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Electron-transfer MALDI MS methodology for microalgae/phytoplankton pigments analysis

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ABSTRACT

The method describes pigment analysis from microalgae/phytoplankton extracts using electron-transfer Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (ET MALDI MS). Current microalgae/phytoplankton pigment analysis requires resource- and time-intensive chromatographic methods due to the broad polarity range of the target analytes. On the other hand, traditional MALDI MS chlorophyll analysis, using proton-transfer matrices such as 2,5-dihydroxybenzoic acid (DHB) or α-cyano-4-hydroxycinnamic acid (CHCA), results in central metal loss and phytol–ester cleavage. ET MALDI MS is an alternative for the rapid screening and detection of pigments in microalgae extracts.

- MALDI matrices with ionization energies above 8.0 eV guarantee electron-transfer processes from photosynthetic and photoprotective pigments whose ionization energies lay below 7.5 eV.
- ET MALDI MS pigment analysis agrees with data gathered from conventional chromatographic techniques (HPLC) and optical microscopy for pigment extracts from *C. vulgaris* cultures and freshwater phytoplankton samples.
- The ET MALDI MS method allows fast and reliable detection of pigments in microalgae cultures and freshwater phytoplankton samples.

Specifications table

Subject area:	Chemistry
More specific subject area:	*Pigment analysis by Electron-Transfer MALDI MS*
Name of your method:	*Electron-transfer MALDI MS methodology for microalgae pigments analysis*
Name and reference of original method:	*N/A*
Resource availability:	*Flexcontrol manual (Software used for data collection)* https://researchservices.pitt.edu/sites/default/files/flexControl%20User%20Manual.pdf *Flexanalysis manual (Software used for data analysis)* *https://researchservices.pitt.edu/sites/default/files/flexAnalysis%20User%20Manual.pdf* *Certified standards* https://c14.dhigroup.com/productdescriptions/mixedphytoplanktonpigments https://c14.dhigroup.com/productdescriptions/phytoplanktonpigmentstandards

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Method details

Chlorella vulgaris culturing and freshwater phytoplankton sampling

1. The handling and manipulation of *Chlorella vulgaris* cultures and freshwater phytoplankton samples should be carried out in a laminar flow hood (Class-2 Biohazard safety cabinet, BSL-2) to avoid bacterial and fungi contamination.
2. *Chlorella vulgaris* were cultured under intermittent lighting (12 V white LED, 12 h of light, and 12 h of darkness) using the BBM medium. The process starts with $1.4x10^6$ cells/mL in 100 mL of culture medium (Fig 1).
3. Freshwater samples -for phytoplankton isolation- were collected using a bottle sampler by gently scooping surface water from a shallow artificial lake located on the main campus of the Universidad Industrial de Santander, Bucaramanga, Colombia (7°8′28.271′′ N, 73°7′14.731′′ W, 1009 mamsl).
4. *Chlorella vulgaris* and phytoplankton, from culture media and freshwater samples, were collected in 50 mL sterile Falcon tubes filled to 3/4 with the liquid samples. The samples were centrifuged at 8000 rpm, at 4 °C, for 15 min (avoid tightly closing the Falcon tube lid), the supernatant was discarded, and the resulting pellet -containing biomass- was stored at -80 °C until extraction (Fig 1). At this point, it is advisable to divide the pellet into several small fractions - and individually store them in amber vials- to avoid thawing the whole pellet when minute samples are required for pigment extraction.

Microscopy analysis

1. Optical microscopy analysis of phytoplankton and *Chlorella vulgaris* cells was performed on an OLYMPUS BX53 optical microscope. Place a 10 µL aliquot of *Chlorella vulgaris* culture or phytoplankton sample on a glass microscope slide previously cleaned with isopropyl alcohol and dried at room temperature.
2. Place a clean and dry coverslip over the liquid sample. Set the slide on the microscope stage and secure it with the stage clips. The sample is ready to observe using a 10x eyepiece and a 40x objective. Microorganisms found in the samples can be photographed with a Canon Professional Digital EOS 5DSR camera in an autofocus setting.

Pigment extraction protocol

1. The microalgae/phytoplankton biomass pellets manipulation should be performed in amber vials under a fume hood with dimmed light to avoid pigment photooxidation.
2. Weigh 2 mg of a previously thawed microalgae/phytoplankton pellet in a 1.5 mL amber vial and add 1 mL of analytical grade acetone at room temperature (avoid thawing large amounts of biomass).
3. Cell lysis was performed under ultrasonic radiation using an ultrasound bath (40 kHz, Branson Ultrasonics™ CPX, Danbury, CT, USA) for 25 min. Cell lysis time depends on microorganism type [1,2] (Fig. 1).
4. Filter the extraction mixture using a 0.45 µm PTFE membrane filter. Store the liquid extracts in amber vials below 4 °C until analysis. It is preferable to perform the analysis immediately after the extraction to avoid pigment decomposition.

