Korean J. Limnol. 41 (4): 485~489 (2008)

Comparison of Phytoplankton Chlorophyll-*a* Extracted with Different Solvents

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Algal chlorophyll-*a* is commonly determined by spectro-photometric method using 90% acetone as solvents. However, acetone has low extraction efficiency without grinding filters, and DMF (dimethyl formamide) was tested for the compatibility with acetone. Chlorophyll-*a* concentration was determined for samples from 5 reservoirs of different trophic states and phytoplankton composition, using acetone extraction with grinding and DMF without grinding. Chlorophyll-*a* measured by DMF and Acetone did not show a significant difference when using trichromatic method of UNESCO and Lorenzen, and therefore, DMF can substitute acetone. But when acidification method was applied, they showed significant difference of 8%. It can be concluded that DMF can extract chlorophylls more effectively without grinding and it can be a better alternative for the standard extraction solvent.

Key words : chlorophyll-*a*, Acetone, DMF, reservoir, solvent

INTRODUCTION

Chlorophyll-*a* is an essential pigment for photosynthesis and widely used as an indicator of phytoplankton biomass or primary productivity (Wetzel and Linkens, 1991). Algal pigments are extracted with solvents and then can be determined by spectrophotometric, fluorometric, or HPLC methods. Spectrophotometric methods are commonly adopted as the standard method (ISO, 1992; U.S.EPA, 1997; APHA, 1998) because the procedure is simple and the required instrument is not expensive. 90% acetone is the common choice of the extraction solvent (APHA, 1998).

Although acetone has some merits that it produces sharp absorption peaks and it does not have toxicity (Arnon, 1949; Jaffrey and Humphrey, 1975; Humprey and Jeffrey, 1997), acetone has low extraction efficiency for many vascular plants and some green algae, and grinding of filters is recommended especially for samples of green algae or cyanobacteria (Sartory and Grobbelaar, 1984; Porra *et al.*, 1989; Porra, 2002; Jeffrey *et al.*, 1997; Wright *et al.*, 1997). However, grinding filter is a tedious laborsome work, and researchers tried to find a better solvent for the extraction of algal pigment. Solvents that have been tested and suggested as good candidates are as methanol, ethanol, DMSO-acetone mixture, and N,N-Dimethylformamide (DMF) (Stauffer *et al.*, 1979; Speziale *et al.*, 1984; Porra *et al.*, 1989; Wright *et al.*, 1997). Of these tested solvents DMF was reported to have high efficiency of extraction even without grinding filters and have similar specific absorption coefficients and spectra for chlorophylls (Speziale *et al.*, 1984).

Many reports showed that DMF results in higher recovery efficiency than acetone when tested without grinding (Suzuki and Ishimaru, 1990; Tada *et al.*, 2004). And therefore, in the use of acetone grinding filters became a common

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practice in order to ensure extraction efficiency.

When grinding was employed, that is DMF without grinding and acetone with grinding were compared, the two solvents has produced conflicting results, even though complete extraction can be assumed for both solvents. Speziale *et al.* (1984) compared extraction by DMF without grinding and acetone with grinding, using natural freshwater phytoplankton samples and green algae and cyanobacteria-spiked samples. They had higher recovery with acetone for diatom/chrysophytes-dominated natural samples, but on the contrary had higher recovery with DMF for chlorophytes/cyanobacteria-dominant samples.

Since absorption spectra of chlorophylls in DMF is similar, researchers are using the same equations of chlorophyll determination as in acetone. But the absorption spectra of various algal pigments in DMF may not be exactly same as in acetone, and it needs to be further tested for various algal compositions. Two spectrophotometric methods are commonly used for chlorophyll-*a* determination; the trichromatic method (SCOR-UNESCO, 1966) or acidification method (Lorenzen, 1967). Both two methods are listed as standard methods (APHA, 1998) and widely used. Speziale *et al.* (1984) used acidification method of Lorenzen, while Tada *et al.* (2004) used trichromatic method in the test of DMF. Since the trichromatic method use only extracts of neutral pH for the calculation, the absorption spectrum of chlorophylls needs to be identical only in neutral pH in order to use the same equation with acetone. On the other hand, acidification method acidifies extracts to degrade chlorophylls into pheophytins, the spectra of pheophytins as well as chlorophylls also needs to be identical both in neutral and acidic conditions.

In this study we tested the feasibility of substituting DMF for acetone for chlorophyll-*a* determinations, and tested the compatibility of equations for chlorophyll-*a* calculation. Chlorophyll-*a* concentrations were determined by using acetone with grinding and DMF without grinding and compared. The compatibility of two spectrophotometric methods with DMF was also tested.

