

Comparison of different solvent extraction compositions for the analysis of phytoplankton pigments and lipids using a rapid and sensitive LC-MS method

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Method Article

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Abstract

Background: Carotenoids and chlorophylls are important pigments as they have antioxidant properties and some such as lutein, astaxanthin and β -carotene are used as nutraceuticals. Pigment profiles are also used as taxonomic markers for phytoplankton. There are many solvent compositions published for the extraction of phytoplankton pigments. Here we report the testing of five different commonly used solvents and compare the overall pigment and lipid profiles and abundances using liquid chromatography-mass spectrometry (LC-MS) for three microalgal species with different pigment profiles.

Results: A fast and sensitive LC-MS method was developed using a C18 column with a gradient consisting of isopropanol, acetonitrile and water. A total of 18 pigments along with phosphatidylcholines (PCs), triacylglycerols (TAGs) were separated in 25 mins. An extraction solvent composition of 90% acetone provided the broadest-coverage of pigments and lipids, while ethanol/hexane resulted in the highest extraction efficiency for chlorophyll and the non-polar triacylglycerol lipids. Overall, the result for the different mixtures shows the significant impact that solvent polarity has on the overall pigment and lipid profile and that no single mixture was optimal for all targets. Complete cell lysis using mechanical disruption such as bead beating is also an important step for quantitative extraction as chemical extraction on its own does not provide reproducible results.

Conclusion: A 90% acetone extraction solvent mixture provides the broadest extraction of pigments from the species *Haematococcus pluvialis*, *Desmodesmus subspicatus* and *Chlorella variabilis* when coupled with bead beating. This extraction solvent is comparatively safe, polar and less toxic than other solvents. It was also found that none of the common solvent mixtures tested here provided the highest extraction efficiencies across all compound groups

Background

Carotenoids and chlorophylls are the major lipophilic pigments that are responsible for the absorption and transfer of light energy for photosynthetic processes of phytoplankton (1). Carotenoids including both carotenes and xanthophylls act as photoprotective pigments by suppression of reactive oxygen species (ROS) under excess light in algae (2).

Pigment markers are used in taxonomic studies of phytoplankton assemblages. There are a range of cell lysis methods and solvent compositions reported. Cell lysis techniques range from straight solvent extraction (3–5), freeze thawing cells (6), maceration (6–9), ultrasonication (8, 10–14) to bead-beating (8, 15, 16). Depending on the cell wall composition of the species, some of these procedures may not provide adequate cell lysis which impacts the extraction efficiency and reproducibility.

Extraction efficiency depends on multiple factors such as cell wall composition, pigment polarity, the duration of the extraction protocol and the mechanical disruption technique used (17). In this study, a range of five different solvent compositions were used including methanol (11), acetone (12), ethanol and

hexane (18) and the classic Folch extraction for lipids (19) to determine how solvent composition impacts the measured concentrations of pigments and lipids from microalgae.

High performance liquid chromatography (HPLC) has been used for decades to fractionate and quantify phytoplankton pigments (20–25). In fact, the term chromatography was coined by the Russian botanist Mikhail Tswett in 1906 when he developed adsorption chromatography while working on the separation of plant pigments by passing a leaf extract through a column of calcium carbonate, sucrose and alumina, using petroleum ether/ethanol mixture as an eluent (26). Modern separation techniques now generally employ reversed-phase chromatography for pigments and lipids. For example, reverse-phase HPLC with a C8 monomeric column was used for the successful separation of forty phytoplankton pigments including the partial resolutions of chlorophylls from their respective divinyl (DV) forms (20, 27–30). However, it is difficult to identify pigment isomers due to their similar structures and co-elution (e.g., lutein and zeaxanthin). More recently, a pentafluorophenyl octadecyl silica column was used for the complete resolution of mono- and divinyl forms of chlorophyll a, b, and c in phytoplankton (12). To obtain sufficient pigment resolution the typical run-time for HPLC methods is ca. 30 minutes or more which is time-consuming for analytes. Long runtimes can be problematic due to sample throughput and stability issues (12, 31, 32). Degradation products can also result in interferences in an HPLC analysis, particularly when pigments are in low concentration (33).

With the advent of ultra-high performance liquid chromatography coupled with mass spectrometry (LC-MS), some novel pigments and their derivatives (e.g., [8-ethyl]-chlorophyll c3) have been identified in a single chromatographic run (34–37). There is a considerable benefit in the application of LC-MS to pigment analysis from the characterization of novel compounds in phytoplankton cultures or compound confirmation during routine analysis. LC-MS also enables the detection of pigments at trace/ultra-trace levels which is important for complex environmental samples where some species may not grow abundantly.

Many studies have been carried out using LC-MS for the analysis and identification of phytoplankton pigments (4, 11, 14, 15, 18, 38). There is no one study that describes a universal extraction procedure. This study uses five different solvents for pigment extraction. The solvents range from the highly polar methanol to the least polar hexane. Here we have summarised the common pigment extraction techniques to validate these approaches and recommended the most comprehensive extraction procedure for a range of pigments and lipids for LC-MS analysis along with the most suited cell lysis method. We also tested different mobile phase and column chemistries and report an optimized integrated protocol for phytoplankton pigment analysis by LC-MS.