Electron-transfer Matrix-Assisted Laser Desorption Ionization (ET MALDI MS)

1. Prepare a 4 mM (1 mg) solution of *trans*-2-[3-(4-tert-butylphenyl)-2-methyl-2-propenylidene] malononitrile (DCTB), MALDI matrix Sigma-Aldrich (St Louis-MO) in 1 mL of acetonitrile (ACN gradient grade for HPLC). If necessary, use an ultrasound bath -for two minutes- to completely dissolve the solid (40 kHz, Branson UltrasonicsTM CPX, Danbury, CT, USA).
2. Prepare a calibration sample using a pigment standard (chlorophyll a, chlorophyll b, β-carotene, diadinoxanthin, and astaxanthin) and the MALDI matrix solution to reach an analyte-to-matrix (A:M) ratio of 1:1000. Use a vortex at 1000 rpm for

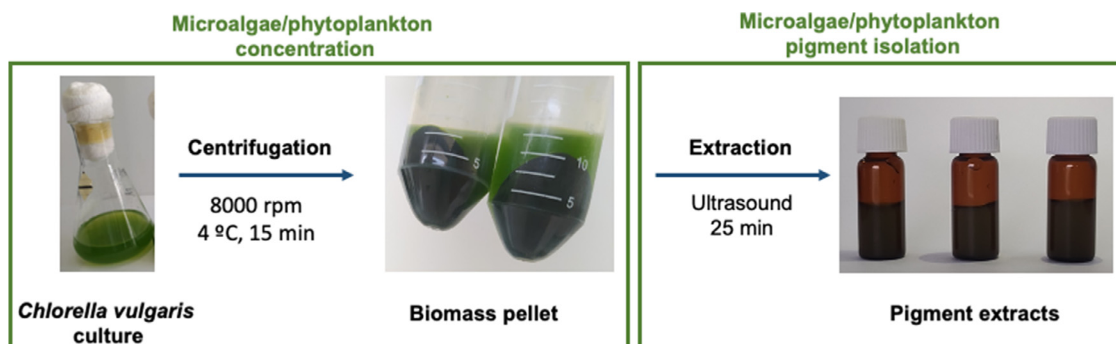


Fig. 1. Microalgae/phytoplankton concentration and pigment extraction.

Table 1
Suggested pigment signals for checking instrument calibration and sample behavior.

Peak label	Ref *m/z*
Chlorophyll a, $M^{+\bullet}$	892.53531
Chlorophyll b, $M^{+\bullet}$	906.51457
Pheophytin a, $M^{+\bullet}$	870.56592
Chlorophyllide a, $M^{+\bullet}$	614.23796
Chlorophyllide b, $M^{+\bullet}$	628.21722
DCTB, $M^{+\bullet}$	250.14700

two minutes to ensure proper mixing. The standard was purchased from DHI (Horsholm, Denmark) with known amounts of individual pigments.