MATERIALS AND METHODS

Sampling sites covers a broad spectrum of trophic state, lake depth, and dominant phytoplankton species. Water samples were collected three times (April, June, August) in 2005 at the surface of the reservoirs (Table 1). In April we could collect samples with dominant phytoplankton of diatoms, chrysophytes, and chlorophytes. In June we had samples with chrysophytes, cryptophytes, and cyanobacteria. In August we had mostly cyanobacteria-dominant samples. Trophic state of each reservoir was evaluated using trophic state index (Carlson, 1977) and lake classification criteria of Kratz and Brexonik (1981).

Phytoplankton samples were collected by sampling water in a 500 mL polyethylene bottle and preserved with Lugol's solution. Dominant phyto-

Table 1. Limnological characteristics of sampling sites and dominant phytoplankton.

Lake name	Depth & Trophic state	Dominant phytoplankton		
		Apr. 2005	June 2005	Aug. 2005
Juam	Deep, large Oligotrophic	*Stephanodiscus* sp. *Cryptomonas* sp.	*Microcystis* sp. *Dinobryon* sp.	*Microcystis* sp. *Cyclotella* sp.
Daechong	Deep, large Mesotrophic	*Asterionella formosa*	*Cryptomonas* sp.	*Microcystis* sp.
Youngsan	Large, estuarine dam, Eutrophic	*Cryptomonas* sp. *Chlamydomonas* sp. *Scenedesmus* sp.	*Pandorina morum* *Cryptomonas* sp. *Phormidium* sp. *Anabaena* sp.	*Microcystis* sp. *Oscillatoria* sp. *Cyclotella* sp.
Wangkoong	Small, shallow Eutrophic	*Cryptomonas* sp.	*Microcystis* sp.	*Microcystis* sp. *Phormidium* sp.
Sungam	Small, shallow Hypertrophic	*Anabaena* sp. *Aphanizomenon flos-aquae*	*Microcystis* sp. *Oscillatoria* sp.	*Merismopedia elegans* *Phormidium* sp. *Spirulina* sp.

Table 2. Comparison of two spectrophotometric methods for the determination of Chl.-*a* concentration.

	Lorenzen	SCOR-UNESCO
Adoptation	EPA Standard Methods	Korea, Japan, Standard Methods
Pheophytin-a	Calibrate pheo-a	Can not distinguish between chl.-*a* and pheo-a
Interference of Chl-*b*, -*c*	Not calibrated	Calibrated by measuring at 647 nm, 630 nm
Measurement procedure	More complicated than UNESCO method	Simpler than Lorenzen method
Empolyed	664, 665, 750 nm	630, 647, 663, 750 nm

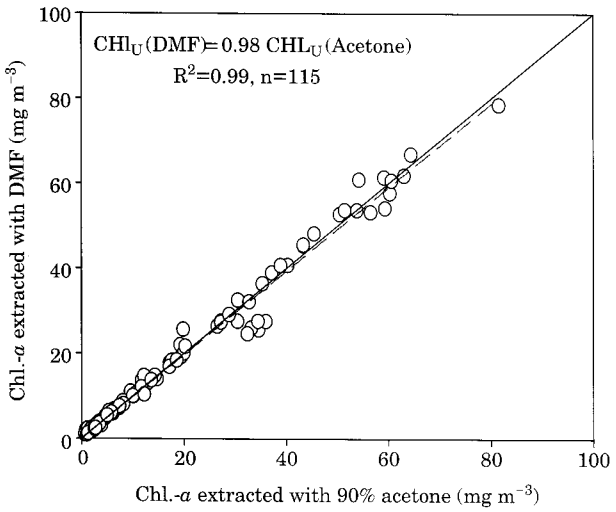

Fig. 1. Comparison of chlorophyll-*a* concentration extracted by DMF without grinding and 90% acetone with grinding. Chlorophyll-*a* was determined by the trichromatic method (SCOR-UNESCO, 1966). Solid line is the line of slope=1, and the dashed line is the regression line.

plankton was examined under microscope and enumerated by using Sedgwick-Rafter counting chamber. Species were identified according to Mizuno (1964) and Jung (1993)'s method.

For chlorophyll-*a* analysis water samples were transported to the laboratory and filtered with GF/C glass fiber filter, and the filters were kept frozen until analysis. Six replicates of filter were prepared for each sample. Three were used for acetone method, and three were used for DMF method. Chlorophyll-*a* was analyzed according to

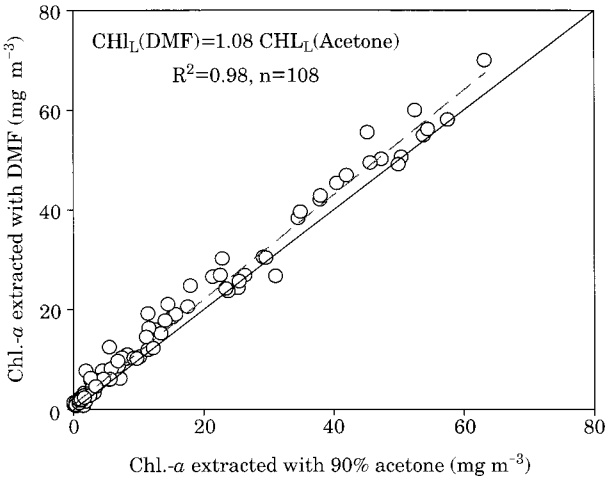

Fig. 2. Comparison of chlorophyll-*a* concentration extracted by DMF without grinding and 90% acetone with grinding. Chlorophyll-*a* was determined by the acidification method (Lorenzen, 1967). Solid line is the line of slope=1, and dashed line is the regression line.