Results And Discussion

Three different microalgal species *Haematococcus pluvialis* (H), *Desmodesmus subspicatus* (D) and *Chlorella variabilis* (C) were selected due to their different cell wall and pigment composition. The cell wall of *C. variabilis* is comprised predominantly of chitin, glycosaminoglycans and sialic acid (39, 40), *H.*

pluvialis has cellulose and non-hydrolysable sporopollenin-like material (41) and *D. subspicatus* has a pecto-cellulosic and glycoproteic cell wall (42). Typically, the pigment composition of *H. pluvialis* consists of high concentrations of astaxanthin, canthaxanthin, β -carotene and lutein (43). Both *C. variabilis* and *D. subspicatus* also have similar pigment composition that varies depending on the culture conditions but both lack astaxanthin (44, 45).

An optimised LC-MS method based on (11, 46) was initially developed, followed by an examination of different cell lysis techniques and finally a comparison of five commonly used solvent compositions was carried out to test their extraction efficiency across a range of pigments. (Table 1). The five solvent extraction systems tested were 100 % methanol (method 1), 90% acetone (method 2), ethanol:hexane (2:1) (method 3), chloroform:methanol:water 8: 4: 3 (method 4) and ethanol:hexane (1:1) (method 5). The optimised procedure for the three key steps in LC-MS analysis of algal pigments included cellular lysis, solvent extraction and chromatographic separation is presented with a particular focus on the impact that these extraction solvents have on the overall pigment profile. The method can also be extended to include lipid analysis so data presented on lipid extraction efficiency has also been included.

S.No.	Extraction solvent	Run time (min)	Column used	Detector used	Pigments detected	Reference
1	Methanol	15	C8	UHPLC-MS/MS	14	(11)
2	Acetone	42	C18 PFP	HPLC-PDA	70	(46)
3	Ethanol/hexane (2:1)	20	C18	UPLC-UV-MS	37	(18)
4	Chloroform/methanol/water*	-	-	-	-	(47)
5	Ethanol/hexane (1:1) +10% Na ₂ SO ₄	50	C30	HPLC/APCI-MS/MS	7	(10)

Table 1. Comparison of extraction techniques used in this study; *classical Folch method that has no chromatography involved

Eighteen pigments were selected for measurement using the multiple reaction monitoring (MRM) described in a method reported elsewhere (11). The MRM collision energies were optimised using standards of each compound (Table 2). Standards for the following pigments were used for quantification (astaxanthin, canthaxanthin, zeaxanthin, lutein, chl-a and chl-b) while relative values were used for the other pigments. Two key lipid classes were also chosen phosphatidylcholine (PC) and triacylglycerol (TAG). Using only 1 μ l and 25 mins run time separation of 18 pigments was achieved (Fig 1). Some peaks are not fully visible as the concentration is very low compared to others.

PIGMENT	RETENTION TIME	PRECURSOR ION (m/z)	PRODUCT ION (m/z)	COLLISION ENERGY
Peridinin	4.8	631.4	553.4	10
Nile Red (IS)	6.7	319	275	35
Neoxanthin	8.2	601.4	221.0	18
Fucoxanthin	8.3	659.5	109	26
Violaxanthin	8.4	601.4	221.0	18
Prasinoxanthin	8.5	601.4	583.4	10
Astaxanthin	8.6	597.4	147.1	25
Alloxanthin	9.3	565.5	157.1	23
Zeaxanthin	9.9	569.4	145	29
Lutein	10.1	569.4	339.1	15
Canthaxanthin	10.5	565.4	203.1	25
Diadinoxanthin	10.7	583.5	157.1	34
Chlorophyll b	12.1	907.5	569.1	38
19-but-fucoxanthin	12.7	745.5	109	31
Chlorophyll a	13.1	893.5	555.0	38
Cryptoxanthin	13.2	552.4	460.4	25
DV Chlorophyll a	13.3	891.5	553.0	38
Lycopene	15.3	537.4	444.4	25
19-hex-fucoxanthin	15.7	773.5	109	30
beta-carotene	15.8	536.4	444.4	25

Table 2. Phytoplankton pigments and internal standard with their MRMs for analysis

Optimised chromatographic separation for tandem mass spectrometry analysis

Reversed phase columns ranging from C8 – C30 bonded phases have been employed in pigment analysis (48). The C30 columns provide the highest selectivity, however, the higher hydrophobicity results in the requirement for stronger non-polar mobile phase solvents and longer run times to facilitate elution (49). Both Fraser et al. (50) and Soares et al. (10) reported efficient separation of pigments in a single run

but with analysis time of 42 minutes and 50 minutes, respectively. In their study, Gupta et al. resolved the issue of longer analysis time by separating 15 major carotenoids in 20 minutes (51). Using C18 column, Chauveau-Duriot et al. provided method for separation of carotenoids with a 45-minute run (52). The issue was again the longer run time, whereas Fu et al. could separate 37 pigments in 20 minutes using C18 column (18).

