Journal of Chromatography A, 1217 (2010) 6704–6708

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Supercritical fluid extraction of microbial phospholipid fatty acids from activated sludge

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ARTICLE INFO

Article history:
Available online 24 May 2010

Keywords:
Supercritical carbon dioxide extraction
Phospholipid fatty acids profile
Microbial community
Activated sludge
Environmental assessment

ABSTRACT

Supercritical carbon dioxide ($scCO_2$) extraction was applied for the determination of microbial phospholipid fatty acids (PLFA) in activated sludge. Quantification was performed by using gas chromatography–mass spectrometry (GC–MS). The highest extraction yields of PLFA, at a concentration of 7.28 nmol/mg-dry activated sludge, was obtained at a temperature of 80 °C, pressure of 25 MPa and 10% (v/v) methanol for a 15-min extraction time. $ScCO_2$ extraction results obtained in these conditions were comparable with those obtained by liquid organic solvent extraction (LSE) based on diversity and equalibility indices. The repeatability test showed that the relative standard deviation values were less than 13%. The experimental results show that the $scCO_2$ extraction saves time and uses much less organic solvent. In addition, $scCO_2$ extraction is a promising and alternative method for the analysis of microbial community structure in environmental assessment using the PLFA profile.

1. Introduction

Characterization of microbial communities is crucial to understanding environmental health. Compositions and functions of microbial community structure have been effectively used in bioremediation [1–4], biomonitoring [5,6], biological wastewater treatment [7–9], anaerobic digestion systems [10–12] and microbial taxonomy [13,14].

Recent methodological advances in microbial ecology research are increasing our understanding of the composition and function of microbial communities in a wide variety of environmental samples. Many of the new methods extract the cellular constituents of microorganisms directly from the sample, eliminating the bias inherent in culture-based methods [15,16].

One of the most commonly used culture-independent tools for investigating microbial populations in environmental research is the analysis of PLFA profiles. Phospholipids are major cell membrane constituents, and their fatty acyl side-chains vary in composition (i.e., length, alkyl-branches and number of double bonds) between eukaryotes and prokaryotes, as well as among many prokaryotic groups. Therefore, PLFAs are used as biomarkers to determine the presence and the abundance of specific microbial groups in their habitats. Since PLFAs are quickly degraded upon microbial death, they represent a 'fingerprint' of the viable microbial community and do not function as storage compounds [17,18].

Analysis of PLFAs is traditionally performed by liquid organic solvent extraction (LSE) with a chloroform–methanol mixture [15,19,20]. The lipids are separated with silicic acid column chromatography into neutral lipids, glycolipids and phospholipids using chloroform, acetone and methanol as solvents. Fatty acids from the phospholipidic fraction are usually transformed into their less polar methyl ester derivatives (FAMEs) for analysis by gas chromatography. As a general standard procedure, this method has some disadvantages, such as the use of large quantities of hazardous solvents and being time consuming.

To overcome these problems, supercritical fluid extraction (SFE) has gained popularity as a common extraction technique in many areas because it offers a faster extraction method and lower solvent volume [21,22]. Carbon dioxide is the preferred extraction fluid in SFE because it has relatively low critical values (31.1 °C and 7.4 MPa), is non-toxic, and it does not create environmental problems when used at the analytical scale.

In our previous study, extraction using $scCO_2$ was successfully applied to determine the microbial ubiquinones and menaquinones from activated sludge [23,24]. However, little research has been reported regarding the application of SFE for PLFA analysis. $ScCO_2$ extraction has been used to extract fatty acids from whole bacterial cells by chemical derivatization [25,26]. An integration system with

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doi:10.1016/j.chroma.2010.05.027

sequential $scCO_2$ extraction for the identification of lipid biomarker including PLFA has also been proposed elsewhere [27].

The objective of the present work is to study the suitability of SFE as an alternative method to extract microbial PLFAs from activated sludge. The extraction conditions were optimized by varying the following: extraction time, extraction temperature, extraction pressure, and modifier concentration. The effect of various modifiers on the total amount of extracted PLFA was also studied. The liquid organic solvent extraction method was used to evaluate the reliability of the SFE method.

