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RESEARCH ARTICLE

EXTRACTION OPTIMIZATION PRIOR TO HPLC ANALYSIS FOR PHYTOPLANKTON PIGMENTS

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ABSTRACT

Extracting marine phytoplankton pigments is a frequently-used analytical technique for determining the main groups present in samples. Determining these compounds (carotenoids and chlorophylls) is done mainly by high pressure liquid chromatography (HPLC), with visible UV detection and fluorescence, using a range of published methods. But the preliminary extraction required to determine them is a little-studied step that requires optimising. This paper analyses several factors that affect the extraction process, using ultra-sound microprobe as the main technique, validating the method with culture samples and real samples harvested at different depths.

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INTRODUCTION

Phytoplankton is the most abundant vegetable fraction in the ocean that is vitally important to the marine ecosystem, as it is the base of the food chain. It is comprised of a set of microscopic organisms, mostly single-cell and photosynthetic, characterised by their low capacity for movement, although some do have a certain power of locomotion, using flagellates and other mechanisms to move around (Lee, 2008). They are especially interesting to study because their photosynthetic capacity makes them one of the greatest producers of oxygen in the ocean (Gibb *et al.*, 2000; Marañón *et al.*, 2001; Barlow *et al.*, 2002; Aiken *et al.*, 2007; Moreno-Ostos *et al.*, 2010). Photosynthetic pigments are molecules with a large capacity to absorb energy from the sun and convert it into organic matter through photosynthesis. Most pigments found in the water column are in the phytoplankton, but they can also be found in benthonic algae communities, phototrophic bacteria and aquatic plants or macrophytes (Carpenter, 1986; Yacobi *et al.*, 1990; Bianchi *et al.*, 1993). Knowing the concentrations of photosynthetic and photoprotective pigments makes it easier to determine the CO_2 absorption properties of the phytoplankton (Matorin *et al.*, 2004) and the rate of photosynthesis in natural waters.

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It also allows the biomass generated and the composition of algae communities present to be determined (Zapata *et al.*, 2004). These measurement provide an indirect view of the physiological condition of the phytoplankton (Gibb *et al.*, 2000; Barlow *et al.*, 2002, 2008; Vega-Moreno *et al.*, 2012). Directly determining the kind of phytoplankton present and their abundance in aqueous samples (in both sea and fresh water) is something that must be done visually using a microscope, making it far more tedious and very much more difficult to automate than the indirect method of chemical analysis of the pigments found. The pigments found in phytoplankton include numerous groups of compounds with different physical and chemical characteristics (Rogers, 1998). However, they can be divided basically into three major groups: chlorophylls, carotenoids (carotenes and xanthophylls) and phycobiliproteins (allophycocyanins, phycocyanins and phycoerythrins) (Lee, 2008). The most widely-used techniques for extracting and determining carotenoids and chlorophylls from phytoplankton are ultrasound extraction and high-pressure liquid chromatography (HPLC) with Visible UV and fluorescence for determining them (Vega-Moreno *et al.*, 2012). HPLC has been widely used to determine phytoplankton pigments (van Leeuwe *et al.*, 2006) because it allows a multitude of phytoplankton groups to be determined and identified in a single sample analysis, and it can also be automated. This technique is accepted in the *Joint Global Ocean Flux Studies (*JGOFS) protocols, for determining ocean

parameters (Knap *et al.*, 1996). Apart from those conducted by the JGOFS, several later studies published have focused on optimising the determination of phytoplankton pigments, including chlorophylls and the main carotenoids by HPLC with visible ultraviolet (visible UV) detection, or fluorescence detection (Zapata *et al.*, 2004). Fluorescence detection only allows chlorophyll derivatives to be detected, which is why vis-UV detection is more widely used (Wright *et al.*, 1991; Rogers, 1998; Szymczak-Zyla, Louda and Kowalewska, 2008; Halim *et al.*, 2010). The chromatographic columns, the conditions of the analysis method and the gradient of the mobile phase are varied in these methods, allowing the number of secondary carotenoids that can be detected to be increased depending on the limitations of each method.

