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Extraction of pigments and fatty acids from the green alga *Scenedesmus obliquus* (Chlorophyceae)

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Abstract

In this paper, the efficiency of pigment and fatty acid extraction from resistant algae using *Scenedesmus obliquus* as an example was examined. We found that adding quartz sand and solvent to freeze-dried algal material and subsequent extraction in an ultrasound bath for 90 min at −4 °C resulted in excellent extraction of these compounds. This extraction method was compared with a method regularly used for extraction of fatty acids and pigments, i.e. addition of solvents to algal material with subsequent incubation. Our extraction using the ultrasound and sand method was about twice as efficient as this method for both pigments and fatty acids. The ultrasound method is simple, extracts over 90% of the different substances in one step and conserves the relationships of pigments and fatty acids. In addition, no alteration- or breakdown products were observed with the new method. Thus, this method allows accurate quantitative extraction of both pigments and fatty acids from *Scenedesmus obliquus* and other algae. The method was also been found to be as effective for *Cryptomonas erosa* (Cryptophyceae), *Cyclotella meneghiniana* (Bacillariophyceae), *Microcystis aeruginosa* (Cyanophyceae), and *Staurastrum paradoxum* (Chlorophyceae, Desmidiaceae) and is thus applicable to a wide spectrum of algae.

Introduction

Green algae, particularly members of the genus *Scenedesmus*, have become the equivalent of laboratory rats in many fields in limnology. They are commonly used as standard organisms in numerous areas of aquatic research, technology, and water management (e.g., Zachleder et al., 1986). Apart from its omnipresent role in research on algal growth, morphology and life cycles (e.g., Trainor, 1995), *Scenedesmus* is also one of the most popular food sources in experiments with herbivorous zooplankton (e.g., Boersma & Vijverberg, 1995). Moreover, it has also become the standard alga in the growing research area on inducible defences, where researchers use this alga to study its reaction to the presence of chemicals excreted by its predator (e.g., Lürling & van Donk, 1997; Wiltshire & Lampert, 1999). In many of these research applications, the quantitative determination of the biochemical composition of algal cells is very important and especially so, where the pigment and fatty acid composition of algae are concerned. Pigments are of fundamental importance in algal cell physiology (Deventer & Heckman, 1996), and lipids or fatty acids are important components of algal nutritional value (Drevon et al., 1993).

Substantial problems associated with extraction and analysis complicate the determination of the pigment composition of algae (Wright et al., 1997; Wiltshire et al., 1998). The analytical problems are well documented, and it is now widely accepted that High Performance Liquid Chromatography (HPLC) is the only accurate means of quantifying chlorophyll and other photosynthetic pigments in aquatic systems (AWWA-APHA, 1985; Wright et al., 1997; Wiltshire et al., 1998). However, exact and uncomplicated extraction of pigments still proves difficult. In an excellent review Wright et al. (1997), have recommended extraction methods for pigments from marine algae. However, these methods are still not adequate

for many of the biochemical and physiological questions involving *Scenedesmus*, where exact quantitative information is required.

The subject of lipid analysis has been extensively reviewed in the literature (Christie, 1982; Parrish, 1999), and many different preparative and analytical techniques have been published for lipids and fatty acids (Folch et al., 1956; Bligh & Dyer, 1959; Kattner & Fricke, 1986). However, the classical work on lipid extraction efficiency was carried out on relatively large samples of animal tissue. (Folch et al., 1956; Bligh & Dyer, 1959). Given the absence of cell walls in animal material, blending or mixing samples during the extraction sufficed for quantitative lipid extraction. In contrast, the extraction procedures and efficiencies for plant material, especially for algae, are less well established. Indeed, the literature on fatty acid and lipid content of microalgae contains no standard extraction method. Published extraction methods include the addition of solvents and subsequent incubation (Ackman et al., 1968), agitation (Cartens et al., 1996), stirring (Whyte, 1988), homogenisation (Ben-Amotz et al., 1985), grinding (Rai et al., 1997), and sonication (Napolitano, 1994).

