for the assessment of complex microbial communities in environmental samples because it is applicable for simultaneous analysis of prokaryotic and eukaryotic microorganisms with the technical superiority in term of simplicity, high reproducibility and ease in data interpretation [34]. In addition, the species with a smaller fractional content (usually as small as about 0.001 in mole fraction) in an ecosystem could also quantified with a higher reliability using lipoquinone analysis. This made it possible to evaluate the microbial community without a loss of information

about the species with a smaller fractional, which also play an important role in an ecosystem [21, 35].

Lipoquinone profile has been used for microbial community analysis in wide range of environmental samples. Hedrick *et al.* proposed the lipoquinone analysis using liquid chromatography [33]. Hiraishi *et al.* uses the lipoquinone profile as a tool for identifying bacterial populations in activated sludge, which has a wide variety of microorganisms [32, 36]. Fujie *et al.* apply the lipoquinone profile to identify the microbial composition and diversity among the soils [21, 37, 38]. The application of lipoquinone profile has also been used to monitor the microbial community in composting process and the compost product [6, 16, 17, 34]. In those studies, lipoquinone was post from the matrix using organic solvent extraction method [20]. Since organic solvent extraction takes long extraction time and a large volume of organic solvent, which is not consistent with the principles of green analytical chemistry, the supercritical fluid extraction was introduced [16-18].

1.5 Perspectives of SFE and Studies on Lipoquinone Analysis using SFE

The SFE is the process of extracting one component from another (the matrix) using supercritical fluids as the extracting solvent. Recently, SFE has been emerging as a superior alternative technique for extraction of natural products and other environmental samples because of its high diffusivities, being suitable for thermo-sensitive substance, producing the cleaner extracts and environmental benignity [22, 39]. Particularly, the $scCO_2$ is the most widely used since it is nontoxic, non-flammable, non-corrosive, and environmentally friendly for analytical scale usage. In addition, it is cheap and readily available in bulk quantities with a high degree of purity [40].

1.5.1 *Definition of Supercritical Fluid*

A supercritical fluid is an element or compound above its critical pressure and critical temperature [39, 40]. The definition of a supercritical fluid is best described by using a typical pressure-temperature phase diagram as shown in

Figure 1.4. There are four main phases: the solid, liquid, gas, and supercritical which is control by the environmental temperature and pressure conditions.

When the combination of temperature and pressure is such that the substance can exist as a gas, liquid, and solid, simultaneously, it is called triple point. In the region above the critical temperature and pressure, a substance can no longer be classified as either a gas or a liquid since it has properties of both. A change of temperature or pressure in the supercritical region changes the phase properties of the compound. In this region above the critical temperature and pressure, a substance is said to be a supercritical fluid.

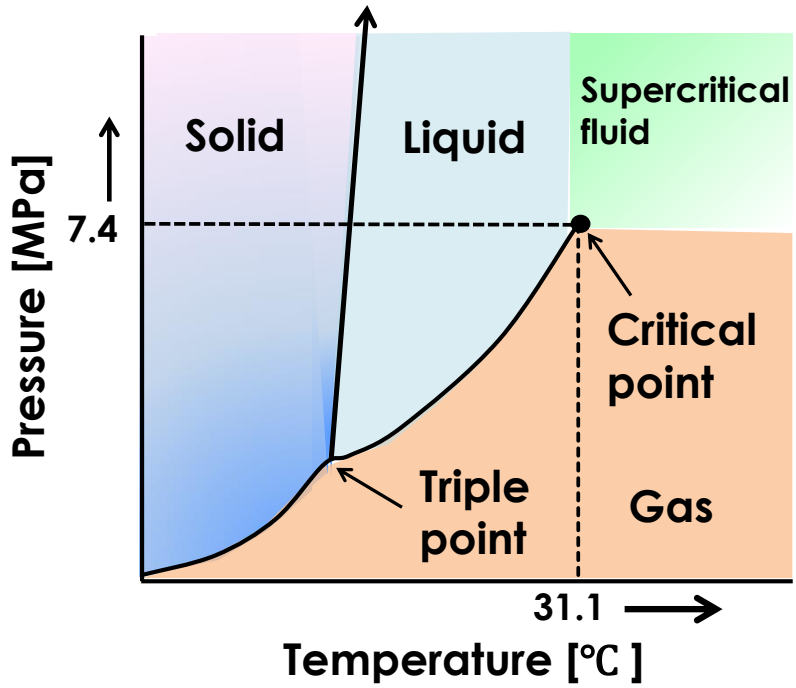

Figure 1.4

Pressure-temperature phase diagram for CO_2 [39, 40].

If the pressure increases at given temperature, the gas density becomes higher, however, the inter-molecular distance becomes shorter, so that the intermolecular effects is impossible to disregard. It can be said that from the microscopic standpoint, they are in a state close to liquid. If the molecular movement can be directly observed, it is assumed to be as violent as in the gaseous state. This is the reason that supercritical is the intermediate state

between gas and liquid [22, 41]. The low critical value of CO_2 (31.1 °C and 7.38 MPa) allows the supercritical operation at low pressures and near room temperature. It can be used with or without the addition of a polar co-solvent (such as alcohols or other organic chemicals) to enhance extraction efficiency, and widen the range of what is solvated in the supercritical fluid [22].

1.5.2 *Characteristics of Supercritical fluids*

It is both the liquid-like and gas-like characteristics of supercritical fluids that make them unique for chemical separation. In particular, supercritical fluid densities, diffusivities, and viscosities fall into ranges between those of liquids and gases. The critical pressure and temperature for various solvents classified according to their chemical nature, as well as the fluid density at critical point which called the critical density are summarized in **Table 1.1.**

The physical properties of gases, supercritical fluids (SCF), and liquids are shown in **Table 1.2**. The hydrodynamic properties of a supercritical fluids also lie between a liquid and a gas, imparting favorable qualities for extractions to the SCF in this respect. The dynamic viscosity of a SCF is comparable to that of a gas at the same temperature. However, the density of a SCF near its critical point is at least 2 orders of magnitude higher than that of a gas, resulting in a very low kinematic viscosity. This is very advantageous for mass transfer, since natural convection effects are inversely proportional to the square of the kinematic viscosity. The following sections elucidate the several properties of supercritical fluids, namely: diffusivity, solubility, and density. The properties of supercritical fluids are frequently expressed in terms of reduced rather than absolute values. A reduced value is defined as the ratio of actual absolute value to the critical point value.

Table 1.1

Feature of various solvents at critical point [39]

Solvents	Critical temperature [℃]	Critical pressure [bar]	Critical density [g/ml]
Inorganic			
CO_2	31.1	72	0.47
N_2O	36.5	70.6	0.45
NO_2	158	98.7	0.27
Ammonia	132.5	109.8	0.23
Water	374.2	214.8	0.32
Helium	-268	2.2	0.07
Hydrogen	-240	12.6	0.03
Xenon	17	56.9	1.11
Hydrogen Chloride	51	83.3	0.45
Sulphur Dioxide	157	76.8	0.52
Hydrocarbons			
Methane	-82	46	0.169
Ethane	32.3	47.6	0.2
Propane	96.7	42.4	0.22
n-Butane	152	70.6	0.228
n-Hexane	234.2	28.9	0.23
Benzene	288.9	98.7	0.302
Toluene	319	41.1	0.292
Alcohols			
Methanol	239	78.9	0.27
Ethanol	243.4	72	0.276
Isopropyl alcohols	235.3	47.6	0.27
Miscellaneous			
Acetone	235	47	0.279
Acetonitrile	275	47	0.25
Pyridine	347	56.3	0.312

Table 1.2 Comparison of physical properties of gases, SCFs and liquid [39]

Properties	Gas	SCFs	Liquid
Density [kg/m^3]	1	100-800	1000
Viscosity [cP]	0.01	0.05-0.1	0.5-1
Diffusivity [mm^2/s]	1-10	0.01-0.1	0.001

Diffusivity

The diffusivity in supercritical CO_2 are describes in **Figure 1.5**. Diffusivity is classically defined as the mass of solute transferred per unit area per unit time under unit concentration gradient. The diffusivity of a solute defines the rate of transfer of the solute in a given fluid under the driving force of a concentration gradient. The mass transfer process is called diffusion.

The diffusivity of a solute in a supercritical fluid always exceeds that in an ordinary liquid solvent. Diffusivity in a supercritical fluid decreases with increase in the pressure. Diffusivity increases with increasing temperature, especially in vicinity of the critical point. The slopes of diffusivity-temperature isobars increase with decreasing temperature and pressure.

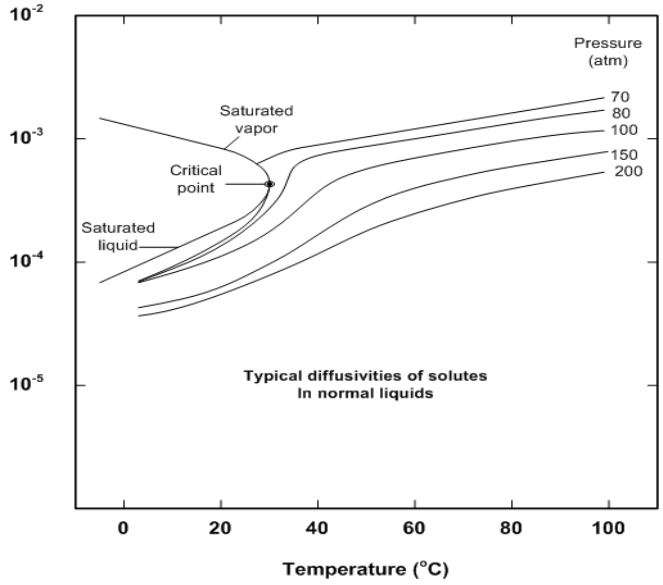

Figure 1.5

Variation of the diffusivity in CO_2 as a function of temperature at several pressures [39].

Solubility

Among the unique features characteristic of the solubility behavior of a solute in an SCF solvent are the exponential solubility enhancement. The solubility behavior of solute in a SCF solvent is analyzed in terms of pressure and temperature. At higher densities, the molecular interactions between the solvent and the solute are enhanced and as a result, more solute is dissolved.

By changing the pressure and temperature of the fluid, the properties can be "tuned" to be more liquid or more gas like. Solubility in a supercritical fluid tends to increase with density of the fluid (at constant temperature). Since density increases with pressure, then solubility also tends to increase with pressure. At constant density, solubility will increase with temperature [39].

The dependence of solubility on solute structure is the main factor for estimating the relative effectiveness of SFE as a sample preparation method. The solubility in the binary CO_2/solute system suggests that the nonpolar, lipophilic solute showing the largest solubility in scCO_2. For the qualitative trends in solute solubility suggests that an increase in the molecular mass of a homologous or oligomeric series of solutes lead to a decrease in solubility in the supercritical fluid phase [42].

Density

The density of a material is its mass per unit volume. The density of a supercritical fluid is markedly dependent on its pressure and temperature. The variation of the density of a supercritical fluid with the pressure at a constant temperature (an isotherm) is typically non-linear. Density in the supercritical region increases sharply with increasing pressure at a constant temperature; also, it decreases with increasing temperature at a constant pressure. The density of a supercritical fluid can be altered over a wide range by changing the pressure, the temperature or both. The dissolving power of a given fluid depends on its density [39].

Dielectric Constant

The dielectric constant is one of the physical-chemical properties for defining the solubility of a substance in fluids. For the CO_2, the dielectric constant increases with pressure as its density. The dielectric constant of CO_2 in a very dense state (200 bar and 40°C) is 1.5, so it can be assimilated to highly non polar solvent which is appropriate for dissolving nonpolar substances [39].

Since the lipoquinone has higher polarity than CO_2, the addition of a polar co-solvent as modifier was needed to adjust the polarity of the extraction solvent. By adjusting several parameters, the SCF can extract the quinone selectively. The dielectric constant of several solvent are shown in **Table 1.3**. The higher the dielectric constant, the higher the polarity of the solvent.

Table 1.3 The dielectric constant of several solvent [43, 44]

Compound	Dielectric Constant at temperature (°C)
Water	78 (25)
Methanol	33 (20)
Ethanol	25 (25)
Acetone	21 (25)
Chloroform	4.4 (20)
Toluene	2.4 (20)
Carbon tetrachloride	2.2 (20)
Hexane	2.0 (20)
CO_2	1.5 (40)

Studies on Lipoquinone Analysis using SFE

The lipoquinone profile method is widely used for analyzing microbial community structures because it is a relatively simple procedure, quantitative, and highly reproducible [35]. Since lipoquinone profile is useful as a biomarker in microbial community analysis, and the potential application of supercritical fluid extraction in sample pretreatment, the study of lipoquinone analysis using SFE is growing interest.

Irvan *et al*. investigated SFE conditions for activated sludge and found that methanol is the best modifier for extraction of lipoquinone using SFE. The SFE conditions for lipoquinone extraction from activated sludge are as follows: 25 MPa; 55°C, flow rate of 3 mL min^{-1}, using methanol as a modifier (10%) for 0.1 g sample. Comparable lipoquinone profile obtained between organic solvent

extraction method and SFE method showed the possibility of SFE in replacement of organic solvent extraction for analysis of lipoquinone [23, 24].

Another previous studied on lipoquinone using SFE is investigating the effects of the solid phase trapping to the total extracted lipoquinone from activated sludge. Using conditions obtained by Irvan *et al.*, Hanif *et al*. obtained that the solid phase cartridge could trap lipoquinone and simplify the procedure of lipoquinone extraction [25]. Therefore, the application potential of SFE and the capability of solid phase trapping system to simplify the SFE step was the motivation of development on-line SFE-HPLC. The development of on-line SFE-HPLC has beneficiary in simplify the method, reduce in time of analysis, improve the reproducibility of the method, and high feasibility of automation in analysis.

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Chapter 2

Materials and Methods

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2.1 Chemicals and Reagents

All chemicals were of analytical grade. Standard for UQ-10 and MK-7, acetone, chloroform, hexane, diethyl ether, diisopropyl ether, and methanol were obtained from Wako Co. (Osaka, Japan). The standard solutions were prepared and diluted to the desired concentration with acetone. The CO_2 from Taiyo Nippon Sanso Co. was used in all of the SFE experiments. The Sep-Pak plus silica cartridges (10 mm inner diameter (id), 20 mm long, 600 mg of silica, particle diameter of 55–105 μm) were purchased from Waters Co. (Milford, MA). Chromafil filters 20/15 MS (pore size 0.20 μm, filter diameter of 15 mm) were obtained from Macherey-Nagel (Düren, Germany).