However, these publications have not made a deep dive into the procedures prior to determination, mainly, taking samples and extracting these compounds. In eutrophic zones, the pigment extraction process is not a critical step, because small compound recovery percentages are enough to obtain good chromatographic signals that are easily determined experimentally. But in zones like the region of the Canary Islands, oligotrophic with little primary production and low phytoplankton concentrations (Arístegui, 1990), this step can be decisive for obtaining conclusive results. The volumes of sea water filtered to determine phytoplankton pigments vary between 0.5 and 4 litres, depending on the concentration of phytoplankton expected in them (Barlow *et al.*, 2002; Moreno *et al.*, 2012). Some authors recommend filtering a minimum of 4 litres per sample for oligotrophic waters, and this can reach as much as 10 litres, but in the logistics of an oceanographic campaign, it is not always possible to collect so much water from each Niskin bottle. Furthermore, even in cases in which the sample volume is high, the concentration is so low that chromatographic signals can be close to the method's limits of detection (LOD) when detecting these compounds. That is why it is important to optimise the extraction procedure for these compounds from the cellulose filters where they are harvested (Claustre *et al.*, 1994), especially for oligotrophic regions, to guarantee their recovery and concentration in the extract, thus enhancing their chromatographic signal while reducing the noise from interference with the matrix or degradation products.

MATERIALS AND METHODS

Materials

This study worked with two different kinds of samples: phytoplankton obtained from cultures and oceanic phytoplankton. The oceanic samples were harvested in 20 litre Niskin bottles, with an array of 12 bottles. The phytoplankton cultures used were of the genus *Nichia* and *Phaeodactylum* (Spanish Algae Bank, BEA in Spanish). Artificial cultures were necessary to obtain a large number of identical samples for the analytical study and to optimise the parameters in a reproducible manner. The filters used for recovering and concentrating them were 47mm diameter Whatman GF/F filters, and the pigments were extracted with the application of an ultrasound microtip, using methanol as extractant. The ultrasonic sonifier used (Branson Digital Sonifier, model 450) is fitted with a micro-tip for extracting small quantities of the sample in vials and test tubes. This study used 10ml total volume conical test tubes with a screw thread. After extraction, each sample is clarified using centrifugation (3000 rpm for 5 minutes) and filtration (with 0.22 µm syringe filters) before being injected into the HPLC. Methanol was the extractant selected (top quality, ®Panreac methanol) to facilitate compatibility with the determination system (Vega-Moreno *et al.*, 2012). The extracts were analysed by HPLC, following the methodology of the JGOFS (W) protocols (Wright *et al.*, 1991) with a Waters Spherisorb 5 µm ODS2 4.6x250 mm C18 chromatographic column. For determining the individual concentrations of each compound, chlorophyll a and chlorophyll b standards obtained in Sigma-Aldrich were used, and the carotenoid standards were obtained in the DHI Institute for Water and Environment (Denmark). The analysis was performed with a VARIAN ® Pro Star 230 HPLC Chromatograph, with a 410 auto-sampler that guarantees replication of the volume injected into the HPLC for each sample (200 µl).

Methods

The culture samples were homogenised and filtered to give a total of 60 filters with the same concentration of phytoplankton from the lot, with a view to optimising the extraction procedure with comparable samples. The same kind and volume of phytoplankton sample was used for all of them, and once filtered, they were kept at -80ºC. Prior to each extraction, the filters necessary for each optimization points were tempered 30 minutes before each study. The extraction procedure was performed under controlled light conditions to prevent photo-oxidation of the pigments (Nelson, 1993; Kowalewska and Szymczak, 2001). The optimised parameters for extracting the phytoplankton pigments from these samples were: amplitude of the ultrasound tip, extraction time, refrigeration between successive extractions (extraction pulses per ultrasound), prior fractioning of the filter and volume of extractant. HPLC with visible UV at 440nm and 680nm was used for determining the phytoplankton pigments.

The optimization study was conducted under a series of initial working conditions, based on previous experiences and the preliminary results of this study (Vega-Moreno *et al.*, 2012): extractant: 4 ml of methanol at 4ºC, and initial sonication time: 30 seconds (this parameter was later optimised). To complete the samples, they were centrifuged at 3000 rpm for 7 minutes at 0 ºC and then they were filtered before the HPLC-UV analysis.

Determining pigments by high resolution liquid chromatography (HPLC-UV)

The pigment extracts were analysed by high resolution liquid chromatography with visible ultraviolet detection (HPLC-vis-UV), following the methodology of Wright et al., 1991. The mobile phase is tertiary, constituted by the mixture of mobile phase A (mixture of methanol and a ®Sigma-Alorich 99.0% aqueous solution of ammonium acetate with a concentration of 0.039 g/ml, in a ratio of 80:20), mobile phase B comprised of a mixture of high quality ®Panreac acetonitrile and MiliQ water at a ratio of 90:10, and a mobile phase C of 100% high quality ®Panreac ethyl acetate. The chromatographic determination was performed using an automatic sampler, but with a refrigeration accessory (4ºC) to prevent the samples from degrading in the time taken in the analysis sequence. The carotenoids were analysed at 400nm for the visible-UV detector, and the chlorophylls at 436nm agitation and 680nm emission with the fluorescence detector.

