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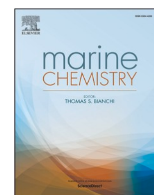

Design of experiment guided development of an UHPLC method for phytoplankton pigments analysis

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ABSTRACT

Phytoplankton pigment analysis by high-performance liquid chromatography (HPLC) is widely used to characterize community composition, support satellite algorithm validation, and advance aquatic biogeochemical research. However, conventional HPLC methods often rely on toxic solvents, require relatively long analysis times, and may have difficulty resolving structurally similar pigments such as mono- and divinyl chlorophylls and xanthophyll isomers. In this study, we developed and validated a novel ultra-high-performance liquid chromatography (UHPLC) method optimized for the separation of ecologically significant phytoplankton pigments. The method used a core–shell C8 column (100 × 4.6 mm, 2.6 μm) maintained at 60 °C with a linear gradient of ethanol and solvent B (56 mM tetrabutylammonium hydroxide in water adjusted to pH 7) over 22.5 min, followed by a 3-min hold at 100% ethanol, reducing solvent toxicity while maintaining chromatographic performance. Method development was guided by a Design of Experiments (DoE) approach to optimize the resolution of challenging pigment pairs, such as divinyl/monovinyl chlorophyll *a* and lutein/zeaxanthin. The final method achieved identification of 33 pigments and showed excellent reproducibility, with retention time variation below 0.6% and a peak area variation below 12%. Validation was performed using pigment standards, laboratory cultures, and natural samples from the northern Adriatic Sea, and by comparison an established validated method, demonstrating robust performance across a wide range of concentrations and matrix complexities. Overall, the study provided a rapid, reproducible, and environmentally safer alternative UHPLC method for phytoplankton pigment analysis in marine research and operational monitoring programs.

1. Introduction

Marine phytoplankton contribute nearly half of global primary productivity and play a central role in biogeochemical cycles and climate regulation (Behrenfeld et al., 2001; Demarcq et al., 2012; Alvain et al., 2013). Chlorophyll *a* (chl *a*), a key pigment present in all phytoplankton, is commonly used to assess biomass and primary production (Falkowski et al., 1998; Harding et al., 2016). However, chl *a* alone provides limited insight into community composition. Pigment-based chemotaxonomy is a robust approach that takes advantage of diagnostic pigments characteristic of specific taxonomic groups to infer phytoplankton functional composition (Mackey et al., 1996; Jeffrey et al., 1997; Vidussi et al., 1996; Uitz et al., 2006; Brewin et al., 2010; Roy et al., 2011). High-performance liquid chromatography (HPLC), and more recently ultra-performance liquid chromatography (UHPLC), are widely recognized as reference techniques for the qualitative and quantitative analysis of phytoplankton pigments in aquatic environments (Roy et al., 2011; Suzuki et al., 2015). Their high specificity and sensitivity, coupled with their ability to resolve complex pigment mixtures, make them fundamental tools for validating satellite-derived estimates of chl *a* (Joint Global Ocean Flux Study, 1994). Despite these strengths, pigment analysis via HPLC presents several analytical challenges. Phytoplankton pigments vary widely in polarity and include numerous structural isomers, which complicates their simultaneous separation and

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quantification. The mono- and divinyl forms of chlorophylls *a*, *b*, and *c* are particularly important due to their chemotaxonomic value, yet they are difficult to resolve chromatographically (Goericke and Repeta, 1993; Barlow et al., 1997). Over than thirty HPLC methods have been reported for pigment analysis (Roy et al., 2011), with most of which employ C8 or C18 reversed-phase columns and diode array detectors (Gieskes and Kraay, 1986; Wright et al., 1991; Goericke and Repeta, 1993; Barlow et al., 1997; Van Heukelem and Thomas, 2001; Zapata, 2005). Over the past few decades, the methods proposed by Wright et al. (1991), Zapata et al. (2000), and Van Heukelem and Thomas (2001) have been widely adopted in oceanographic research and in protocols for validating satellite data (Joint Global Ocean Flux Study, 1994; Jeffrey et al., 1997; Mueller et al., 2003; Roy et al., 2011). However, these methods traditionally involved relatively long analysis times (i.e., longer than 30 min), large sample injection and solvent volumes, and the use of toxic organic solvents such as methanol and acetonitrile. This raised concerns regarding throughput, environmental impact and operator safety. In response, to these concerns, recent advances have explored more efficient, environmentally friendly workflows. For instance, ethanol has been suggested as a safer alternative to methanol, achieving comparable extraction performance (Sanz et al., 2015), while UHPLC systems have enabled faster analyses without compromising pigment resolution (Suzuki et al., 2015).

As said, a key challenge in HPLC-based pigment analysis is achieving the simultaneous resolution of ecologically important pigment pairs. Examples include Chlorophyll *c1* (chl *c1*) and Chlorophyll *c2* (chl *c2*), and the monovinyl (MV) and divinyl (DV) forms of Chlorophyll *a* and *b* (MVchl *a* vs. DVchl *a*, and MVchl *b* vs. DVchl *b*). Divinyl Chlorophylls *a* and *b* are the primary photosynthetic pigments in the marine cyanobacterium *Prochlorococcus marinus*, which is a major contributor to phytoplankton biomass in subtropical and tropical ocean regions (Goericke and Repeta, 1993). Moreover, in order to avoid overestimating chlorophyll *a* in satellite ocean color observations, it is essential to chromatographically separate DVchl *a* from its MV counterparts (Jeffrey et al., 1997; Mueller et al., 2003). Comparative studies have assessed the performance of different stationary phases, including octylsilica (C8) and octadecylsilica (C18), in pigment separation. C8 phases often provide enhanced resolution of chlorophyll *c* variants and divinyl analogues (Mendes et al., 2007). On the other hand, C18 columns allow shorter run times and lower flow rates, offering advantages for detecting pigments at trace concentrations. Advancements in column chemistry have further improved pigment selectivity. Pentafluorophenyl (PFP) phases, combined with octadecyl spacers, have demonstrated enhanced selectivity toward structurally similar pigments. This configuration has enabled the simultaneous separation of nearly 70 pigments—including mono- and divinyl chlorophylls, as well as many taxonomically relevant carotenes and xanthophylls—in under 42 min (Sanz et al., 2015). Additionally, Jayaraman et al. (2011) used a C16-amide column method that successfully resolve several MV/DV pairs of chlorophylls *a* and *b*, as well as isomeric carotenoids such as lutein (lut) and zeaxanthin (zea).

The aim of the present study was to develop and validate a novel UHPLC method for phytoplankton pigment analysis that (i) reduces the toxicity of extraction and elution solvents, (ii) minimizes sample and solvent requirements, and (iii) shortens analytical run times, while maintaining or improving the resolution and reproducibility achieved by established HPLC methods used in satellite validation and chemotaxonomic studies. In a recent review, Sahu et al. (2018) highlighted the benefits of Design of Experiments (DoE) in HPLC method development and validation. In the present study, the UHPLC method development was guided by a DoE approach, optimizing key chromatographic parameters using pigment standards representative of typical phytoplankton pigment profiles, including the MV/DV chl *a* and zea/lut pairs. A variety of column chemistries and solvent systems were evaluated, including two core-shell C8 columns and one C18-phyenyl column. Following optimization, the method was validated using both laboratory cultures and natural samples collected during a year-long monthly monitoring campaign in the northwestern Adriatic Sea. The newly developed method was further validated against the widely used Van Heukelem protocol (2001) as implemented by Canuti (2023) to ensure compatibility with established reference procedures. This study contributes to the modernization of pigment analysis workflows, by offering a more sustainable, rapid, and scalable alternative for aquatic research, ecological monitoring, and satellite algorithm development.