3. Mix the *Chlorella vulgaris*/phytoplankton pigment extracts with the MALDI matrix solution using a vortex at 1000 rpm as described above to reach an A:M ratio of 1:1000. The ratio was calculated using literature reports of chlorophyll-a concentration in *Chlorella vulgaris*. Alternatively, an approximate chlorophyll-a concentration can be derived using UV-vis measurements [3].
4. Using the dried droplet method, seed the mixture (1 µL) on a polished steel MALDI target at room temperature and allow it to dry. Place at least three spots per sample to improve data reproducibility and reduce sample-to-sample variations due to sweet spots. Also, we recommend using only freshly prepared matrix solutions for the MALDI experiments. Test the matrix purity and stability by taking an LDI spectrum before performing any MALDI experiments. Purify the matrix, by recrystallization or sublimation, in case of degradation.
5. Prepare a saturated solution of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 30:70 v/v acetonitrile: 0.1% trifluoroacetic acid (TFA) in water. Prepare a 200 ppb solution of standard peptides (leu-enkephalin, bradykinin, bombesin, and renin substrate) in water, mixing with a vortex at 1000 rpm for one minute. Mix the two solutions prepared above in 1:1 v/v proportions, and deposit 1 µL of the mixture on a polished steel MALDI target. Run at least five spots of the calibrants to improve data reproducibility and reduce sample-to-sample variations due to sweet spots.
6. Insert the polished steel MALDI target in the ionization source of an Ultraflextreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). Wait until the source reaches a working pressure (set point: 3.0*x*10-6 mbar). Start by analyzing the calibration mixture using the following conditions (note that these conditions may vary depending on instrument type and years of use, particularly parameters related to the laser energy-per-pulse): PIE (pulsed ion extraction) 100 ns, laser spot size 100 µm, laser attenuation 40%, laser frequency 1000 s-1, source voltage 20 kV. Accumulate a total of 3000 spectra corresponding to three shots per spot. The TOF mass analyzer was operated in reflectron mode (25 kV).
7. The laser energy per pulse was varied from 0.15 to 2.74 µJ using the instrument's attenuation setting. The actual laser's energy output was measured using a PowerMax-USB UV/Vis Wand (Coherent, Santa Clara, USA); all laser energy-per-pulse measurements were performed in triplicate. However, if there is no means of measuring the laser energy-per-pulse, use the instrument attenuation setting to obtain reproducible signals with high S/N ratios and resolution. Use the following α-CHCA related ions for calibrating the spectrum low mass region: $[M+H]^+$ at *m/z* 190.04987, $[M+Na]^+$ at *m/z* 212.03181, $[2M+H]^+$ at *m/z* 379.09246, $[2M+Na]^+$ at *m/z* 401.07048, and $[3M+H]^+$ at *m/z* 568.13506. Use the $[M+H]^+$ peptide signals at *m/z* 556.27657, 1060.56868, 1619.82234, and 1758.93260 for leu-enkephalin, bradykinin, bombesin, and renin substrate, respectively, to calibrate the medium and high mass range. Note that pigments are low-molecular-weight metabolites by MALDI-TOF standards; thus, the mass range for the MS method should be set to *m/z* 3000.
8. Calibrate the instrument using the standard internal calibration protocol (see Resource availability section, FlexControl 3.4 User Manual section 3.7.6). Once internal calibration was performed, start running the pigment standards and the pigment extracts using the same conditions described above for the calibration samples. If necessary, adjust (increase or decrease) the laser attenuation to improve signal descriptors (abundance/relative intensity, S/N, resolution). Check the signal abundance in different parts of the spot (you can use the auto setting in the instrument). We recommend building a table including expected charged species such as chlorophyll a ($C_{55}H_{72}O_5N_4Mg$, $M^{+\bullet}$ *m/z* 892.5353), chlorophyll b ($C_{55}H_{70}O_6N_4Mg$, $M^{+\bullet}$ *m/z* 906.5145), and derivatives such as pheophytin a ($C_{55}H_{74}N_4O_5$, $M^{+\bullet}$ *m/z* 870.5659), chlorophyllide a ($C_{35}H_{34}N_4O_5Mg$, $M^{+\bullet}$ *m/z* 614.2379), and chlorophyllide b ($C_{35}H_{32}N_4O_6Mg$, $M^{+\bullet}$ *m/z* 628.2172), and the MALDI matrix DCTB ($C_{17}H_{18}N_2$, $M^{+\bullet}$ *m/z* 250.1470). See Table 1 for an example.

Beware: DCTB is primarily an electron-transfer matrix: thus, radial cations are the species expected in the MALDI experiment. However, DCTB has an acidic β-H and can produce protonated molecules even when using aprotic solvents for sample preparation.

9. Store the data using a consecutive code; these files can be used, compressed, or exported for data processing.
10. Data analysis was performed using the Flex Analysis software (Bruker Daltonics, Billerica MA, USA). After external calibration, use the peak finding setting for an automatic report of ion abundances, S/N ratios, resolution, peak area, and monoisotopic masses. Mass spectra were not smoothed, or baseline corrected before analysis.
11. Pigment identification involved comparing the *m/z* values of the signals detected in the extracts to the standard mixture of pigments and a database previously built by our research group from literature reports of pigments identified in microalgae using other analytical techniques. Furthermore, experimental, and theoretical isotope patterns, calculated with ChemCalc [4],

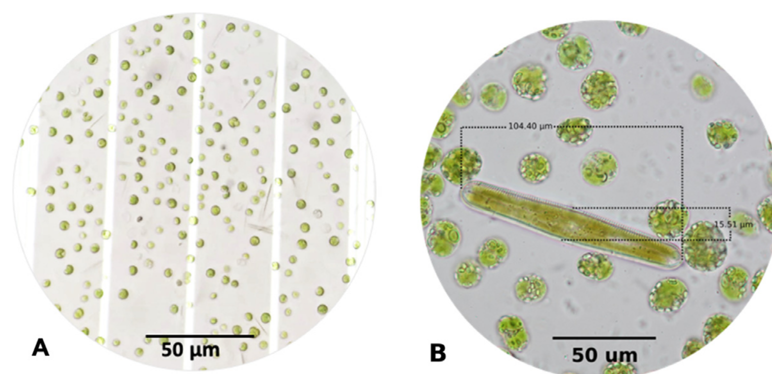


Fig. 2. Optical *microscopy* of microalgae/phytoplanktonic species in liquid samples, magnification *1000x*. **A**-*Chlorella vulgaris* cells in BBM culture media. **B**-Specimen of the genus *Phacus*, with a clear eyespot and a conical tail, and specimen of diatom *Bacillariophyceae* subclass showing the frustule: a rigid cell wall made of opaline silica characteristic of diatoms**.**

were compared to verify compound identification. Establishing a minimum relative intensity threshold (0.01%) for assigning signals in the mass spectrum is suggested. The mass spectra were plotted in Origin Pro 9.0 64-bit.