Optimising ultrasound extraction

In situ refrigeration in the extraction

An increase in temperature would affect the composition of pigments, as they are thermo-sensitive (Lee, 2008). Applying the ultrasound tip to the sample generates heat and therefore, produces an increase in the temperature of the sample. There are studies that refrigerate the sample with ice after a certain time in the ultrasound microtip and attempts have been made to assess its influence (van Leeuwe *et al.*, 2006). The results for three different amplitudes (20, 40 and 60W) were compared, refrigerating the sample with ice every 10 seconds (with a total of 3 10-second pulses, refrigerating after each pulse) and without cooling (30 seconds of extraction without any intermediate refrigeration). Concentrations were better in the samples that were refrigerated during the extraction process (Figure 1). The *chlorophyll a* and the *b-carotenes* where the compounds most affected by the increase in temperature in extraction due to lack of refrigeration, because they are more sensitive to high temperatures and solar irradiation (Simon and Helliwell, 1998; Barlow *et al.*, 2002; van Leeuwe *et al.*, 2006; Halim *et al.*, 2010).

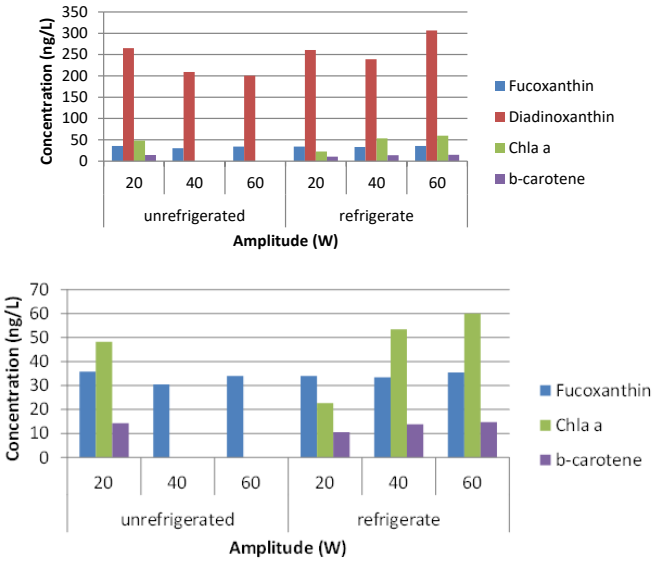

Figure 1: Amplitude - Refrigeration Optimization

Optimising the amplitude

After these results, extraction was set at 3 10-second pulses of ultrasound tip with refrigeration after each pulse, and the study was broadened for amplitudes of between 40 and 60W (Figure 2).

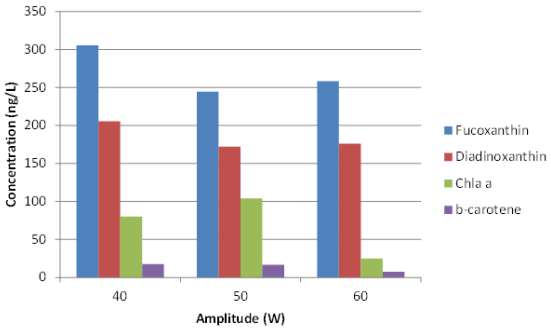

Figure 2. Amplitude Optimization

The results vary for each compound studied, but an intermediate amplitude (50W) was chosen, as this presented good results and a better signal/noise ratio in the HPLC chromatographs. The amplitude chosen is the same as the one mentioned in related articles (Wright *et al.*, 1991; Vega-Moreno *et al.*, 2012)

Optimising extraction time

The total extraction time was optimised for an amplitude of 50W. A total extraction time range of between 10 and 60 seconds was determined, refrigerating after each pulse, which is the equivalent of between 1 and 6 pulses of sonication. The results over 30 seconds were better than for very short times, especially for the more apolar compounds (including chlorophyll a and b-carotene). A total extraction time of 50 seconds was chosen as the optimum value, carried out in 5 10-second pulses. Longer times were more satisfactory than shorter times, and 50 seconds was the ideal time for this study.