2. Materials and methods

2.1. Seawater samples

Surface seawater samples were collected monthly from November 2023 to October 2024 at a depth of 0.5 m using Niskin bottles, approximately 3000 m offshore from the Foglia River (F3000: 43°56′55″N, 12°56′18″E; maximum depth 13 m) in the northwestern Adriatic Sea. Water samples were filtered onto Whatman GF/F filters (nominal pore size 0.7 μm; 25 mm diameter; Whatman, Dassel, Germany). In this study, filtration volumes ranged between 300 and 700 mL. More generally, the appropriate filtration volume should be selected according to the trophic conditions of the water body. When available, in situ measurements at the time of sampling—such as the absorption coefficient at 412 nm (Canuti, 2026)—can be used to guide the selection of an optimal filtration volume. Depending on phytoplankton biomass, suitable filtration volumes may range from approximately 50 mL under bloom conditions in small lakes, to up to 4000 mL in ultra-oligotrophic waters, thereby ensuring reliable pigment quantification across a wide range of environmental conditions. Filters were flash-frozen in liquid nitrogen and stored at −80 °C until extraction.

2.2. Algal cultures

Microalgal strains were selected for their diversity in pigment composition and for representing a variety of marine phytoplankton species, including some unique to the Adriatic Sea. These strains are also relevant for pigment-based chemotaxonomic and ecological applications. The cultured strains—listed with their designation indicated in parentheses—used in the present study were: *Navicula* sp. (CBA 2 (D1')), *Chaetoceros* sp. (CBA 13), *Skeletonema marinoi* (CBA 3D), *Cylindrotheca fusiformis* (CBA 82), *Prorocentrum micans* (CBA 21), *Scripsiella trochoidea* (CBA 2- F500), *Gymnodium impudicum* (GY6V), *Alexandrium minutum* (CNR-AMI V1), *Pavlova gyrans* (CCMP 608) and *Heterosigma akashiwo* (HA2V). Bacillariophyceae cultures were maintained in F/2 medium at 18 ± 1 °C. Dinoflagellate cultures were grown in F/4 —Si medium, while the classes Pavlovophyceae and Raphidophyceae were cultured in L1 medium at a temperature of 23 ± 1 °C. Illumination was provided by cool-white fluorescent bulbs, with a photon flux density of 100 μE m^{-2} s^{-1}, under a standard 14:10 h light–dark photoperiod. In addition, the *Synechococcus* (PCC 6911) was grown under dim lighting conditions and at a temperature of 22 °C (Wojtasiewicz and Stoń-Egiert, 2016). The strains were cultured at the Laboratory of Marine Ecology, University "Carlo Bo" of Urbino, Italy. For each strain, 10 mL of culture aliquots was added and gently mixed with sea water samples (1 L). An amount of 250 mL of this mixture was filtered under low vacuum through 25 mm diameter glass fiber filters (0.7 μm pore size) and immediately flash frozen in liquid nitrogen and preserved at −80 °C until analysis.

2.3. Pigment standards

Certified pigment standards used for method calibration, along with a pigment mixture (Mix-128, Lot No.) employed for method development and retention time verification, were obtained from DHI Laboratory Products (Hørsholm, Denmark). Each standard was accompanied by a certificate of analysis to ensure identity, purity, and concentration accuracy.

2.4. Pigment extraction

In the present study, we aimed to evaluate a less toxic extraction solvent that is more compatible with the mobile phase of the newly developed UHPLC method. The extraction time itself was not subject to optimization. Two extraction solvents were compared: acetone and ethanol. To this end, the extraction procedure described by Van Heukelem and Thomas (2001) and implemented by Canuti (2023) for acetone was adopted and applied to ethanol, with the only modification being the concentration of the internal standard. The procedure involved a total extraction time of at least 3 h, as recommended by Wasmund et al. (2006), and was also time-efficient.

To assess pigment recovery throughout the extraction process, α-tocopherol was used as an internal standard due to its high stability and absence of degradation products in both methanol and acetone, regardless of extraction time (Van Heukelem and Thomas, 2001; Esteban et al., 2009). The filters were cut into pieces and transferred into 10-mL polypropylene tubes (Corning Inc., Arizona, USA) containing 2.5 mL of an internal standard solution—either 0.025 g/L α-tocopherol (Honeywell, North Carolina, USA) in acetone or 0.0125 g/L α-tocopherol in ethanol (HPLC gradient grade, Merck, Darmstadt, Germany)—along with 150 μL of Milli-Q water. The samples were soaked for 1 h at −20 °C, sonicated for 90 s on ice using a probe sonicator (BANDELIN electronic, Berlin, Germany), and then soaked for an additional 3.5–4 h at −20 °C. After soaking, the resulting extract was filtered through a 0.2-μm Teflon syringe filter into an amber vial, vortexed, and transferred into an UHPLC amber vial. The samples were then stored at 4 °C in a thermostated autosampler until analysis. Wasmund et al. (2006) reported that, for ethanol extraction, extraction time has no significant influence within the range of 3 to 24 h. A similar assumption can be made for acetone extraction (Canuti et al., 2022). Based on this, all extracts were analyzed within 24 h from the start of the procedure to minimize the risk of sample degradation.

2.5. UHPLC Equipment

Pigment separation was carried out using an Agilent Infinity II 1260 UHPLC system, equipped with a quaternary pump (G7104A), diode array detector (G7115A DAD WR equipped with a 10 mm pathlength capillary cell), fluorescence detector (G1321A FLD), autosampler (G7129B), and a thermostated column compartment. Chromatograms were analyzed using OpenLab CDS software, with absorbance monitored at 436, 440, and 665 nm. Three different chromatographic columns were tested for performance and selectivity (specifications provided in Table 1). The mobile phase consisted of solvent A (ethanol, HPLC grade, Merck, Germany) and solvent B (28 mM tetrabutylammonium hydroxide [TBA-OH] in Milli-Q water, adjusted to pH 6 or 7 with acetic acid). The importance of flow rate, buffer pH, and column temperature in achieving efficient pigment separation is well documented (Snyder and Dolan, 2009). These parameters, along with gradient time and initial ethanol percentage, were systematically optimized using a DoE approach (see Section 2.6). A linear gradient elution was applied, and the experimental conditions were varied according to the DoE matrix. The linear gradient was followed by of 3-min at 100% of solvent A for all the analysis. A 3-min of re-equilibration step at initial conditions was included between injections. Sample injection was performed by sequentially loading 40 μL of sample followed by the ion-pairing reagent (28 mM TBA-OH in Milli-Q water) to reduce peak distortion, as recommended in previous studies (Zapata and Garrido, 1991; Latasa et al., 1996; Van Heukelem and Thomas, 2001). The amount of ion-pairing added to the sample was also matter of optimization in the DoE approach. All samples were equilibrated at the temperature of 4 °C in the UHPLC autosampler compartment before the injection.

Chlorophylls and carotenoids were detected using diode-array spectroscopy across the 270–800 nm range. Pigment identification was performed by comparing the retention times and spectral features of chromatographic peaks with those of authenticated standards or reference extracts from phytoplankton cultures. Peak purity was assessed by evaluating spectral homogeneity. The resolution, R_s, between two adjacent peaks (a and b) was calculated using the eq. [1]:

$$Rs, (a/b) = \frac{2(Rtb - Rta)}{(Wa + Wb)} \quad (1)$$

where Rt_a and Rt_b are the retention times of peaks a and b, respectively, and W_a and W_b are their corresponding baseline peak widths (Snyder and Dolan, 2009). Resolution of 1 is considered acceptable, 1.5 is considered desirable (Van Heukelem and Thomas, 2001; Snyder and Dolan, 2009).