In this study, various mobile phases were tested starting with 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). Solvent system consisting of isopropanol (50%) and methanol (50%) (B) was also tested. Finally, the mobile phase consisting of 10 mM ammonium formate in water (A) and 75% isopropanol, 20% acetonitrile and 5% water (10 mM ammonium formate) (B) was found to be the most suitable as it facilitated the ionisation of pigments and non-polar lipids (triacylglycerols) as ammonium adducts (Fig. 1). The details of the final method used are in the method section below with runtimes of less than 30 mins. The use of a mass selective detection provides a second dimension for separation therefore complete resolution of each pigment is not as necessary.

Cell lysis using bead-beating and ultrasonication

Many cell lysis techniques are utilised in pigment analysis including maceration, ultrasonication, high-pressure assisted extraction and bead beating, depending on the microalgal species. In this study, two extraction techniques were tested: straight solvent extraction with sonication and solvent extraction with bead-beating using silica beads. The pigment recoveries with the different cell lysis techniques were then compared (Fig. 2). Bead-beating recovered significantly higher concentrations for all pigments: chl-b was 380-fold higher than ultrasonication followed by chl-a which was 9.5-fold higher, and both zeaxanthin and lutein were ~3.4-fold higher in *C. variabilis* (Fig 2). This clearly shows that complete cell lysis using mechanical maceration is an important first step in the overall extraction protocol and that solvents will not efficiently extract pigments through non-lysed cells. The same results were observed for the other two species (Fig 2). Similar findings have been reported in microalgal species *Scenedesmus obliquus* (53). It was also reported that bead-beating pre-treatment with ethanol led to higher carotenoid extraction than ultrasound and freeze-thawing (54). Some contradicting results have been reported for astaxanthin extraction, an 81% astaxanthin extraction efficiency from *H. pluvialis* biomass was reported that was pre-treated by sonication at 750 W coupled with chemical lysis using 2 M NaOH solution followed by methanol extraction (55). However, in contrast a relatively low astaxanthin recovery of 19.8 mg/g cell from dried *H. pluvialis* biomass by two-step treatment with 4 M HCl at 70 °C and ultrasonic extraction for 20 min in acetone was documented (56). Similarly, a very low astaxanthin extraction efficiency (12%) from intact *H. pluvialis* cysts, despite the use of a high-power 600 W horn-type sonicator (1 s on and 3 s off for 30 min) with methanol was also reported (57).

Selection of optimum extraction solvent for separation of pigments

Another important parameter for extraction efficiency is the solvent used (58). The goal of this study was to provide a universal extraction protocol from the many existing solvent systems in literature depending on the microalgae species and target pigments (59, 60).

The following data is based on the quantitative estimates for 18 pigments and 2 lipid classes. The ethanol:hexane (1:1) (method 5) solvent mixture provided the highest extraction efficiency for most of the pigments (zeaxanthin, lutein, chl-b, astaxanthin, Div Chl a, canthaxanthin, alloxanthin, β -carotene) in *H. pluvialis*. Chl-a made an exception that was extracted by ethanol:hexane (2:1) (method 3) Folch method is routinely used for lipidomics extractions, here it is shown that PCs have a greater extraction efficiency with the folch (method 4) that corroborates with the literature (Fig 3). PCs were extracted best through folch whereas TAGs through ethanol:hexane (2:1) (method 3) in *C. variabilis* and *D. subspicatus*. Similarly, the PCs were extracted most efficiently using the Folch mixture in *H. pluvialis* closely followed by acetone (method 2). Pigments from *D. subspicatus* showed a maximum recovery using the ethanol:hexane solvent system (zeaxanthin, lutein, Div Chl a, β -carotene, cryptoxanthin and lycopene). Chlorophylls (chl-a and chl-b) were extracted best by ethanol:hexane solvent systems in all the three species. Ethanol:hexane solvent system worked best for extraction of pigments in *C. variabilis* as well. The pigments neoxanthin, prasinoxanthin, violaxanthin, diadinoxanthin were maximally extracted through Folch (method 4) in *H. pluvialis*. Neoxanthin, prasinoxanthin, violaxanthin were also equally extracted through methanol (method 1) in *D. subspicatus* and through acetone (method 2) in *C. variabilis* (Supplementary Fig 1).