Method validation

Optical microscopy analysis of Chlorella vulgaris cultures and freshwater phytoplankton

Chlorella's studies initiated algae research in the 1950s. *Chlorella vulgaris* is a green, spherical (2-10 µm), unicellular microalgae belonging to the *Chlorellaceae* family, *Chlorophyta* taxon. Commonly found in freshwater, *C vulgaris* has a high nutritional value due to its pigments (chlorophyll and carotenoid, 1-2 wt%) and protein (42-58 wt%) contents [5]. In addition, *Chlorella vulgaris* presents an interesting lipid composition (structural and signaling lipids) ranging between 5 and 40 wt% depending on the medium and culture conditions such as pH, temperature, nutrient availability, and light exposure. Fig. 2A shows an optical microscopy image of *Chlorella vulgaris* cells. We observe *Chlorella vulgaris* as spherical cells with diameters from 5 to 10 µm, a nucleus of about 2.1 µm, and a chloroplast occupying a third or, in some cases, half of the cell. The morphological characteristics reported above agree with previous literature reports [5,6]. *C. vulgaris* cells are much smaller than other phytoplankton microalgae, such as the ones in Fig. 2B.

On the other hand, phytoplankton is a diverse community of autotrophic organisms that live in freshwater and marine bodies. There is a consensus that phytoplankton communities are composed of eukaryotic algae, such as diatoms, dinoflagellates, and prokaryotic cyanobacteria. Phytoplankton communities are the first link in the trophic chains that make up aquatic ecosystems and serve as food for primary consumers such as zooplankton [2,7,8]. The photosynthetic organisms of phytoplankton are biological pumps that capture up to 40% of atmospheric CO_2, fixing inorganic carbon in the form of organic matter. Additionally, because of photosynthetic reactions, more than 50% of atmospheric oxygen is produced by phytoplankton [9–11]. Due to the fundamental role of these organisms in the carbon, water, and oxygen cycles, monitoring their activity and composition is vital in oceanographic, ecosystemic, and climate change studies. We identified seven specimens in freshwater phytoplankton samples using morphological observations from optical microscopy and taxonomic keys available in the literature [12–14]. Four specimens were identified up to the genus taxonomic level and the other three up to subclass (*Bacillariophycidae* and *Fragilariophycidae* subclasses). The identified species were *Phacus salinus* cf., *Tetradesmus lagerheimii* cf., *Stauridium tetras* cf., and *Pediastrum duplex* cf. Fig. 2B show optical microscopy images of some of the observed specimens.

HPLC analysis of pigment standards and phytoplankton pigment extracts

HPLC is the "gold standard" employed to perform chemotaxonomical analysis of phytoplankton populations using pigments as biomarkers [15–17]. Qualitative and quantitative HPLC data is used, together with oceanographic/aquatic variables such as dissolved oxygen, temperature, pH, salinity, and conductivity, to feed algorithms that allow estimating phytoplankton class abundances from chemical markers.

We performed HPLC analysis of pigment standards and microalgae pigment extracts using the method reported by Heukelem et al. [15]. The pigment standard sample, used as a reference compound to identify pigments in freshwater phytoplankton extracts, contains chlorophyll a, chlorophyll b, β-carotene, diadinoxanthin, and astaxanthin. In short, an Eclipse XD8 C18 column (150 × 3.9 mm) was used for pigment separation with gradient elution involving solution A (0.001% formic acid in H_2O) and solution B (0.001% formic acid in MeOH) at a flow rate of 1 mL·min−1 as follows: 0–0.5 min, 95% B; 0.5–3.00 min, 95%–100% B; 3.00–50.00 min, 100% B. Colum equilibration, before sample injection, was achieved by flushing with MeOH for 15 min and pigment detection was performed using a DAD (λ= 450 nm). Fig. 3 shows the chromatographic trace and identity of the pigments in the freshwater phytoplankton sample.