2. Experimental

2.1. Chemicals

All solvents were HPLC grade, and all other chemicals were analytical grade. Methanol, acetone, chloroform, ethanol and hexane, K_2HPO_4 and 1N HCl were purchased from Wako Co., Japan. Standards fatty acid methyl esters (Supelco 37-component FAME mix; Cat. No. 47885-U) and 0.5N Methanolic HCl (Supelco; Cat. No. 33095) were purchased from Sigma–Aldrich Co., Japan. The Sep-Pak Plus Silica cartridges were purchased from Waters Co., Japan. The Sep-Pak is a column, 10 mm in inner diameter and 20 mm in length, packed with 600 mg of silica gel with particle diameters of 55–105 μm.

2.2. Sample preparation

The activated sludge used in this study was obtained from the aeration tank (ca. 380 m^3) of the domestic wastewater treatment plant at Toyohashi University of Technology, Japan. Prior to the PLFA analysis, the activated sludge samples were dried in a vacuum-freeze dryer for 24 h, crushed and sieved to collect particles smaller than 500 μm for extraction. Dried activated sludge samples were stored at −20 °C until analysis. A freeze-dried sample of 0.1 g was used for LSE and SFE.

2.3. Liquid solvent extraction

Liquid organic solvent extraction was carried out according to Bligh and Dyer method [19], as modified by White et al. [15]. In this study, liquid solvent extraction protocol was completed in 3 days. PLFAs were extracted from dried activated sludge by addition of a mixture 1.2:0.8 (v/v/v) containing chloroform, methanol and phosphate buffer. The supernatant (upper layer) was transferred into a 100-ml separatory funnel using a glass Pasteur pipette. Chloroform, methanol and phosphate buffer were added to the supernatants to a final ratio of 1: 1: 0.9 (v/v/v) to split the phases (organic and aqueous). In order to split the phases (organic and aqueous), and then the mixture was left overnight at darkness. The organic phase (bottom layer) was filtered into a round bottom flask using Whatman #2 filter paper, reduced using a rotary vacuum evaporator (< 37 °C) and transferred to a glass test tube and stored at −20 °C.

Total extracted lipid, obtained with the extraction method described above, was fractionated into neutral lipids, glycolipids and phospholipids, with chloroform (5 ml), acetone (5 ml) and methanol (5 ml), on silicic acid columns. The fraction containing phospholipids was collected in 15-ml glass test tubes, evaporated to dryness under a stream of nitrogen and stored at −20 °C until required for derivatization.

2.4. Supercritical fluid extraction

All supercritical fluid extractions were performed using an in-house constructed supercritical fluid extraction system. This system is equipped with a high-pressure pump (SCF-201, Jasco Co.), a back-pressure regulator (880-81, Jasco Co.) and an oven (GC A353, GL Sciences Inc.). Freeze-dried activated sludge samples were placed in a 1-ml stainless steel extraction vessel. The liquid CO_2 was compressed with a cooler and then allowed to flow into the extraction vessel. The liquid CO_2 and modifier were continuously mixed in-line. The modifier is a polar or non-polar miscible solvent, and it is used to modify the polarity and solvent strength of the SFE. All operations were performed in dynamic extraction mode at the various desired conditions. The dynamic mode means the mixture was passed over the activated sludge sample inside the extraction vessel. During the extraction, extracted lipids were collected in a dark glass tube to prevent their photodecomposition and then dried down gently under a N_2 gas stream.

2.5. Derivatization procedure

Prior to GC–MS analysis, the collected lipid was fractionated to PLFA. The PLFAs were derivatized to produce their fatty acid methyl esters (FAMEs), as described elsewhere [20]. Briefly, phospholipid extracts were incubated at 80 °C in a water bath for 2 h after having added methanolic HCl solution (0.5N). The tubes were vortex-mixed every 10 min during the 2 h of incubation and after that the samples were cooled for approximately 20 min. Then, phospholipid extracts were vortex-mixed for 15 s in 3 ml of hexane and centrifuged for 5 min at 2000 rpm. The hexane phase containing the FAMEs was transferred to an amber vial, evaporated to dryness under nitrogen and stored at −20 °C.