In short, no standard technique exists to quantitatively extract pigments and fatty acids from microalgae. Moreover, the choice of *Scenedesmus* as the standard alga for the applications mentioned above seems unfortunate. Species from this genus have particularly resistant cell walls (Bisalputra & Weier, 1963; van Donk et al., 1997) and the extraction of pigments and fatty acids is notoriously difficult (Wood, 1985; Mouget et al., 1993). Consequently, we set out to develop a method for the complete and quantitative extraction of pigments and fatty acids from the green alga *S. obliquus*. The criteria we used were maximum extraction efficiency in one step, ease of handling, and use of solvents of low toxicity. The methods were subsequently tested with different algal species to assess their general applicability.

Materials and methods

Scenedesmus obliquus (strain no: SAG 276-3a, Göttingen culture collection) was cultured in chemostat cultures at 20 °C and at 300 μmol m^{-2} s^{-1} of light in WC (Woods Hole MBL) culture medium. The lysis of cell membranes and cell walls is the rate-limiting step in the extraction of pigments from *Scenedesmus* and was determined under the microscope (% of cells counted lysed) throughout this work. After trying several quite laborious alternatives (e.g., hand grinding or French Press, Table 1), we chose the following method, for its ease of use and its lysis percentage of over 90%. We centrifuged 2–10 ml of algal suspension, at 4 °C for 10 min at 4000 RPM. After checking for errant cells, the overlying water was carefully removed, and the algal pellet frozen at −80 °C. Subsequently, the algae were freeze-dried in the dark, and the pellet weighed. The algae were freeze-dried, as the presence of water can cause the breakdown of chlorophylls via chlorophyllase (Wright et al., 1997). Directly after drying, the algae were covered with the appropriate solvents (pigments: 1 ml of 100% nanograde acetone for pellets under 0.5 mg, 3 ml for heavier pellets; fatty acids 4 ml of 2:1 dichloromethane/methanol mixture), and 0.2 g of analytical grade quartz (particle size 10–30 μm) added. The samples were subsequently placed in an ultrasound bath (35 kHz; 80 W) at −4 °C (using saltwater), and sonicated for 90 min (shorter sonication times resulted in lower lysis percentages). This 'maximum extraction' method was compared with the method where the appropriate solvent was added to freeze-dried algae, shaken manually for one minute (Ahlgren & Merino, 1991), and left standing. All extractions were followed up with a second extraction. The samples were centrifuged, the original solvent removed, and new solvent added to establish the percentage of the pigments and fatty acids remaining in the algae. The experimental procedures of the second extractions were identical to the first extractions. Relevant controls (with only sand and solvent) were also analysed. All extractions were done in quintuplet. Once the maximum extraction method was established for the chemostat culture of *Scenedesmus*, we applied it to batch cultures of other algae: *Cryptomonas erosa* (Cryptophyceae) (CE), *Cyclotella meneghiniana* (Bacillariophyceae) (CM), *Microcystis aeruginosa* (Cyanophyceae) (MA), and *Staurastrum paradoxum* (Chlorophyceae, Desmidiaceae) (SP). Duplicate samples were taken, and the extraction efficiencies checked against the control method.

Many different solvents have been compared for the extraction of pigments in marine algae (Wright et al., 1997) with the general conclusion being that extraction with dimethylformamide (DMF) was the most efficient. However, due to its toxicity this solvent cannot be recommended. Although alcohols (methanol and less frequently ethanol) are usually better solvents for extraction than acetone, these are known

Table 1. Initial experiments to establish the percentage lysis of *Scenedesmus obliquus* cells. All cells were frozen. For all treatments the algae were frozen both at −180 °C and at −10 °C. No differences were found between the two temperatures. The percentage of lysed cells is indicated, with the time of exposure to the break-up method in minutes in brackets. The percentage of lysed (ruptured) cells was determined microscopically