Ethanol:hexane (method 3 and 5) system gave the highest amount for most of the pigments and also for TAGs except for the pigments neoxanthin, prasinoxanthin, violaxanthin and diadinoxanthin. PCs overall were maximised using Folch (method 4) and acetone (method 2). Acetone and ethanol are generally regarded as safe solvents (GRAS) (61) and therefore provides a better option than the ethanol/hexane combination due to the toxicity of hexane. Furthermore, it has been shown that methanolic pigments are degraded more quickly than other solvents (62, 63). Several reports have also shown that methanol promotes formation of allomers of chlorophyll (64, 65). Acetone therefore provides a more stable system in comparison to methanol. For these reasons, 90% acetone (method 2) was selected as the system which provides the best compromise as it's less volatile so easier to handle, less toxic moderately polar, simple solvent system that provides a broad spectrum of pigments. However overall, it was shown that no single solvent system is optimal for all pigments, this is due to the relative polarities of the solute and solvents. The key to selection therefore is the ease of handling, toxicity and target pigments of interest.

Overall Pigment profile analysis

The whole data set contained 18 pigments and 2 lipids. To visualise the impact of solvent composition on the whole profile, principal components analysis (PCA) was used. A PCA described 83% of the variance in the first two components. Clear separation of each of the species (C, D and H) can be observed along PC1 based on the overall pigment profiling where D and H are slightly closer to each other (Fig 4(a)). Most variation can be seen across PC1 (47.2%) and therefore, C, H and D have been grouped separately and interestingly is based on different pigments rather than lipids. Across PC2 (36%), clustering among different species can be seen based on the extraction solvents and the compounds extracted. A clear gradient from high polarity to low polarity solvent can be seen in PC2. The loading plot also show this (Fig4(b)). Therefore, C3 -C5 and C1- C4 are clustered together wherein C2 separates

out as an intermediate group that justifies the use of acetone as an extraction solvent with intermediate polarity. Similarly, D1 -D4 and D3- D5 are clustered together and D2 seem to separate out as a subgroup closer to D3- D5. Same pattern can be observed for H where H3 -H5 are close to each other and so as H2- H1 and H4 separate out as a different cluster. The species of the extraction methods 3 and 5 cluster together, this is due to the similar composition of the extraction solvent with different ratios of ethanol: hexane. The compound group mainly responsible for the separation from the other extraction methods are the non-polar TAGs. The PCs are responsible for clustering 1,2,4 in C and D together. Canthaxanthin, astaxanthin, alloxanthin and diadinoxanthin cause separation between H (Fig 4(b)).

Conclusions

An LC-MS method coupled with C18 column was developed for fast separation of 18 pigments as well as phospholipids and TAGs. An optimised sample preparation method utilising cell lysis with bead beating and 90% acetone extraction provided the broadest coverage of lipophilic compounds in three unique microalgal species based on the cell wall and pigment composition. It was also found that none of the common solvent mixtures tested here provided the highest extraction efficiencies across all compounds groups so this is an important consideration for chemical analysis of non-polar compounds from phytoplankton.

Materials And Methods

Microalgal cultures and pigment standards

Algal cultures used for studies were freshwater species- *Desmodesmus subspicatus*, *Chlorella variabilis*, and *Haematococcus pluvialis*. All cultures were grown in MLA medium and maintained at light irradiances of 50 $\mu\text{mol}/\text{m}^2/\text{s}$ (16:8 light-dark cycles) at 23°C. Samples were centrifuged at 10,000 rpm for 5 min to collect cell pellets. Pure pigment standards of canthaxanthin ($\geq 95\%$), astaxanthin ($\geq 98\%$), zeaxanthin ($\geq 95\%$), lutein ($\geq 96\%$), chlorophyll-a ($\geq 95\%$), and chlorophyll-b ($\geq 95\%$) were purchased from Sigma Aldrich (Sydney, Australia). Internal standard (Nile red) was purchased from Sigma. All solvents used in the experiments were LC-MS grade (Honeywell, Melbourne, Australia). Deionised water (18.2 ΩM) was produced using a Milli-Q system (Merck, Darmstadt, Germany).

Calibration curves for the standards were prepared at concentrations of 0.01, 0.1, 1, 10, and 100 $\mu\text{g}/\text{mL}$.

Instrumentation

Sample analysis was performed on triple quadrupole mass spectrometer (Shimadzu LC-MS 8050) with positive electrospray ionisation. A 2.1 x 100 mm 1.8 μm C18 column was used for chromatographic separation (Agilent Eclipse Plus C18 RRHD). Three binary mobile phase solvent systems tested were: (1) 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B); (2) isopropanol (50%) and methanol (50%) (B); (3) 10 mM ammonium formate in water (A) and 75% isopropanol, 20% acetonitrile and 5% water (10mM ammonium formate) (B). Mobile phase system 3 was selected and the optimal gradient is

in Table 3. The mobile phase flow-rate was 0.4 mL/min and the column temperature was 50 °C. A 1 µL injection volume was used throughout.

TIME	% Mobile phase B
0	25
15.00	100
20.00	100
21.00	25
25.00	25

Table 3. Gradient profile of the LC-MS method.

The temperature of the autosampler compartment was 4°C to minimise sample degradation. The mass spectrometer was operated in positive ion MRM (Multiple Reaction Monitoring) (Table 2).