2.6. Gas chromatography analysis of PLFA

PLFA concentrations were quantified on a Hewlett-Packard 6890 GC Series system with Agilent 5973 N Mass Selective Detector. The GC was operated with the following configuration: column: Supelco (SP-2560 column) 100 m × 0.25 mm i.d. × 0.20 mm thick film; oven: 140 °C (hold 5 min) to 240 °C at 4 °C/min, hold for 15 min; helium carrier gas, 0.9 ml/min; injector at 260 °C. A 37-component FAME standard (Supelco # 47885-U) was used to identify and quantify FAME in the samples by comparing their retention times to those of the FAME standard. The solvent delay was 4 min, and the total run time was 60 min. The transfer line was kept at 290 °C. MS data were collected in a scan mode (29–520 m/z). The standards progress through the gas chromatograph was recorded on a computer fitted with NIST 98 MS software library for confirmative identification of FAME and discrimination of the non-ester interferences. FAMEs are named according to the standard nomenclature (A:BωC) (IUPAC-IUB, 1977) [28].

3. Results and discussion

Activated sludge is a typical mixed culture of microorganisms [7]. The study of activated sludge microbial community structure can provide useful information to solve many problems in wastewater treatment plants operation [29]. Dried activated sludge samples with particles smaller than 500 μm was used in this study. Generally, the larger surface area of small particles results in an increase in the extraction efficiencies. In addition, excessive grinding of sample can severely impede the extraction due to re-adsorption of the analytes onto matrix surfaces and pressure drop inside the extraction vessel [30].

The chromatograms of the PLFAs extracted from the activated sludge by using LSE and SFE are shown in Fig. 1. Ten PLFAs were identified from the activated sludge using both extraction procedures, showing that SFE technique is sensitive enough to extract phospholipids from this type of samples. The same number and types of PLFAs were obtained for each method. The results of the

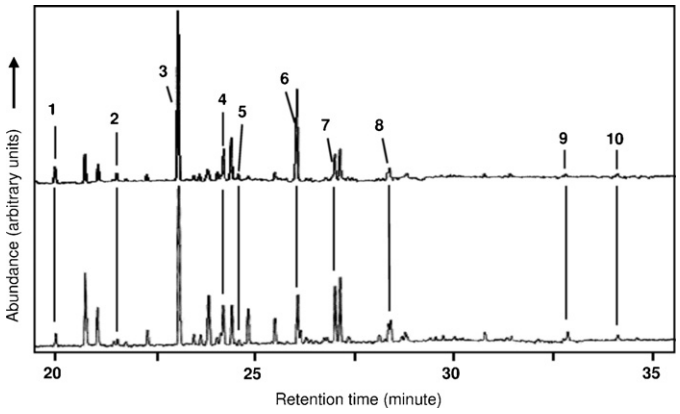

Fig. 1. GC–MS chromatograms of PLFA extracted from the activated sludge by using liquid organic solvent extraction (top) and supercritical fluid extraction (bottom) with methanol as modifier at pressure 25 MPa and temperature 80 °C. Ten PLFAs were (1) C14:0, myristic acid; (2) C15:0, pentadecanoic acid; (3) C16:0, palmitic acid; (4) C16:1, palmitoleic acid; (5) C17:0, heptadecanoic acid; (6) C18:0, stearic acid; (7) C18:1ω9c, oleic acid; (8) C18:2ω6c, linoleic acid; (9) C23:0, tricosanoic acid and (10) C24:0, lignoceric acid.

two methods show that similar shapes of chromatogram peaks were obtained with nearly identical retention times.

3.1. Effect of the modifier on the extraction yields of PLFAs from activated sludge

The experiments on the effect of various modifiers on the extraction yields of PLFAs were performed for 15 min at a temperature of 80 °C, pressure of 25 MPa, $scCO_2$ flow rate of 2.7 ml/min and methanol concentration of 10% (v/v). The extraction yields of PLFAs are expressed as nmol/mg-dry activated sludge. Various solvents, such as n-hexane, chloroform, ethanol, acetone and methanol were investigated in this study. These solvents were selected based on the order of their polarity index in Burdick and Jackson's solvent guide [31]. Methanol has the highest polarity index among the tested solvents in this study. The experimental results are presented in Fig. 2. The supercritical fluid extraction of PLFAs from activated sludge was initially performed by using pure $scCO_2$ without any modifier. The extraction yields of PLFAs were found to be 0.5 nmol/mg-dry activated sludge when pure $scCO_2$ was used as an extraction solvent. Low yields of PLFAs were obtained using this procedure probably due to the limited ability of pure CO_2 to dissolve polar compounds. Thus, the addition of a modifier was necessary