2.2 Sample Preparation

2.2.1 *Sample Preparation for SFE of Lipoquinone from Compost for Microbial Community Analysis*

The sample used in this study was provided by the Komasuya Composting Company, located in Toyota City, Aichi Prefecture, Japan. It is a commercial full scale composting site which produces 3250 ton compost/month, consisting of a mixture of food waste (50% v/v) and wood chips. Prior to analysis of lipoquinone, the sample were dried in a vacuum-freeze dryer for 24 h, crushed and then sieved to collect particles smaller than 500 μm. Freeze dried compost samples were stored at -30°C until analysis. Approximately 0.3 g of freeze dried compost sample was used in each extraction experiment.

2.2.2 *Sample Preparation for Development of On-line SFE-HPLC for Lipoquinone Analysis in Activated Sludge*

Activated sludge samples for development of on-line SFE-HPLC was obtained from three different wastewater treatment plants: TUT domestic wastewater treatment plant (capacity of 3.8×10^2 m^3 day^{-1}), NK wastewater treatment plant (capacity of 8×10^4 m^3 day^{-1}), and TG wastewater treatment plant (capacity of 10.4×10^4 m^3 day^{-1}). The NK and TG plants are typical wastewater treatment facilities that treat sewage discharged from residential and industrial areas. However, the treatment methods at these wastewater treatment plants are different. The NK plant uses an aerobic system. The TG plant has two types of activated sludge systems: one uses only an aerobic system and the other uses a combined anaerobic-aerobic system. Samples from TG plant were taken from the mixing effluent of both systems. On the other hand, the TUT plant uses an intermittent aeration system, it is on-off period of aeration process in the same tank. Sample from TUT plant was used for optimizing the analytical conditions of on-line SFE-HPLC. The samples from NK and TG were then analyzed under the optimized conditions as the application of on-line SFE-HPLC. The samples were dried in a vacuum freeze dryer for 24 h, and then homogenized by crushing and sieving to collect particles smaller than 500 μm. Freeze-dried samples were stored at -30°C until analysis.

2.2.3 *Sample Preparation for Effects of Static Extraction to the Extraction Efficiencies using On-line SFE-HPLC for Lipoquinone Analysis in Activated Sludge*

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

2.3 Apparatus

2.3.1 *SFE of Lipoquinone from Compost for Microbial Community Analysis*

The extractions were performed using the SFE system, as shown in **Figure 2.1**. This system consists of a cooler (Scinics CH-201, Tokyo, Japan), pumps (PU-1580 HPLC and PU-2086 HPLC, Jasco, Tokyo, Japan), a preheating coil (0.25 mm id × 1.59 mm od × 2 m), an oven (GC-353B, GL Sciences, Tokyo, Japan), and a back-pressure regulator (BPG 880-81, Jasco). A freeze dried compost sample was then placed into a 1 ml stainless steel extraction vessel (SUS316, Jasco).

The Water Acquity UPLC system (Milford, MA, USA) that is equipped with a binary solvent delivery manager, a sample manager and a photo-diode array detector (PDA-2996, Waters), was used for the quantification of lipoquinone during all parts of the study. A Waters Acquity UPLC™ BEH C18 column (2.1 mm id × 50 mm, particle size 1.7 µm) was used as the analytical column.

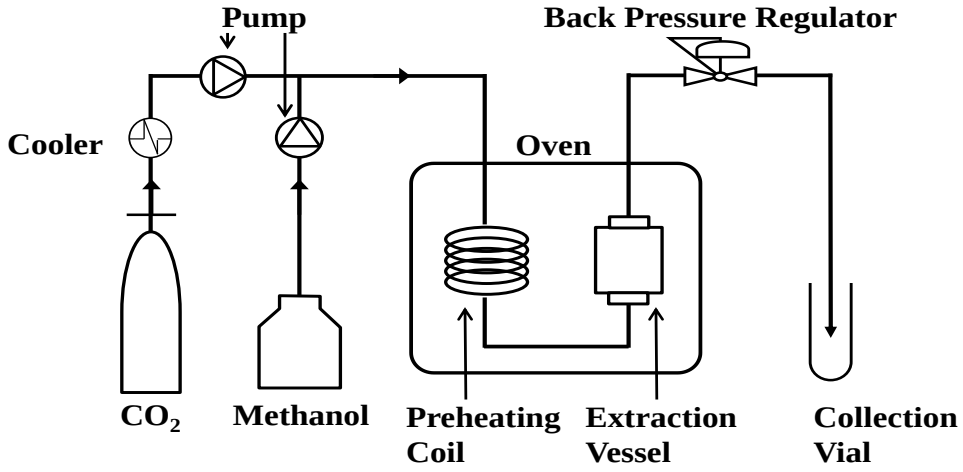

Figure 2.1

Schematic diagram of SFE apparatus for lipoquinone extraction.

2.3.2 *Development of On-line SFE-HPLC for Lipoquinone Analysis in Activated Sludge*

A schematic diagram of the on-line SFE-HPLC system is shown in **Figure 2.2**. As mentioned in the previous section, the SFE consisted of a cooler (Scinics CH-201, Tokyo, Japan), pumps (PU-1580 HPLC and PU-2086 HPLC, Jasco, Tokyo, Japan),

preheating coil (0.25 mm id ×1.59 mm od × 2 m), oven (GC-353B, GL Sciences, Tokyo, Japan), 1 mL inner volume of extraction cartridge (SUS316, Jasco) for sample extraction, and backpressure regulator (BPG 880-81, Jasco). In the on-line SFE-HPLC, water pump (PU-2080, Jasco) was placed between SFE and HPLC to pump the water at a certain flow rate to the trapping column. The extraction and analysis system were connected via a guard-column (Zorbax SB C18, Agilent, United States; 4.6 mm id × 12.5 mm, particle size 5 µm) as a trapping column, which was connected to a six-port valve (Rheodyne, Cotati, CA) in the HPLC system. The six-port valve provided two switching modes: extraction mode and analysis mode. This valve was linked to the injection valve in the HPLC.

The HPLC was also equipped with a degasser (DGU-14A, Shimadzu, Kyoto, Japan), two high-pressure pumps (LC-10AD VP, Shimadzu), analytical column (CD-C18, Cadenza, Imtakt Technologies, United States, 4.6 mm id × 250 mm, particle size 3 µm), an oven (CTO-10AS VP, Shimadzu), a UV-Vis detector (SPP 10A VP, Shimadzu) and a PDA detector (SPD M10A VP, Shimadzu).

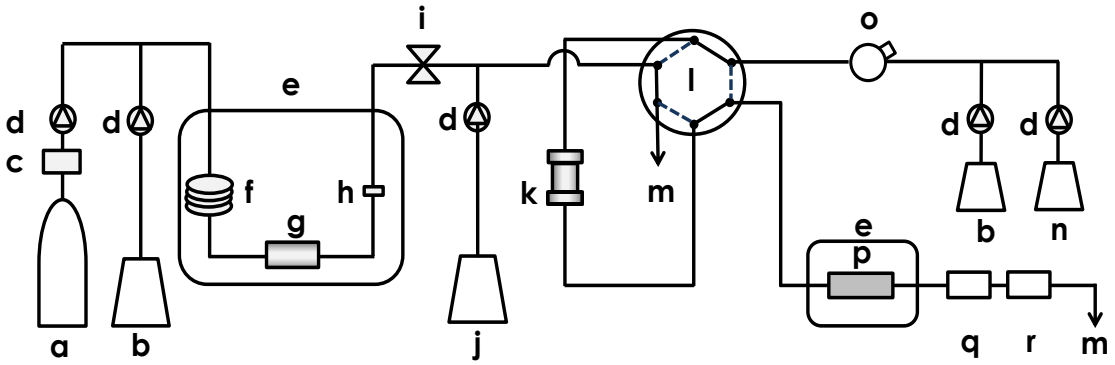

Figure 2.2

Schematic diagram of on-line SFE-HPLC for lipoquinone; (a) CO_2; (b) Methanol; (c) Cooler; (d) Pump; (e) Oven; (f) Preheating Coil; (g) Extraction Vessel; (h) Filter; (i) Back Pressure Regulator; (j) Water; (k) Trapping Column; (l) Six-port Valve; (m) Waste; (n) Diisopropyl Ether; (o) Injector; (p) Separation Column; (q) Uv-vis Detector; (r) PDA detector.

2.3.3 *Effects of Static Extraction to the Extraction Efficiencies using On-line SFE-HPLC for Lipoquinone Analysis in Activated Sludge*

A schematic diagram of the simplified on-line SFE-HPLC system is shown in **Figure 2.3**. In this system, two pumps are eliminated. Therefore, the SFE consisted of a cooler (Scinics CH-201, Tokyo, Japan), pump (PU-1580 HPLC, Jasco, Tokyo, Japan), preheating coil (0.25 mm id ×1.59 mm od × 2 m), oven (GC-353B, GL Sciences, Tokyo, Japan), 1 mL inner volume of extraction cartridge (SUS316, Jasco) for sample extraction, and backpressure regulator (BPG 880-81, Jasco). The extraction and analysis system were connected via a guard-column (Zorbax SB C18, Agilent, United States; 4.6 mm id × 12.5 mm, particle size 5 µm) as a trapping column, which was connected to a six-port valve (Rheodyne, Cotati, CA) in the HPLC system. The six-port valve provided two switching modes: extraction mode and analysis mode. This valve was linked to the injection valve in the HPLC.

The HPLC that equipped with a degasser (DGU-14A, Shimadzu, Kyoto, Japan), two high-pressure pumps (LC-10AD VP, Shimadzu), analytical column (CD-C18, Cadenza, Imtakt Technologies, United States, 4.6 mm id × 250 mm, particle size 3 µm), an oven (CTO-10AS VP, Shimadzu), a UV-Vis detector (SPP 10A VP, Shimadzu) and a PDA detector (SPD M10A VP, Shimadzu) was used for the quantification of lipoquinone.

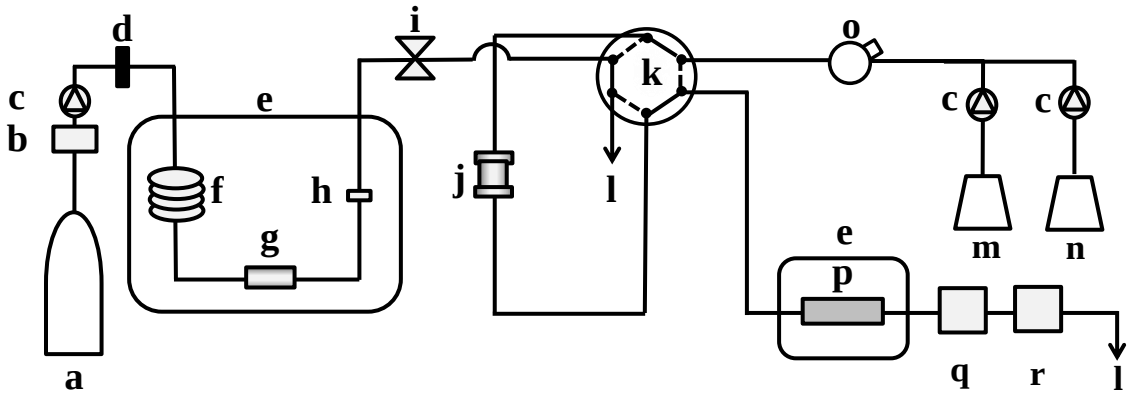

Figure 2.3

Schematic diagram of simplified on-line SFE-HPLC for lipoquinone. (a) CO_2; (b) Cooler; (c) Pump; (d) CO_2 Valve; (e) Oven; (f) Preheating Coil; (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.

2.4 Organic Solvent Extraction

Organic solvent extraction was performed to extract lipoquinone, as reported elsewhere [1-2]. For extraction of lipoquinone from compost, the 0.3 g freeze-dried compost was used, on the other hand, for extraction of lipoquinone from activated sludge, the 0.1 g of freeze-dried activated sludge was used. Lipoquinone were extracted from the activated sludge by using chloroform: methanol (2:1 v/v), followed by filtration and evaporation. To remove the impurities, the crude lipoquinone extract was extracted with hexane and water, and then fractionated using Sep-Pak plus silica cartridges in series at a fixed flow rate of 24 mL min^{-1} before HPLC analysis [2]. Triplicate experiments were carried out to determine the precision of the experiments.

2.5 Analytical Methods

2.5.1 Optimization SFE for Lipoquinone Extraction in Compost and Analysis using UPLC

The extractions were conducted in dynamic mode. The CO_2 was used as an extraction solvent at a flow rate of 2.7 mL min^{-1} and methanol as a modifier at 0.3 mL min^{-1} were continuously mixed in the line to extract the lipoquinone from the compost sample. The extracted lipoquinone was purified, as described elsewhere [3]. The hexane extract was loaded into the Sep-Pak plus silica cartridge and the Chromafil before quantification on UPLC. Triplicate experiments were carried out to determine the precision of all experiments.

Methanol was used for the mobile phase in determination of lipoquinone using UPLC, with a flow rate at 0.3 mL min^{-1}. The analysis was performed at 35 ± 1°C with a chromatographic run time of 50 min. The auto sampler temperature was set at 4.0 ± 1°C and the sample injection volume was 10 µL [4].

The UQ and MK species were identified based on the retention time on the column and the spectrum of each peak observed in the PDA detector. The PDA detector is a multi-channel UV detector that facilities the accurate determination of quinones composition [5]. The MK observed at 270 nm and the UQ observed at 275 nm.

The linier correlation between the logarithm of retention time of lipoquinone and an equivalent number of isoprenoid unit (ENIU) was used to identify the lipoquinone species [6, 7]. UQ-10 and MK-7 were used as quantitative standards for UQ and MK, respectively. The ENIU can be approximated by the following equation:

$$ENIU_k = a + b\log\left(\frac{ET_k}{ET_{std}}\right) + c\left[\log\left(\frac{ET_k}{ET_{std}}\right)\right]^2 \quad \text{.....(2.1)}$$

where ET_k represents the elution time of a quinone species k, and Et_{std} represents the elution time of standard quinone. The constants are shown as a, b, and c, which are

empirically obtained for each UPLC system [7]. The amounts of lipoquinone were calculated from the peak area based on the mole absorption coefficients (ubiquinone 14.4 $mM^{-1}cm^{-1}$, menaquinone 17.4 $mM^{-1}cm^{-1}$) [8]. The lipoquinone mole fraction was calculated as a ratio of the lipoquinone content in the species k to the total lipoquinone content.

