Rapid Detection and Quantification of Haptophyte Alkenones by Fourier Transform Infrared Spectroscopy (FTIR)

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Number of tables, figures, and supplementary materials:

Four tables (Table 1–4), six figures (Fig. 1–6), and one supplementary table (Supplementary Table 1).

27

28 **Abstract**

29 Several haptophyte algae produce unique neutral long-chain (C₃₇-C₄₀) ketones (alkenones) with
30 two to four *trans*-carbon-carbon double (C=C) bonds and a keto-group. These molecules are of
31 great biological interest and may have substantial commercial relevance as biofuel sources.
32 Unfortunately, their detection and quantification, involving extraction from cells with organic
33 solvents and fractionation of lipids through columns, are rather cumbersome. Hence, we developed a
34 method for the rapid and reliable quantification of alkenones in intact cells using Fourier transform
35 infrared spectroscopy (FTIR). The method is based on the absorption band at 962.5 cm⁻¹,
36 which corresponds to a *trans*-C=C bond that is unequivocally attributable to alkenones. This peak
37 was exclusively observed in alkenone-producing haptophytes such as *Emiliania huxleyi* NIES-837,
38 *Emiliania huxleyi* CCMP 2090, *Tisochrysis lutea* (former *Isochrysis galbana* T-iso), *Tisochrysis*
39 *lutea* CCMP 463, and *Chrysotila lamellosa* CCMP 1307, as well as in appropriate standards. We
40 compared our FTIR method with a typical GC analysis method. The alkenone quantity determined
41 by these two methods showed similar values. Nevertheless, the FTIR method developed in this
42 study does not require complex extraction procedures and is therefore a much easier and rapid
43 quantification method. This FTIR method can also be used for the screening of strains and the
44 optimization of culture conditions for alkenone production.

45

46 **Keywords**

47 Alkenones; Alkenone-producing haptophyte; *Emiliania huxleyi*; FTIR; Lipid; *trans*-carbon-carbon
48 double bond

49

50 **1. Introduction**

51

Alkenones are long-chainunsaturated ketones (C₃₇-C₄₀) produced by several species of haptophyte algae [1]. An alkenone molecule contains two to four *trans*-carbon-carbon double (C=C) bonds (Fig. 1), whereas a fatty acid molecule contains *cis*-typedouble bonds. Interestingly, the number of double bonds in alkenones is known to increase from two to three or four when growth temperaturedecreases, and vice versa [2, 3, 4]. Based on this feature of alkenones, the relationship between the number of double bonds and temperature has been applied in geosciences to estimate sea surface paleotemperature whenmarine sediments are produced[5].

The biological role of alkenones is only partially understood. This is mostly because there is no easy and rapid quantification method to determine the production rate and cell content of alkenones using intact cells. Generally, in a biological laboratory, extraction of lipids and column fractionation of alkenones are rather complicated and time-consuming procedures when the laboratory is not well-prepared for alkenone analysis. The complexity of these quantification methods is the reason effective culture management is challenging and not very cost-effective. Nevertheless, several cellular functions of alkenones have been proposed. First, alkenones may regulate the formation of membrane vesicles, and within membranes, they may regulate the physicochemical properties of membranes [6]. Second, alkenone accumulation may contribute toward buoyancy regulation by influencing the specific gravity of cells [7]. Third, alkenones may serve as storage compounds because of their carbon abundance and energy in small hydrated (thus occupying a smaller volume) carbon chains [8, 9, 10]. The storage function of alkenones is also supported by a recent finding that showed the lipid bodies of the alkenone-producing haptophyte *Tisochrysis lutea* mostly contain alkenones (about 75%, w/w), with only small proportions of alkenes (1%, w/w) and other lipids such as sterols (24%, w/w); these lipid bodies were named "alkenone bodies" [11].