Pigment extraction methods

For extraction of the pigments, five different solvents were tested commonly reported in the literature: (1) 100% methanol; (2) 90% acetone; (3) Ethanol/hexane (2:1) with water; (4) Chloroform/ methanol/water based on Folch method, and (5) Ethanol/hexane (1:1) with 10% sodium sulphate (Table 1). All these extractions were carried out in ice with minimal exposure to light in the fume hood.

1. Extraction method One: Methanol

A 1 mL aliquot of 100% methanol (with 0.001 µM Nile red) was pipetted into each Eppendorf tube (5 replicates for each species) consisting of silica beads-combination of 0.5 micron and 0.1-micron sizes (roughly 30% of the tube) in the fume hood. The tubes were then vortexed ensuring that the cell pellets were resuspended then put back on ice. The samples were then lysed in the bead-beater (MpBio, Sydney, Australia)-two rounds of 30 sec bead-beating (5.5m/sec) for improved extraction followed by centrifugation at 10,000 g for 1.5 mins. The supernatant was transferred to a fresh tube and evaporated using a vacuum concentrator until dry (Labconco acid/resistant CentriVap Concentrator coupled with a -105 °C CentriVap cold trap, Melbourne, Australia). Samples were stored at -80 °C and re-dissolved in 1 mL mobile phase B for analysis. For ultrasonication, 1 mL samples were placed in sonicating water bath (POWER SONIC 410- Thermoline Scientific™, Melbourne, Australia) for 15 minutes and similarly evaporated using a vacuum concentrator until dry. These samples were stored and reconstituted as described above.

2. Extraction Method 2: Acetone

A 1 mL aliquot of 90% of acetone (with 0.001 µM Nile red) was pipetted into each Eppendorf tube (5 replicates for each species) consisting of silica beads-combination of 0.5 micron and 0.1-micron sizes.

The tubes were then vortexed ensuring that the cell pellets get resuspended. The samples were then lysed in the bead-beater (MpBio)-two rounds of 30 sec bead-beating (5.5m/sec) for improved extraction followed by centrifugation at 10,000g for 1.5mins and the supernatant was transferred to a fresh tube and evaporated using the method described above. Similar protocol was followed for ultrasonication as given above using 90% acetone.

3.Extraction method 3: Ethanol hexane (2:1) with water

A 3 mL aliquot of ethanol (2 mL) and hexane (1mL) was pipetted into each Kimble tube followed by 2 mL water and 4 mL hexane (5 replicates for each species) consisting of silica beads-combination of 0.5 micron and 0.1-micron sizes. The tubes were then vortexed ensuring that the cell pellets get resuspended. The samples were then lysed and the hexane layer was evaporated as described above. Similar protocol for ultrasonication was followed using ethanol: hexane (2:1).

4. Extraction method 4: Folch method

Chloroform/methanol/water (8:4:3) was pipetted into each Eppendorf tube (5 replicates for each species) consisting of silica beads-combination of 0.5 micron and 0.1-micron sizes. The tubes were then vortexed ensuring that the cell pellets get resuspended. The samples were then lysed and the chloroform layer was evaporated as described above. Similar protocol was followed for ultrasonication as given above using chloroform:methanol:water (8:4:3).

5. Extraction method 5: Ethanol/hexane (1:1) with 10% sodium sulphate

A 1 mL aliquot of ethanol and 1 mL of hexane with 7.5 mL of 10% sodium sulphate was pipetted into each Eppendorf tube (5 replicates for each species) consisting of silica beads-combination of 0.5 micron and 0.1-micron sizes. The tubes were then vortexed ensuring that the cell pellets get resuspended. The samples were then lysed and the hexane layer was evaporated as described above. Similar protocol was followed for ultrasonication as given above using ethanol: hexane (1:1).

Data analysis and Statistics

LC-MS data was normalized to the internal standard and standard curves. Bar graphs and box and whisker plots were made using GraphPad Prism 8.0.2. For overall pigment analysis among the different species, PCA was performed on log-transformed data using Metaboanalyst 5.0. Lab Solutions Insight was used for processing of the data. Welch's t-test was performed using GraphPad Prism 8.0.2 (La Jolla, California, USA).

Abbreviations

HPLC: High-performance Liquid Chromatography, LC-MS: Liquid Chromatography-Mass spectrometry, MRM: Multiple Reaction monitoring, PCA: Principal component analysis, PC: Phosphatidylcholine, ROS: Reactive oxygen species, TAG: Triacylglycerol

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article in graphical form. The numerical datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Competing interests

The authors declare they have no competing interests.

Author's contributions

The extractions, pigment analysis and LC-MS method development were done by US and DLC. DLC, LA and SKL helped in planning of experiments and writing of manuscript. All authors read and approved the manuscript.