2.6. Method development and validation

A structured DoE approach was used to develop and optimize a UHPLC method for separating chlorophylls and accessory pigments in phytoplankton samples, using ethanol as component of the mobile phase. The experimental design included both continuous and categorical variables. The categorical factors comprised column type—two C8 core-shell and one C18 reversed-phase columns (see Table 1)—and mobile phase pH (levels 6 and 7). These were selected to ensure adequate separation performance while preserving pigment stability, particularly for monovinyl chlorophylls.

Continuous variables were tested across defined ranges: gradient time (5–25 min), initial ethanol concentration (20–60%), column temperature (25–60 °C), flow rate (0.8–1.3 mL/min), and ion-pair volume added to sample prior to injection (0–40 μL of 28 mM TBA-OH).

An I-optimal design was selected to minimize the average prediction variance across the factor space, allowing robust model building while limiting the number of experimental runs. This design enabled the evaluation of key chromatographic performance metrics, including resolution between critical peak pairs (e.g., MV/DV chl *a* and chl *b*, lut/zea), peak symmetry, and pigment detectability (i.e., number of compounds effectively separated). The approach also contributed to a more resource-efficient method development process, aligned with the study's sustainability objectives. The experimental matrix and run randomization were generated using Design-Expert® software (see Supplementary Table S1).

Following optimization, the UHPLC method selected through DoE was evaluated for performance and robustness using a pigment mix-standard and pigment extracts from pure algal cultures. The strains included diatoms and dinoflagellates, as well as representative strains of cyanobacteria (*Synechococcus*), haptophytes (*P. gyrans*), and raphidophytes (*H. akashiwo*). Some of the selected strains were chosen to represent taxa typically found in the Adriatic Sea, the source of the

Table 1
Characteristics of the chromatographic columns used in this study.

Columns	Bonded phase	Dimensions (mm)	Pore size (μm)	Particle size (Å)	Surface area (m^2/g)	Carbon load
Kinetex Core-shell	C8	100 × 4.6	2.6	100	200	8%
Kinetex Core-shell	C8	150 × 4.6	5	100	200	8%
ACE C18-AR	C18-Phenyl	150 × 4.6	3	100	300	15.5%

seawater samples used for method evaluation. Moreover, the new method was compared with an existing validated method (Canuti, 2023). Natural seawater samples were analyzed using both methods, and statistical comparisons were conducted to assess linearity, limit of detection, precision, and reproducibility across pigment classes.

3. Results and discussion

3.1. Criteria for conditions used in the experimental design

Columns were selected based on their demonstrated or expected ability to resolve critical pigment pairs, focusing on two chemistries: C8 core-shell and C18-phenyl phases. The tested columns differed in bonding chemistry (monomeric C18-phenyl vs. core-shell C8), particle size, pore size, and surface area—factors that collectively affect retention strength and separation efficiency. Core-shell columns feature a solid core surrounded by a porous shell, where only the outer layer provides active sites for analyte interaction. This design reduces analyte diffusion paths and travel distances, producing sharper, narrower peaks with improved resolution and sensitivity. Their enhanced efficiency and lower backpressure enable faster analysis times while maintaining high separation performance—comparable to sub-2 μm fully porous columns, but with reduced solvent consumption. C18-phenyl columns, on the other hand, were expected to perform well particularly with ethanol-based mobile phases and in separating the critical co-eluting compounds (Sanz et al., 2015). In typical marine pigment analyses, sample injection volumes range from 100 to 170 μL (Wright et al., 1991; Zapata et al., 2000; Van Heukelem and Thomas, 2001; Mendes et al., 2007; Jayaraman et al., 2011; Sanz et al., 2015). The injection volume was reduced to 40 μL, which contributed to conserve the sample and also to achieve better peak shape and faster injections, supporting higher-throughput workflows. Ethanol was selected as the organic phase in a UHPLC system capable of withstanding higher pressures. As a stronger elution solvent with twice the viscosity of methanol (Snyder, 1978), ethanol allows the use of an alternative and less harmful mobile phase composition (Sadek, 2002; Plotka et al., 2013). Although the method followed a DoE approach, which prevented full optimization of each column individually, the main goal was to systematically evaluate how key parameters influence pigment separation while minimizing runs and allowing subsequent fine-tuning. The primary aim was to optimize pigment separation using a TBA-OH-based buffer while replacing methanol with ethanol as solvent A. Column temperature is known to influence retention and selectivity (α) in reversed-phase systems (Van Heukelem and Thomas, 2001; Snyder and Dolan, 2009; Sanz et al., 2015). Prior studies showed that changes of 20 °C or more can significantly impact α and resolution (R_s), as α does not vary linearly with temperature (Van Heukelem and Thomas, 2001; Snyder and Dolan, 2009). In particular, lower temperatures (30–40 °C) have been reported to improve the resolution of certain pigment classes on specific columns (Roy et al., 2011). Based on these findings, temperature ranging from 25 to 60 °C were tested. Gradient elution times ranged from 5 to 25 min, with a linear gradient consistently applied across all runs to isolate the effects of column performance and factor interactions from those related to mobile phase composition. Solvent B consisted of 28 mM TBA-OH, adjusted to pH 6 or 7, with ethanol used exclusively as solvent A to ensure sufficient elution strength for strongly retained analytes. Initial organic solvent content (20–60%) was also varied to assess its impact. Additional variables included the volume of buffer (0–40 μL) added to the sample prior to injection, which can influenced peak shape and reproducibility in ion-pairing systems, and flow rate (0.8–1.3 mL/min), which affected resolution and run time. Chromatographic performance was evaluated by examining the peak symmetry, the number of compounds separated and resolution of critical pigment pairs such as MV/DV chl *a* and chl *b*, zea/lut and prasinoxanthi/violaxanthin/19'-Hexanoyloxyfucoxanthin (pras/viol/hex). A total of 63 chromatographic runs were performed across the three columns (see Supplementary Table S1). These experiments showed insight into how critical method parameters, such as column temperature, gradient time, buffer addition, and initial organic content, interact with column chemistry and pH to affect the efficiency of pigment separation. The use of pigment standard mixtures was essential for benchmarking method performance and identifying resolution limitations before applying method to natural samples. These mixtures provided a controlled system, free from interference by unknown or minor peaks, making them particularly useful during the initial stages of method development. Chromatograms obtained from these mixtures allowed understanding the separation performance for several key pigment pairs thus providing a solid foundation for the accurate quantification and identification of pigments in complex phytoplankton assemblages (Van Heukelem and Thomas, 2001; Sanz et al., 2015; Suzuki et al., 2015). Mixtures of 28 pigments (i.e., mix-128 from DHI) were used to evaluate and refine the conditions for the optimal separation of taxonomically relevant pigments.

3.2. Method development: DoE results and method of choice

The results of the ANOVA from the DoE showed that the most influential factor affecting the separation of MV/DV chl *a* and chl *b* was the type of column used. For the separation of zea and lut, the initial percentage of solvent B (28 mM TBA-OH) was identified as the primary influencing factor, while the column type also had a moderate but statistically significant effect ($p = 0.0035$). Three factors were found to be significant regarding the total number of compounds separated: the elution gradient time, the initial percentage of solvent A and the flow rate. The latter showed a moderate yet statistically significant influence ($p = 0.0029$). In contrast, peak symmetry did not exhibit any significant dependence on the tested experimental factors. Among the tested columns, the C18-AR column provided the best separation for the α,β- and β,β-carotene isomers, as well as for the pras-viol-hex group. However, priority was given to the separation of MV/DV chlorophylls, which are rarely resolved in C18-phase columns under the short chromatographic run times tested in this study. Additional methodological considerations were made to improve pigment separation and sample compatibility. A comparison of pigment extraction in ethanol versus acetone was conducted using microalgal strains. Previous extraction test under identical condition and analyzed using the Canuti (2023) method, showed that comparable results in terms of pigment quantification can be achieved (Kratzer et al., 2022). In the present case, injecting the extracts in acetone led to a low front tailing effect in the early elueters (i.e., chl *c*3 and chl *c*2), whereas this effect was not observed when the ethanol extracts were injected in a more compatible solvent mobile phase (i.e., ethanol and buffer). Overall, the ethanol extracts showed improved chromatographic behavior and higher reproducibility (see section 3.4.).