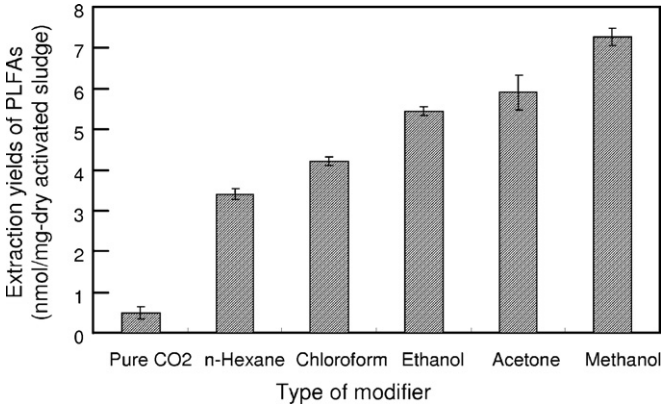

Fig. 2. Effect of the modifiers on the extraction yields of PLFAs from the activated sludge. Extraction time: 15 min; pressure: 25 MPa; temperature: 80 °C; CO_2 flow rate: 2.7 ml/min; modifier concentration: 10% (v/v). All experiments were performed in triplicate.

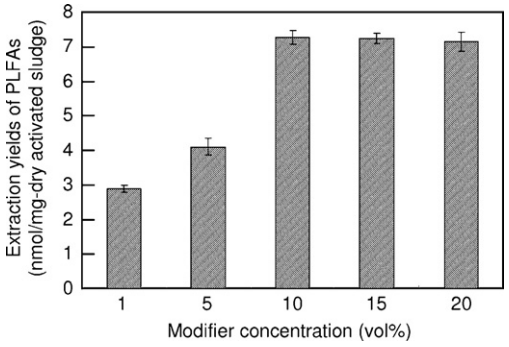

Fig. 3. Effect of modifiers concentration on the extraction yields of PLFAs from the activated sludge. Extraction time: 15 min; pressure: 25 MPa; temperature: 80 °C. All experiments were performed in triplicate.

to overcome the solubility limitation of pure CO_2, thereby facilitating the extraction of PLFAs from activated sludge. The highest extraction yields of PLFAs were achieved using methanol as modifier, reaching a value of 7.28 nmol/mg-dry activated sludge. This is most likely due to the fact that methanol has the highest polarity index among the tested solvents. Addition of methanol enhances the ability of $scCO_2$ to extract PLFAs due to increased solubility and desorption of PLFAs from the activated sludge.

The effect of modifier concentration on the extraction yields of PLFAs was also investigated using $scCO_2$ with the addition of 1, 5, 10, 15 and 20% methanol modifier (v/v), as shown in Fig. 3. The addition of polar methanol significantly increased the extraction yields of PLFAs at the 10% methanol modifier level. There was no further improvement above this concentration. The methanol concentration of 10% has also been shown to be effective for the $scCO_2$ extraction of quinones from activated sludge [23]. Considering the same extraction time with the previous work, the methanol concentration of 10% was therefore selected as optimum in this study.

3.2. Effect of extraction pressure on the extraction yields of PLFAs from activated sludge

The experiments on the effect of extraction pressure were performed for 15 min at a constant temperature of 80 °C, using $scCO_2$ at a flow rate of 2.7 ml/min and methanol concentration of 10% (v/v). The pressures were varied from 10 to 30 MPa. The extraction yields of PLFAs increased as the pressure of the system increased, as presented in Fig. 4. It is well known that the supercritical fluid solvating power is primarily a function of density. The higher densities are most readily achieved through increasing pressure result

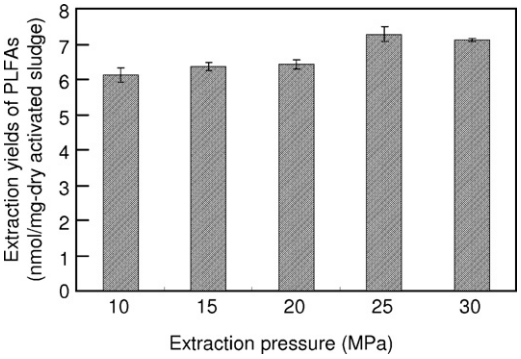

Fig. 4. Effect of extraction pressure on the extraction yields of PLFAs from the activated sludge. Extraction time: 15 min; temperature: 80 °C; CO_2 flow rate: 2.7 ml/min; methanol concentration: 10% (v/v). All experiments were performed in triplicate.