Alkenones are considered potential sources of renewable fuels because their pyrolysis of dry cells of alkenone-producing microalgae generates hydrocarbons that are very similar to crude oils [12, 13, 14]. The interest in alkenones as potential sources of biofuels was further increased after the dry cells of the alkenone-producing haptophyte *E. huxleyi* were shown to contain more lipids and

hydrocarbons than that in the cyanobacterium *Arthrospira*, the green alga *Dunaliella*, and the euglenophyte *Euglena* [15]. Alkenones were also shown to be ideal substrates in butenolysis with second-generation Hoveyda-Grubbs catalyst for the production of short-chain hydrocarbons that can be used as biodiesels or jet fuels [16]. Alkenone producers are mostly marine algae because of the rapidly increasing global water shortage; this increases their suitability for biorefinery applications and biofuel production (e.g., see [17]).

A few genera of haptophytes, such as *Emiliania* and *Gephyrocapsa* (family Noëlaerhabdaceae, formerly Gephyrocapsaceae), and *Tisochrysis*, *Isochrysis* and *Chrysotila* (family Isochrysidaceae), have been reported to produce alkenones [2, 18]. Although the phylogenetic relationships between Isochrysidaceae and Noëlaerhabdaceae have not been fully elucidated, 18S rDNA sequence analyses confirmed that *Emiliania*, *Gephyrocapsa*, and *Isochrysis* are monophyletic [19]. At present, it is still unknown whether other alkenone producers exist and how alkenone production is regulated physiologically. The identification of other alkenone producers through a broad screening of algae strains could reveal very interesting information relevant to not only cell biology but also geology and paleontology. Alkenones, in fact, are excellent biological markers and geological paleothermometers, whose presence in sediments can greatly help to elucidate algal paleodistribution and variations of global temperature over geological time scales. As existing analytical methods are often time consuming and technically challenging, the development of new rapid and non-extractive methods would represent a substantial leap forward for research in the field.

Fourier transform infrared spectroscopy (FTIR) exploits the fact that different chemical functional groups absorb at specific wavenumbers in the medium infrared range (i.e. 4000–400 cm⁻¹; [20]). Therefore, FTIR spectra analysis provides information on functional groups and molecular structures that can be useful for the identification of specific compounds [21]. FTIR has been applied in the study of eukaryotic algae since 2001 [21]. It subsequently became a common methodology in algal biology and has been used to observe growth rates by determining organic matter production [22], changes in elemental carbon allocation [8], physiological responses to changes in

nutrient availability [8, 23, 24, 25], evolutionary trends of phytoplankton [26, 27, 28, 29], and to predict and monitor biomass quality for commercial utilization [23, 30].

The aim of this study was to develop a method for the rapid, easy, and reliable detection and quantification of alkenones in whole cells using FTIR. This analytical approach would eliminate costly, complicated, and time-consuming extraction procedures. It would greatly facilitate studies on alkenone physiological functions and alkenone distribution among microalgae. Furthermore, a rapid detection method would allow the monitoring of cultures for alkenone production.

2. Materials and methods

2.1. Strains and culture conditions

The haptophytes *Emiliania huxleyi* NIES-837, *E. huxleyi* CCMP 2090, *Isochrysis affinis galbana* (recently renamed as *Tisochrysis lutea* T-iso; [31]), *Isochrysis* sp. CCMP 463 (recently renamed as *T. lutea* CCMP 463; [31]), *Chrysotila lamellosa* CCMP 1307, which are known as alkenone producers, and *Pleurochrysis carterae*, which is known as a triacylglycerol (TAG) but non-alkenone producer, were used in this study (see Table S1 for the origin of the strains).

These strains were maintained in 100 mL Erlenmeyer flasks at 20°C, at an irradiance of 20–30 $\mu\text{mol m}^{-2} \text{s}^{-1}$ from fluorescent lamps with a 16-h light/8-h dark photoperiod. The growth medium composed of natural seawater enriched with nutrients according to the modified Erd-Schreiber's medium recipe (commonly referred to as natural seawater-ESM), with the substitution of soil extracts with 10 nM disodium selenite [32]. The medium also contained 10 mM Tris-buffer, and the pH was adjusted to 8.2. In the experimental cultures, growth conditions were similar except that the medium was prepared with Marine Art SF1 artificial seawater (Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) dissolved in deionized water (referred to as MA-ESM) in 500 mL flat-oblong glass vessels, and the cultures were continuously illuminated by fluorescent lamps at an

intensity of $100 \mu\text{mol m}^{-2} \text{s}^{-1}$ and bubbled with filter-sterilized air at a rate of 100 mL min^{-1} .