	Acetone added (ml)		
	1.5	3	5
Hand grinding	–	40–60 (10)	20–40 (10)
Mechanical grinding	–	40–60 (10)	20–40 (10)
Ultrasound microtip	70 (8)	50–70 (8)	50 (8)
Ultrasound microtip & sand	–	70–80 (5)	70–80 (5)
Ultrasound bath −4 °C	<20 (90)	<20 (90)	<40 (90)
Ultrasound bath & sand −4 °C	>90 (90)	>90 (90)	>90 (90)

to promote the formation of allomers of chlorophyll (Strain & Svec, 1966; Bowles et al., 1985). A mixture of 90% acetone and 10% water has been the solvent of choice in many studies, but chlorophyllase activity is still substantial in this mixture, hence 100% acetone is the preferred solvent. The HPLC method used was optimised for the separation of the xanthophylls, chlorophylls and carotenes. Extracts were filtered through a 0.45 μm pore-size cellulose filter and then 60 μl of sample was packed in between two 20 μl water 'plugs' (Villerius et al., 1996) in the injection loop and injected in duplicate via a cooled autosampler straight into an HPLC system. This system consisted of a low-pressure pump and autosampler (Waters Alliance), a column oven and a diode array detector (Waters 996). The flow rate used was 1 ml min^{-1}, the column used was a reversed phase $5C_{18}$, (Vertex, Knauer) column, 25 cm long. This column was kept thermostated at 15 °C in a column oven. The gradient required three solvent mixtures: A (80:10:10 methanol:water:ammonium acetate), B (90:10 methanol:acetone), and C (10:7.7 methanol:propanol). The initial solvent was A, after 5 min, this was changed to a 1:1 A:B mixture, held there for 5 min, thereafter changed linearly 100% B at 15 min. Then the solvent was changed back to 100% A at 27 min and then this converted linearly to 100% C. After 29 min C was reduced back to A. Finally, after 35 min the system was set back to the initial solvent conditions (A). All solvents were degassed nanograde HPLC solvents (Baker). The identification of the pigments was carried out using retention times (Francis et al., 1973; Fawley, 1991) in combination with commercial standards (VKI & Sigma), which were checked against the spectra from the diode array detector. The instrument was calibrated for the relevant xanthophylls and chlorophyll pigments using a five-point calibration every 100 samples with commercial standards in 100% acetone (chlorophyll *a* and *b*; Sigma) with a regression coefficient of between 0.98 and 0.99 in the range of 0.01 mg l^{-1} and 5 mg l^{-1}. A two-point calibration was carried out every 15 samples. The accuracy and purity of the commercial standards were checked using spectrophotometric measurements. The absolute detection limit of the system was 0.08 μg l^{-1} of chlorophyll in acetone extract. The instrument standard error is no more than 1% for five replicate measurements.

Most current fatty acid extraction methods are based on the extraction methods of Bligh & Dyer (1959), where mixtures of chloroform and methanol have been used to extract lipids. As there is growing concern about the potential health hazards associated with the use of chloroform, we substituted the chloroform with the less toxic dichloromethane (methylene chloride) (Chen et al., 1981; Parrish & Wangersky, 1987). Dichloromethane is also more volatile than chloroform and evaporation of the solvent is more easily achieved. To avoid auto-oxidation of the unsaturated fatty acids, 200 mg l^{-1} of butylated hydroxytoluene (BHT; Sigma) was added to the CH_2Cl_2/MeOH mixture (Christie, 1982). Kattner & Fricke (1986) showed that for fatty acids a washing step with a NaCl solution (Folch et al., 1956) is not essential. Therefore, the extraction mixture with the dissolved lipids was evaporated to dryness under N_2, and trans-esterified with 2 ml of 3% H_2SO_4 in methanol (Kattner & Fricke, 1986) for four hours at 70 °C. The

samples were then cooled, and shaken twice with 2 ml of iso-hexane. The solvent was evaporated, and 50 μl of iso-hexane were added.

The Fatty Acid Methyl Esters (FAMEs) were analysed on a Hewlett Packard 5890 gas chromatograph with a HP-225 silica-fused column (30 m; 0.25 mm I.D.). The sample volume was 1 μl; the temperature of the injector was 175 °C. We used a splitless injection technique in a split/splitless liner, with a purge after 40 s. The temperature program was one minute at 45 °C; then a temperature increase to 180 °C at a rate of 25 °C min^{-1}, followed by a 3 °C min^{-1} increase to 220 °C and finally a hold at 220 °C for 18 min. Helium was used as the carrier gas (1 ml min^{-1}). The temperature of the flame-ionisation detector was 250 °C. FAMEs were identified by the comparison of their retention times with retention times from single standard FAMEs. As internal standards we used a series of odd-chained fatty acids (C13:0–C21:0; Restek). Differences in response factors of the detector for different FAMEs were established with the help of quantitative mixtures of different FAMEs (Supelco). HP-chemstation software was used to identify and quantify different FAMEs.