2.5.2 Analytical Methods for Development of On-line SFE-HPLC for Lipoquinone Analysis in Activated Sludge

Optimization on-line SFE-HPLC for activated sludge was performed under the following conditions: pressure of 25 MPa, extraction temperature of 45°C, and extraction time of 15 min [3]. The CO_2 extraction solvent and methanol modifier were supplied at a flow rate of 1 mL min^{-1} because there was little difference in the amount of lipoquinone detected at flow rates of 3 and 1 mL min^{-1} (data not shown). The freeze dried activated sludge (0.1 g) was put in a 1 mL stainless steel extraction vessel and placed in the SFE system. The CO_2 and methanol were continuously mixed in the line and passed through the extraction vessel to the outlet of the SFE. The extracted lipoquinone was carried through the regulator, mixed with water at a particular flow rate, and collected in the trapping column, which was the interface between the SFE and HPLC systems. The two switching valve modes allowed the standard solution to be determined in the analysis mode by using the injection valve (20 µL sampling loop), which was part of the HPLC system. In the on-line SFE-HPLC, when the extraction was complete, the valve was switched to the analysis mode to connect the trapping column to the HPLC system.

In HPLC, an isocratic mixture of methanol: diisopropyl ether (4:1 v/v) was used as the mobile phase with a flow rate of 1 mL min^{-1}. 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 80 min. The UQ and MK species were identified based on the retention time and the spectrum. The correlation between the logarithm of the quinone retention time and the equivalent number of isoprenoid units was used to identify the quinone species, as previously mentioned [6, 7]. The amount of quinone was calculated from the peak area based on the molar absorption coefficient (UQ: 14.4

mM^{-1} cm^{-1}, MK: 17.4 mM^{-1} cm^{-1}) [8]. Triplicate experiments were carried out to determine the precision. The effects of water flow rate and temperature on trapping efficiency were investigated in on-line SFE-HPLC for activated sludge.

2.5.3 *Analytical Methods for Effects of Static Extraction to the Extraction Efficiencies using On-line SFE-HPLC for Lipoquinone Analysis in Activated Sludge*

The on-line SFE-HPLC was performed at the pressure of 25 MPa with the CO_2 flow rate of 1 mL min^{-1} and the extraction temperature of 45°C. Samples was put into a 1 mL stainless steel extraction vessel and methanol as a modifier was spiked to the extraction vessel in a certain volume. The extraction vessel then set in the SFE system. After reaching the corresponding supercritical fluid conditions, the static extraction was performed and in the end of the static extraction, the dynamic extraction with the constant flow rate of CO_2 was carried out. In this study, the effects of static and dynamic extraction time as well as methanol volume were optimized. The extracted lipoquinone was then collect in the trapping column which was connected to six-port valve in HPLC system. When the extraction was completed, the six-port valve was switched to the analysis mode to connect the trapping column into the HPLC system. The extracted lipoquinone were then analyzed in the HPLC system using the same conditions as mentioned in the on-line SFE-HPLC. The schematic diagram of simplified on-line SFE-HPLC system for quinone is shown in **Figure 2.3.**

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Chapter 3

Supercritical Fluid Extraction of Lipoquinone from Compost for Microbial Community Analysis

Chapter 3

Supercritical Fluid Extraction of Lipoquinone from Compost for Microbial Community Analysis

Summary

Supercritical fluid extraction (SFE) was used to extract lipoquinone from compost to monitor the microbial community dynamics during composting. The 0.3 g of dried compost was extracted using 3 mL min^{-1} of carbon dioxide (90%) and methanol (10%) at 45°C and 25 MPa for a 30 min extraction time. The extracted lipoquinone were analyzed using ultra performance liquid chromatography (UPLC) with 0.3 mL min^{-1} of methanol mobile phase for a 50 min chromatographic run time. A comparable detected amount of lipoquinone was obtained using the developed method and an organic solvent extraction method; being 36.06 µmol kg^{-1} and 34.54 µmol kg^{-1}, respectively. The significantly low value of dissimilarity index (D) between the two methods (0.05) indicated that the lipoquinone profile obtained by both methods was considered identical. The developed method was then applied to determine the maturity of the compost by monitoring the change of lipoquinone during composting. The UQ-9 and MK-7 were predominant lipoquinone in the initial stage of composting. The diversity of lipoquinone became more complex during the cooling and maturation stages. This study showed that SFE had successfully extracted lipoquinone from a complex matrix. The developed method offered not only simplification of the analysis but also the rapidity that is beneficial for routine analysis.

Keywords: compost; lipoquinone profile; supercritical fluid; extraction; UPLC

3.1 Introduction

Composting is one of the effective method for the conversion of organic waste into valuable products that can be used in agricultural. Compost can improve the physical properties of the soil, increase the organic matter content and cation exchange capacity, as well as the crops production [1, 2]. Nonetheless, in some cases, the utilization of immature compost may generate destructive effects, including delay in germination and inhibition of grain yield [3, 4].

Immature composts might contain much easily decomposable organic matters such as organic acids [4]. The organic matters provides a high microbial activity that requires a large intake of oxygen and nitrogen. High oxygen and nitrogen consumption by microorganisms often cause those deficiencies to grow crops. Therefore, the maturity of compost is essential for the soil environment and the crop production.

The microbial community plays an important role in composting, therefore, the evaluation of compost maturity based on it has attracted an increased focus [1, 5]. One of the convenient methods for the assessment of microbial community is the lipoquinone profile method due to its simplicity, quantitative and high repeatability [1, 6-8]. The evaluation of the microbial community in compost is based on the changes of lipoquinone profile. In general, one species or genus of microbial community has only one dominant type of lipoquinone [1, 9, 10]. The identification of classes of lipoquinone provides a snapshot of the diversity of microbes present. Therefore, a lipoquinone profile could represent the microbial community structure during composting.

The lipoquinone profile method has been applied to monitor the microbial community structure in composting. Tang et al. applied the lipoquinone profile method to detect the microbial community structure in various compost product [1]. Hiraishi et al. used this method to analysis the microbial community structure in composting of a solid biowaste for fed-batch treatment [11-12]. Yu et al. also

applied lipoquinone profile method to identify the succession of microbial community and lignocellulose degradation on agricultural waste composting [6].

Lipoquinone determination is consists of the extraction of lipoquinone from the matrix and analysis using HPLC [8, 13]. Organic solvent extraction using a mixture of chloroform and methanol has been used as a sample preparation for extracting lipoquinone [8, 13]. However, the procedure is tedious and time consuming. Moreover, this method requires a large quantity of organic solvent.

In order to overcome these disadvantages, a sample preparation with SFE has been developed [14, 15]. SFE has been successfully used as an effective, rapid and quantitative extraction technique for lipoquinone from activated sludge samples [14, 15]. Irvan et al. reported that the optimum conditions for extraction of lipoquinone from activated sludge were at a pressure of 25 MPa, a temperature of 55°C, and 10% (v/v) methanol for a 15 min extraction time [14].

In this study, the SFE was used to extract lipoquinone from commercial compost of food waste and wood-chips, as a substitute for organic solvent extraction. Since the compost contained a more complex matrix if compare to activated sludge, the amount and the diversity of the microbial community in compost also higher than in the activated sludge. This might make the extraction of lipoquinone from compost difficult, therefore, the extraction conditions including the extraction time and temperature was examined to obtain optimized conditions. The method was validated by comparing the lipoquinone profile with those obtained from an organic solvent extraction method. Furthermore, this method was then used to determine the lipoquinone profile for daily monitoring of the composting process.

3.2 Results and Discussion

3.2.1 *Effects of Extraction Temperature on the Detected Amount of Lipoquinone from the Compost*

Extraction temperature is one of the essential parameters in SFE with respect to the complexity of the matrix in the compost and decomposition of lipoquinone. In this study, the effects of extraction temperature was examined at a constant pressure of 25 MPa over a temperature range from 35°C to 55°C, using CO_2 for solvent extraction at a flow rate of 2.7 mL min^{-1} and methanol as a modifier at a flow rate of 0.3 mL min^{-1} for a 30 min extraction time. **Figure 3.1** shows the effects of the extraction temperature on the detected amount of lipoquinone from compost. The results showed that the detected amount of lipoquinone was highest at a temperature of 45°C.

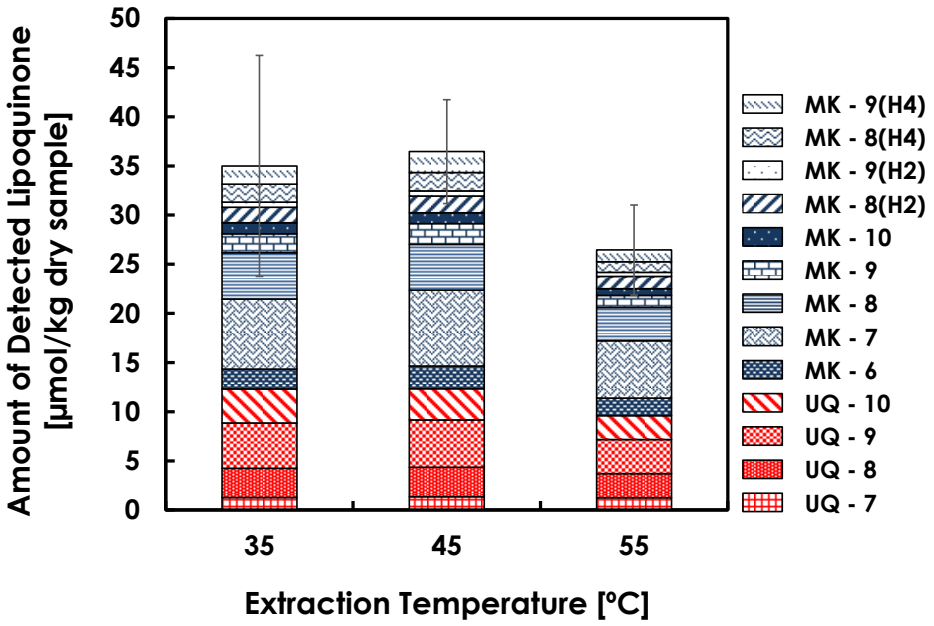

Figure 3.1

Detected amount of lipoquinone obtained on different extraction temperature. Sample: 0.3 g dry compost of food waste and wood chips. SFE conditions: 25 MPa; 90% CO_2-10% methanol with flow rate of 3 mL min^{-1}; 30 min; number of samples examined = 3.

The increase of extraction temperature at the constant pressure causes an enhancement in diffusivity and supercritical fluid which increases the solubility of the lipoquinone. When the extraction temperature was higher than 45°C, the detected amount of lipoquinone decreased due to the decomposition of lipoquinone at temperatures above 45°C. Based on the results shown in **Figure 3.1**, a temperature of 45°C was the optimum temperature over the examined temperature range because the detected amount of lipoquinone was highest and the error bar was relatively small, due to the minimal loss of lipoquinone during the extraction. Therefore, a temperature of 45°C is considered as the most appropriate temperature to extract lipoquinone from compost.

3.2.2 *Effects of Extraction Time on the Detected Amount of Lipoquinone from the Compost*

Compost contains a complex matrix which might provide difficulty in the process and that requires extra energy to extract lipoquinone from compost. Therefore, the extraction time is another crucial parameter in SFE. Varied extraction times of between 10 to 40 min were evaluated to determine the suitable extraction time for lipoquinone from compost with SFE. **Figure 3.2** shows the detected amount of lipoquinone at a different extraction time.

A comparable detected amount of lipoquinone was obtained at an extraction time of 20 min, 30 min, and 40 min. However, at the shorter extraction time, a high error bar was obtained. This may be due to the inhomogeneous nature of the system. When the extraction time was increased to 30 min, the balance of extraction was reached to enable extraction of the analytes. At the extraction time of 40 min, the high error bar might be caused by the increased amount of CO_2 releasing from the restrictor, which could lead to higher lipoquinone loses.

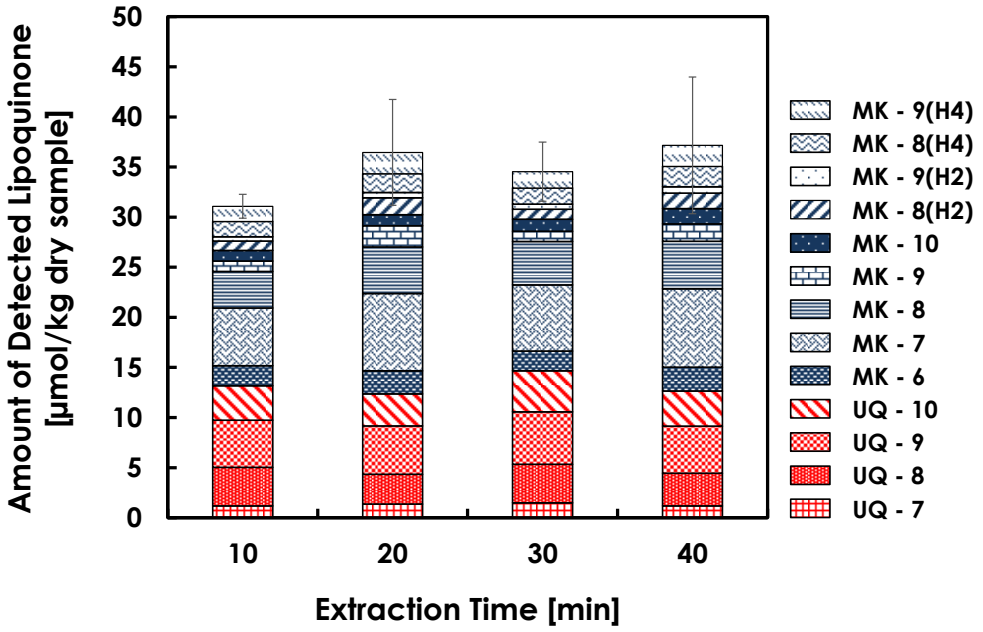

Figure 3.2

Detected amount of lipoquinone obtained in different extraction time periods. Sample: 0.3 g dry compost of food waste and wood chips. SFE conditions: 25 MPa; 90% CO_2-10% methanol with flow rate of 3 mL min^{-1}; 45°C, number of samples examined = 3.