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Figures

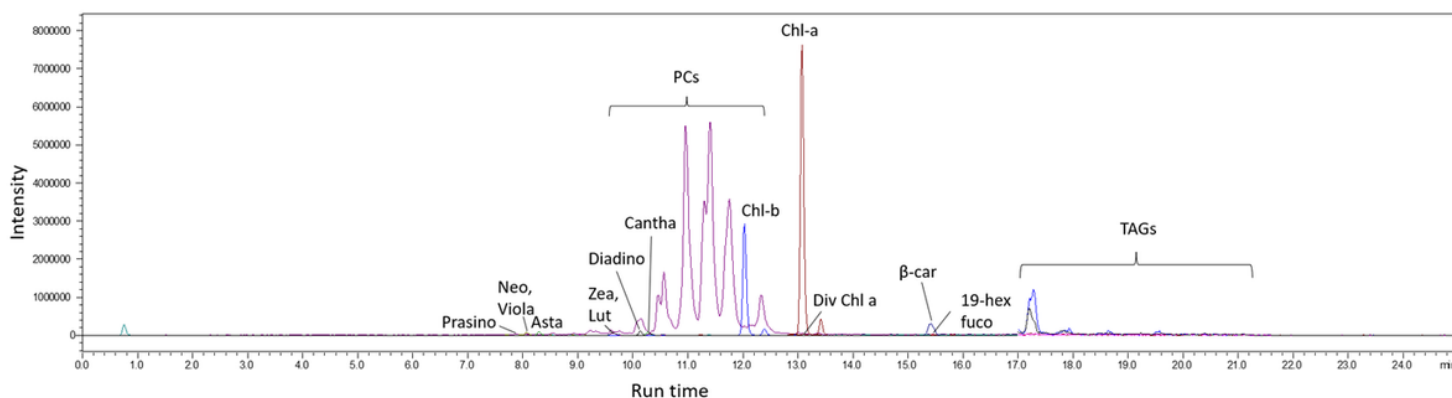


Figure 1

Chromatograms of the pigments in standard mixture (TIC)- Asta, astaxanthin; β-car, β-carotene, Cantha, canthaxanthin; Chl-b, chlorophyll b; Chl-a, chlorophyll a; Diadino, diadinoxanthin; Div Chl a, Divinyl chlorophyll a; Prasino, prasinoxanthin; Neo, neoxanthin; Viola, violaxanthin; 19-hex fuco, 19-hex fucoxanthin; Zea, zeaxanthin; Lut, lutein; TAG, triacylglycerol; PCs, phosphatidylcholines