2.2. Sample preparation

Samples were collected at the stationary growth phase when cell alkenone content is known to be the highest. Cells were harvested by centrifugation ($1,720g$, for 5 min), and the algal pellet was then washed three times with 0.5 M ammonium formate to remove residual culture medium (for microscope images of whole cell pellet containing Nile Red-staining for neutral lipids, see Fig. 1A, B). The washed algal pellet was resuspended in 0.5 M ammonium formate and then dried at 80°C for 3 h on silicon windows (diameter: 15 mm, thickness: 2mm, S.T. Japan Inc., Tokyo, Japan). The same volume of 0.5 M ammonium formate was used as a blank. The whole cell samples and blanks were directly used for FTIR analysis without any further manipulations [33].

Lipids were extracted from two *E. huxleyi* strains (CCMP 2090 and NIES-837) and from *P. carterae* and then fractionated according to Sawada et al. [34] with some modifications. Total lipids were extracted with methanol and dichloromethane (F1 fraction). The extract was loaded onto a silica gel column and further divided into two fractions: the alkene fraction (F2 fraction; hexane as eluent) and the alkenone fraction (F3 fraction; hexane/ethyl acetate (95:5) and hexane/ethyl acetate (9:1) as eluents). Finally, fatty acids were eluted with methanol/ethyl acetate (1:1) (F4 fraction). After the evaporation of solvents, samples from all fractions were resuspended in methanol, deposited on the silicon windows, and dried in an oven at 80°C . The purified alkenones extracted from *E. huxleyi* NIES-837 (white paste of F3 fraction in Fig. 1C) were also resuspended in hexane/ethyl acetate (9:1) and treated similarly to the samples in methanol; this was done to assess the effect of solvent on the FTIR spectra (for results, see Fig. 4).

For standards, three compounds were used: (A) commercially available *trans*-9, 12-octadecadienoic methyl ester (Sigma-Aldrich Co. LLC., St. Louis, Mo), (B) methyl linoleate (Sigma-Aldrich Co. LLC.), and (C) squalene (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Compound A was dissolved in heptane (10 mg mL⁻¹); B and C were directly used because these compounds are liquids at around 25 °C. All standards were dissolved or mixed with 100% methanol (30% v/v final concentration) and then dried in an oven at 80°C for 3 h on the silicon windows.

2.3. FTIR analysis

FTIR spectra of dried samples were acquired with an Affinity-1S IR spectrometer (Shimadzu Corporation, Kyoto, Japan) over the 4000–500 cm⁻¹ wavenumber range at a resolution of 4 cm⁻¹ (32 averaged scans). A He–Ne laser operated at a wavelength of 632.8 nm was used as the excitation source. The Shimadzu system was controlled by the inbuilt high performance LabSolutions IR software (Shimadzu Corporation). Bands were attributed to functional groups according to Guillen and Cabo [35] and Giordano et al. [21].

In previous reports, organic compounds such as carbohydrates and proteins were semi-quantified with FTIR [8, 36]. In the present study, we applied principles of the same method described in those reports to estimate alkenones semi-quantitatively using whole cell samples. The spectra were deconvoluted using the “Peak Separation” option of the LabSolution IR software; the peaks at 962.5, 1057, 1655, and 1737 cm⁻¹ were selected for relative quantification of alkenones, carbohydrates, proteins, and lipids (except alkenones), respectively. Each peak area was calculated using the LabSolution IR software.

In order to calculate alkenone content (% of total organic matter), the sum of the contents of alkenones, carbohydrates, proteins, nucleic acids, and lipids (except alkenones) were semi-quantitatively calculated, and the value obtained was the total organic matter by assuming that most of the organic materials were involved (for data, see Table 2).

In addition, the value of alkenone content (%) determined by FTIR was used to calculate the semi-quantity of alkenones by assuming that the total organic matter is equal to the total organic carbon (TOC) (for data, see Table 4).