Therefore the extraction time of 30 min was selected as the optimum extraction time to extract lipoquinone from a 0.3 g freeze dried compost sample under the tested conditions. In addition, using an extraction time of 30 min will reduce energy usage when compared to an extraction time of 40 min. The chromatogram of lipoquinone obtained from compost sample by SFE and UPLC is shown in **Figure 3.3.**

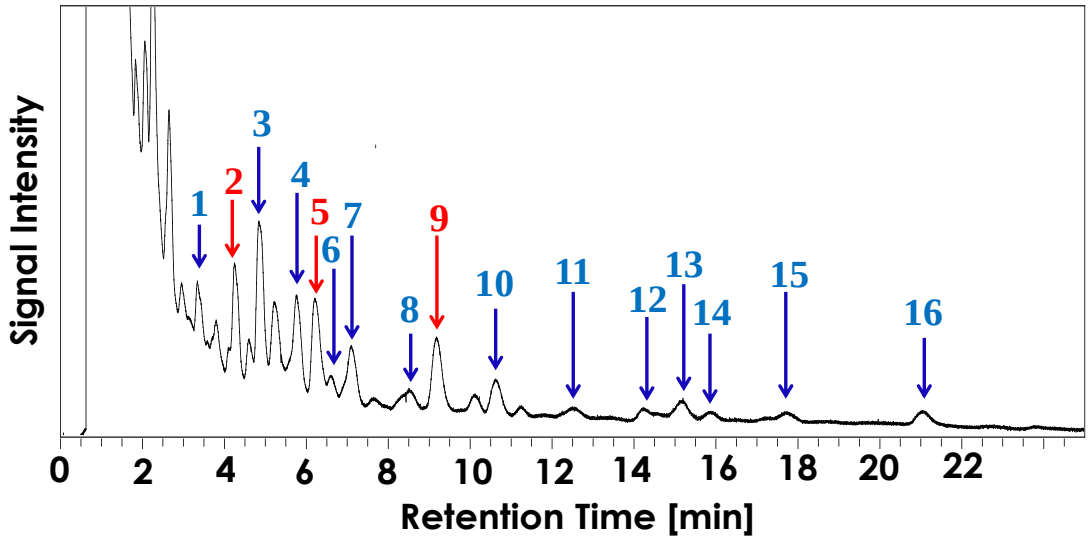

Figure 3.3

Chromatogram of lipoquinone obtained from compost by SFE and UPLC. Sample amount: 0.3 g-dried compost. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 1 mL min^{-1}. UPLC conditions: column: 2.1 × 30 mm, 1.7µm ACQUITY BEH C18 at 35°C; methanol mobile phase at flow rate of 0.3 mL min^{-1}; 10 µL sample injection volume; PDA. Descriptions: 1 = MK-6; 2 = UQ-8; 3 = MK-7; 4 = MK-7(H2); 5 = UQ-9; 6 = MK-7(H4); 7 = MK-8; 8 = MK-8(H2); 9 = UQ-10; 10 = MK-9; 11 = MK-9(H2); 12 = MK-9(H4); 13 = MK-10;14 = MK-9(H6); 15 = MK-10(H4); 16 = MK-10(H8).

3.2.3 *Comparison of Lipoquinone Profile between SFE and Organic Solvent Extraction Method*

The detected amount of quinone obtained by SFE method based on the optimized conditions was compared to those obtained by organic solvent extraction method. Both SFE and organic solvent extraction method were conducted thrice for each 0.3 g freeze dried compost sample. Four types of UQs (UQ-7, UQ-8, UQ-9, and UQ-10) and nine types of MKs (MK-6, MK-7, MK-8, MK-9, MK-10, MK-9(H2), MK-10(H4), MK-9(H6), and MK-other) were obtained by using the two methods. The predominant of MK-7 in compost was also confirmed by another

author [1, 6]. The amount and the composition of each lipoquinone species obtained by the two methods are shown in **Figure 3.4**. Total detected amount of lipoquinone obtained by SFE and organic solvent extraction were 34.54 μmol kg^{-1} dry sample and 36.06 μmol kg^{-1} dry sample, respectively. These results showed that the detected amount of lipoquinone by both methods were comparable. The detected amount of lipoquinone were usually varied [1, 16] depending on the composition of the raw materials and the conditions of composting. Therefore, there are no particular tendencies on the detected amount of lipoquinone in composting.

The dissimilarity index (D) value between the two methods was evaluated; it is the number that indicated degree of differences in lipoquinone patterns between samples. It is calculated by equation as follow:

$$D_{(i,j)} = {}^{1}/_{2} \sum_{k=1}^{n} |f_{ki} - f_{kj}| \quad \text{.....(3.1)}$$

where f_{ki} and f_{kj} are molar fractions of lipoquinone species k for samples i and j, respectively. Two lipoquinone profile are considered to be different if the value of D between them is equal or greater than 0.1 [7]. In this experiments, the low value of D (0.05) indicates that the lipoquinone profile obtained by both methods were similar.

The comparable values of detected amount of lipoquinone and low value of D showed that the development of SFE can substitute the organic solvent extraction with shorter time for analysis, lower cost and less usage of organic solvent. Furthermore, this simplified method is suitable for routine analysis with a large number of samples. In this study, the SFE was used for monitoring the lipoquinone profile in the composting of food waste and wood-chips.

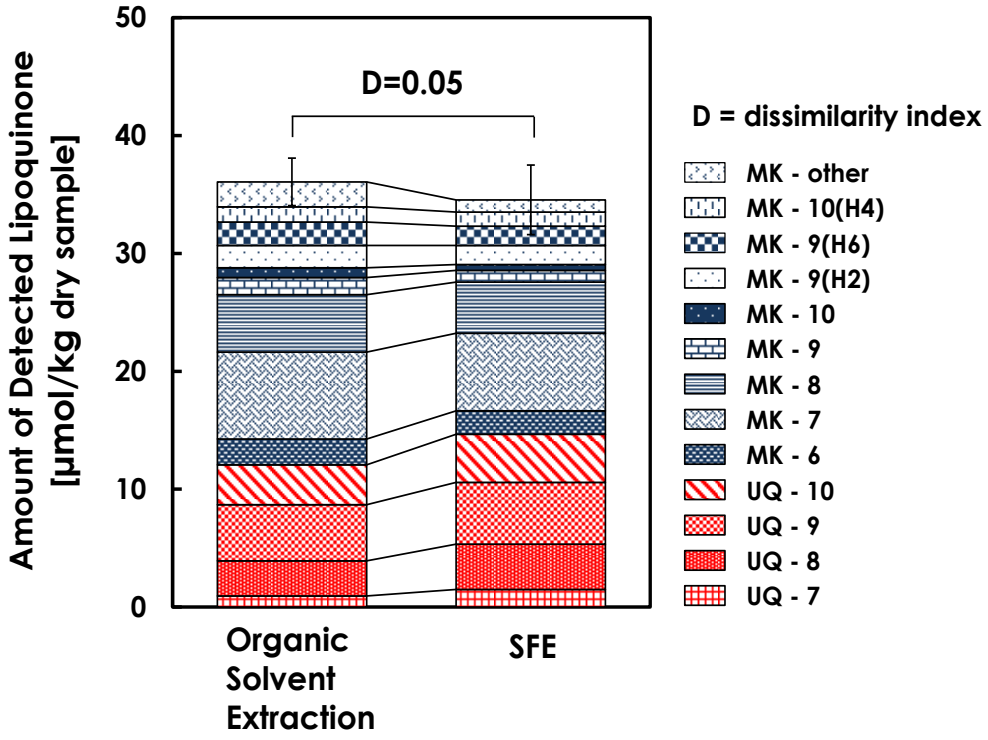

Figure 3.4

Comparison of lipoquinone profile between SFE and organic solvent extraction method. Sample: 0.3 g dry compost of food waste and wood chips for both methods. Organic solvent extraction conditions: chloroform: methanol= 2:1 (20 ml); 15 h. SFE conditions: 25 MPa; 90% CO_2-10% methanol with flow rate of 3 mL min^{-1}; 45°C; 30 min; number of samples examined = 3.

3.2.4 *Changing of the Lipoquinone Profile during Composting from Food Waste and Wood Chips*

The correlation between the changing of the lipoquinone with the temperature and C/N ratio during composting of food waste and wood chips is shown in **Figure 3.5.** The composting was divided into an initial phase with a rapidly increasing temperature, a second phase with a higher temperature, and a cooling process as

the final phase. According to the company who provided samples for this study, during the initial and the second phase of composting, the raw materials were turned once a week without aeration supply. In addition, on the second phase, the material was moved into several small outdoor piles. The aeration was applied after ten weeks of composting when the material moved into several small indoor composting areas before it was finally packed.

As shown in **Figure 3.5**, the temperature increased from the first week of composting to as high as 65°C, while the C/N ratio decreased from 28 to 22.5. The increase in temperature and the decrease of the C/N ratio were correlated to the activities of the microbial community in the degradation of organic matters [17, 18]. On the other hand, the detected amount of lipoquinone increased and reached maximum in the 4th week of composting, which shows the growth of the microbial community to decompose the easily decomposable organic matter. The predominant lipoquinone at this stage were MK-7 (20.5%), followed by UQ-9 (13.1%) and UQ-10 (12.5%). Several studies have reported that the microbial community of Proteobacteria was the common microbial community in the initial phase of composting and that Proteobacteria was known to contain the types UQ-9 and UQ-10 [6, 17]. The increasing temperature, the decreasing C/N ratio and the presence of those types of lipoquinone on this stage were also found in other studies, indicating that composting in this study was working properly [1, 19]. In addition, it shows that the developed lipoquinones analysis was acceptable for composting monitoring.

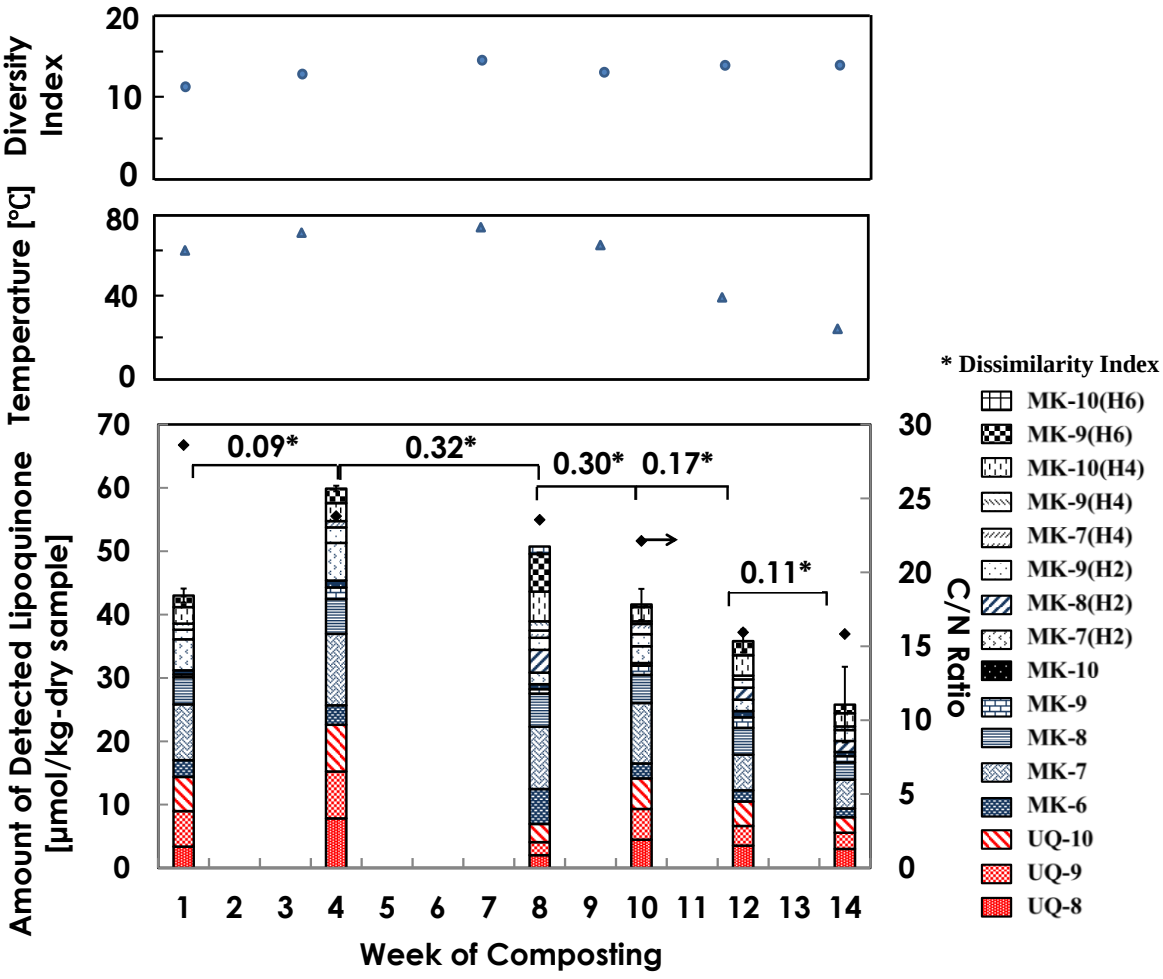

Figure 3.5

Change of lipoquinone profile, C/N ratio, temperature and diversity index of compost from food waste and wood chips. The number represents the dissimilarity index of lipoquinone profile. Number of samples examined = 3

In the second phase, the temperature rises 70°C in the 8th week of composting. In order to maintain the increasing temperature, the material was moved into a smaller pile to ensure a sufficient oxygen supply. The tendencies of the C/N ratio decreased, which is in agreement with other studies [17-19]. The detected amount of lipoquinone was gradually decreased, which indicated a decrease of biomass. The changes of biomass and lipoquinone profile were caused by a change in

composting conditions, particularly the temperature and oxygen amount. The major lipoquinone in this stage was MK-7, which is in agreement with other studies [1, 6, 18]. Other studies report that the microbial community structure from a genus of *Bacillus* where common bacteria exist at high temperatures in composting and is known to contain MK-7 as the major lipoquinone [1, 6, 18].