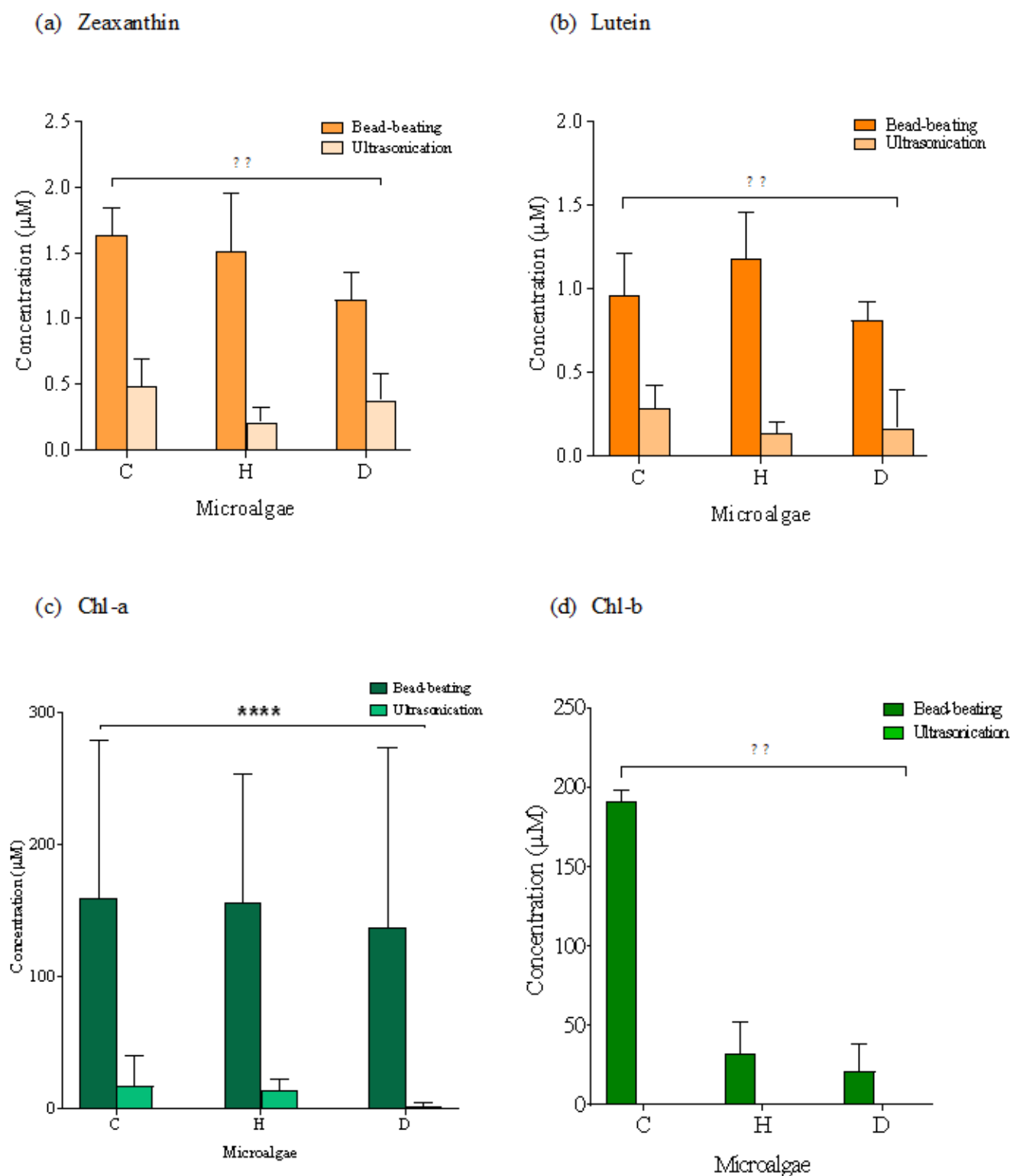


Figure 2

Comparison of cell lysis methods. Extraction efficiency was compared between bead-beating and ultrasonication for pigments (n=5) (a) zeaxanthin, (b) lutein, (c) chlorophyll a, (d) chlorophyll b for microalgae *Chlorella variabilis* (C), *Haematococcus pluvialis* (H) and *Desmodesmus subspicatus* (D); note the error bars represent standard deviation. Statistical significance was analysed by Welch's t-test.

Black asterisks represent significant difference between bead-beating and ultrasonication (**p < 0.05, ****p < 0.0001)