The method selected based on the DoE analysis used a Kinetex C8 column (100 × 4.6 mm, 2.6 μm particle size) at 60 °C. The flow rate was set to 1.3 mL/min, and a linear ethanol–solvent B gradient was applied over 22.5 min, followed by a 3-min hold at 100% ethanol and a 1.5-min re-equilibration phase (Table 2). The initial proportion of solvent A was 40%. A volume of 40 μL sample was injected and mixed with an additional 40 μL of buffer. Both solvent A and the buffer used for sample dilution were adjusted to pH 7. Total UHPLC analysis time was of 25.5

Table 2
Gradient profile of the UHPLC used for the separation of chlorophylls and accessory pigments in microalgal cultures and seawater sample extracts (flow rate: 1.3 mL/min).

Time (min)	% Solvent A (Ethanol)	% Solvent B (Water Phase: 28 mM TBA-OH)
0	40	60
22.5	100	0
25.5	100	0
27	40	60

min.

Compared to the Van Heukelem and Thomas (2001), as implemented in Canuti (2023), this approach reduced solvent consumption by 25% and shortened the total chromatographic analysis time (including column equilibration and injection) from 40 min to 25.5 min. The method successfully identified 33 pigments (Fig. 1): the retention times, the wavelength of quantification and the other main information were reported in Table 3. The pigments elution order (Table 3) was similar to other method using C8 as stationary phase and methanol and/or ethanol as mobile phases (i.e., Zapata et al., 2000; Van Heukelem and Thomas, 2001; Sanz et al., 2015). Using this method, good resolution (i.e., $R_s > 1.5$) of zea and lut (peak 20 and 21 respectively in Fig. 1) was achieved, as well as of MV and DV chl *a* (peak 28 and 29 in Fig. 1). In addition, several other challenging and taxonomically important pigment pairs were successfully resolved, including phaeophorbide *a* and peridinin ($R_s > 1.5$), 19′-butanoyloxyfucoxanthin ($R_s > 1.5$), and fucoxanthin and 9′-cis-neoxanthin ($R_s > 1.5$). For chlorophyll *c*1 and *c*2, which were not both present in the mix-128 lot, a mixture of the two standards was used for checking the separation. However, pigment such as viol and pras, MV/DV chl *b* ($R_s = 0.58$), and α,β-carotene and β,β-carotene ($R_s = 1.02$), displayed only partial resolution under the tested gradient conditions, indicating some degree of co-elution. Further optimization was required for these pairs, such as fine adjustments in gradient slope (i.e., longer analytical time) and adjustments to the ion-pairing solution to achieve full baseline separation (see section 3.3).

3.3. Method optimization

Targeted tests were conducted using extended run times (32.5 and 40 min) and three different Solvent B buffer molarity (56 mM, 112 mM, and 224 mM TBA-OH) to enhance the separation of overlapping pigments, particularly pras, viol, and hex which partially co-eluting in the method of choice, and MV/DV chl *b*. These trials aimed to optimize the resolution of these critical pigment groups, especially in complex phytoplankton matrices. Increasing the gradient time to reach 100% ethanol in phase A from 22.5 to 32.5 min only partially resolved the pras/hex co-elution, with the resolution improving from 0.47 to 0.66, while viol and pras remained co-eluting. Similarly, a further extension of the gradient time to 40 min did not resolve the viol/pras co-elution. In addition, testing longer run times was considered out of the scope of the present study.

An additional test was conducted using the method of choice (25.5 min run time) with different Solvent B molarities. Specifically, 56, 112, and 224 mM TBA-OH, adjusted to pH 7, were tested using the mix-128 standard. The use of 56 mM TBA-OH at pH 7 resulted in improved resolution between viol, pras, and hex. Compared to the initial co-elution of viol/pras and a resolution of 0.47 for pras/hex, resolutions of 1.3 (viol/pras) and 0.88 (pras/hex) were achieved, respectively (Fig. 2). Under these conditions, the resolution between MV/DV chlorophyll *b* also slightly improved, from 0.58 (with 28 mM TBA-OH) to 0.72 (with 56 mM TBA-OH). Further increases in ion-pairing reagent concentration did not improve the separation of viol, pras, and hex, instead reduced the separation of acidic chlorophylls (data not shown). Moreover, higher ion-pairing molarities caused noticeable peak broadening.

3.4. Method performance: calibration and reproducibility

Calibration was performed using certified pigment standards (DHI Laboratory Products, Denmark) diluted at four concentration levels with the lowest level corresponding to approximately three times the signal-to-noise ratio (SNR). The limit of detection (LOD) for each pigment was determined based on the lowest quantifiable concentration from the standard dilutions. The average LOD across 22 pigments was 0.42 ± 0.26 ng/injection (mean ± standard deviation), corresponding to 0.012 ± 0.006 mg/L of standard.

Calibration curves exhibited strong linearity, with coefficients of determination (R^2) greater than 0.99 for all 24 pigments, except for DVchl *b* ($R^2 = 0.89$). The response factors (RFs), expressed as slope values, varied between compounds, ranging from 1.22 for DVchl *b* to 9.37 for viol, reflecting intrinsic differences in detector response and molar absorptivity. For MVchl *a*, a RF of 3.13 was observed, in agreement with values previously reported for the Van Heukelem method (Van Heukelem and Thomas, 2001) (Supplementary Fig. S1).

Residuals were calculated as the difference between the measured peak area and the theoretical peak area predicted by the calibration curve at the corresponding concentration. This residual analysis was used to assess both the goodness of fit of the calibration model and potential systematic deviations across the concentration range. As shown in Supplementary Fig. S1, residuals were generally symmetrically distributed around zero, indicating the absence of significant bias and supporting the validity of the linear calibration for most pigments. The magnitude of the residuals was quantified using the root mean square error (RMSE). Low RMSE values were obtained for most compounds (e.g., MVchl *b*: 0.69; chl *c*1: 0.73), reflecting high analytical precision and stable detector response. In contrast, MVchl *a* (RMSE: 9.75) and alloxanthin (Allo; RMSE: 8.63) showed higher dispersion, particularly at the lower end of the concentration range. This behavior was consistent with increased relative uncertainty near the detection limit, where signal-to-noise ratios are lower. These trends were clearly visible in the residual plots, which showed larger deviations from zero at low concentrations (Supplementary Fig. S1), while residuals remained small and randomly distributed at medium and high concentrations.

Method performance was evaluated using two sets of replicate injections of a pigment standard (Mix-128): one undiluted set of six injections and one diluted set of five injections at ratio of 1:10. In the undiluted set, pigment concentrations ranged from 5.1 to 269.9 ng per

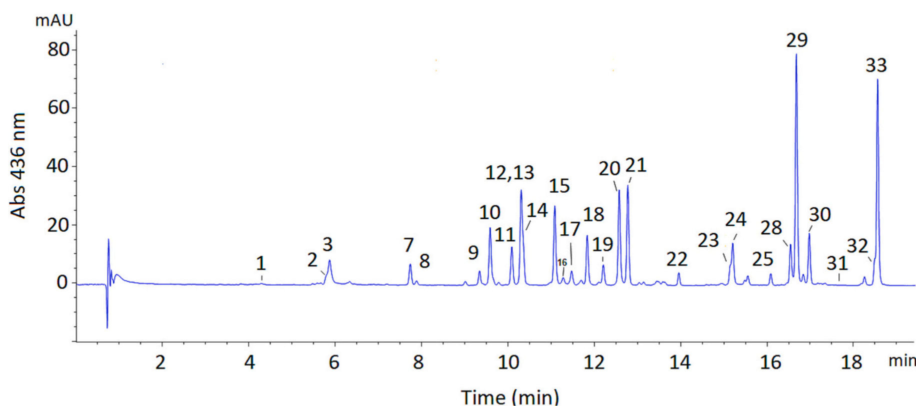

Fig. 1. UHPLC chromatogram of the DHI mix-128 pigment mixture for DoE the selected method. Refer to Table 3 for pigment peak identification.