The final phase of composting was characterized by a gradual decrease of temperature to 40°C and the temperature of the compost product reaching 28°C. The C/N ratio was 15.9 by the end of composting. In this stage, aeration was applied to stabilize the quality of the product. The detected amount of lipoquinone decreased with the more complex profile. The gradually decrease in the detected amount of MK-7 could be an indication of the succession of the microbial community. The increase in the detected amount of MK-8(H2), MK-9(H6), MK-10(H4) and some other MK-n(Hx) were also found in this phase which suggested that these types of lipoquinones played an important role during the degradation of remaining organic matters in composting. The tendencies of temperature decrease and C/N ratio which were accompanied by an increase in the complexity of the lipoquinone obtained in this study, are considered to be the maturation of compost since the evidence was similar to those studied elsewhere [6, 17, 18]. Therefore, the physical and chemical properties of compost were significantly related to the microbial properties and it could be characterized by the lipoquinone profile that was performed by the developed method.

The dissimilarity index (D), which quantitatively indicate a change of lipoquinone profile between samples, showed that the value of D from the 1st to 4th week of composting was low. It means that the types of lipoquinones were not significantly change for the easily decomposable organic matter. However, the value of D was high from 4th to 8th week and also from 8th to 10th week of composting, which shows the significantly changing in the microbial community due to the changing of physical and chemical properties of composting. At the maturation of compost, the value of D was low that indicates the stability of the microbial community.

The complexity of lipoquinones defined by diversity index (DQ) value, which is calculated using the following equation [7]:

$$DQ = \left(\sum_{k=1}^{n} \sqrt{f_k}\right)^2 \quad \text{(3.2)}$$

where the f_k is the mole fraction of the k lipoquinone species and n is the number of lipoquinone species. As shown in **Figure 3.5**, the DQ in the initial phase of composting was 11.8 and reaching a value of more than 13 at the final phase. This indicated that the microbial community structure was developed to be more complex in the mature stage. These results were also confirmed with the previous studies, in which microbial diversity increases during cooling and maturation stage of composting [6, 14].

3.3 Conclusions

The SFE and UPLC had successfully determined the microbial community dynamic during composting based on lipoquinone profile. The comparison of this method and the conventional organic solvent extraction method, with the indication of dissimilarity index, showed that this method should be considered as a substitute of the organic solvent extraction method. Since the SFE and UPLC that proposed in this study could be used as an alternative method, the assessment of microbial community dynamics in compost should be more fully covered in future studies to collect more information in the evaluation of the maturity of compost.

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Chapter 4

Development of On-line Supercritical Fluid Extraction-High Performance Liquid Chromatography for Lipoquinone Analysis in Activated Sludge

Chapter 4

Development of On-line Supercritical Fluid Extraction-High Performance Liquid Chromatography for Lipoquinone Analysis in Activated Sludge

Summary

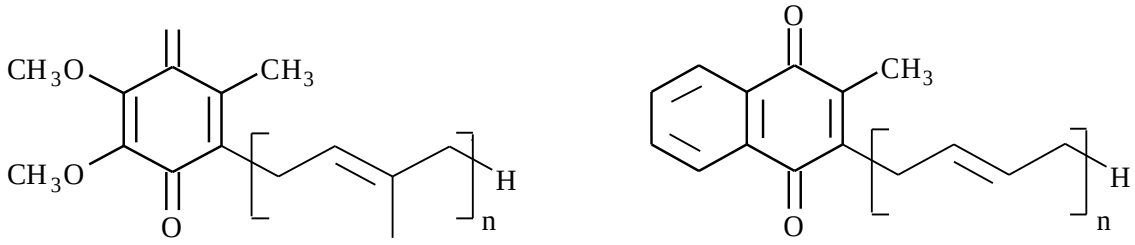
An on-line supercritical fluid extraction-high performance liquid chromatography (on-line SFE-HPLC) was developed to determine the microbial community dynamics based on lipoquinone profile. The SFE, with carbon dioxide as an extraction medium and methanol as a modifier was connected to high performance liquid chromatography (HPLC) by using trapping column as an interface for collecting lipoquinone. The method was fast (less than 2 h) and high reproducibility (1.6% in coefficient of variation). The comparable results were obtained by this method and the organic solvent extraction method, it was 603.4 μmol kg^{-1} dry sample and 594.1 μmol kg^{-1} dry samples, respectively. The low dissimilarity index (0.07) between the two methods indicates that the lipoquinone profile obtained by both methods were considered similar. The results show that the on-line SFE-HPLC method offers simplification for a rapid and routine analysis such as monitoring the performance of biological process.

Keywords: microbial community, lipoquinone profile, SFE, coupling technique, activated sludge

4.1 Introduction

Microbial community has an important role in environmental biological processes [1-7]. One of the way to control and ensure the process working properly and to improve its performance is by monitoring the microbial community. Clearly, compared with a scientific study, the routine monitoring of biological processes has different requirements and constraints for the procedure, costs, and acquisition time. Therefore, selecting an appropriate analysis method is important for monitoring environmental biological processes.

One of the straightforward analysis and culture independent method for microbial community is lipoquinone profile method. Lipoquinone are constituents of the bacterial plasma membrane that are essential for electron transport. There are two main types of lipoquinone: UQ and MK, as shown in **Figure 4.1** [8, 9]. 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 [8-10].



Ubiquinone (UQ-n(Hx)) Menaquinone (MK-n(Hx))

Figure 4.1
Chemical structure of UQ and MK.

The lipoquinone profile method is widely used for analyzing microbial community structures because it is a relatively simple procedure, it is quantitative, and highly reproducible [8, 11-15]. The lipoquinone profile provides the composition of the microbial community and their biomass concentration [9]. Since monitoring the

biological process requires many samples to be analyzed, the lipoquinone profile method is considered to be an appropriate method for the routine analysis.

Since the conventional method for quinone determination is tedious and uses the hazardous organic solvent which is against the principles of green analytical chemistry [11, 16, 17], the SFE has been used to extract lipoquinone from environmental samples [18, 19]. CO_2 has been used as an extraction solvent in lipoquinone extraction with methanol as a modifier to adjust its polarity. Therefore, the extracted lipoquinone was obtained in the methanol solution. The re-extraction using n-hexane and water was used to extract lipoquinone from the methanol. The hexane extract was then fractionated using Sep-Pak Plus silica cartridges joint in series. The lipoquinone extract was then eluted with a mixture of hexane and diethyl ether (9:1), followed by evaporation to dryness by using a rotary vacuum evaporator. The crude of lipoquinone extract was then diluted in acetone before analysis using HPLC [18-20].

In the previous study, Hanif *et al.* obtained that solid phase could trap the extracted lipoquinone and it simplify the procedure [21]. They used the Sep-Pak Plus silica cartridges to trap the extracted lipoquinone. Therefore, the re-extraction procedure can be eliminated. The extracted lipoquinone was then directly eluted from Sep-Pak Plus silica cartridge using acetone, before analysis using HPLC. This study shows the possibility of using solid phase as the interface to trap the extracted quinone in the on-line SFE-HPLC system. However, this solid phase trapping is not suitable for the operating pressure in on-line SFE-HPLC. On the other hand, a Zorbax guard-column is a common column in chromatography system. It was selected as a trapping column because it is suitable for the operating pressure, stable to most organic solvents, reusable, and has excellent acid and alkali resistance, making it useful under various demanding separation conditions. Moreover, it can be used in a wide range of temperatures.

On-line SFE-HPLC could have several potential advantages for the qualitative and quantitative determination of lipoquinone. An on-line system can improve the

performance and cost-effectiveness of analysis. In addition, on-line systems are valuable for analytes that are labile. Lipoquinone and their derivatives are photosensitive and susceptible to oxygen [9], therefore, direct transference to the chromatography system from the extraction step can minimize lipoquinone loss through degradation. Also, an on-line system is beneficial for a limited amount of analytes because all the extracted analytes are transferred to the chromatography system without a tedious sample pretreatment. Therefore, this can improve the sensitivity and reproducibility of analysis.

The SFE-liquid chromatography (LC) coupling system has been used for sample preparation and analysis of various compounds. Khorassani *et al.* used SFE coupled on-line to reversed phase LC to analyze polymer additives [22]. They used a packed bed to trap extracted analytes with high flow rates of CO_2 and modifier. Pól *et al*. used on-line SFE-HPLC with a single monolithic column to determine lycopene in food [23]. Recently, on-line SFE-UPLC with a PDA was used to detect solid piperine in pepper [24]. Zougagh *et al.* used the SFE in conjunction with LC and fluorimetric detection to screening and confirmation of polycyclic aromatic hydrocarbons (PAHs) in vegetable oil samples. They used a C18 column (250 mm × 4.6 mm ID stainless steel column filled with Bond Elut C18 sorbent, 47-60 μm particle size) as the interface to adsorb the extracted PAHs from the vegetable oil samples [25]. Ramsey *et al.* used the direct aqueous SFE coupled on-line with liquid chromatography-tandem mass spectrometry for the analysis of polyether ionophore antibiotics in water. In their study, the two coupled guard columns (Phenomenex 3 mm × 4.6 mm ID C_1 cartridge) were used as the SFE solid phase trapping system in the SFE-LC-MS-MS [26]. Although coupling of SFE-LC has been used for determining many substances, on-line SFE-HPLC has not been used to analyze lipoquinone from environmental samples.

In this study, the on-line SFE-HPLC was developed to analysis lipoquinone from activated sludge. The trapping column Zorbax SB-C18 was used as an interface between the SFE and HPLC system. The trapping column, as a collector of the extracted lipoquinone, was associated with six-port valve in HPLC system, therefore,

all the extracted lipoquinone could be directly transferred to the HPLC system. Using the trapping column as the interface, an on-line SFE-HPLC system was developed into a fast, simple, highly reproducible method for lipoquinone analysis. Optimizing the extraction conditions can increase the selectivity of the method. It is also important to optimize the trapping system type and conditions for determining the amount of lipoquinone, because lipoquinone are sensitive to the temperature. The method was validated by comparing the lipoquinone profile with those from the organic solvent extraction method. Our method was then used to determine the lipoquinone profile in several activated sludge samples.

4.2 Results and Discussion

4.2.1 Effects of Water Flow Rate on Trapping Efficiency in On-line SFE-HPLC

In the on-line SFE-HPLC system, the SFE was connected to a trapping column, which collected the extracted lipoquinone. The chemical structure of the trapping column interacted with the extracted lipoquinone.

The trapping column was optimized to achieve the best trapping efficiencies. Water was added to the extraction fluid to adjust the solubility of lipoquinone in the trapping column. To evaluate the effects of additional water flow on the trapping efficiencies, a standard solution (UQ-10 and MK-7) was used to examine the amount of lipoquinone detected at a water flow of 0.05 mL min^{-1} on the on-line SFE-HPLC system. **Figure 4.2** shows that the recovery of MK-7 increased substantially, from 29.9% to 86% at a water flow of 0.05 mL min^{-1}, which was almost three times the recovery of MK-7 without a water flow in the trapping system. Conversely, the recovery of UQ-10 decreased slightly, from 92.6% to 84.1%. MK-7 has a smaller molecular weight than UQ-10 and higher solubility in the methanol compared to UQ-10, that is why MK-7 is more difficult to be trapped in the non-polar trapping column Zorbax SB C-18. Introducing water to the extraction fluid altered the solubility of extraction fluid, so that MK-7 was easily trapped in the guard-column.

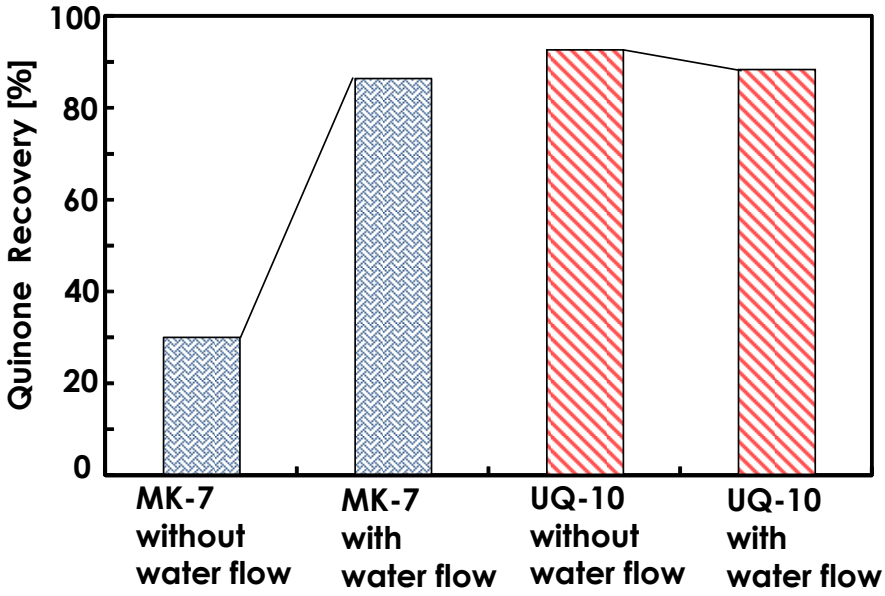


Figure 4.2

Effects of water flow rate on trapping efficiency in on-line SFE-HPLC. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 3 mL min^{-1}. Trapping conditions: 4.6 mm id ×12.5 mm, Zorbax SB-C18 column, 5 µm; 45°C; 0.05 mL min^{-1} water flow.

The trapping efficiency for water flow in the trapping column was determined for flow rates from 0 to 0.06 mL min^{-1} for dried activated sludge. **Figure 4.3** shows that the diversity of lipoquinone obtained at all water flow rates was almost similar. However, the highest amount of lipoquinone detected was obtained at a water flow rate of 0.04 mL min^{-1}, and this was used as the optimum flow rate in on-line SFE-HPLC for the activated sludge.