We analysed our data in two way ANOVAs with treatment (ultrasound-control), and extraction (First-Second) as fixed factors, and the different pigments and fatty acids as the dependent variables. Because within one sample the different compounds are not independent, we used a repeated measure design, where all compounds were analysed in one ANOVA. Significant interactions of treatment with compounds would indicate that the different compounds are extracted with different efficiencies. To corroborate this, we subsequently carried out the same analysis with ratios to chlorophyll *a* (pigments), or total fatty acids.

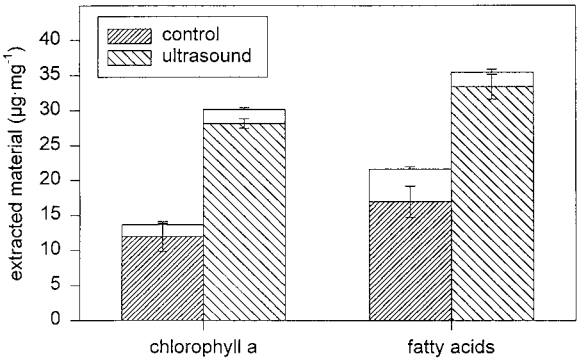

Figure 1. Comparison of the extraction efficiency of the ultrasound and passive (control) extraction for chlorophyll a., and total fatty acids from *Scenedesmus obliquus*. Error bars indicate standard errors ($n = 5$). The amounts extracted in the second extractions (open bars) are stacked on the amounts extracted in the first extraction (filled bars).

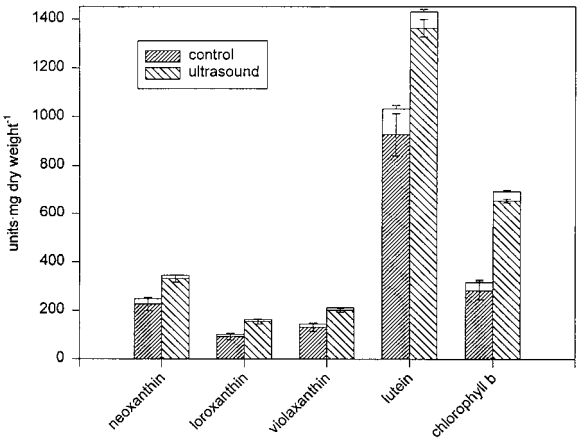

Figure 2. Amounts of different pigments extracted using the two extraction techniques from *Scenedesmus obliquus*. All differences between first and second extraction are highly significant (Table 2). Error bars indicate standard errors ($n = 5$). The amounts extracted in the second extractions (open bars) are stacked on the amounts extracted in the first extraction (filled bars).

Results

The main carotenoid pigments present in *S. obliquus* were neoxanthin, loroxanthin, violaxanthin, and lutein. The main porphyrin pigments present were chlorophyll *a*, *b* and their allomers. No chlorophyll breakdown products (chlorophyllides and phaeopigments) were found. In the continuous cultures of *Scenedesmus obliquus* we observed no carotenes, although these are usually found in green algae. The lack of carotenes is not an artefact of extraction but was specific to the culturing conditions. In fact, in the experiment with the different algal species, the batch culture of *S. obliquus* contained β-carotene. We observed a highly significant effect of all of the effects in the ANOVA (Table 2a). The extraction efficiency of the ultrasound method was significantly higher than that of the control treatment. For example, for chlorophyll *a*, the first extraction using the ultrasound method yielded more than double the amount of the control treatment (Figure 1). The other pigments were also extracted to a significantly higher degree using the ultrasound treatment (Figure 2). In the second extraction, a significant difference between the two methods was found for one substance only, caused by the fact that most of the pigments were extracted in the first

Table 2. Summary ANOVA tables of two way ANOVAs with treatment and extraction as fixed factors, and the different substances (pigments, fatty acids) as repeated measures of one sample. Absolute amounts as well as the ratio to chlorophyll *a* (pigments) or total fatty acid amount (fatty acids) were analysed