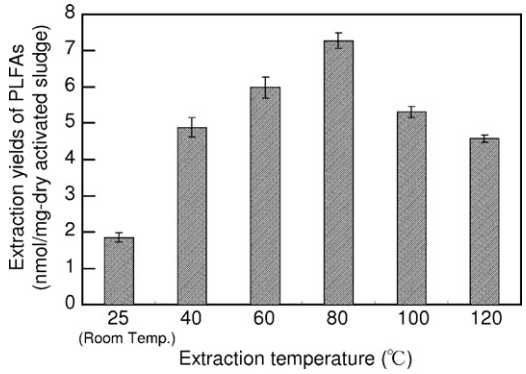

Fig. 5. Effect of extraction temperature on the extraction yields of PLFAs from the activated sludge. Extraction time: 15 min; pressure: 25 MPa; CO_2 flow rate: 2.7 ml/min; methanol concentration: 10% (v/v). All experiments were performed in triplicate.

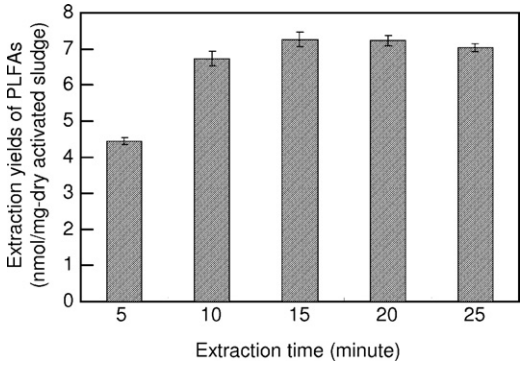

Fig. 6. Effect of extraction time on the extraction yields of PLFAs from the activated sludge. Pressure: 25 MPa; temperature: 80 °C; CO_2 flow rate: 2.7 ml/min; methanol concentration: 10% (v/v). All experiments were performed in triplicate.

in higher solvating power under the same temperature. However, high pressure is not always recommended in practical application. When the pressure is elevated, the higher solubility of solutes can cause the extract becomes very complex and the analysis becomes very difficult [30]. The results showed that 25 MPa should be the most suitable pressure for PLFA extraction.

3.3. Effect of extraction temperature on the extraction yields of PLFAs from activated sludge

The effect of extraction temperature on the extraction yields of PLFAs is shown in Fig. 5. The extractions were performed at a constant pressure of 25 MPa, over a temperature range from room temperature (25 ± 1 °C) to 120 °C, using $scCO_2$ at a flow rate of 2.7 ml/min and methanol concentration of 10% (v/v) for 15 min. The results demonstrated that temperature played an important role in PLFA extraction. The extraction yields of PLFAs was first enhanced with the increase in temperature, reached a maximum value at 80 °C and then decreased with a further increase in temperature up to 120 °C. The total amount of extracted PLFA obtained at 100 and 120 °C was lower than that at 80 °C. To date, the effect of temperature on PLFA extraction has not been well studied. But, it is assumed that some decomposition of the PLFA was occurring at these higher temperatures (above 80 °C) in the extraction vessel. At a constant pressure, it can be noticed that the increase in extraction temperature enhances the vapor pressure of PLFAs, but conversely decrease the density of the scCO2. As a result, the solubility and the extraction efficiency of PLFAs reach a maximum value. It has also been reported that the effect of a temperature elevation is difficult to predict because of its dependence on the nature of the sample [30]. In the present study, an extraction temperature of 80 °C can be considered the most appropriate temperature to extract PLFAs from activated sludge.