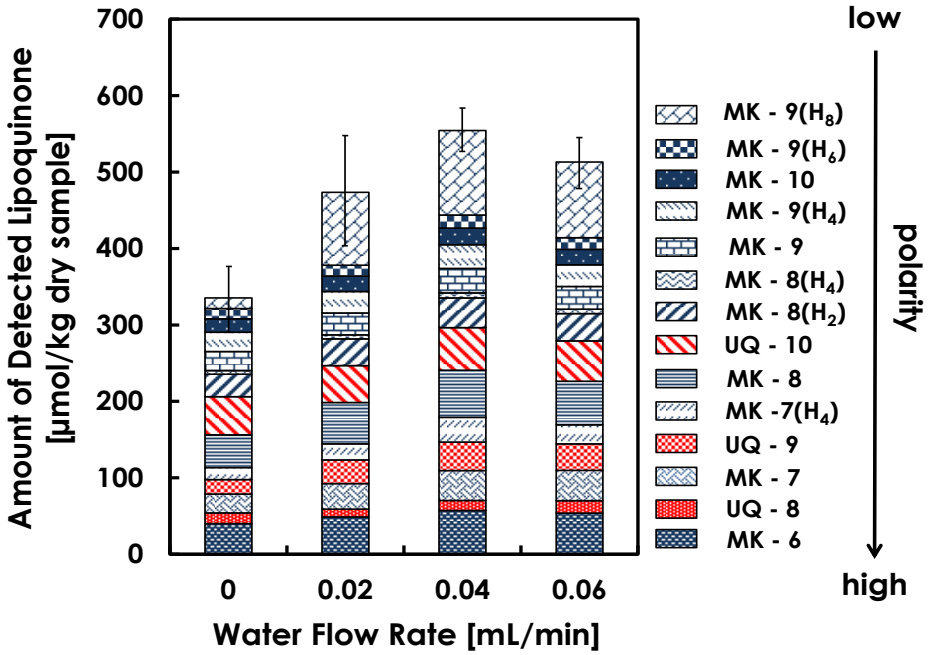


Figure 4.3

Detected amount of lipoquinone obtained at different water flow rate in on-line SFE HPLC. Sample amount: 0.1 g freeze dried activated sludge. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2 -10% methanol with flow rate of 1 mL min^{-1}; number of samples examined = 3. Trapping conditions: 4.6 mm id ×12.5 mm, Zorbax SB-C18 column, 5 µm; 45°C.

4.2.2 Effects of Trapping Temperature on Trapping Efficiency in On-line SFE-HPLC

The trapping temperature is important for trapping efficiency. Increasing the temperature increases the solubility of the lipoquinone in the extraction fluid, which could cause difficulties in trapping the lipoquinone. Therefore, the effects of trapping temperature on trapping efficiency was examined.

The trapping column was tested at room temperature (24°C) to 45°C with a water flow rate of 0.04 mL min^{-1}. **Figure 4.4** shows that the detected amounts of lipoquinone were not substantially different, although the amount of lipoquinone detected was slightly higher at room temperature, and thus room temperature was used as the trapping system temperature.

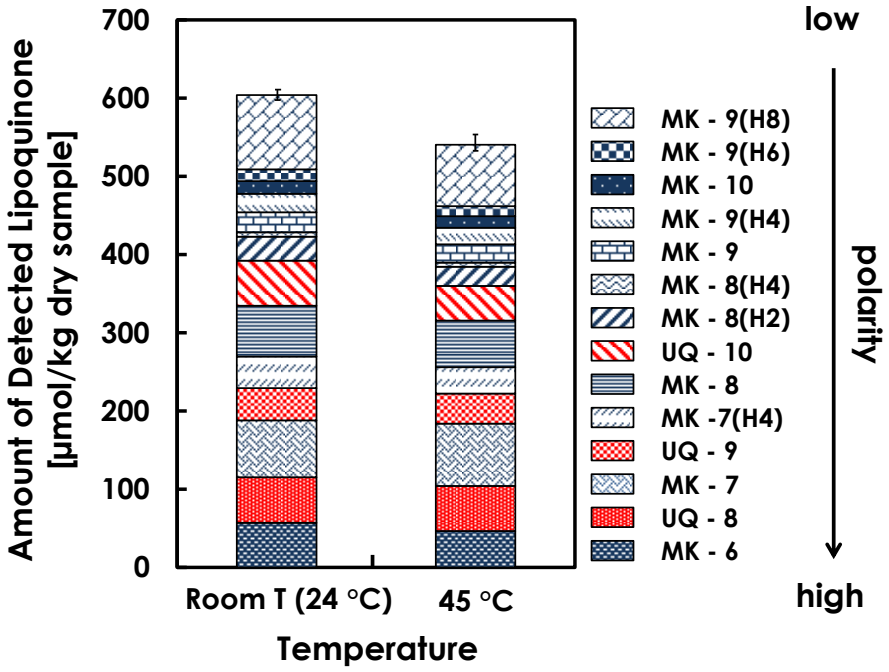


Figure 4.4

Detected amount of lipoquinone obtained at different trapping temperature in on-line SFE-HPLC. Sample amount: 0.1 g freeze dried activated sludge. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 1 mL min^{-1}; number of samples examined = 3. Trapping conditions: 4.6 mm id × 12.5 mm, Zorbax SB-C18 column, 5 µm; 0.04 mL min^{-1} water flow.

4.2.3 Comparison of Lipoquinone Profile for Organic Solvent Extraction Method and On-line SFE-HPLC Method

Since the organic solvent extraction method is tedious and uses a large volume of organic solvent, the coupled SFE-HPLC method was developed. The lipoquinone profile obtained from the on-line SFE-HPLC method and the organic solvent extraction method were compared based on the dissimilarity index (D) value. The D indicates the degree of difference between lipoquinone patterns of samples and is calculated as previously described in equation (3.1). A *D* value equal to or greater than 0.1 would indicate that the methods produced different profiles [11, 12].

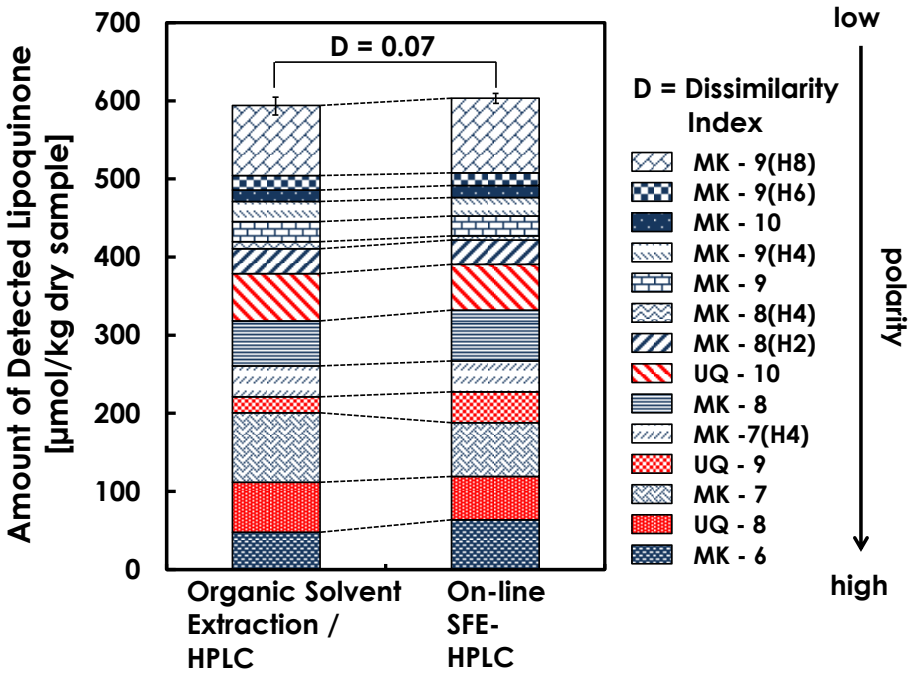


Figure 4.5

Comparison of lipoquinone profile for organic solvent extraction method and on-line SFE-HPLC method; Sample amount: 0.1 g freeze dried activated sludge. Organic solvent extraction conditions: chloroform: methanol = 2:1 (20 mL); 24 h. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 1 mL min^{-1}; number of samples examined = 3. Trapping conditions: 4.6 mm id × 12.5 mm, Zorbax SB-C18 column, 5 µm; 24°C; 0.04 mL min^{-1} water flow.

Each experiment was conducted three times for 0.1 g freeze dried activated sludge samples with the organic solvent extraction method and on-line SFE-HPLC method. **Figure 4.5** shows that three types of UQ (UQ-8, UQ-9, UQ-10) and eleven types of MK (MK-6, MK-7, MK-7(H4), MK-8, MK-8(H2), MK-8(H4), MK-9, MK-9(H4), MK-10, MK-9(H6), MK-9(H8)) were detected with the two methods. The amounts of lipoquinone detected were calculated from the peak area based on the mole absorption coefficient of UQ and MK [17, 27]. The peak heights obtained by organic solvent extraction and HPLC were smaller than the peak heights obtained by on-line SFE-HPLC because only a small quantity of the extracted lipoquinone was injected into the HPLC system.

The total detected amount of lipoquinone obtained by the on-line SFE-HPLC method (603.4 μmol kg^{-1} dry sample) was comparable to that obtained by the organic solvent extraction method (594.1 μmol kg^{-1} dry sample). In addition, the D value for the two methods was 0.07, indicating that the lipoquinone profile was similar. This showed that the on-line SFE-HPLC method could replace the organic solvent extraction method.

4.2.4 Application of On-line SFE-HPLC for Lipoquinone Analysis in Environmental Sample

The on-line SFE-HPLC method was used to determine the lipoquinone profile of activated sludge discharged from different wastewater treatment plants. The results are shown in **Figure 4.6**. Three types of UQ (UQ-8, UQ-9, UQ-10) existed with the UQ-8 being predominant in all samples. It is in agreement with other studies that UQ-8 is the most abundant of the UQs in activated sludges [8]. Among the MK, the MK-9(H8), MK-7, MK-8 are predominant in the samples from TUT, NK and TG plants, respectively. Those MKs also reported as predominant components in other activated sludges, depending on the characteristic of influent wastewater [8].

It is known that UQ and MK are specific indicators of aerobic and anaerobic bacteria, respectively [11, 14]. In the ratio of MK/UQ value, the sample from the TG plant has the highest MK/UQ value (5.2) followed by samples from the TUT plant (2.92) and the NK plant (1.82). The NK plant is operated an aerobic system; therefore, the ratio of UQ in the total amount of lipoquinone is relatively higher than the others.

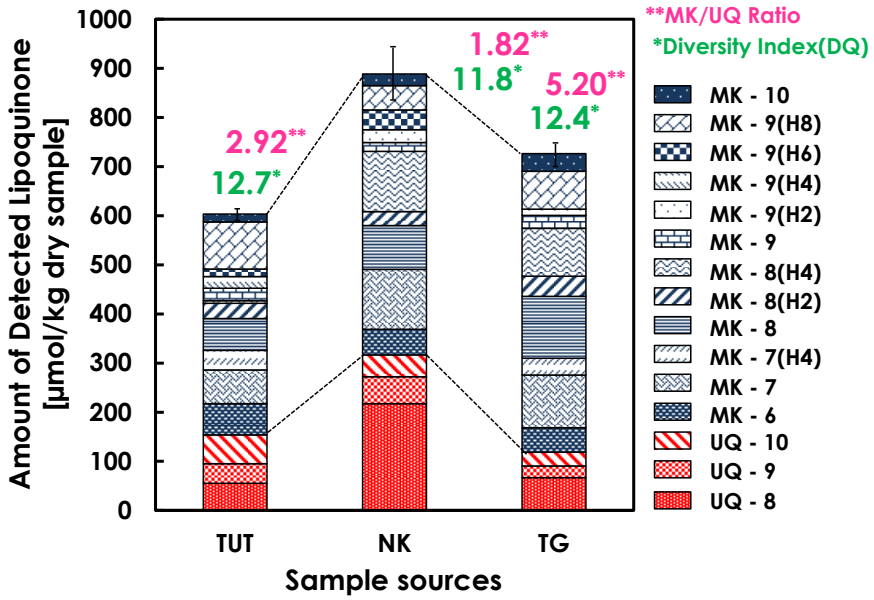


Figure 4.6

Application of on-line SFE-HPLC to sludge samples. TUT: activated sludge obtained from Toyohashi University of Technology; NK: activated sludge obtained from NK wastewater treatment plant; TG: activated sludge obtained from TG wastewater treatment plant. Sample amount: 0.1 g freeze dried activated sludge. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 1 mL min^{-1}; number of samples examined = 3. Trapping conditions: Zorbax SB-C18 (4.6 mm id x 12.5 mm, 5 µm); 24°C; 0.04 mL min^{-1} water flow.

The diversity of microbial communities was calculated as previously described in equation (3.2). The diversity of the microbial community among these samples was 12.7 for samples from the TUT plant, 12.4 for samples from the TG plant and the lowest

is 11.8 for samples from the NK plant. The TUT plant is operated with an intermittent aeration system, having anaerobic-aerobic processes in the same tank, therefore, the existence of microbial community in this samples is the most complex within these three samples. It means the calculated diversity is highest in the others. On the other hand, the NK plant uses only the aerobic process which is simpler than the others means that this sample has the lowest diversity.

4.2.5 Advantages of On-line SFE-HPLC

The comparison of performance between organic solvent extraction / HPLC method and on-line SFE-HPLC method are shown in **Table 4.1**. The determination of lipoquinone using the on-line SFE-HPLC method reduced the total analysis time to less than 2 h by simplifying the method. In the organic solvent extraction method, a long extraction time was required for each sample. Additionally, purification was required after extraction because the organic solvent extracted impurities with the lipoquinone.

In the on-line SFE-HPLC method, the high diffusivity and selectivity of the extraction solvent and using the trapping column under optimum conditions reduced the extraction time and avoided purification step. The trapping column also increased the reproducibility and sensitivity of the method because all the extracted analytes were transferred directly to the HPLC without any pretreatment. Moreover, it reduced the amount of lipoquinone lost during preparation through oxidation and photoreactions. Furthermore, the on-line SFE-HPLC method reduced the use of hazardous organic solvent.

Table 4.1
Comparison of performance between organic solvent extraction / HPLC method and on-line SFE-HPLC method on the analysis of 0.1 g dried activated sludge sample

	Descriptions	Organic Solvent Extraction / HPLC	On-line SFE-HPLC
1.	**Analytical time in hour (excluded freeze dried time for one sequence of 0.1 g sample)**	**< 25**	**< 2**
2.	**Chemicals (mL)**		
	▪ **Chloroform**	**40**	**0**
	▪ **Methanol**	**20**	**1.5**
	▪ **Diethyl ether**	**1**	**0**
	▪ **Hexane**	**80**	**0**
	▪ **Acetone**	**5**	**0**
	▪ **CO_2**	**0**	**13.5**
3.	**Reproducibility (% coefficient of variation)**	**5.8**	**1.6**
4.	**Ratio of sample amount**	**(5)**	**(1)**

In the term of sample amount, the on-line SFE-HPLC can reduce the use of sample amount for one analysis until one fifth because in the on-line SFE-HPLC all the extracted lipoquinone could directly transfer to HPLC system. On the other hand, when the extracted lipoquinone was diluted in 100 µL acetone before analysis using HPLC, only 20 µL of 100 µL of the extracted lipoquinone was injected into the HPLC system in the organic solvent extraction method, as shown in **Figure 4.7**. Therefore, when 0.1 g of sample was required for analysis of lipoquinone in organic solvent extraction method, the 0.02 g sample was sufficient enough to obtain similar results as the organic solvent extraction. The chromatogram of lipoquinone obtained from TUT activated sludge by organic solvent extraction and on-line SFE-HPLC was shown in **Figure 4.8** as the example.