Table 3
Phytoplankton pigments and their identification sources, retention time (t_r), resolution (R_s) and maximum wavelength (λ_{max}) values as determined with the UHPLC. If not indicated, R_s is assumed to be >1.5.

Peak no.	Pigment name	SCOR WG 78	Source*	t_R (min)	R_s	Absorption max (nm)			Quantified (nm)
1	Chlorophyll *c*3	Chl *c*3	DHI-mix, DHI-std, A	4.26		454	592	638	436
2	Chlorophyll *c*2	Chl *c*2	DHI-mix, DHI-std, A- I	5.82		446	582	634	436
3	Mg 2,4 divinyl pheoporphyrin a_5 monomethyl ester	Mg-DVP	DHI-mix, DHI-std	5.86	0.41 (2/3)	442	632		436
4	Chlorophyll *c*1	Chl *c*1	DHI-std, B—C, H-I	6.02		442	581	634	436
5	Chlorophyllide *a*	Chlide *a*	DHI-mix, DHI std., C	6.32		434	468		665
6	Pheophorbide *a*	Pheo *a*	DHI-std, B-C	6.47		412	668		665
7	Peridinin	Peri	DHI-mix, DHI-std, D-G	7.61		480			436
8	Peridinin isomer	Peri iso	DHI-mix, D-G	7.70	1.45 (7/8)	484			436
9	19'-Butanoyloxyfucoxanthi	But	DHI-mix, DHI-std	9.37		450			436
10	Fucoxanthin	Fuco	DHI-mix, DHI-std, A-D, G-I	9.53		456			436
11	Neoxanthin	Neo	DHI-mix, DHI-std	10.04		438	468		436
12	Prasinoxanthin	Pras	DHI-mix, DHI-std	10.25	co-elute (Pras, Viol)	460			436
13	Violaxanthin	Viol	DHI-mix, DHI-std, I	10.26	co-elute (Pras, Viol)	442	472	458	436
14	19'-Hexanoyloxyfucoxanthin	Hex	DHI-mix, DHI-std	10.32	0.47 (13/14)	450	470	466	436
15	Diadinoxanthin	Diad	DHI-mix, DHI-std, A-H	11.05		448	478	464	436
16	Dinoxanthin	Dino	DHI-mix, DHI-std, D-F	11.26		444	472	460	436
17	Antheraxanthin	Anth	DHI-mix, DHI-std	11.40		448	464	476	436
a	Unknown (myxo-xantholike spectra)		L	11.50					436
18	Alloxanthin	Allo	DHI-mix, DHI-std, C	11.81		454		482	436
19	Diatoxanthin	Diat	DHI-mix, DHI-std, C, F, H	12.18		454	480	472	436
20	Zeaxanthin	Zea	DHI-mix, DHI-std, C, I, L	12.53		458	480	470	436
21	Lutein	Lut	DHI-mix, DHI-std	12.74		446		474	436
22	Gyroxanthin	Gyro	DHI-mix, DHI-std	13.95		448		472	436
23	DV Chlorophyll *b*	DVchl *b*	DHI-mix, DHI-std	15.18		474	652		436
24	MV Chlorophyll *b*	MVchl *b*	DHI-mix, DHI-std	15.20	0.58 (23/24)	464	650		436
25	Crocoxanthin	Croco	DHI-mix,DHI-std	16.06		448	474		436
26	Chlorophyll c2-monogalactosyldiacylglyceride	Chl-c2-MGDG	DHI-std, H	16.10		460	590	638	436
27	Echinenone	Echi	DHI-std	16.12		466			436
28	DV Chlorophyll *a*	DVchl *a*	DHI-mix, DHI-std	16.51	1.48 (27/28)	442	622	666	665
29	MV Chlorophyll *a*	MVchl *a*	DHI-mix, DHI-std, A-L	16.65		416	432	666	665
30	Chlorophyll *a* epimer	Chl *a*'	DHI-mix, DHI-std, A-H	16.98		416	432	666	665
31	Pheophytin *a*	Phy *a*	DHI-mix, DHI-std	17.27		410	664		665
32	β,ε-carotene	β,ε-car	DHI-mix, DHI-std	18.50		446	474	462	436
33	β,β-carotene	β,β-car	DHI-mix, DHI-st, A-L	18.53	1.02 (32/33)	452	478	470	436

* DHI-Mix: DHI standard of mixed phytoplankton pigments, DHI-Std: DHI quantitative standard; A: *Navicula* sp. (CBA 2 (D1')); B: *Chaetoceros* sp. (CBA 13); C: *Skeletonema marinoi* (CBA 3D); D: *Gymnodium impudicum* (GY6V); E: *Alexandrium minutum* (CNR-AMI V1); F: *Scripsiella trochoidea* (CBA 2- F500); G: *Prorocentrum micans* (CBA 21); H: *Pavlova gyrans* (CCMP 608); I: *Heterosigma akashiwo* (HA2V); L: *Synechococcus* (PCC 6911).

injection, covering a range of retention times from 5.8 to 18.5 min. The relative standard deviation, RSD% (also known as coefficient of variation, CV%) was considered as metric. The overall variation in peak area quantification across all pigments was 12.2%, with lower variability observed for major pigments such as MVchl *a* (0.9%) and primary pigments (7.8%). Retention time reproducibility across three separate sequences was also high, with a maximum variation of 0.6% observed for all pigments.

For the diluted standard (1:10), the analysis included 20 quantified pigments, covering a range of retention times from 5.8 to 18.5 min. The concentration in the injected samples ranged from 0.3 to 7.6 ng per injection. Retention time reproducibility remained high, with an average RSD% of 0.7% (range: 0.0–3.5%). Peak area reproducibility was more variable, with an average RSD% of 14.6% (range: 2.2–32.7%).

Overall, the method proved robust and reproducible for the majority of pigments, with negligible bias and tight residuals, supporting its applicability for quantitative pigment profiling in environmental samples.

3.5. Method validation on pigments mixture: comparison with HPLC methods

The pigment concentrations obtained using the new UHPLC method for the pigment mix-128 were compared with the mean values reported by the quality reference group participating in the international intercomparison exercise on phytoplankton pigments (HIP-8; Canuti et al., 2025). Relative percent differences (RPD%) between the two datasets were calculated. For chl *a* (defined as the sum of MVchl *a,* DVchl *a* and chlide *a*), the RPD was only 7.9%, while the average deviation across all other pigments was approximately 25%. The highest deviations were observed for chl *b* (defined as the sum of MVchl *b* and DVchl *b*), hex, and *peri*dinin (peri), each exceeding 35%. For the remaining pigments, the deviations remained below 25%. These results further supported the suitability of the method as a candidate reference method for phytoplankton pigment analysis. (Table 4).