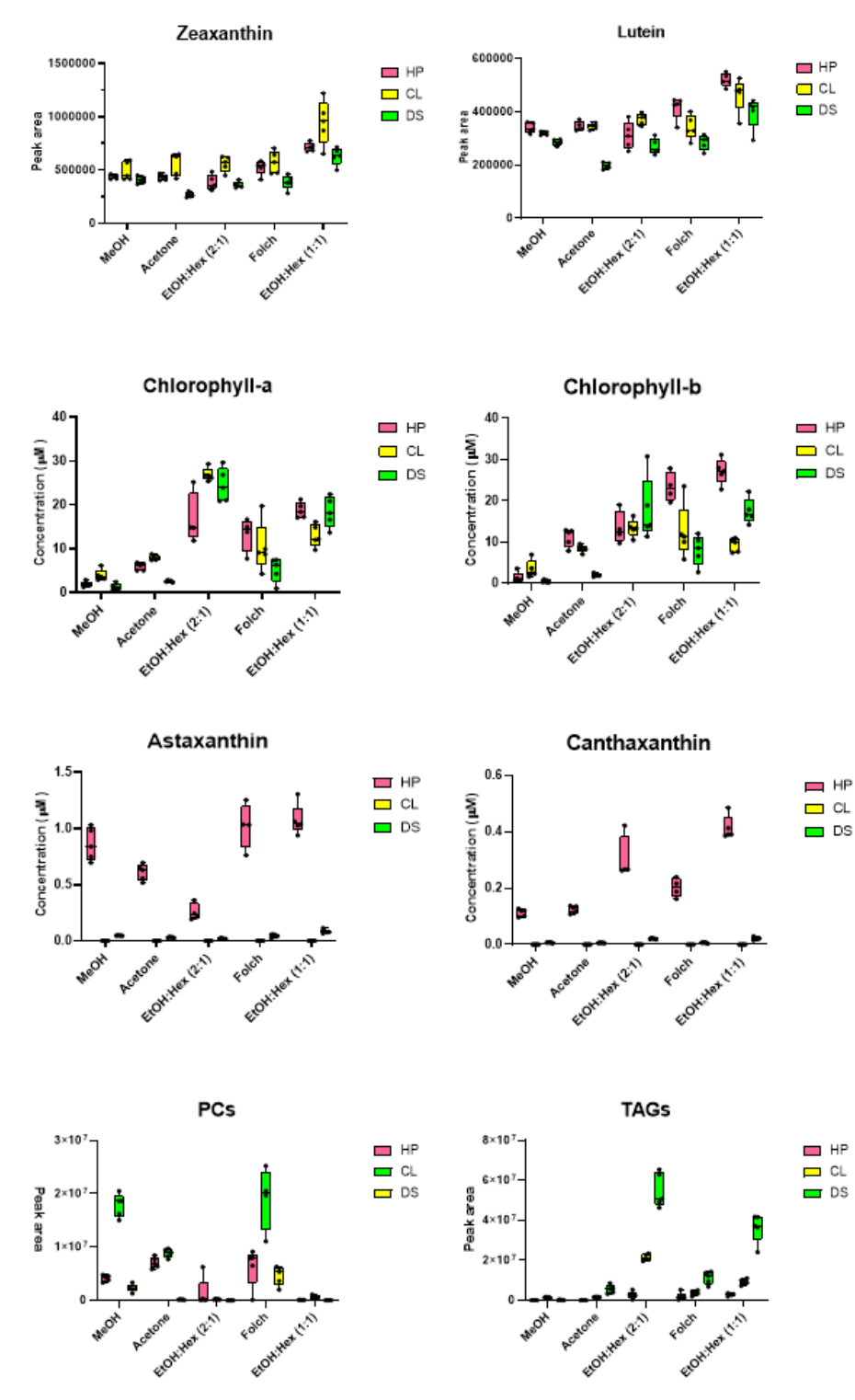


Figure 3

Box and whisker plots for the pigments (n=5) with standards-zeaxanthin, lutein, chlorophyll-a, chlorophyll-b, astaxanthin, canthaxanthin and lipids-phosphatidylcholine (PC), triacylglycerols (TAGs) for all the three

microalgal species *H. pluvialis* (HP), *C. variabilis* (CL) and *D. subspicatus* (DS) with different extraction solvents

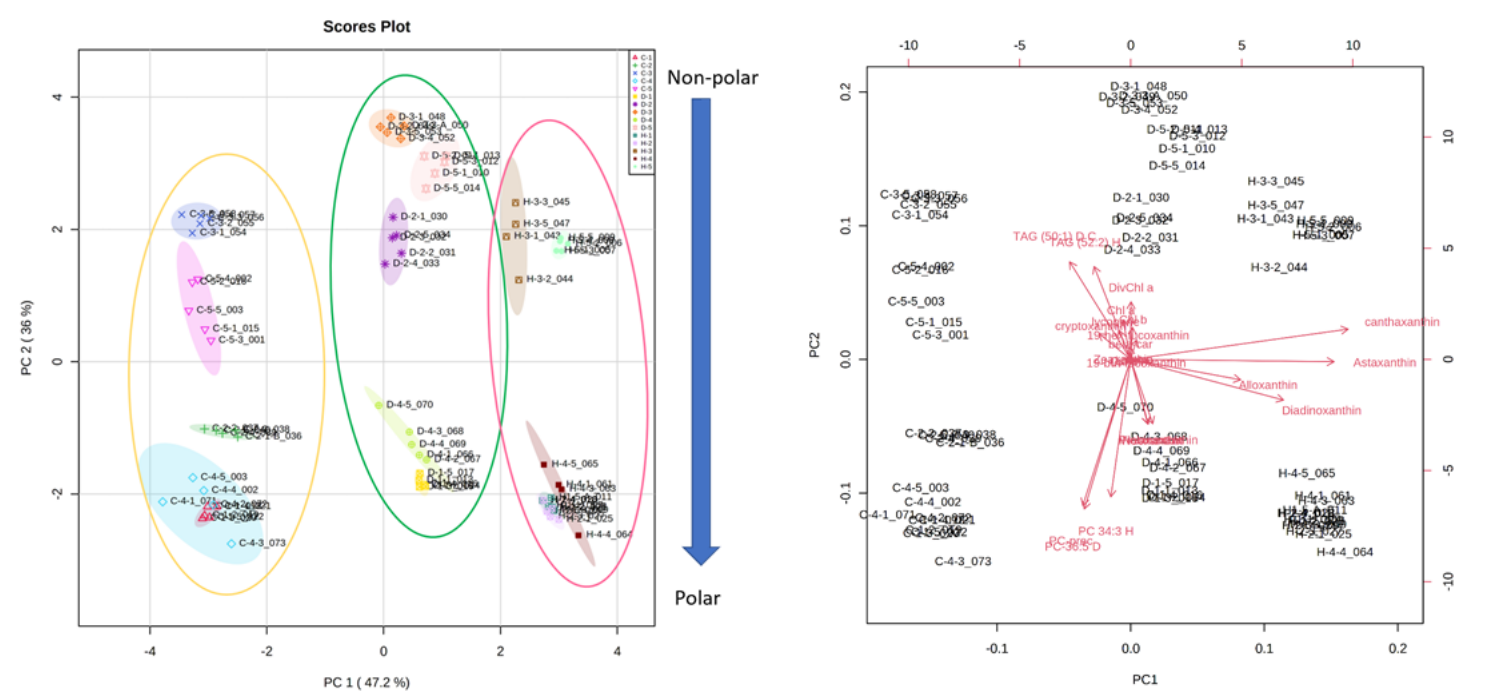


Figure 4

Principal component analysis (PCA) (a) score plot and (b) biplot of microalgal species *C. variabilis* (C), *D. subspicatus* (D) and *H. pluvialis* (H) in five replicates and for the five solvent compositions. The species have been encircled as yellow, green and pink for C, D and H respectively. Blue arrow represents increasing polarity of the solvent systems across PC2. The first number in the nomenclature indicates the extraction method followed by the replicate number i.e. C-4-3 means *C. variabilis* extracted by method 4 (Folch) and replicate 3.

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