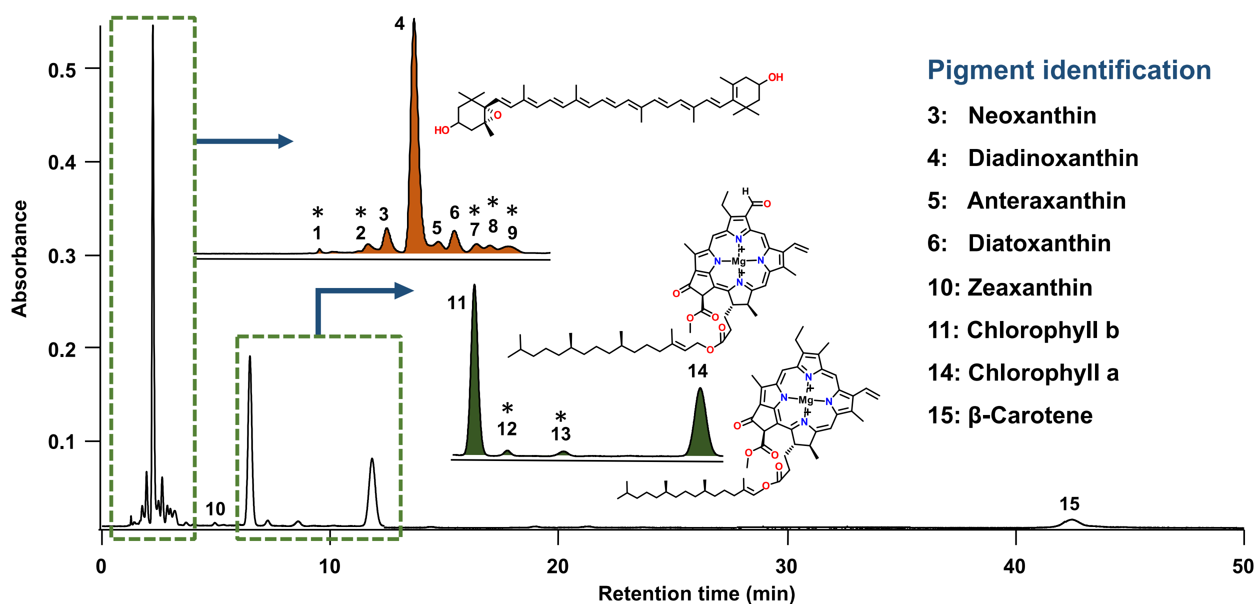


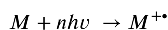
Fig. 3. HPLC chromatogram of the pigments extracted from a freshwater phytoplankton sample. Eclipse XD8 C18 column (150 × 3.9 mm). Peaks marked with an asterisk (*) were not identified.

Four of fifteen signals detected in the phytoplankton pigment sample were identified using certified standards (diadinoxanthin, chlorophyll a, chlorophyll b, and β-carotene), and four by comparison with retention times reported in the literature (neoxanthin, antheraxanthin, diatoxanthin and zeaxanthin). The remaining chromatographic peaks were not identified; however, with lower retention times than chlorophyll a and b, these may correspond to polar derivatives, e.g., chlorophyllide and pheophorbide. The results shown in Fig. 3 are consistent with Brotas & Plante-Cuny [18], who reported the presence of diadinoxanthin, antheraxanthin, and diatoxanthin, in methanolic extracts of *Euglena* sp grown under controlled conditions of temperature and sunlight. *Euglena* sp. is a microalga typically found in freshwater. The authors also reported chlorophyllide b and pheophytin b in *Euglena* sp. Additionally, other authors have identified carotenoid pigments such as neoxanthin, diadinoxanthin, diatoxanthin, α-carotene, and β-carotene in freshwater microalgae, *e.g., Euglena gracilis* [11,19]. In this work, we identified neoxanthin, diadinoxanthin, diatoxanthin, and β-carotene by HPLC.

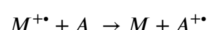
Regarding analytical resources, the pigment extract HPLC analysis took over 50 minutes, from running the standard mix to assigning individual retention times. Also, the volume of solvents used in the HPLC analysis was close to 60 mL per sample after column and detector stabilization. These facts highlight the resources required for analyzing a phytoplankton pigment extract by HPLC. However, despite the operational costs involved in HPLC analysis, separating all pigments in the mixture remains an analytical challenge.

ET MALDI MS analysis of a pigment standard and microalgae/phytoplankton pigment extracts

Several charging schemes are viable in MALDI MS. Proton transfer/abstraction and cation exchanges are typically used for biomolecule analysis. However, using proton-transfer matrices like α-CHCA, 2,5-DHB, and SA is not recommended for pigment analysis because acid matrices provoke chlorophyll demetallation and phytol hydrolysis [20]. On the other hand, electron transfer (ET) in MALDI MS is predominantly used in material analysis applications where proton or cation exchanges are challenging [21]. ET begins with the formation of primary matrix ions through a "pooling" mechanism - as described in the CPCD model (Coupled Physical and Chemical Dynamics) [22,23]. Once formed, the matrix ions ($M^{+\bullet}$) can abstract an electron from a neutral analyte (A) to form a secondary ion ($A^{+\bullet}$). This reaction is only possible if the analyte ionization energy $E_{i(A)}$ is lower than the matrix $E_{i(M)}$ [24–26]. The ET reactions proceed according to the following mechanism:



$$M + nh\upsilon \rightarrow M^{+\bullet}$$



$$M^{+\bullet} + A \rightarrow M + A^{+\bullet}$$

$$E_{i(Matrix)} > E_{i(Analyte)} \therefore \Delta E_i > 0.5\ eV$$

Previously, various researchers reported using the ET MALDI approach to detect chlorin-type pigments such as bacteriochlorophyll a, chlorophyll a, and carotenoids in microorganisms and food. For these analyses DAN and DCTB exhibited the best performance as