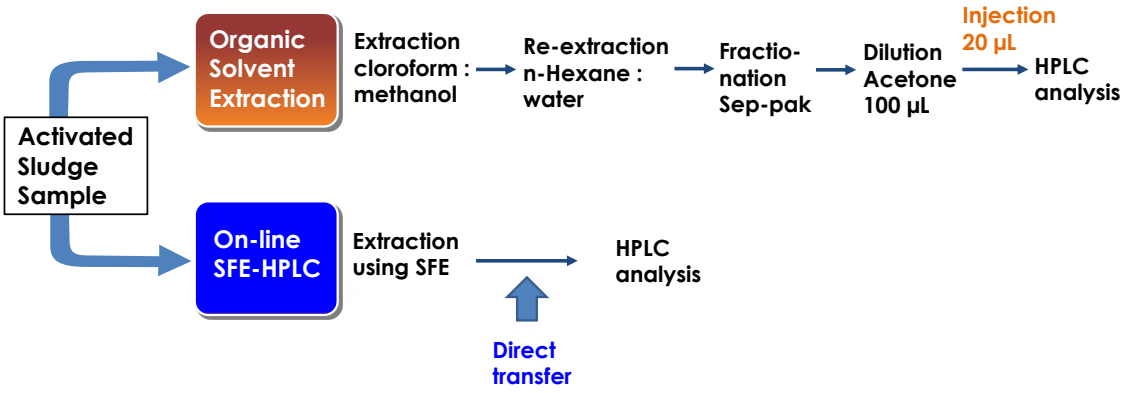
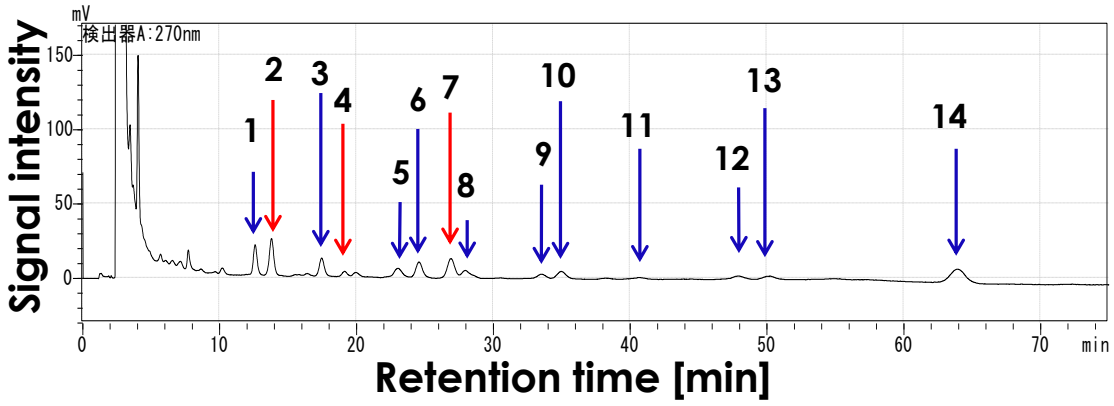


Figure 4.7

Schematic of comparison between on-line SFE-HPLC and organic solvent extraction/ HPLC

The success of the on-line SFE-HPLC method on determining the microbial community structure in several environmental samples based on the lipoquinone profile, shows that this method could replace the organic solvent extraction method and would be essential for routine analysis for large numbers of samples.

(a)



(b)

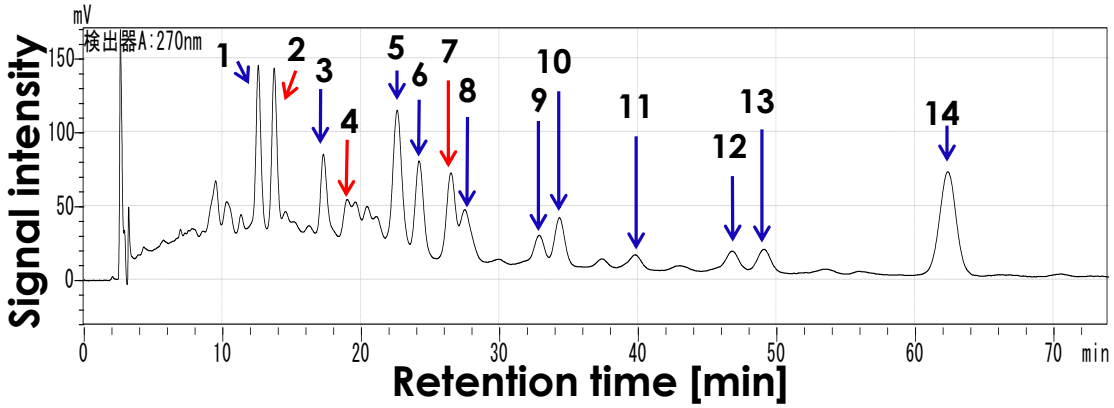


Figure 4.8

Chromatograms of lipoquinones obtained from TUT activated sludge obtained by using (a) organic solvent extraction, (b) on-line SFE-HPLC. Sample amount: 0.1 g-freeze dried activated sludge. Organic solvent extraction conditions: chloroform: methanol = 2:1 (20 mL); 24 h. SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 1 mL min^{-1}. Trapping conditions: 4.6 mm id × 12.5 mm, Zorbax SB-C18 column, 5 µm; 24°C; 0.04 mL min^{-1} water flow. HPLC conditions: 4.6 mm id × 250 mm packed column, Cadenza CD-C18, 3 µm; methanol/diisopropyl ether mobile phase; 1.0 mL min^{-1} flow rate; 270 nm; PDA. Description : 1 = MK-6; 2 = UQ-8; 3 = MK-7; 4 = UQ-9; 5 = MK-7(H4); 6 = MK-8; 7 = UQ-10; 8 = MK-8(H2); 9 = MK-8(H4); 10 = MK-9; 11 = MK-9(H4); 12 = MK-10; 13 = MK-9(H6); 14 = MK-9(H8).

4.3 Conclusions

Our on-line SFE-HPLC method successfully determined the microbial community structure in environmental samples based on the lipoquinone profile. The method is simple, making it suitable for analyzing a large number of samples. In addition, it has the total analysis time of less than 2 h, low organic solvent usage, and high reproducibility and sensitivity. Furthermore, the lipoquinone profile obtained with this method were similar to those obtained by the organic solvent extraction method according to the low dissimilarity index. The results obtained by the on-line SFE-HPLC were comparable to other study obtained by conventional method. The lipoquinone profile obtained in this study could be explained based on the category of wastewater treatment process. Therefore, the on-line SFE-HPLC method is expected to be seen as a breakthrough technology for monitoring the performance of wastewater treatments by accumulating lipoquinone profile information.

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Chapter 5

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

Chapter 5

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

Summary

The effects of static extraction on extraction efficiencies using on-line SFE-HPLC has been investigated for lipoquinone analysis in activated sludge. This is the simplified system of previous study using on-line SFE-HPLC. The combination of static extraction and dynamic extraction with methanol spiked directly into the sample was optimized to eliminate the water pump and methanol pump from the system. The 0.1 g of freeze dried activated sludge was spiked with methanol in 1 mL extraction vessel using CO_2 as solvent extraction. The lipoquinone was collected in the trapping column, then directly transferred to HPLC system for determination. The best results in terms of extraction yield were obtained at 25 MPa, 45°C, 10 min static extraction with 500 µL methanol spiked, and 25 min dynamic extraction 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, SFE, static extraction, dynamic extraction

5.1 Introduction

The environmental biological processes such as wastewater treatment, anaerobic digestion, composting, and bioremediation are complex processes which involve the dynamic of microbial community structure [1-5]. 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 low running cost and technical skills is a basic requirement in the analysis.

There are many methods for microbial community analysis. A culture independent method is preferred to use because the microbial community does not need to cultivate in this method. In addition, not all of the microorganisms could be cultivated. For the monitoring environmental biological process, the lipoquinone profile method is a convenient method because it is more reliable and high reproducibility.

The lipoquinone, which is mainly consist of UQ and MK is component of microorganisms that essential for electron transporter. Generally, a species or genus in a microbial community produces one dominant type of quinone and any change in the quinone profile reflects a change in the microbial community [6-8]. The lipoquinone is not only provides an understanding of the composition of microbial community, but also the biomass concentration of the samples [6-8]. Therefore, there is a growing interest in the analysis of microbial community using lipoquinone profile method in environmental samples.

The conventional method for determination of lipoquinone from environmental sample consists of direct extraction using organic solvent extraction and analysis using HPLC [9]. Since the long extraction time and the large volume of organic solvent uses in this method, the $scCO_2$ was introduced to be a potential alternative method to extract lipoquinone [10-12]. The CO_2 under supercritical condition was used as extraction solvent to extract lipoquinones and methanol was the best

modifier to adjust its polarity. 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 lipoquinones 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 switching valve in HPLC system. Therefore, using on-line SFE-HPLC, all the extracted lipoquinones 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 [7], the direct transferring of extracted lipoquinones 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.

5.2 Results and Discussion

5.2.1 Effects of Static Extraction on the Extraction Efficiency using Simplified On-line SFE -HPLC

The static extraction was examined in the range of 0 to 25 min and followed by 15 min dynamic extraction. As shown in **Figure 5.1**, the different static time did not significantly affect the detected amount of lipoquinone. The highest detected amount of lipoquinone with the lowest error bar was obtained at the 10 min of static extraction. Therefore, 10 min was chosen as static extraction time.

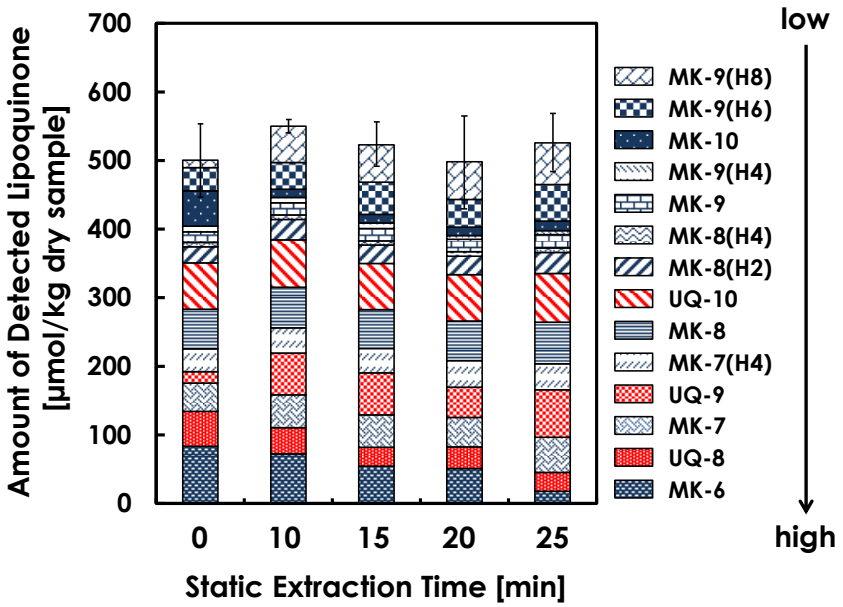


Figure 5.1

Effects of static extraction time on the detected amount of lipoquinone using simplified on-line SFE-HPLC. Sample: 0.1 g freeze dried activated sludge. SFE conditions: 45°C; 25 MPa; 0.9 mL min^{-1} (in dynamic extraction); 15 min dynamic extraction; 500 µL methanol spiked; number of samples examined = 3. Trapping conditions: Zorbax SB-C18 (4.6 mm id x 12.5 mm, 5 µm); room temperature (24°C).

The high error bar before the 10 min of static extraction might cause by the inhomogeneous nature of the system. On the other hand, 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, long time in high extraction temperature (45°C) that could cause decomposition of lipoquinone, or lipoquinone lost by leaking from the system.

5.2.2 Effects of Dynamic Extraction on the Extraction Efficiency using Simplified On-line SFE-HPLC

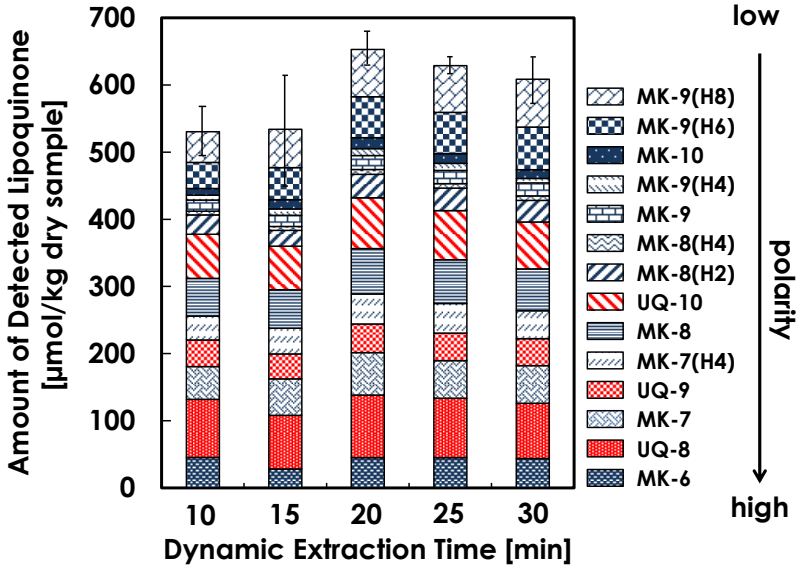


Figure 5.2

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

With the 10 min of static extraction time, the effects of dynamic extraction time on the extraction efficiency was studied in the range of 10 to 30 min. The results were shown in **Figure 5.2**. 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

5.2.3 Effects 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 5.3.**

The detected amount of lipoquinone with the methanol spiked volume of 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. The high error bar was obtained when the volume of methanol is less than 500 µL. It might be due to the insufficient of methanol to adjust the polarity of the extraction solvent, so that not all the lipoquinone was extracted well.