3.6. Sample extraction: solvent evaluation

The choice of solvent for chlorophyll extraction—such as acetone, ethanol, or methanol—has long been debated (Jeffrey et al., 1997). In

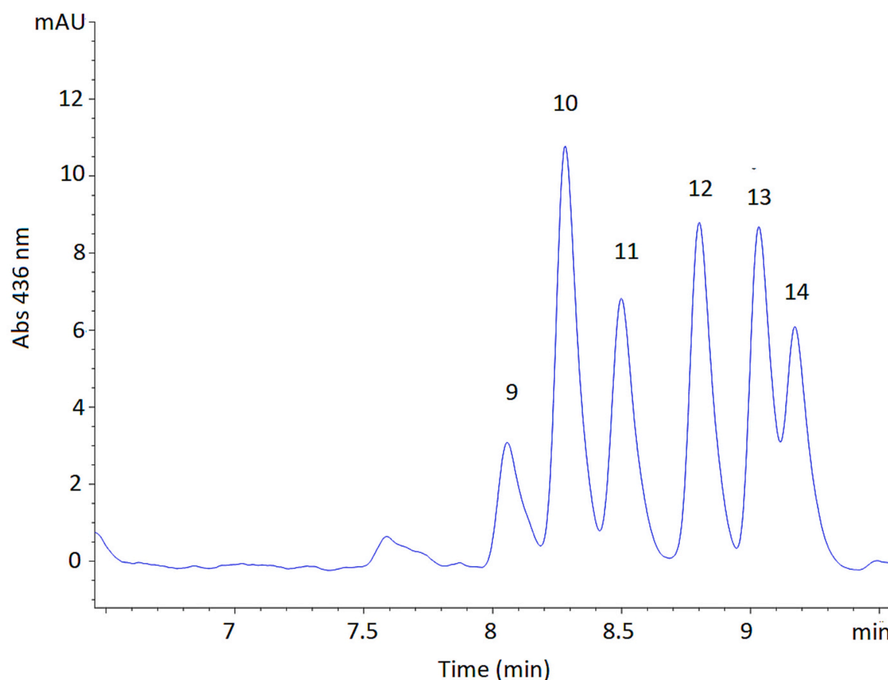

Fig. 2. Improved separation of co-elutes by increasing in the solvent A the molarity of TBA-OH to 56 mM.

Table 4
Comparison of pigment concentrations (mg/L) in the standard mix-128 between the HIP-8 inter-comparison quality reference group (Canuti et al., 2025) and the new UHPLC method expressed as relative percent difference (RPD%).

	mix-128 [mg/L]		
	HIP-8 inter-comparison quality reference group	new UHPLC method	RPD%
Chl *a*	2.912	3.153	7.9
Chl *b*	1.113	0.779	−35.3
Chl *c*	0.217	0.175	−21.5
Caro	0.876	0.677	−25.6
But	0.083	0.070	−17.1
Hex	0.128	0.192	39.7
Allo	0.178	0.205	13.9
Diad	0.283	0.342	18.9
Diat	0.069	0.075	8.0
Fuco	0.292	0.349	17.7
Peri	0.273	0.404	38.6
Zea	0.436	0.395	−9.8

marine research, 90% acetone has traditionally been used (Jeffrey et al., 1997; Roy et al., 2011), while 96% ethanol is recommended by HELCOM (2001). For freshwater samples, 90% ethanol is the prescribed solvent according to DIN 38412 (DIN 38412, 1985) and ISO 10260 (1992). Wasmund et al. (2006) observed that generally, chl *a* concentrations tend to be lower in acetone extracts compared to ethanol, particularly in *Phaeodactylum* and *Microcystis*. Fernandes et al. (2024) reported higher carotenoid, xanthophyll and chlorophyll concentrations in ethanol extracts of *Scenedesmus obliquus* and *Arthrospira platensis* than in acetone extracts. Overall, the extraction efficiency of the various solvents seems to depend on factors, such as the taxonomic composition of the algal community (Wasmund et al., 2006).

To evaluate the reproducibility of pigment extraction from cultures, chl *a* concentration was measured on duplicate samples from cultures extracted using ethanol and acetone. The RSD% for the MVchl *a* peak area was 3.5% for ethanol extracts and 8.7% for acetone extracts of *C. fusiformis* culture. Similar results were obtained for the MVchl *a* quantified in the *Pavlova* strain, having 3.6% for ethanol and 7.4% for the acetone. When considering all four replicates (two for each solvent), the overall inter-replicate variation was 14.6%, with values of 7.1% and 8.7% for the *C fusiformis* and the *P. gyrans* respectively. This indicated that there were no systematic differences between the extraction methods. Ethanol yielded slightly more consistent results, and notably, did not lead to any degradation in reproducibility.

It is important to note that pigment extractions from algal cultures is inherently variable and technically challenging. Therefore, these values should be regarded as approximate indicators of performance rather than absolute benchmarks (Canuti et al., 2025). Nevertheless, the observed variability remains within 10% for the MVchl *a,* supporting the suitability of ethanol as an extraction solvent (Table 5). Furthermore, pigment-specific reproducibility was evaluated for key chemotaxonomic markers: fuco for diatoms, peri for dinoflagellates, and chl *c*2-MGDG for haptophytes. These pigments were selected because they provide a link between pigment profiles and taxonomic groups (i.e., markers). As shown in Table 5, ethanol extractions generally resulted in lower mean variation for these markers compared to acetone, supporting its suitability for accurate quantification across major phytoplankton groups. However, an exception was observed for *A. minutum*, where acetone yielded a lower RSD for chlorophyll *a* (2.2%) compared to ethanol (10.2%), suggesting that extraction efficiency may still vary by species and pigment composition. Moreover, for all the species considered, the extraction in ethanol yielded higher recovery of both MVchl *a* (from 3% in *Navicula* to 62.6% of *Chaetoceros*) and pigments, with the only exception of haptophytes, where the acetone was more efficient in extracting pigments (Table 5). These findings are consistent with the previous results reported (Wasmund et al., 2006; Kratzer et al., 2022; Fernandes et al., 2024), which reinforce ethanol's applicability in the phytoplankton analysis for microalgal samples. As final remark, the formation of degradation products was not observed in the ethanol-extracted samples, in comparison with acetone-extracted samples.

Table 5
Pigment quantification reproducibility in algal cultures using ethanol (Eth-OH) and acetone (Ac) as extraction solvents. Mean variation between replicates (expressed as RSD%) are reported for (A) MVchl *a* and (B) for selected taxon-specific marker pigments: fuco (diatoms), peri (dinoflagellates), and c2-mgdvp, (haptophytes). For each pigment, the values obtained with ethanol and acetone in all extracts are shown. The difference in extraction efficiency between Eth-OH and Ac was expressed as Peak Area Relative Difference [%].

(A) Solvent / Metric	*Navicula* sp.	*Chaetoceros* sp.	*C. fusiformis*	*P. micans*	*S. trochoidea*	*G. impudicum*	*A. minutum*	*P. gyrans*
RSD% Eth-OH	1.3	7.8	3.4	6.8	1.9	6.7	10.2	3.7
RSD% Ac	11.3	8.4	8.6	8.4	9.8	9.7	2.2	7.4
RSD% All	6.7	18.4	14	6.9	6.3	6.3	6.3	7.5
Peak Area RD [%]*	3	62.6	20.4	−4	4	12.6	12.4	−9.2
(B) Solvent / Metric	***Navicula* sp.**	***Chaetoceros* sp.**	***C. fusiformis***	***P. micans***	***S. trochoidea***	***G. impudicum***	***A. minutum***	***P. gyrans***
Main Pigment	Fuco	Fuco	Fuco	Peri	Peri	Peri	Peri	c2-MGDG
RSD% Eth-OH	5	4.8	4.3	3.6	3.9	4.9	10.1	4.6
RSD% Ac	11.2	9.3	9.1	4.3	8.6	8.8	2.9	7.8
RSD% All	10.3	26.8	18.8	8.2	14	14	14	6.8
Peak Area RD [%]*	12.6	37	26.9	12.2	20	30.3	21.2	−7.6

* Peak Area Relative Difference [%] = (ethanol − acetone) / max(ethanol, acetone) × 100

3.7. Analytical performance assessment: quantification of algal cultures

To assess the analytical performance of the UHPLC method in resolving taxonomically relevant pigments, species-specific strain cultures of phytoplankton representing key functional groups were quantified (Fig. 3).

