Short Contribution

HPLC Determination of Phytoplankton Pigments Using N,N-Dimethylformamide

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The suitability of N,N-dimethylformamide (DMF) as an extractant for the standard reverse-phase HPLC method was examined using algal cultures. Good pigment separations and recovery were achieved with 20% (volume %) addition of an ion-pairing solution in an injection. While slight amounts of degradation products of chlorophyll a, i.e., chlorophyllide a, allomeric and epimeric forms, were produced, adequate attention to filtration and extraction prevents the formation of degradation products, confining them to an acceptable level. Because of its strong extractability, which expedites the extraction process, DMF is an efficient solvent for HPLC analysis of phytoplankton pigments.

Keywords:
- Pigments
- HPLC
- N,N-dimethylformamide
- phytoplankton
- chlorophyll a

1. Introduction

High-performance liquid chromatography is proving to be an extremely useful tool in studying a range of aspects of phytoplankton ecology. Quantification of chlorophyllous and carotenoid pigments provides information on biomass, taxonomic composition, physiological state, photoadaptation, grazing processes and detritus of algal origin (e.g., Gieskes, 1991). Their accurate measurement involves a number of procedures. Pigment extraction is one of the most important steps. Among various solvents for chlorophyll a (Chl a), N,N-dimethylformamide (DMF) is known for its power of extraction and efficiency in various taxonomic groups (e.g., Moran and Porath, 1980; Speziale et al., 1984; Suzuki and Ishimaru, 1990). Furthermore, DMF shows the best extractive ability for accessory pigments among the commonly used solvents, such as 90% acetone or methanol (Suzuki et al., 1993; Wright et al., 1997). Although 90% acetone is most widely used as an extractant in both conventional fluorometry and HPLC, it can lead to an underestimation of Chl a. The underestimation is serious in extraction by soaking, and even mechanical treatments of samples, such as homogenization and sonication, result in some losses of materials (Wright et al., 1997).

In spite of its high performance, DMF is at present seldom used as an extractant for HPLC, probably due to safety considerations in view of its toxicity. Although Suzuki et al. (1993) described a simplified HPLC method using DMF, separations of some polar pigments were insufficient. This was mainly because the method did not use ion-pairing reagents (Mantoura and Llewellyn, 1983). In this communication we examine the suitability of DMF for the standard reverse-phase HPLC method using ion-pairing reagents, paying special attention to the degradation of chlorophyll a.

The test was conducted using phytoplankton cultures including a diatom Phaeodactylum tricornutum with a high chlorophyllase activity.

2. Materials and Methods

The examinations were done using clonal cultures of P. tricornutum, a cryptophyte Chroomonas salina, a haptophyte Pleurochrysis carterae and a chlorophyte Dunaliella tertiolecta. Cells were grown in the f/2 medium (Guillard and Ryther, 1962) at 20°C under illumination of ca. 60 μmol photon m⁻² s⁻¹ supplied by daylight type fluorescence tubes with a photoperiod of 12:12 h LD. The cultures were harvested in the mid to late exponential growth phase. Care was taken to take the cell suspension into a flask before filtration, avoiding residual materials around culture bottles. Cell suspensions were filtered onto 25 mm Whatman GF/F filters using gentle suction of 150 mmHg (vacuum). Immediately after the filtration the filter papers were folded once and blotted between pieces of filter paper to reduce the excess water content. From the filtration through the whole assay process the samples were handled under dim light or in darkness to prevent photodegradation of the pigments.

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Pigments were extracted from the cultures immediately after the filtration (hereafter referred as Fresh). For *P. tricornutum*, which showed high chlorophyllase activity (Barret and Jeffrey, 1971), an additional extraction was done to examine the effects of chlorophyllase during extraction, either after freezing at -82°C for 2 h (Frozen), freeze-dried after the frozen treatment (F-D), or Fresh treatment with densely filtered algal materials (Dense). The F-D treatment was done to examine the effects of the filters water content. Three filters were used for each treatment. In the Dense treatment, about three times the amount of algal materials was filtered on a 25 mm GF/F. Before the Frozen treatment the filters were dipped in liquid nitrogen to ensure rapid freezing, and then transferred to a freezer. The F-D treatment was done just before subsequent extraction and the F-D filters were not retained. For extraction all the filters were ground in 2 mL of DMF with a glass homogenizer until the filters were completely crushed.

The extract was mixed with 1-M ammonium acetate as the ion-pairing reagent and filtered through a 0.2 µm PTFE filter (Millipore, USA). This mixing and filtration was done immediately before injection. The mixture was injected into a Shimadzu HPLC system (a pump LC-9A with a low pressure gradient unit FCV-9AL, an on-line degasser DGU-3A, a photodiode array UV-vis detector SPD-M10AV) fitted with a 5-µm LiChrosphere ODS column (4 x 125 mm, 100RP-18e, Merk, USA) and a guard column (4 x 4 mm, LiChrosphere 100RP-18e, Merk, USA). A binary solvent system, modified from Mantoura and Llewellyn (1983), was used with 1-M ammonium acetate solution as a buffer (Zapata et al., 1987). Solvent A was 80% methanol and 20% 0.5-M ammonium acetate, and solvent B was 70% methanol and 30% acetone. Solvent delivery was programmed by four successive linear gradients: (1) from 100% solvent A to 75% solvent A and 25% solvent B for 4 min; (2) to 50% solvent A; 50% solvent B for 4 min; (3) to 25% solvent A: 75% solvent B for 4 min; and (4) to 100% solvent B for 4 min, then isocratically until after appearance of β-carotene. The flow rate was 1 mL min⁻¹. Methanol and acetone were HPLC grade and all the other reagents were analytical grade (Wako, Japan). The water used throughout was prepared using a Milli-Q system (Millipore, USA).

The pigments separated were identified based on retention time and on their retention time and on-line absorption spectra of the detector for the other pigments. Chl a and β-carotene were quantified by weight from peak area calibrated against that of the standard solution, the concentrated which was determined spectrophotometrically. Allomeric and epimeric forms of Chl a were determined using the same calibration factor as Chl a. Chlorophyllide a (Chlide a) was prepared from the culture of *P. tricornutum* (Barret and Jeffrey, 1971) and purified by HPLC. The weight of Chlide a is expressed as the equivalent amount of Chl a.

### 3. Results and Discussion

An ion-pairing agent in injections yielded good separations of pigments (Fig. 1). The improvement of the separation was obvious in polar pigments, such as Chlide a and Chl c as found by Mantoura and Llewellyn (1983). The separation of polar pigments was better with an increase in relative amount of ammonium acetate solution added to the pigment extract. However, recovery of non-polar pigments became worse with an increase of ammonium acetate solution (Fig. 2). Three portions of ammonium acetate solution added to seven portions of DMF extract showed a low recovery of Chl a, although no loss was observed for pigments eluted before Chl a (data now shown). Recovery of β-carotene, which is a less polar pigment than Chl a, was much worse at this mixing ratio (Fig. 2). A similar change of recovery was also obtained both for Chl a and β-carotene when the ammonium acetate solution was replaced by pure water (data not shown). This confirmed the instability and loss of non-polar pigments in highly aqueous extracts (Wright et al., 1991). Therefore, careful attention should be paid to the water content of the injection. A 20% content of the ion-pair solution appears to be most appropriate in terms of both the separations and recovery. A glassfiber filter is commonly used to collect cells, and since such filters tend to retain excess water on filtration, blotting is an important step to...