2.4. TOC and GC-flame ionization detector (GC-FID) analyses

E. huxleyi CCMP 2090 culture samples (50 mL) were harvested at the stationary growth phase (after 8–13 days). Half of the cell suspension (25 mL) was centrifuged (1,720g, for 5 min). The supernatant was filtered through a Millex HA filter (pore size 0.45 µm, Merck Millipore, Darmstadt, Germany) to obtain a culture medium deprived of cells (culture medium fraction). TOC values of both cell suspension and culture media fraction (25 mL each) were measured with a TOC analyzer (Shimadzu Corporation, Japan). The cells organic C quota was calculated with the following equation:

$$\text{Cell C} = (\text{TOC in cell suspension}) - (\text{TOC in culture medium fraction})$$

where the TOC value is expressed as TOC of cells contained in a 1-L culture (mg L⁻¹).

Identification and quantification of various molecular species of alkenones in the F3 fraction were performed with a GC-2014 AFSC GC-FID (Shimadzu Corporation) equipped with a capillary column (length 50 m, internal diameter, 0.32 mm; CP-Sil 5 CB; Agilent Technologies, Santa Clara, CA). Helium was used as carrier gas. The temperature of the capillary column was programmed in pressure control mode (109.7 kPa, flow rate of 2 mL min⁻¹) as follows: 60 °C for 3 min; increasing to 180 °C at a rate of 15 °C min⁻¹; then increasing to 310 °C at 10 °C min⁻¹; followed by 310 °C for 20 min. GC-FID spectra containing various alkenone molecular species were analyzed by a peak analysis program; the amount of each alkenone molecule was calculated from the peak area normalized on an internal standard (hexatriacontane).

2.5. Statistics

All results were obtained from three independent experiments. For the FTIR spectra in each experiment, one typical result is shown from measurements of three different cultures. Except for

the FTIR spectra, experimental results in Tables 1–4 are expressed as the mean and standard deviation values of the measured samples (mean $\pm$ SD) from three different cultures.

3. Results and discussion

Figure 2 shows representative FTIR spectra of the alkenone-producing haptophytes (*E. huxleyi* CCMP 2090 and *E. huxleyi* NIES-837) and of the non-alkenone-producing haptophyte *P. carterae* cells. Various peaks derived from cell components such as polysaccharides, proteins, and lipid bands were detected in both alkenone and non-alkenone producers [21, 33], as shown in Table 1. Two peaks, at 1705.5 cm^{-1} and 962.5 cm^{-1} , were exclusively observed for the two alkenone producers (Fig. 2A, B) but not for the non-alkenone producing haptophyte *P. carterae* (Fig. 2C). The peak at 1705.5 cm^{-1} (dotted arrow between peaks e and f) is compatible with the saturated C=O stretching mode of ketones [37]; the peak at 962.5 cm^{-1} (solid arrow) is compatible with the C-H vibrational mode of a *trans*-C=C bond [37] (also see [38], where the presence of a similar band was reported for chemically synthesized alkenones). Both features (C=O and C=C) are strongly indicative of alkenone molecules (see Fig. 1 for molecular formula.). Since the absorption intensity at 962.5 cm^{-1} was much higher than that at 1705.5 cm^{-1} (Fig. 2A, B), we focused on this peak.

In order to confirm that the peak at 962.5 cm^{-1} is indeed indicative of alkenones, we first compared the FTIR spectra of the total lipid (F1), alkene (F2), alkenone (F3), and fatty acid (F4) fractions. In the extracts from two alkenone-producing *E. huxleyi* strains, the peak at 962.5 cm^{-1} was clearly visible in the F1 and F3 fractions; however, it was not observed in the F2 and F4 fractions (Fig. 3A, B). In the non-alkenone-producing haptophyte *P. carterae*, no peak was detected at 962.5 cm^{-1} in all fractions (Fig. 3C).