A. Pigments	Absolute amounts				Ratios to chlorophyll *a*			
	MS	df	F	*P*	MS	df	F	P
Treatment	212620	1	25.7	<0.001	0.130	1	29.4	<0.001
Extraction	3458335	1	417.3	<0.001	0.003	1	0.7	0.40
Pigment	975727	5	659.8	<0.001	0.387	4	176.5	<0.001
Treat × Extract	259371	1	31.3	<0.001	0.005	1	1.1	0.31
Treat × Pigment	35894	5	24.3	<0.001	0.047	4	21.5	<0.001
Extract × Pigment	718933	5	486.2	<0.001	0.001	4	0.5	0.77
3-way interaction	44935	5	30.4	<0.001	0.002	4	0.8	0.52
Error	1479	80			0.002	64		
B. Fatty acids	**Absolute amounts**				**Ratios to total fatty acids**			
	MS	df	F	*P*	MS	df	F	P
Treatment	16.8	1	29.5	<0.001	0.0001	1	0.3	0.60
Extraction	166.6	1	293.2	<0.001	0.0001	1	5.8	0.03
Fatty acid	42.5	12	167.5	<0.001	0.2094	12	276.5	<0.001
Treat × Extract	31.5	1	55.4	<0.001	0.0001	1	0.3	0.60
Treat × Fatty acid	2.6	12	10.2	<0.001	0.0005	12	0.6	0.83
Extract × Fatty acid	25.0	12	98.5	<0.001	0.0078	12	10.4	<0.001
3-way interaction	5.0	12	19.9	<0.001	0.0012	12	1.6	0.10
Error	0.3	168			0.0007	168		

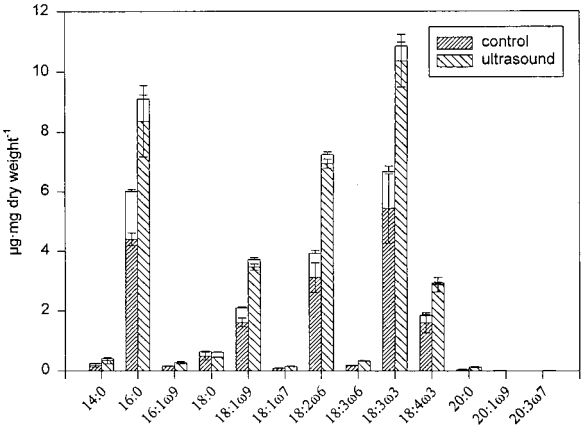

Figure 3. Amounts of different fatty acids extracted using the two extraction techniques from *Scenedesmus obliquus*. All differences between first and second extraction are highly significant (Table 2). Error bars indicate standard errors ($n = 5$). The amounts extracted in the second extractions (open bars) are stacked on the amounts extracted in the first extraction (filled bars).

extraction with the ultrasound treatment and the control treatment extracted less. To assess the effect of the different extractions on the total pigment signature, we computed the ratios of pigment peak areas to the peak area of chlorophyll *a*, the ubiquitous pigment. Table 2a shows a significant treatment, pigment and interaction (treatment × pigment) effect. This was mainly caused by the higher extraction of chlorophyll *a* due to the ultrasound method (Figure 1).

As was the case with the pigments, we observed highly significant treatment, extraction, and compound effects for the fatty acids (Table 2b). Higher extraction efficiencies were found for the fatty acids with the ultrasound-extraction (Figures 1 and 3). We assessed changes in the fatty acid signature by computing the ratios of single fatty acids to the total amount of fatty acids found. Because of the fact that substances with very low initial concentrations were not detected in the second extraction, we observed a significant extraction effect for the ratios of the fatty acids to the total of the fatty acid pool. With the exception of one fatty acid (18:0), the amounts relative to the total amount of fatty acids extracted showed no differences between the ultrasound method and the controls, resulting in a non-significant treatment effect for the ratios. Hence, for fatty acids both methods extract the same spectrum, but are quantitatively different.