All diatom strains (*Navicula* sp., *Chaetoceros*, *S. marinoi*,) exhibited prominent fucoxanthin peaks, confirming the method's ability to identify this primary carotenoid marker with high specificity. Additionally, chls *c1* and *c2* were clearly resolved in both *Chaetoceros* and *S. marinoi*, demonstrating the method's efficiency in separating closely eluting chlorophyll derivatives. Only *Navicula* presented chl *c*3, while chl *c*1 was not detected (Méléder et al., 2003; Costa et al., 2022). Chlorophyllide *a* and pheophorbide *a*, were detected in *S. marinoi* and in *Chaetoceros,* both in ethanol and acetone extract. Among the dinoflagellates, *P. micans*, *S. trochoidea*, *G. impudicum*, and *A. minutum* presented *peri*, which was clearly resolved and exhibited excellent peak symmetry, even at lower concentrations. These findings provide further support for the suitability of the method for detecting group-specific pigments across a range of retention times. Both the haptophyte *P. gyrans* and the raphidophyte *H. akashiwo* contained chls *c*1 and *c*2, with a high level of resolution between these components. A minor peak, which was attributed to chl *c*2-MGDG was uniquely observed in *Pavlova*, highlighting the method's sensitivity to lesser-known accessory pigments. Zeaxanthin was identified in both *Heterosigma* and *Synechococcus*, consistent with their known pigment composition. As expected, *Synechococcus* lacked chlorophyll *c* pigments, confirming its cyanobacterial lineage. These results were consistent with previous analyses on these algal cultures performed with different analytical methods (Wright et al., 1991; Van Heukelem and Thomas, 2001; Méléder et al., 2003; Zapata et al., 2004; Sanz et al., 2015; Costa et al., 2022; Nogueira et al., 2022; Wang et al., 2022; Bérard et al., 2024), demonstrating the robustness and taxonomic resolution offered by the UHPLC method. The clear separation of major and minor pigment components across phylogenetically diverse cultures supports the use of this method for identifying functional groups and species in complex natural phytoplankton assemblages.

3.8. Method validation on natural samples: comparison between UHPLC and HPLC methods

The agreement between UHPLC and HPLC methods for pigment quantification was assessed in 10 natural phytoplankton samples collected from the Adriatic Sea using both linear regression and Bland–Altman analyses (Bland and Altman, 1999). Only pigments for which at least four paired measurements were available from both methods were considered. The statistical results, including slope, intercept, R^2, mean bias, RPD%, and absolute percent differences (APD %), were summarized in Table 6. These metrics were calculated for each compound, accounting for the range of concentrations observed, and further visualized through Bland–Altman plots (Supplementary Figs. S2 and S3), to evaluate consistency and potential systematic differences across concentration levels.

Strong agreement between UHPLC and HPLC was observed for pigments with high numbers of measurements (n) and low dispersion, including fucoxanthin (fuco, $n = 10$), diadinoxanthin (diad, $n = 10$), MVchl *a* ($n = 10$), chl *c*2 ($n = 9$), and β,β-carotene (β,β-caro, $n = 9$). For these pigments, R^2 values were high (0.95–1.00), slopes were close to unity (0.87–1.08), bias was low (≤0.17 μg/L), and APD% values were mostly below 20% (except chl *c*2, APD% = 41.5%, reflecting moderate variability). Bland–Altman plots confirmed narrow 95% limits of agreement and minimal proportional error, indicating robust inter-method reproducibility. Fuco, despite a slightly reduced slope (0.89), maintained excellent agreement, supported by minimal bias (−0.03 μg/L) and RPD% (9.9%). MVchl *a* showed an excellent correlation (R^2 = 1.00), but a consistent negative bias (−0.17 μg/L) and increasing variability at higher concentrations, as seen in its Bland–Altman plot. This suggests a systematic underestimation by UHPLC relative to HPLC, potentially due to matrix effects. Nevertheless, the APD% for chlorophyll *a* was 15.5%, which is within the acceptable threshold for satellite validation applications (i.e., 15%), as suggested by Hooker and McClain (2000). Therefore, despite the observed underestimation, UHPLC quantification of chl *a* remains suitable for satellite data product validation purposes. Pigments with moderate agreement included *peri*, (n = 6), MVchl *b* ($n = 6$), hex ($n = 5$), and diatoxanthin (diat, $n = 5$). These pigments showed generally good R^2 values (0.84–0.98) but higher RPD % (up to 75.1% for peri) or moderate bias, and Bland–Altman plots indicated wider limits of agreement or slight proportional errors. These results suggest that quantification was reasonably reliable but should be interpreted with caution in sensitive applications. Pigments with lower confidence due to limited data points included chl *c*3 ($n = 4$) and chlorophyllide *a* (chlide *a*, $n = 4$). Although R^2 values ranged from 0.84 to 1.00, the small number of paired measurements and moderate APD% (28.4–48%) reduce confidence in the reproducibility of these compounds.

Overall, UHPLC provided excellent agreement with HPLC for 5 pigments with both high n and low APD% (fuco, diad, MVchl *a*, β,β-caro, chl *c*2), moderate agreement for 5 pigments (allo, peri, MVchl *b*, hex, diat), and limited reliability for pigments with low n (chl c3, zea, chlide *a*).