Association of the 962.5 cm^{-1} peak with alkenones was confirmed using a purified alkenone fraction (F3) prepared from *E. huxleyi* NIES-837 cells (Fig. 4). When alkenones were dissolved in hexane/ethyl acetate (9:1), the peak at 962.5 cm^{-1} became prominent (Fig. 4A). The same peak was

also observed for samples dissolved in 100% methanol; however, the signal intensity (peak height) was much smaller, and a large signal of methanol appeared at 1105 cm^{-1} (Fig. 4B, C).

For alkenones dissolved in hexane/ethyl acetate (9:1), other major peaks were observed at 719, 1460, 1705, 2845, and 2912 cm^{-1} (Table 1). The 719 cm^{-1} peak is compatible with the C-H bending vibration of alkanes [37]. Alkenones dissolved in methanol showed major peaks at 962, 1105, 2845, and 2912 cm^{-1} but not at 719, 1460, and 1705 cm^{-1} . These results can be explained by the low solubility of alkenones in methanol.

Further unequivocal confirmation of the indicative band at 962.5 cm^{-1} was based on the analysis of internal standards. Unfortunately, no commercial alkenone standard is available; thus, we had to utilize standards of molecules that contain functional groups similar to alkenones. However, not all functional groups were in the same molecule. Three standards were used: (A) *trans*-9,12-octadecadienoic methyl ester (contains a *trans*-type double bond structure), (B) methyl linoleate (contains a *cis*-type double bond), and (C) squalene (contains a trisubstituted double bond). A peak was clearly observed in the $970\text{--}960\text{ cm}^{-1}$ range in the spectrum of standard A (Fig. 5). In contrast, peaks attributable to a *cis*-type carbon double bond and a trisubstituted double bond were observed at 723.3 cm^{-1} and 835.2 cm^{-1} , respectively, in standard B and C.

Peaks at $970\text{--}960\text{ cm}^{-1}$ are due to the C-H vibrational mode of *trans*-C=C bonds [37], and they were observed in the standards of oils with *trans*-type double bonds [35]. Therefore, we concluded that the peak at 962.5 cm^{-1} is the result of a *trans*-double bond and can be attributed to alkenones. Our conclusion was supported by the clear detection of the 962.5 cm^{-1} peak in other major alkenone-producing haptophytes, *T. lutea* (T-iso), *T. lutea* CCMP 463, and *C. lamellosa* CCMP 1307 (Fig. 6A-C), but not in the non-alkenone-producing haptophyte *P. carterae* (Fig. 2C, 3C).

Computational methods have been developed to use FTIR data for the semi-quantification of cell carbohydrates, proteins, and lipids [8, 36]. In the present study, we applied the same procedures to estimate the abundance of alkenones in whole cells of alkenone-producing haptophytes (Figs. 2–6). By using this approach, we roughly estimated that the alkenone content was ca. 17% of cell

organic matter in the alkenone-producing haptophyte *E. huxleyi* CCMP 2090 harvested at the stationary growth phase (8–13 d of culture), assuming that the cellular components include lipids, carbohydrates, nucleic acids, and proteins (Table 2).

Various kinds of alkenones were also quantified by GC-FID analysis (Table 3). These analytical data of individual alkenone components can be provided only by GC analysis, but not by FTIR analysis. According to analytical data, C₃₇- and C₃₈-alkenones with two C=C bonds (C_{37:2} and C_{38:2}) were the most abundant molecular species, followed by those with three C=C bonds (C_{37:3} and C_{38:3}); C₃₉-alkenones were minor components. The calculated total alkenone amount was 1.3–1.5 pg cell⁻¹ (equal to 15–27 mg L⁻¹ of culture) in three independent experiments. The carbon amount in alkenones was 12.8–20.5% (16.5% average) of TOC (Table 3). In photosynthetic ¹⁴C-fixation experiments, Tsuji et al. [10] reported that ca. 17% of total fixed ¹⁴C was constantly incorporated into alkenones during 24-h ¹⁴C-labeling experiments that were performed separately, using *E. huxleyi* NIES-837 cells harvested at the stationary growth phase. Interestingly, the alkenone contents determined by FTIR, GC-FID/TOC, and ¹⁴C-labeling experiments are all around 17% of ¹⁴C-TOC (Table 4). These results are good examples to demonstrate the usefulness of FTIR analysis for alkenone semi-quantification using intact cells.