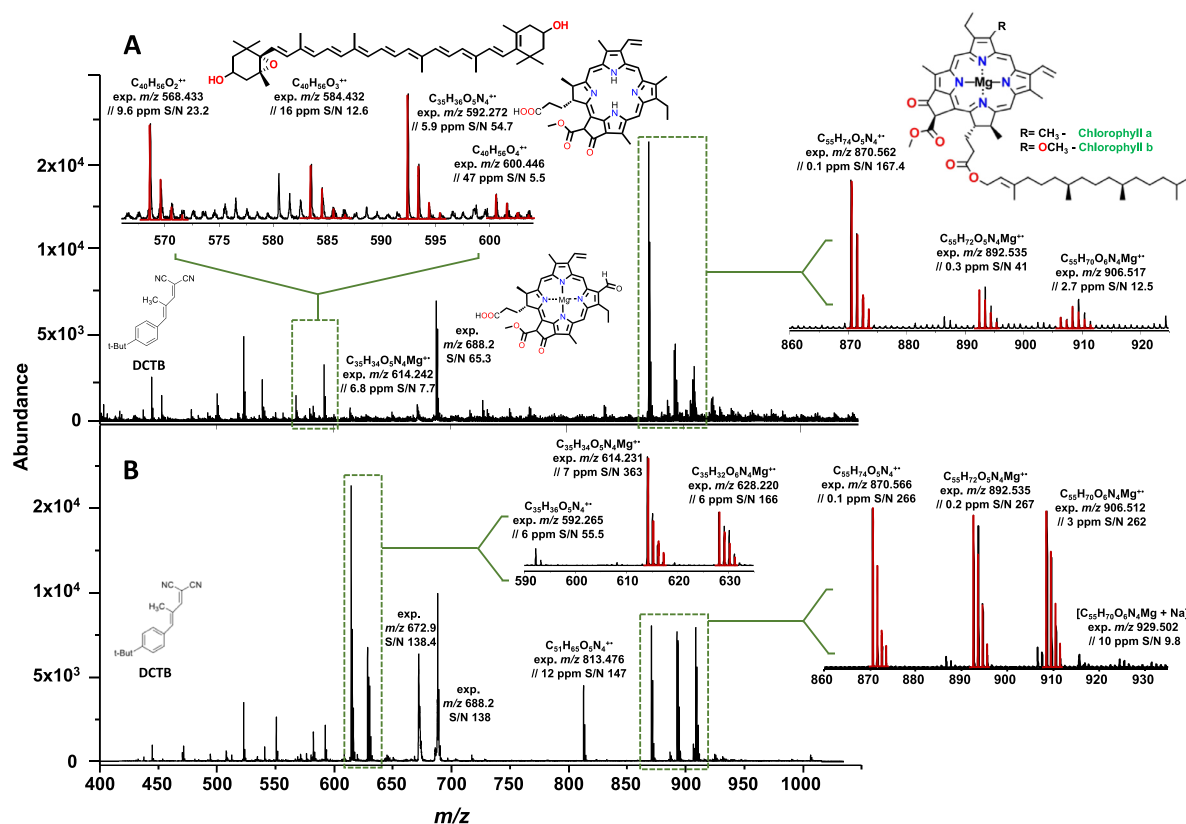


Fig. 4. ET MALDI MS mass spectra of **A**, *Chlorella vulgaris* cells pigment extract and **B**, freshwater phytoplankton pigment extract. Insets show molecular formula, experimental mass, mass accuracy, signal/noise ratio, and theoretical/experimental isotopic pattern for the identified pigments.

ET matrices [27–29]. During the method development we also tested *α*-cyanophenylenevinylenes (*α*-CNPVs) derivatives, reported by our research group as efficient ET-MALDI matrices for nanoparticle, polymer, petroporphyrin, and fullerene analysis [30–33]. Unlike DCTB, *α*-CNPVs exhibit high vacuum stability meaning a low vapor pressure under the MALDI source conditions, which is an advantage for imaging applications. However, we observed that the vacuum instability of DCTB is beneficial for intact chlorophyll desorption since a very low laser pulse energy (0.42 µJ) is required to transfer this labile molecule to the gas phase. On the other hand, chlorophyll ionization with *α*-CNPV matrices requires higher laser pulse energy (> 0.5 µJ) than with DCTB which ultimately causes decomposition.