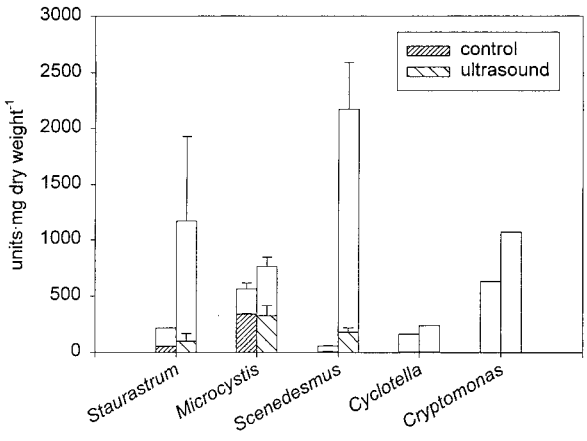

Figure 4. Amounts of different pigments extracted using the two extraction techniques from different algae, exemplified for chlorophyll *a* (open bars) stacked on β-carotene (hatched bars). Error bars indicate standard errors ($n = 2$). Other pigments found in the different species were: Alloxanthin (CE), Chlorophyll b (SO, SP) , Chlorophyll c2 (CE), Crocoxanthin (CE), b-cryptoxanthin (MA, SO), α-carotene (MA), Diadinoxanthin (CM), Diatoxanthin (CM), Fucoxanthin (CM), Echinone (MA), Loroxanthin (SO, SP), Lutein (SO, SP), Monadoxanthin (CE), Myxoxanthophyll (MA), Neoxanthin (SO, SP), Violaxanthin (SO, SP), Zeaxanthin (MA).

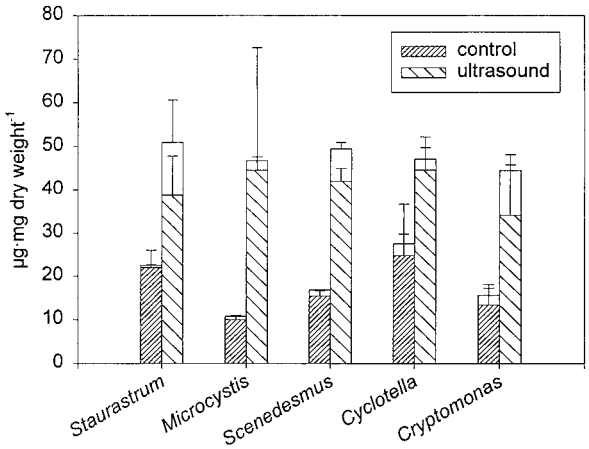

Figure 5. Amounts of unsaturated fatty (open bars) stacked on saturated (hatched bars) acids using the two extraction techniques from different algae. Error bars indicate standard errors ($n = 2$).

Figure 4 shows the extraction efficiencies of the ultrasound method relative to the control method, for pigments in a variety of algae with different structures. Different pigments were found in the different algae; for all of them the extraction efficiencies were higher for the ultrasound method. This is exemplified by Figure 4 for β-carotene (filled bars) and chlorophyll *a* (open bars). For most of the identified pigments and fatty acids the extracted amounts were higher with the ultrasound method. After *Scenedesmus obliquus*, the desmid *Staurastrum paradoxum* proved to be most difficult to extract showing the greatest differences between the two methods for pigments, whereas for fatty acids the extraction from *Microcystis* proved very difficult (Figure 5). The extraction efficiency of each compound also varied for the different algae. Chlorophyll c2 and oleic acid (18:1ω9) were examples of substances, which extracted very badly from *Cryptomonas* and *Cyclotella*, using the control method.

Discussion

Under ideal extraction conditions, solid substances should dissolve in a solvent rapidly and totally in a short time period. However, this cannot be assumed when dealing with complex mixtures of substances, let alone substances in algal cells bound to membranes and associated with protein complexes. Thus, in work on the pigment and fatty acid composition of *Scenedesmus* and other algae under different environmental conditions one cannot simply assume total extraction. Consequently, as the first rate-limiting step in an extraction is cell lysis and having found a method that provided maximum cell lysis, we investigated the efficiency of pigment and fatty acid extraction using two sequential extractions. Both absolute amounts and relative amounts were considered.