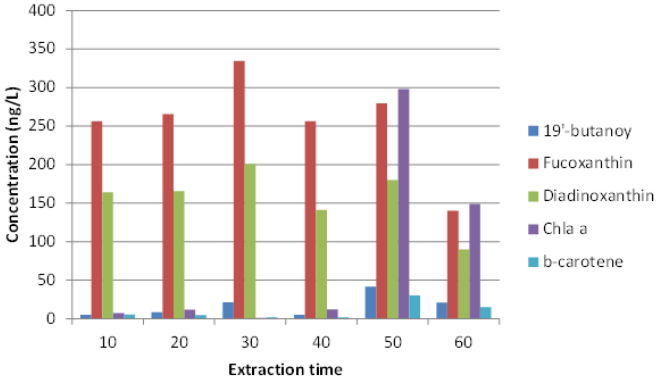

Figure 3: Time Optimization

Optimising pieces of filter

What applying ultrasound microtip to the sample does is facilitate the breakdown of phytoplankton cells to extract the pigments. These cells adhere to the cellulose filter. That is why fragmenting the filter before starting extraction with the microtip can facilitate the process, or make it more efficient, by increasing the concentrations of pigments obtained. In order to study this effect, the filter that contained the sample was chopped up before extraction began. The results were analysed for a whole filter and with the filter chopped into 2, 4 and 6 pieces. The results in figure 4 show that fragmenting the sample before extraction facilitates the extraction process and maximises the results.

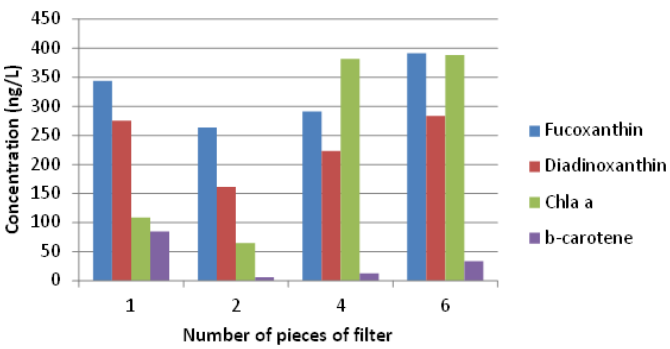

Figure 4: Chopped Optimization

Optimising extractant volume

To complete the study, and based on previously optimised data, extractant volume was optimised. The extractant was methanol at 4ºC. Up until this optimization, the volume of extractant set for optimising the other parameters was 4 ml, but a large volume implies a lower pre-concentration of the sample, but a small volume may not be enough to obtain the actual extraction of the compounds. The volume of methanol was studies in a range of between 2 and 6ml.

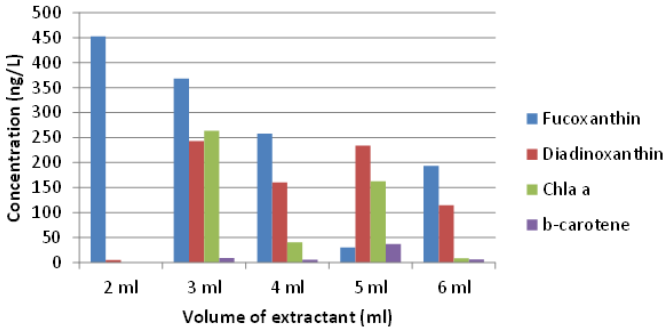

Figure 5: Extractant Volume Optimization

The results in Figure 5 show that a volume of 2 ml is not enough to extract the more apolar compounds, but it produces the highest pre-concentration. In an attempt to maximise both factors, a volume of 3ml was chosen.

Summary of the optimised pigment extraction method

The first step in optimising the extraction process is to obtain sixty identical filters with the same concentration and kind of phytoplankton from marine cultures, to provide replicable samples that allow the extraction parameter optimization study to be performed. The parameters optimised in the study were:

- Drying the sample beforehand
- Chopping the Whatman GF/F filter before extraction
- Volume of extractant added (pure, refrigerated methanol)
- Extraction amplitude and time using ultrasound microtip
- One-step or consecutive-step extraction with rest times between steps
- Study cooling of the sample during rest times during extraction to minimise degradation loss
- Centrifugation and clarification of the sample
- Filter before HPLC-visible-UV and fluorescence determination

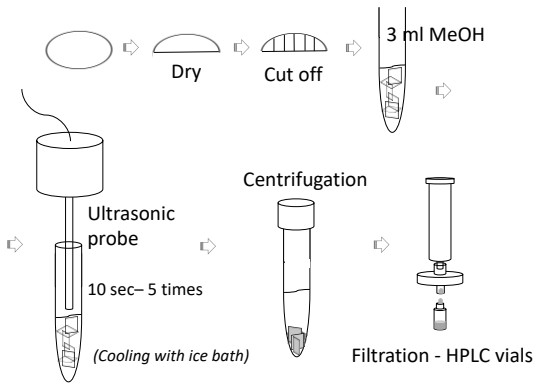

Illustration 1. final pigment extraction process

RESULTS

Validation

The procedure was repeated 6 times under the same conditions to study the validity of the protocol. The results varied depending on the pigment; chlorophylls presented a greater percentage of standard deviation (%RSD), which could be due to the fact that they are more sensitive and could have been affected by the extraction time. The pigments that showed the least variation were Fucoxanthin and Diadinoxanthin, with 12% and 25% respectively.