ET ionization is a valuable strategy for the selective ionization of structurally related compounds with similar ionization energies, such as detecting petroporphyrins in complex mixtures, as previously reported [30–33]. In microalgae, photosynthetic (chlorine derivatives) and photoprotective (isoprenoid derivatives) pigments share common architectures, and consequently, their ionization energies (amongst the members of each group) are similar. Thus, we selected the commercially-available electron transfer matrix (DCTB) with an ionization energy of 8.54 eV to perform the ionization step [31–34]. According to literature reports and our calculations, the E_i values of photosynthetic and photoprotective phytoplankton pigments range between 6.2 to 7.1 eV; thus, electron transfer from neutral analytes to primary matrix ions is thermodynamically feasible according to the mechanism shown above [35]. Considering the scope of this method, which is to detect a wide range of chlorophylls, carotenoids, and xanthophylls in a single run, DCTB is the most suitable electron-transfer matrix.

Fig. 4 shows the ET MALDI TOF mass spectra of pigment extracts from *C vulgaris* and freshwater phytoplankton, using DCTB as a matrix, with an A:M ratio of 1:1000, 0.42 µJ of laser energy, and 3000 shots per spot. We observe two distinct regions in the MS; photoprotective pigments -carotenoids- are distributed between *m/z* 500 and *m/z* 600, while intact photosynthetic pigments -chlorophylls-signals occur between *m/z* 850 and *m/z* 1000. Pigment molecules readily form radical cations under the MALDI conditions used. Signal clustering at different spectral regions facilitates data interpretation. However, keep in mind that some pigments are labile -particularly chlorophylls- and even under MALDI's soft ionization conditions, fragments can be formed and detected at low *m/z* values. In the chlorophyll region, typical ions correspond to chlorophyll a ($C_{55}H_{72}O_5N_4Mg$, *m/z* 892.535), chlorophyll b ($C_{55}H_{70}O_6N_4Mg$, *m/z* 906.514), and derivatives such as chlorophyllide a ($C_{35}H_{34}N_4O_5Mg$, *m/z* 614.237), chlorophyllide b ($C_{35}H_{32}N_4O_6Mg$, *m/z* 628.217), and pheophytin a ($C_{55}H_{74}N_4O_5$, *m/z* 870.565). Interestingly, the identification of chlorophyll derivatives (chlorophyllides and pheophorbides) could not only be associated with the breakdown of the chlorophyll molecule during the extraction or purification process but also with a biological response. Chlorophyll derivatives are naturally present in water bodies as by-products of metabolism

in fish; they are vital indicators of ecosystemic processes connected to the functioning of trophic chains. Also, using mutant photosynthetic microorganisms, Vavilin et al. found that chlorophyllide and pheophorbide are products of chlorophyll degradation in the photosystems I and II [36]. We observed chlorophyll a ($C_{55}H_{72}O_5N_4$Mg-K) and -b ($C_{55}H_{70}O_6N_4$Mg-K) adducts at *m/z* 931.499 and 945.478, respectively. Some authors have previously reported the affinity of chlorophylls with different cations [37–40].

Chlorophyll a experiences fragmentation reactions resulting in the fragments at *m/z* 614.237, *m/z* 555.147, and *m/z* 481.098. Pheophytin also undergoes fragmentation resulting in the species at *m/z* 592.578, *m/z* 533.165, and *m/z* 459.157 [41]. Also, some chlorophyll b fragments are detected at *m/z* 553.256 (loss of –(phytyl-H)OCH_3-CO_2), *m/z* 495.146 (loss of -CHO-COOphytyl-$COOCH_3$), *m/z* 476.128 (loss of -CHO-CH_2CH_2COOphytyl-$COOCH_3$) [42]. In MALDI MS approaches, the number and type of fragments detected from chlorophyll depend on the measurement conditions and sample treatment. In this work, electron-transfer matrix used (DCTB, 8.54 eV, vapor pressure $9.09x10^{-7}$ mm Hg at 25°C [43]) reduces chlorophyll fragmentation during the MALDI process by facilitating the desorption of labile intact species. We believe some of the unidentified signals in the spectrum may be due to acyl glycerides. Identifying and quantifying pigments, such as chlorophyll a and chlorophyll b, is vital to calculate parameters like Net Primary Production (NPP) in water bodies essential to determine the health of the trophic chain in aquatic ecosystems [44].