By using the developed FTIR analysis using *E. huxleyi* CCMP 2090 as the main algal material in this study, the alkenone contents (%) are semi-quantitatively calculated as total alkenone carbons/TOC although no data of GC-FID and other methods. The values are 16.7, 16.6, 6.7, 7.2, and 9.9 in intact cells of *E. huxleyi* CCMP 2090 (calculated from data in Fig. 2A), *E. huxleyi* NIES-837 (Fig. 2B), *T. lutea* (formerly *I. galbana* T-iso) (Fig. 6A), *T. lutea* CCMP 463 (Fig. 6B) and *C. lamellose* CCMP 1307 (Fig. 6C), respectively. However, in order to improve data quality of the semi-quantification of alkenones, we need to improve the analytical method of FTIR for not only alkenones but also other cellular components such as membrane lipids, proteins, carbohydrates, etc., as shown in Table 2.

Further validation of FTIR analysis can be useful toward the quantification of alkenones in the

intact cells of other alkenone-producing organisms. This will offer the distinct advantage of not requiring any extraction procedures in comparison with the other methods that require extraction for analysis.

4. Conclusion

We showed that the FTIR peak at 962.5 cm^{-1} allows the time- and cost-effective identification of alkenones in algal cells. A comparison with GC-FID results demonstrates that FTIR combined with TOC analysis can be used to reliably estimate the amount of alkenones in whole alga cells. A distinct advantage of our FTIR method is that it does not require complex extraction procedures. Our method would be extremely useful for monitoring alkenone production in commercial scale cultures, optimizing conditions for alkenones production, and identifying beneficial alkenone-producing strains from the field. Furthermore, the efficiency of the method will facilitate the investigation of the kinetics and regulation of alkenone production in algal cells.

Acknowledgements

This work was supported by Core Research for Evolutional Science and Technology (CREST) of the Japan Science and Technology Agency (JST) [CREST/JST] to YS (FY2010-2015). AP was supported by the *Campusworld* program funded by Università Politecnica delle Marche, Ancona, Italy.

Appendix A. Supplementary data

Supplementary data for this article can be found online at:

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Figure Captions

Fig. 1.(A, B) Microscope images of cell pellets of the alkenone-producing haptophyte *E. huxleyi* NIES 837, (C) alkenones highly purified by fractionation with organic solvents, and (D) the molecular structure of C₃₇-alkenones. In Fig. 1C, the alkenone extract was made from the F3 fraction by the evaporation of solvents (for details on fractionation, see Materials and methods). Cell pellets were obtained by centrifugation, and then deposited on a slide and covered with a cover-slip. Fig. 1A (whole cell pellet) was taken under differential interference contrast (DIC); Fig. 1B (0.02 mM Nile Red-stained cells) was taken under fluorescence with U-MWIB3 (Olympus, Tokyo, Japan); both pictures were taken using an Olympus BX52 microscope (Olympus, Tokyo, Japan) with a EOS KISS III camera (Canon, Tokyo, Japan).

Fig. 2.FTIR spectra of intact cells of the alkenone-producing haptophytes (A) *E. huxleyi* CCMP 2090 and (B) *E. huxleyi*NIES-837, and of the non-alkenone-producing haptophyte (C) *P. carterae*. *E. huxleyi* strains and *P. carterae* were grown in MA-ESM and NA-ESM, respectively. Cells were harvested by centrifugation and washed with 0.5 M ammonium acetate solution; they were then deposited on a silicon window and dried in an oven at 80 °C for 3 h. Dried samples were analyzed with an FTIR spectrometer in the 4000–500 cm⁻¹ range (the x-axis scale at 2000–500 cm⁻¹ is drawn little wider than that at 4000–2000 cm⁻¹). The range of 1800–800 cm⁻¹(shown with a black bar in Fig. 2A-C) is also shown with extended scales in Fig. 2A (expanded) and Fig. 2C (expanded). The solid and dotted arrows indicate the bands at 962.5 cm⁻¹ and 1705.5 cm⁻¹, attributed to *trans*-double bond and keto-group of alkenones, respectively. The lower case letters (a-m) indicate the wavelengths of the bands listed in Table 1.