3.4. Effect of extraction time on the extraction yields of PLFAs from activated sludge

To determine the suitable extraction time for PLFA extraction with scCO2, the extraction time was varied from 5 to 25 min. Fig. 6 shows the effect of extraction time on the extraction yields of PLFAs at 80 °C and at a pressure of 25 MPa, using CO_2 at a flow rate of 2.7 ml/min and methanol concentration of 10% (v/v). When the time was extended, the extraction yields of PLFAs slightly increased, especially during the 15-min extraction. However, there was no further improvement after 15-min of extraction. It is considered that under the tested conditions, an extraction time of 15 min is the most suitable time to extract PLFAs from 0.1 g activated sludge.

3.5. Comparison of supercritical CO_2 extraction with liquid organic solvent extraction

The extraction yields and composition of PLFAs obtained by $scCO_2$ extraction, with methanol as a modifier were compared to those obtained by liquid organic solvent extraction, as shown in Table 1. Optimized conditions achieved from previous experiments were applied to the $scCO_2$ extraction experiment. Each experiment was replicated five times for each 0.1-g activated sludge sample, either by $scCO_2$ or organic solvent extraction. The extraction yields of PLFA were 7.28 and 7.44 nmol/mg-dry activated sludge with $scCO_2$ and organic solvent extraction, respectively. The extraction yields of PLFA by $scCO_2$ were comparable to or slightly lower than that resulting after extraction with organic solvent with the same 0.1 g activated sludge sample. The difference in the results obtained in each sample by using the two methods was estimated by comparing the PLFA profile data.

Three samples of dried activated sludge with the same weight were also used for the repeatability test of the developed method. Each run was performed under the same conditions. We observed that the relative standard deviation (RSD) values were less than 13% for each identified PLFA. Considering the changes of complex composition of the microorganisms at any time and affect the activated sludge matrix, the repeatability is reasonably good for PLFA analysis. Furthermore, to enhance the objectivity of the information and to make quantitative estimates, two statistical indices, the Shannon (diversity) index and Evenness (equability) index, were used. The Shannon index and Evenness index were calculated by using the equations, as described elsewhere [32,33]. Recently, this

Table 1
Quantitative comparison of supercritical CO_2 ($scCO_2$) extraction and liquid organic solvent extraction (LSE) for phopholipid fatty acids from the activated sludge.

PLFA[a]	SFE		LSE	
	Concentration (nmol/mg)	SD[b] (nmol/mg)	Concentration (nmol/mg)	SD (nmol/mg)
C14:0	0.50	0.07	0.60	0.05
C15:0	0.57	0.08	0.58	0.07
C16:0	1.42	0.26	1.99	0.21
C16:1	2.45	0.07	1.39	0.16
C17:0	0.35	0.06	0.34	0.03
C18:0	0.59	0.11	1.60	0.08
C18:1ω9c	0.51	0.21	0.33	0.02
C18:2ω6c	0.56	0.17	0.46	0.14
C23:0	0.24	0.04	0.11	0.02
C24:0	0.08	0.03	0.04	0.01

[a] Shorthand designations of the fatty acid methyl esters are mentioned in Fig. 1.
[b] Standard deviations (SD) are based on the results of analysis averaged from five (n = 5) trials.

index has also been used to measure the relative distribution of the microbial PLFAs. Diversity is a function of two main components: species richness or species abundance and species evenness or species equitability. The values of the Shannon and Evenness indices were equal to 1.929 and 0.838 for the method employing $scCO_2$ extraction. These results were similar to those obtained by the organic solvent extraction method, with index values of 1.940 and 0.809, meaning that the two methods have a similar diversity value. Therefore, given these results, the two PLFA profiles could be considered similar to each other, proving that $scCO_2$ extraction is a useful method in the PLFA analysis of activated sludge.

4. Conclusions

In this work, an alternative method for the extraction of microbial PLFAs from activated sludge by using $scCO_2$ extraction has been investigated. The experimental results have demonstrated that the application of $scCO_2$ extraction to microbial PLFA analysis has the potential to drastically reduce the amount of solvent used and extraction time needed, and could simplify the procedure. Further studies are in progress to improve this method as an effective technique for analyzing microbial phospholipid fatty acid in environmental samples, with the possibility of extended application and automation.

Acknowledgement

This work was financially supported by a Grant-in-Aid for Scientific Research (code: B) from the Japan Society for the Promotion of Science (JSPS).

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