The carotenoid ET MALDI spectral region includes signals in the form of radical cations from diadinoxanthin ($C_{40}H_{54}O_3$, *m/z* 582.407), neoxanthin ($C_{40}H_{56}O_4$, *m/z* 600.417), diatoxanthin ($C_{40}H_{54}O_2$, *m/z* 566.412), β-e-carotene ($C_{40}H_{56}$, *m/z* 536.438), zeaxanthin ($C_{40}H_{56}O_2$, *m/z* 568.428), and antheraxanthin ($C_{40}H_{56}O_3$, *m/z* 584.422). We detected an astaxanthin ($C_{40}H_{52}O_4$) sodium adduct $[M + Na]^+$ at *m/z* 619.376. Among these compounds, diadinoxanthin ($C_{40}H_{54}O_3$, *m/z* 582.407) belongs to the xanthophyll cycle, a critical enzymatic cycle that serves as an indicator of a photosynthetic organism's physiological condition. In phytoplanktonic microorganisms, diadinoxanthin is transformed into diatoxanthin (in the case of diatoms) or dinoxanthin (for dinoflagellates) [45]. Using HPLC, other authors have reported the same pigments we identified via our ET MALDI MS methodology in *Chlorella vulgaris* [46], and in phytoplankton from freshwater samples, we also identified these pigments using HPLC. The compounds identified are characteristic biomarkers of algae belonging to the Haptophyte, Chlorophyta, and Euglenophyta phyla [7]. This work offers a rapid methodology for detecting photosynthetic and photoprotective pigments in a single assay, with comparable results to other analytical techniques [46]. For instance, microalgae growth is typically monitored via UV-vis spectroscopy [47]. We have successfully applied ET MALDI to investigate how *Chlorella vulgaris* growth affects pigment profiles (data not shown). Overall, this methodology has the potential to provide valuable insights into the growth status of microalgae and their response to changing environmental factors.

We use isotopic pattern coincidence as an additional criterion for compound identification besides mass accuracy. Fig. 4 includes theoretical/experimental isotopic pattern comparisons using information from the Compass Isotope Pattern software (Bruker Daltonics), and the experimental data from the ET MALDI assays. Isotope patterns correspond to a group of signals related to ions with the same chemical formula but containing different isotopes, also known as isotopologues. Therefore, the isotopic pattern is vital in identifying ionic species in mass spectrometry [48].

The ET MALDI experiments also resulted in metastable ion detection (Fig. 4). Metastable ions are vibrationally excited species that can emerge from the MALDI plume. These ions fragment after the ionization source and typically exhibit broad signals with an apparent mass different from the original ion's. Baldas and Porter [49] reported metastable ions in carotenoids and chlorophylls MALDI studies. Using HRMS, a characteristic M-80 signal was assigned to C_6H_8 loss in carotenoids analysis. Brown and Wilkins [50] indicate that metastable chlorophyll ions arise from excess energy supplied during desorption. Several parameters can influence MALDI's metastable ion formation, such as energy absorption directly from the laser, collision with matrix molecules, ion acceleration through the source's electrical field, and exothermic reactions in the MALDI plume [48,51,52]. The metastable ion at *m/z* 672.9 (Fig. 4) could have a likely structure $[M + 2K - H - (phytyl - H) - H_2O]^+$ [53].

Conclusions

Electron-transfer reactions allow selective ionization of compound families sharing similar architectures (chlorins and carotenoids) with ionization energies below the matrix ionization energy. Compared with the gold standard (HPLC), ET MALDI provides valuable chemical information. HPLC separates pigments based on differential interactions between the analytes, mobile, and stationary phases. Although robust, reliable, and easily adapted, the technique requires excessive sample handling, long run times, and high reagent consumption. For pigment analysis, issues with coelution, complex workouts for low-abundance pigments, and artifact formation are still unresolved.

Electron-transfer MALDI-TOF MS, on the other hand, selectively ionizes groups of compounds and separates them by mass. Complete analysis of pigment extracts can be completed in minutes. In addition to a speedy analysis, ET MALDI has advantages such as high sensitivity, selectivity, tolerance to impurities and contaminants, broad applicability, and the capability of complex mixture analysis [54–56]. Also, the technique's high sensitivity (pico to femtomole levels of the extract on the sample holder) and selectivity allows for detecting low-abundance compounds in the extract [48]. Recent reports show the importance of ultra-low-abundance pigments as vital biomarkers for identifying threatened/valuable/key organisms in phytoplankton communities. On the other hand, tolerance to contaminants is an attractive advantage for microalgae/phytoplankton analysis in MALDI MS, due to the presence of dissolved organic matter in these habitats and the micro and macronutrient requirements for microalgae growth. Finally, we believe ET MALDI can become a robust strategy for pigment analysis paving the way to phytoplankton chemotaxonomic identification using mass spectrometry.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Luis M. Díaz-Sánchez: Conceptualization, Methodology, Investigation, Validation, Writing – original draft. **Cristian Blanco-Tirado:** Funding acquisition, Project administration, Supervision. **Marianny Y. Combariza:** Conceptualization, Methodology, Supervision, Visualization, Resources, Funding acquisition, Project administration, Writing – review & editing.

Data availability

Data will be made available on request.

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