The ultrasound and sand method enables us to quantitatively extract pigments and fatty acids from the difficult-to-extract green alga *Scenedesmus obliquus*, (Chlorophyceae) (cf., Jespersen & Christoffersen, 1987). Even though *Scenedesmus*, and chlorophytes in general, might be especially problematic, we found, that even in the 'easier' algae substantial differences existed between the methods used in this study. Especially *Staurastrum paradoxum* (Chlorophyceae, Desmidiaceae) for pigments and *Microcystis aeruginosa* (Cyanophyceae) for fatty acids showed large differences between the methods. Hence, we do not agree with Ahlgren & Merino (1991), who stated that freeze-drying breaks the cells, and turns algal material into a loose, fine powder, making homogenisation unnecessary.

The current literature (see review of Wright et al., 1997) on the analyses of pigments, and particularly the chlorophylls, points out that a primary criterion in the extraction of substances is their preservation. The production of chlorophyllides and phaeo-pigments from

chlorophyll (for example) is a common problem in extraction of algae. Therefore, no matter which extraction method is applied to substrates containing pigments (from algae through to sediments for example) the preservation of substances should always be addressed and checked. We observed no alteration products resulting from the ultrasound extraction (e.g., phaeo-pigments, chlorophyllides and carotenoid alteration) at any wavelength (330–700 nm). Moreover, we did not observe any alteration products when standards of pigments (chlorophyll *a*, *b* and lutein) were treated identically. This was also corroborated using a Kontron SFM25 fluorescence detector.

Generally, the question as to the amount remaining in a cell after extraction of pigments seems largely to be ignored in the literature. Although authors have been intent on developing effective extraction methods (e.g., Wright et al., 1997), it is often assumed that one extraction is enough and rarely is the error associated with estimations of pigments remaining in biomass even considered when discussing quantitative results. The fact that not all substances extract at the same rate is also generally simply assumed to be of negligible importance. Of course, when developing an extraction method ease of use (i.e., one extraction step) must be balanced against the fact that, for chemical reasons alone, in solute-substrate mixtures no single step will ever be 100% efficient. Multiple extractions of pigments from algae are a rarity in the phycological literature and, when discussed at all, are inconclusive and confusing (see Wright et al., 1997). In developing our method, we checked the extraction efficiency of the methods using a double sequential extraction. Both extraction techniques extracted highly significantly more pigments in the first extraction than the second extraction (ten to twenty times more). In terms of absolute amounts, the treatment with sand and ultrasound was more effective than the treatment without. Between 92–96% of the total extracted amounts of each pigment were extracted using the sand and ultrasound treatment and as low as 70% (chlorophyll *a*) in the treatment without ultrasound and sand. Consequently, less was extracted in the second extraction with the former technique, and more residual pigments were removed in the second extraction in the treatment without sand and ultrasound. There was no significant difference between the extraction efficiency of carotenoids and chlorophylls (ratios) using either method. This is reassuring, as both groups of compounds exhibit different chemical properties and it could have been possible that the extraction method may have been fine for one group of compounds but sub-optimal for the other.

In contrast to the pigment literature, multiple extractions have been regularly applied in the extraction of lipids and fatty acids (Folch et al., 1956; Bligh & Dyer, 1959). However, many of the papers on fatty acid content of biological material are not specific as to the exact extraction procedures (e.g., omitting extraction temperatures and duration, not reporting the amount of extracted material). The original papers by Folch et al. (1956) and Bligh & Dyer (1959) both emphasise the point of sample homogenisation, and mention grinding for tougher tissues. Christie (1982) already suggested the use of clean sand while homogenising difficult tissues, which would certainly include microalgae (Ahlgren & Merino, 1991). Our results show that sonifying samples with sand as an abrasive substantially increases the amount of extracted fatty acids from algal material, as illustrated by the significant difference in extraction efficiency between the ultrasound and the non-ultrasound method. Using the ultrasound and sand method, we were able to extract double the amount of fatty acids from algal material and most of that in the first extraction.

This work represents a major step forward in the extraction of pigments and fatty acids from an alga (*Scenedesmus*) which is notoriously difficult to extract. The method is easy to use, allows the extraction of pigments and fatty acids highly quantitatively (over 90%) in one step, and conserves the relationships of different pigments and fatty acids to one another. The substances are conserved and do not breakdown in the course of the extractions. Moreover, the method is effective for a wide range of other algae, and hence should be generally applicable in research on pigments and fatty acids.

Acknowledgements

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