Fig. 3. FTIR spectra of lipid (F1), alkene (F2), alkenone (F3), and fatty acid (F4) fractions obtained from the alkenone-producing haptophytes (A) *E. huxleyi* CCMP 2090 and (B) *E. huxleyi* NIES-837, and from the non-alkenone-producing haptophyte (C) *P. carterae*. (see text for details on the extraction procedure)

Fig. 4. FTIR spectra of alkenone fraction extracted from the alkenone-producing haptophyte *E. huxleyi* NIES-837. (A) alkenone-containing fraction dissolved in a mixture of hexane/ethyl acetate (9:1). (B) alkenone-containing fraction dissolved in methanol. (C) 100% methanol (blank). The initial amount of samples for extraction was ca. 10 g of dry powdered samples. The procedure for obtaining these fractions is described in the text. The arrow indicates the band at 962.5cm^{-1} , an indication of alkenones.

Fig. 5. FTIR spectra of (A) *trans*-9, 12-octadecadienoic methyl ester (a standard for *trans*-double bond), (B) methyl linoleate (a standard for *cis*-double bond), and (C) squalene (a standard for trisubstituted double bond). (see Fig. 2 for details on the scale, black bar, and arrow)

Fig. 6. FTIR spectra of the intact cells of three alkenone-producing haptophytes (A) *T. lutea* (T-iso), (B) *T. lutea* CCMP 463, and (C) *C. lamellosa* CCMP 1307.

Table 1 Attribution of major bands detected in alkenone producing haptophyte *E. huxleyi* CCMP 2090 (A) and NIES 837 (B), and non-alkenone producing haptophyte *P. carterae*(C) in FT-IR spectrometry.

Peak #	Peaks detected in three strains used in this study ^a	Main absorption bands reported for microalgae in the literature ^b		
		Wavelength (cm ⁻¹)	Band assignment	Functional groups
a	3287(C), 3289(A,B)	3400-3200	ν O-H/ ν N-H	Water, protein
b	2955 (A, B, C)	≈2960	ν_{as} CH ₃	CH ₃ methyl group
c	2922 (A, B, C)	≈2930	ν_{as} CH ₂	CH ₂ methylene group
d	2849 (A, B, C)	≈2850	ν CH ₂ , ν CH ₃	CH ₂ and CH ₃ methyl & methylene groups
e	1732(B), 1734(C), 1737 (A)	≈1745	ν C=O	Ester of lipids and fatty acids
f	1655 (A, C), 1651 (B)	≈1655	ν C=O	Protein (Amide I)
g	1541 (A, C), 1548 (B)	≈1545	δ N-H, ν C-N	Protein (Amide II)
h	No major peak detected	≈1455	δ_{as} CH ₂ , δ_{as} CH ₃	CH ₂ and CH ₃ methyl and methylene groups
i	1375 (B), 1377(A), 1379 (C)	≈1390	δ_s CH ₂ , ν C-O, δ CH ₃ ,	CH ₂ and CH ₃ of proteins/carboxylic groups
j	1242(A), 1244 (C), 1249(B)	≈1240	ν_{as} P=O	Phosphodiester of nucleic acids and phospholipids
k	1057 (A, B), 1060 (C), 1163 (C), 1165 (A, B)	1200-900	ν C-O-C	Polysaccharides/siloxane
l	Not detected	1075, 950	ν Si-O	Siloxane, silicate frustules
m	Not detected	980-940	P-O-P	polyphosphate

^aWavelengths calculated from spectra in Fig. 2.

^bBand assignments are taken from literatures (Mayers et al. 2013). ν =symmetric stretching, ν_{as} =asymmetrical stretching, δ_s =symmetric deformation, δ_{as} =asymmetrical deformation.

Table 2 Percent composition of alkenones and other major cell components determined by FTIR analysis in *E. huxleyi* CCMP 2090 cells which were autotrophically grown until the stationary growth phase.