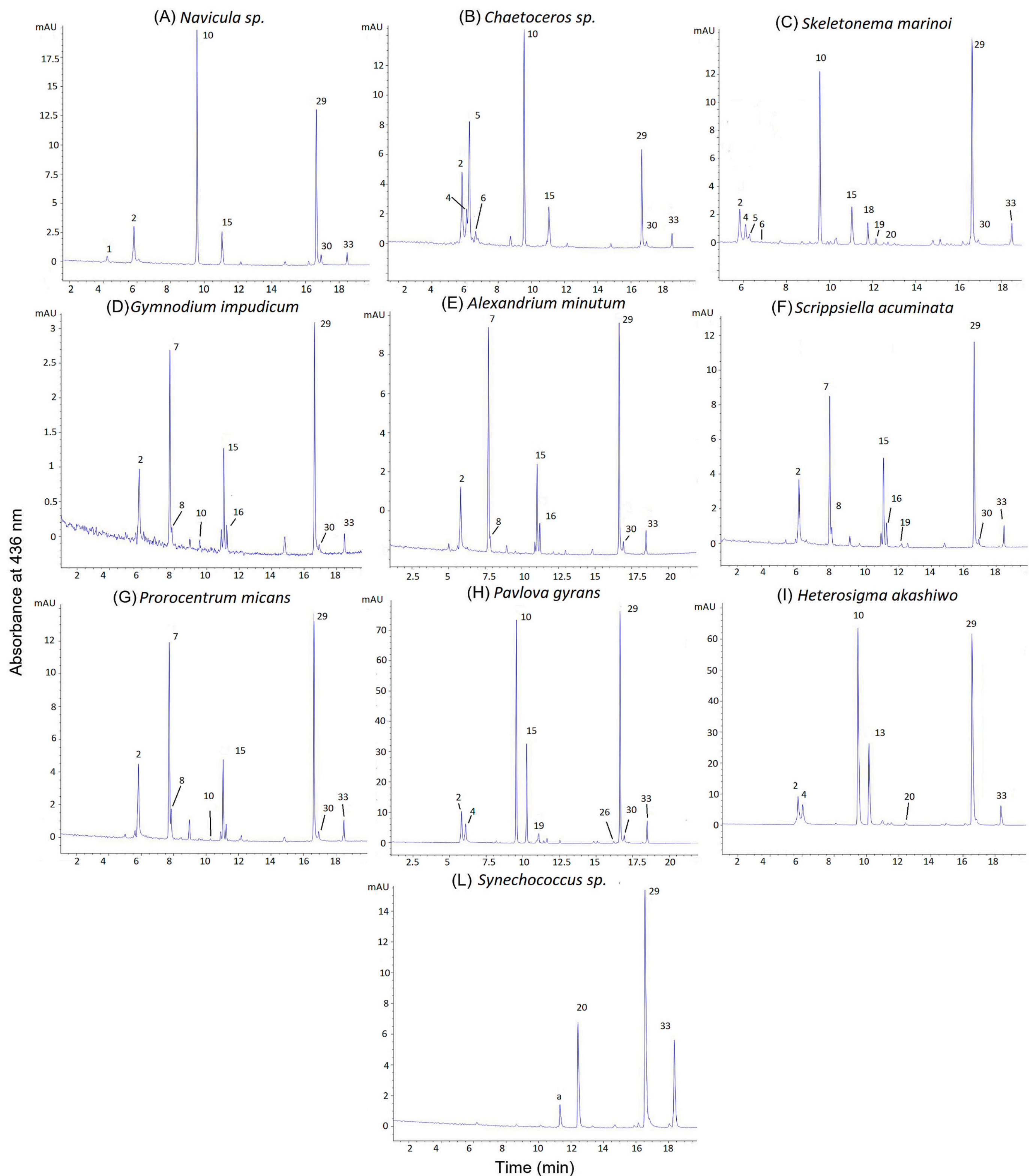

Fig. 3. Chromatograms of pigment extracts in ethanol from selected microalgal cultures. UHPLC chromatograms recorded at 436 nm for 10 different microalgal strains, including diatoms (e.g., (A) *Navicula* sp., (B) *Charatoceros* sp.,(C) *S.marinoi*), dinoflagellates (e.g., (D) *G. impudicum*, (E) *A. minutum*, (F) *S. acuminata,* (G) *P. micans*), haptophytes ((H) *P. gyrans*), raphidophytes ((I) *H. akashiwo*), and cyanobacteria ((L) *Synechococcus* sp.).

Pigments with APD% less than 25% can be considered acceptable for the development of bio-optical algorithms. This applies to several compounds such as MVchl *a*, allo (APD% = 9.6%), fuco (9.9%), diad (19.6%), and β,β-caro (14.2%) all of which showed negligible biases and high correlation values. These findings highlight the importance of considering the number of measurements, APD%, and Bland–Altman analysis when evaluating method performance, particularly for applications such as bio-optical algorithm development or satellite validation.

Table 6
Statistical comparison of pigment concentrations measured by UHPLC and HPLC in natural water samples from the Adriatic Sea.

Pigment	Slope	Intercept	R^2	Bias (μg/l)	Rpd%	Apd%	Min	Max	Mean	N
Chl *c3*	0.24	0.05	0.84	0.03	−48.4	48.4	0.0511	0.0641	0.0571	4
Chl *c2*	1.08	0.01	0.95	0.03	−14.9	41.5	0.0403	1.0482	0.2936	9
Peri	1.4	0.06	0.98	0.08	−73.8	75.1	0.063	0.2077	0.1391	6
Fuco	0.89	0.02	1	−0.03	−1.2	9.9	0.0562	1.024	0.3667	10
Hex	0.77	0.03	0.84	0.01	26.3	59.4	0.0239	0.0527	0.0635	5
Diad	1.04	0.01	0.98	0.01	−19.6	19.6	0.0373	0.278	0.1036	10
Allo	0.78	0.01	1	0	3.2	9.6	0.0155	0.0583	0.0322	6
Diat	0.61	0.01	0.84	0	−11.6	12.7	0.0132	0.0152	0.0197	5
Zea	0.79	0	1	−0.03	25.4	25.4	0.0311	0.0311	0.0945	4
MVchl *b*	0.42	0.06	0.43	0.03	−24.3	32.2	0.0305	0.1478	0.0869	6
β,β-Caro	0.8	0.01	0.97	0	4.1	14.2	0.0191	0.0945	0.0457	9
chlide *a*	0.61	0.05	0.96	−0.04	28.4	28.4	0.0798	0.415	0.2026	4
MVchl *a*	0.87	−0.02	1	−0.17	15.5	15.5	0.2618	3.1168	0.9728	10

4. Conclusion

This study presented a novel UHPLC method for the rapid, reproducible, and environmentally conscious analysis of phytoplankton pigments. The method used a Kinetex C8 column (100 × 4.6 mm, 2.6 μm particle size) thermostatted at 60 °C. The flow rate was set to 1.3 mL/min, and a linear ethanol–solvent B gradient was applied over 22.5 min, followed by a 3-min hold at 100% ethanol and a 1.5-min re-equilibration phase. The initial proportion of solvent A was 40%. A volume of 40 μL sample was injected and mixed with an additional 40 μL of buffer. Solvent B and the buffer used for sample dilution consisted of 56 mM TBA-OH, adjusted to pH 7. Total UHPLC analysis time was of 25.5 min. The method achieves identification of 33 key pigments, including taxonomically important carotenoids and challenging monovinyl/divinyl chlorophyll *a* pair, by replacing methanol with ethanol — a less toxic solvent with lower disposal costs — and employing a DoE approach for chromatographic optimization. Unlike other pigment analysis protocols that relied on ternary gradients (e.g., Wright et al., 1991; Jayaraman et al., 2011) and thus required specialized quaternary pumping systems, this method utilized a binary elution gradient compatible with standard high-pressure binary UHPLC systems. Notably, it enabled the simultaneous separation of both polar (e.g., chlorophyll *c* and protochlorophyllides) and non-polar (e.g., chlorophyll *a*) monovinyl/divinyl pigments. Compared to the widely adopted Van Heukelem and Thomas (2001) method, the proposed approach reduced solvent consumption by 25% and shortened the total analysis time to 25.5 min, without compromising pigment resolution or quantification accuracy. The ethanol extraction proposed procedure for the natural samples maintained the same duration as previously established acetone extraction methods (Van Heukelem and Thomas, 2001; Canuti, 2023), while using a greener solvent. Validation of the method using standards, pigment mixtures, algal cultures, and natural samples from the northern Adriatic Sea confirmed its robustness and applicability across complex aquatic matrices. Pigment-specific uncertainty analysis, based on percentage difference, supported the method's suitability for satellite validation (e.g., for chlorophyll *a*) and bio-optical algorithm development, as the uncertainty remained below 15%. For a subset of minor pigments, including chlorophyll *c*3, chlorophyllide *a*, peridinin, and diatoxanthin, slightly higher variability or proportional bias was observed, while monovinyl/divinyl chlorophyll *b* pair were not fully resolved: this suggests that compound-specific caution may be warranted when precise quantification is required. Increasing the concentration of the ion-pairing reagent improved the resolution of 19′-hexanoyloxyfucoxanthin and successfully resolved the co-elution between prasinoxanthin and violaxanthin. Nevertheless, the proposed UHPLC method offers a robust, sustainable, and scalable solution for phytoplankton pigment analysis. Its balance of efficiency, accuracy, and environmental safety makes it a suitable candidate for routine application in ecological monitoring, chemotaxonomy, and ocean color validation workflows, particularly for the majority of pigments that demonstrated strong inter-method agreement.

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CRediT authorship contribution statement

Elisabetta Canuti: Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Samuela Capellacci:** Investigation. **Silvia Casabianca:** Investigation. **Fabio Ricci:** Investigation. **Antonella Penna:** Writing – review & editing.

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Declaration of competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Data availability

Data will be made available on request.

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