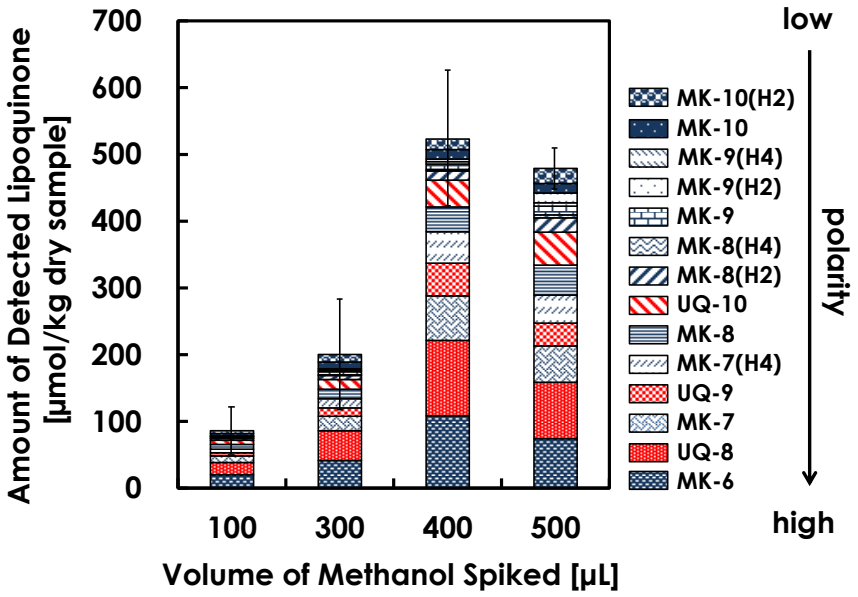
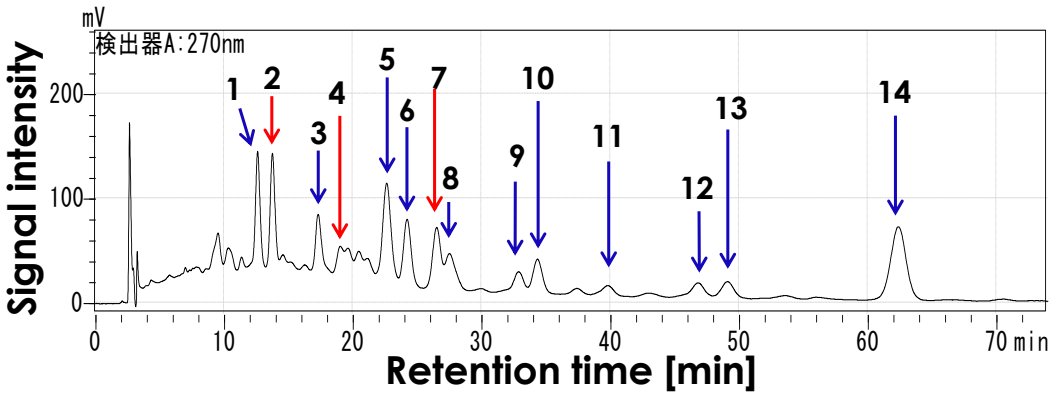


Figure 5.3

Effects of methanol spiked volume on the detected amount of lipoquinone using simplified on-line SFE-HPLC. Sample: 0.1 g freeze dried activated sludge. SFE conditions: 45°C; 25 MPa; 10 min static extraction, 25 min dynamic extraction; 0.9 mL min^{-1} (in dynamic extraction); number of samples examined = 3. Trapping conditions: Zorbax SB-C18 (4.6 mm id x 12.5 mm, 5 µm); room temperature (24°C).

In general, the results obtained between the previous study using on-line SFE-HPLC and this study using simplified on-line SFE-HPLC were similar in terms of detected amount of lipoquinones as well as the type of lipoquinones. It is indicated that this study could be considered as a step forward from the previous study, where the process could be simplified by combining the static and dynamic extraction with methanol spiked directly to the extraction vessel. However, the results could be slightly change due to the season and conditions of the wastewater treatment. **Figure 5.4** shows the chromatogram of lipoquinones obtained from TUT activated sludge by using on-line SFE-HPLC and simplified on-line SFE-HPLC.

(a)



(b)

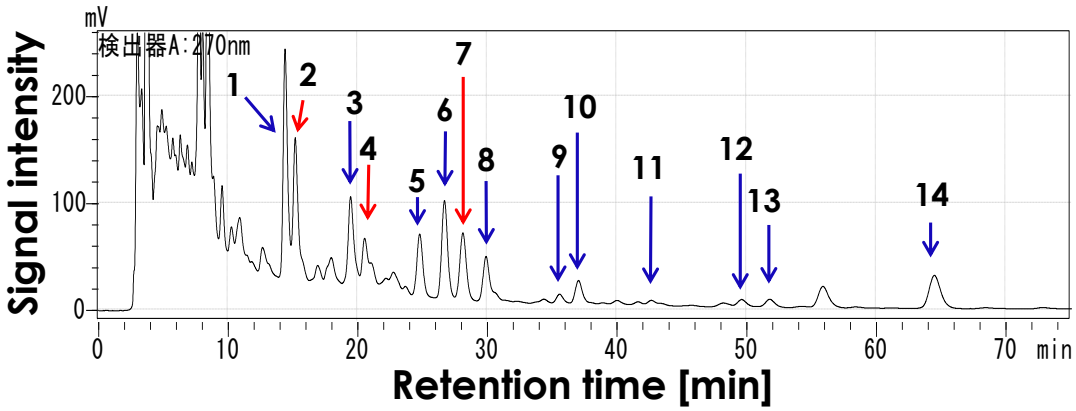


Figure 5.4

Chromatogram of lipoquinones obtained from TUT activated sludge by using (a) on-line SFE-HPLC, (b) simplified on-line SFE-HPLC. Sample amount: 0.1 g freeze dried activated sludge. On-line SFE conditions: 45°C; 25 MPa; 15 min; 90% CO_2-10% methanol with flow rate of 1 mL min^{-1}. Trapping conditions: 4.6 mm id × 12.5 mm, Zorbax SB-C18 column, 5 µm; 24°C with 0.04 mL min^{-1} water flow. Simplified on-line SFE conditions: 45°C; 25 MPa; 10 min static extraction time with 500 µL methanol spiked; 25 min dynamic extraction time with CO_2 flow rate of 0.9 mL min^{-1}. Trapping conditions: 4.6 mm id × 12.5 mm, Zorbax SB-C18 column, 5 µm; 24°C without water flow. HPLC conditions: 4.6 mm id × 250 mm packed column, Cadenza CD-C18, 3 µm; methanol/diisopropyl ether mobile phase; 1.0 mL min^{-1} flow rate; 270 nm; PDA. Description : 1 = MK-6; 2 = UQ-8; 3 = MK-7; 4 = UQ-9; 5 = MK-7(H4); 6 = MK-8; 7 = UQ-10;

8 = MK-8(H2); 9 = MK-8(H4); 10 = MK-9; 11 = MK-9(H4); 12 = MK-10; 13 = MK-9(H6);14 = MK-9(H8).

5.3 Conclusions

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 using 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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Chapter 6

General Conclusions and Future Prospects

Chapter 6
General Conclusions and Future Prospects

6.1 General Conclusions

This study provide a novel analytical method, which is on-line SFE-HPLC, for microbial community analysis based on lipoquinone analysis. The specific conclusion can be written as follows:

1. Conventionally, the extractions are to be done using organic solvent, with tedious pretreatments and purification steps. Not only a lot of time was consumed, but also a quantity amount of organic solvents was used. In order to overcome these disadvantages, a sample preparation with SFE has been developed. The conventional extraction steps were replaced with SFE using CO_2 to extract lipoquinones from compost, and UPLC was used for lipoquinone determination. The significantly low value of dissimilarity index (D = 0.05) between the two methods indicated that the lipoquinone profile obtained by both methods was considered identical. The application of SFE and UPLC method to determine the maturity of the compost by monitoring the lipoquinone profile during composting showed that the method had successfully extracted lipoquinone from a complex matrix.

2. On-line SFE-HPLC could have several potential advantages for the qualitative and quantitative determination of lipoquinone. After the extraction was finished, all the extracted lipoquinone would be trapped into trapping column, and then transferred directly into HPLC, without further pretreatments. The optimum conditions obtained on activated sludge were as follows: 45°C; 25 MPa; 15 min; 90% CO_2 – 10% methanol 1 mL min^{-1}; water 0.04 mL min^{-1}. A summary by comparing conventional method and on-line SFE-HPLC has been showed previously (**Table 4.1**), which gave a more direct understand on how on-line SFE-HPLC could eliminate the time consuming and volume of organic solvents used.

3. To present lipoquinone analysis as a reliable method for analysis of microbial community in environmental processes, the reproducibility of the lipoquinone profile obtained is a key point. Lipoquinones are known to be photosensitive and susceptible to oxygen, and loss of extracted lipoquinones throughout the process in conventional method is one certain aspect that should be considered. Under on-line SFE-HPLC, as mentioned before, no further pretreatments on the extracted lipoquinone are needed. As the extracted lipoquinone would not be exposed to the outside, hence by using on-line SFE-HPLC, the loss of extracted lipoquinone could be reduced until minimum. This leads to higher reproducibility for characterization of microbial community in environmental processes.

4. Running cost and maintenances is one big part that needed to be considered well for any monitoring approach on environmental processes. By the development of simplified on-line SFE-HPLC, in which static extraction was introduced to combine with the dynamic extraction and methanol was spiked directly into the sample, the fewer pumps were used. In addition, the running cost and troubles for maintenances were reduced. The optimum conditions for simplified on-line SFE-HPLC obtained through this study are as follows: 45°C; 25 MPa; 10 min for static extraction time; 25 min for dynamic extraction time; CO_2 0.9 mL min^{-1}; 500 μL methanol spiked.

As a summary, on-line SFE-HPLC could provide a rapid, reliable and simple method in determination of microbial community structure based on lipoquinone analysis. This developed method is expected to be a breakthrough technology for routine analysis in monitoring biological processes. The optimum conditions using on-line SFE-HPLC had been obtained through this study. With shorter analytical time as well as higher reproducibility under on-line SFE-HPLC, the application of lipoquinone analysis should not be far away as comprehensive data in lipoquinone profile that could be obtained in shorter time. The knowledge obtained based on lipoquinone profile using on-line SFE-HPLC could be very useful for understanding and improving the environmental processes.

6.2 Future Prospects

1. In this studies, the dissimilarity index (D) was used to evaluate the degree of difference between the conventional and the proposed method. As a recommendation, the % recovery of the standard lipoquinone in both conventional method and proposed method could be used to determine the accuracy of the method and to understand the matrix interferences.

2. As the on-line SFE-HPLC give the higher sensitivity in the terms of the amount of sample required, the reduction of the sample usage for the extraction step also need to be apply in on-line SFE-HPLC to provide the analysis for the limited amount of samples.

3. The extraction temperature in simplified on-line SFE-HPLC was referring to the previous studies. Since temperature could affect the extraction efficiency, it is recommended to optimize the extraction temperature to understand the effects of extraction temperature to the extraction efficiency in static and dynamic extraction.

4. The identification of lipoquinone was performed by understanding the linear correlation between the logarithm of retention time of lipoquinone and an ENIU. Several lipoquinone have the nearly retention time in the chromatogram that could give difficulties in deciding the lipoquinone species. Therefore, the mass spectrometry (MS) detection would be highly specific technique for addressing the difficulties since the MS detector provided the structural information and molecular weight of the lipoquinone.

5. The samples that used in this study was freeze dried samples. To obtain more reliable method in analysis of lipoquinones, the examination in the possibility of reducing the freeze drying time also recommended in the near future.

6. Soil is the primary material in agriculture. Since this developed method can analysis the microbial community based on lipoquinone profile in a simple and shorter time, it is recommended to maintain or monitor the soil conditions based on analysis of lipoquinones to increase the productivity of the soil.

The method that developed in this study open the road to a breakthrough technology in analyzing microbial community for the environmental assessment and monitoring. Therefore, the extended application of on-line SFE-HPLC could provide useful references for further development of environmental biological process such as wastewater treatment, composting, and anaerobic digestion process. In addition, the developed method has a high possibility for automation in the future, which could motivate researcher to study in this field. Building the lipoquinone profiling database for microbial community analysis by accumulating the comprehensive data of lipoquinone profile is one of the further progression of this study to make worthy understanding and to improve the performance of the environmental biological process.

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ACHIEVEMENTS

List of Papers/Journals with Referee's Review

1. **Ni Luh Gede Ratna Juliasih**, Lee Chang Yuan, Yuki Sago, Yoichi Atsuta, Hiroyuki Daimon, Monitoring Microbial Community Dynamics in Composting using Supercritical Fluid Extraction and Ultra Performance Liquid Chromatography, Journal of Chemistry, Volume 2015 (2015), Article ID 717616.

2. **Ni Luh Gede Ratna Juliasih**, Lee Chang Yuan, Yoichi Atsuta, Hiroyuki Daimon, Development of Coupled Supercritical Fluid Extraction - High Performance Liquid Chromatography for Microbial Community Analysis, Separation Science and Technology, ID No. LSST-2015-8818.R2 (Accepted on August 19, 2015).

1. **Ni Luh Gede Ratna Juliasih**, Lee Chang Yuan, Yoichi Atsuta, Hirotsugu Kamahara, Hiroyuki Daimon, Effect of Static Extraction Time on Extraction Efficiencies using On-line Supercritical Fluid Extraction-High Performance Liquid Chromatography for Lipoquinone Analysis in Activated Sludge, International Journal of Science and Engineering [IJSE, ISSN 2086-5023], http://ejournal.undip.ac.id/index.php/ijse, ID No. 23 (Accepted on August 20, 2015).

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List of Papers at International Conference with Referee's Review

1. **Ni Luh Gede Ratna Juliasih**, Lee Chang Yuan, Yuki Sago, Yoichi Atsuta, Hiroyuki Daimon, Microbial Community Dynamics during Composting Process and Cultivation of Komatsuna, International Symposium on EcoTopia Science 2013, No. 15-5-6 (1184), December 13-15, 2013, Japan.

2. **Ni Luh Gede Ratna Juliasih**, Lee Chang Yuan, Yoichi Atsuta, Hirotsugu Kamahara, Hiroyuki Daimon, Effect of Static Extraction on Extraction Efficiencies using On-line Supercritical Fluid Extraction-High Performance Liquid Chromatography for Lipoquinone Analysis in Activated Sludge, The 8th International Conference of Chemical Engineering on Science and Applications 2015 Proceeding, No. 22 (130-136), September 9-11, 2015, Indonesia.