Contents (%)	
Alkenone	Others ^a
17.29 ± 1.29	82.71± 1.29

^aOther major cell components including carbohydrates, proteins, nucleic acids and lipids except alkenones were estimated from peaks e, f and k in Fig. 2.

Table 3 Composition of various alkenone molecules analysed by GC-FID in *E. huxleyi* CCMP 2090 cells grown autotrophically until the stationary growth phase.

Alkenones	Amounts[mg (L culture) ⁻¹] and Contents (% of total)		
	Exp.1	Exp. 2	Exp. 3
C37:3Me	3.22 (15.6)	3.58 (23.2)	5.99 (22.0)
C37:2Me	8.59 (41.5)	4.37 (28.4)	7.15 (26.2)
C38:3Et	1.11 (5.4)	1.59 (10.3)	2.89 (10.7)
C38:3Me	0.66 (3.2)	0.87 (5.6)	1.28 (4.6)
C38:2Et+Me	6.51 (31.3)	4.25 (27.3)	8.48 (31.2)
C39:3Et	0.10 (0.5)	0.21 (1.4)	0.38 (1.4)
C39:2Et	0.52 (2.5)	0.60 (3.9)	1.10 (4.0)
Total alkenones ^a	20.71 (100)	15.47 (100)	27.27 (100)
Total alkenone/10 ⁹ cells (mg)	1.41	1.53	1.33
Alkenone carbon contents (%) ^b	83.52	83.55	83.55
Total alkenone carbons ^c [mg (L culture) ⁻¹]	17.30	12.92	22.79
Total organic carbons (TOC) [mg (L culture) ⁻¹] ^d	134.9	80.1	111.2
Cell density in algal culture [x 10 ⁹ cells (L culture) ⁻¹]	14.7	10.1	20.5
Total alkenone carbons/TOC (%)	12.82	16.13	20.50
Total alkenone carbons/10 ⁹ cells (mg)	1.18	1.28	1.11

^aThe sum of values of various alkenones contained in each experiment (Exp) listed above.

^bThe values represent C-contents in alkenones in each experiment which was calculated from molecular masses of respective alkenone molecular species and alkenone contents (% of total) determined experimentally.

^cC-contents in alkenones calculated by the equation (a x b/100) in each Lot.

^dThe values were determined by TOC analyzer.

Table 4 Comparison of alkenone contents quantified by GC-FID and FTIR in *E. huxleyi* CCMP 2090 which was grown autotrophically and harvested at the stationary growth phase.

Analytical method	Total organic carbons (TOC) (mg/L)	Total alkenones (mg/L)	Total alkenone carbons/TOC (%)	Total alkenone carbons (mg/L)
TOC	108.73 ± 27.48 ^a	-	-	-
GC-FID	-	21.15 ± 5.92 ^b	16.48 ± 3.85 ^c	17.67 ± 4.95 ^d
FTIR	-	-	17.29 ± 1.29 ^e	19.00 ± 6.10 ^f

^aDetermined experimentally. The value is average of TOC in Exp. 1-3 in Table 3.

^bDetermined experimentally by GC-FID analysis of F3 fraction (alkenone fraction) extracted from whole cells. The value is average of total alkenones in Exp. 1-3 in Table 3.

^cThe value c was calculated by an equation ($d \times 100/a$). The value is average of total alkenone carbons/TOC in Exp. 1-3 in Table 3.

^dThe value d was calculated from b. The value is average of total alkenone carbons calculated in Exp. 1-3 in Table 3.

^eThe alkenone content (%) was estimated experimentally by FTIR analysis (Table 2). The alkenone content (%) determined by FTIR represents "total alkenone carbons/TOC".

^fThe value f was calculated by an equation ($e \times a/100$).

Fig. 2.

Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

▪ FTIR Spectroscopy

Rapid semi-quantification of alkenones in whole cells by FTIR at 962.5 cm⁻¹ peak assigning trans C=C bond.

A large arrow points from the FTIR spectrum towards this text.

Graphical abstract