the procedure of Standard Methods (APHA, 1998). In the acetone method filters were ground in glass homogenizer with the addition of 90% acetone, and then made up to 6.0 mL, and put in a dark refrigerator for 6 hours. In the DMF method filters were not ground, and soaked in DMF for the same period with acetone. Extracts were centrifuged, and 3 mL supernatant was transferred by a spoid to a cuvette and absorption spectrum was scanned from 400 nm wavelength to 750 nm using a slit width of <1 nm. One drop of 0.18 N HCl solution was added to the cuvette to acidify extracts and mixed by converting the cuvette, and absorption spectrum was measured again after 5 minutes. Chlorophyll-*a* concentration was calculated by two methods (Table 2); trichromatic method of SCOR-UNESCO (1966) and acidification method of Lorenzen (1967). For a statistical analysis pared t-test was performed using Microsoft EXCEL 2003.

RESULTS

Chlorophyll-*a* extracted with acetone and DMF showed different results depending on the two calculation methods; the trichromatic method by UNESCO (1966) and the acidification method by Lorenzen (1967). When UNESCO's method was

used, chlorophyll-*a* determined by DMF without grinding (Chl-U-DMF) and by acetone with grinding (Chl-U-acetone) did not show significant difference in a paired t-test ($p > 0.05$). The regression coefficient of Chl-U-DMF on Chl-U-acetone was 0.98. In this study chlorophyll-*a* was determined for various algal compositions of field samples, including samples of various dominant species; chlorophytes, diatoms, and cyanobactria. Therefore, it can be concluded that the two solvents give identical results and can be used interchangeably. Of course, DMF would have the merit of not grinding filters.

On the contrary, when the Lorenzen's method was used for the calculation of chlorophyll-*a* concentration, chlorophyll-*a* extracted by DMF (Chl-L-DMF) and extracted by acetone (Chl-L-acetone) showed significant difference in the paired t-test ($p < 0.01$). The regression coefficient of Chl-L-DMF on Chl-L-acetone was 1.08, which means DMF gives 8% larger values than acetone, and the two solvents can not be used interchangeably.

DISCUSSION

In the measurement of chlorophylls 90% acetone has been used for a long time as the standard solvent since the early years of the analytical method development (Strickland and Parsons, 1972; APHA, 1998). But after the initial establishment of the method acetone has been blamed for its low extraction efficiency with chlorophytes and cyanobacteria. Finally, grinding filters became an alternative, however it is a laborsome procedure. From the result of this study it was confirmed that DMF can be used without grinding instead of acetone, if the trichromatic method is used for the calculation. Therefore, it should be considered to change the standard method from acetone to DMF, especially in countries where the trichromatic method is adopted as the national standard, such as Korea and Japan.

On the contrary, chlorophyll-*a* measured acidification method showed 8% differences for DMF and acetone which can be a significant difference in many cases. In order to use DMF for acidification method it can be calibrated empirically by dividing is by the ratio of Chl-L-DMF/Chl-L-acetone, 1.08. Or we can revise the calculation equation of acidification method in the future by measuring specific absorbance of pheophytin-a.

As for the reason why the two solvents showed discrepancy, the absorption spectrum of pheophytin-a might be different from chlorophyll-*a* in acidic condition. The trichromatic method measures absorbance only in neutral conditions without acidification, whereas acidification method employs acid addition in order to convert chlorophylls to pheophytins. It seems that in acidic condition absorption spectrums of algal pigments are different from in neutral condition. And in order to employ the acidification method using DMF as the solvent for chlorophyll-*a* determination we need to study further spectrums of each pigments in DMF for their specific absorption coefficients.

Another disadvantage of acetone as the extraction solvent is hazard to health (Ritchie, 2006). Acetone is highly volatile, flammable, causative of headache, anesthetic, and skin-irritating. Plastic or latex gloves can not block acetone completely and can even worsen the harmful effect. Acetone can be easily inhaled while using it due to high volatility. On the contrary, DMF seems to have lower risk of hazard because of its low volatility, even though it is more toxic than acetone in the respect of LD50. And if gloves are used, it can block skin contact and lower the risk.

ACKNOWLEDGMENTS

Yunkyoung Lee, Myoungsun Shin, Yukyong Jung and Changwon Jang were supported by the 2nd Brain Korea 21 Project in 2006.

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(Manuscript received 3 November 2008,
Revision accepted 8 December 2008)