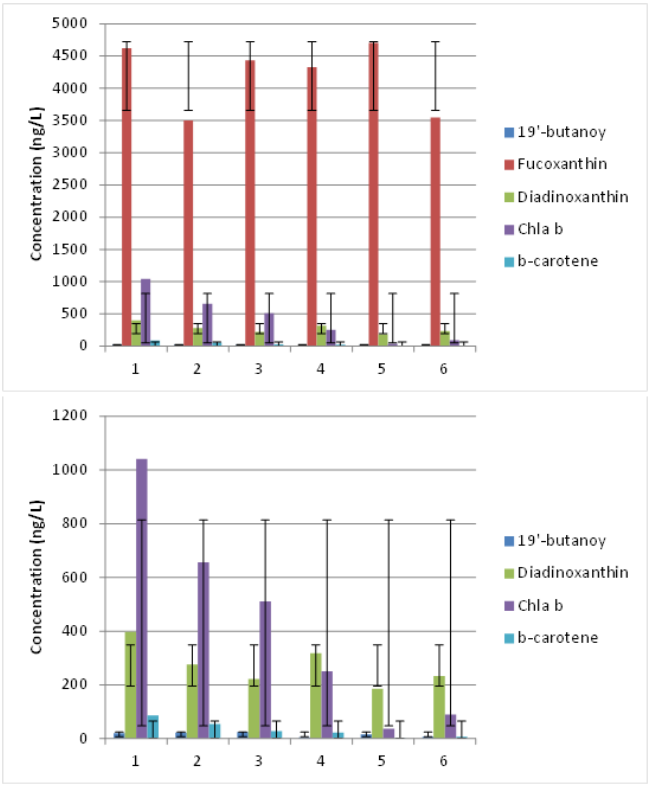

Figure 6: Validation

Real applications

This new optimised method was used to analyse samples of marine phytoplankton in the area of the Canary Islands (Spain). This is an oligotrophic region with low phytoplankton concentration and, thus, limits the chromatographic signal received. Moreover, the on-board logistics of oceanographic campaigns often makes it impossible to filter large quantities of samples that would enable us to enhance the chromatographic signal by increasing the sample volume.

In this case, 500ml of sea water were taken 5 metres from the surface with Niskin bottles. Samples were taken from different sites to the east of the island of Gran Canaria, some two nautical miles of the coast, in September 2015. The samples collected were filtered in Whatman GF/C glass filters with a diameter of 55mm and a particle retention of 1.2 µm, and they were stored at -80ºC until they were analysed. The samples were tempered in the laboratory 30 minutes before extraction and the optimised extraction procedure applied. The results obtained are shown in Figure 6, indicating that the method is valid, for a small sample volume (500ml), for analysing real phytoplankton samples.

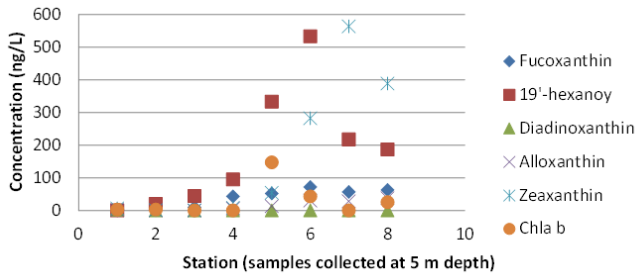

Figure 7: Application to real samples (September 2015)

DISCUSSION

There have been several studies that have analysed and determined marine phytoplankton from their pigments (Abaychi and Riley, 1979; Barlow *et al.*, 2007; Szymczak-Zyla, Louda and Kowalewska, 2008; Halim *et al.*, 2010), and some even propose improvements for analysing and determining phytoplankton pigments using high pressure liquid chromatography (van Leeuwe *et al.*, 2006). However, there is no standard or optimised extraction protocol for these pigments before HPLC determination that guarantees maximising the results, especially in regions of low concentration, or where the volume of the sample that can be taken is low (such as in multi-parameter oceanographic campaigns, where the use of the water contained in each Niskin bottle is limited). The extraction optimization run allows the sensitivity of the process to be enhanced, even for small volume samples, demonstrated with real samples in an oligotrophic region.

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