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EARTH OBSERVATION DATA VALIDATION: IMPLEMENTATION AND PERFORMANCE EVALUATION OF METHODS FOR CHLOROPHYLL*a* RETRIEVAL

Thesis in: Chemical analysis of environmental quality

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Abstract

High-Performance Liquid Chromatography (HPLC) allows determining the concentrations of phytoplankton pigments and is the most used technique to validate Earth Observation (EO) data of Chlorophyll*a* (Chl*a*).

The challenges of current HPLC methods for pigment analysis are the use of toxic solvents and the coelution of important pigment peaks (e.g. mono and divinyl forms of chlorophylls). Despite that, these methods are established in reference laboratories, and its performance metrics were already assessed in previous work conducted by National Aeronautics and Space Administration (NASA) intercalibration exercises.

A more recent method Sanz et al. (2015) is referred as having several chromatographic and instrumental advantages as: a) the use of a simpler binary elution gradient; b) the complete resolution of mono and divinyl forms of chlorophylls; and c) a mobile phase with low toxicity solvents (methanol and ethanol).

In the first part of the thesis, the laboratorial performance of Sanz et al. (2015) methodology for the analysis of phytoplankton pigments was assessed, according to performance metrics defined in NASA HPLC Round-Robin Experiments, such as resolution, injection precision and retention time precision. Considerations about main coelutions and the response to different injection volumes are also discussed. In the second part of the thesis, phytoplankton pigments were quantified in 12 coastal water samples. The followed methodology implemented in CIMA-UAlg laboratorial conditions showed to be easy to implement for a routine level of work and the resolution between mono and divinyl forms of Chlorophyll*a* was achieved. Implications of the results for the pigment quantification were also discussed and are presented.

This study contributes to the overall effort of providing accurate in-situ data to validate satellite EO Chl*a* data, and to accomplish the goal of improving the estimations of phytoplankton distribution.

Keywords: Phytoplankton pigments; High-Performance Liquid Chromatography; Chlorophyll*a*, Performance Metrics, Earth Observation.

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Glossary and Acronyms

Chl*a* – Chlorophyll*a*

CIMA – Centre for Marine and Environmental Research

DAD- Diode array detector

EO – Earth Observation

HPLC - High performance liquid chromatography

UAlg – University of Algarve

UCa – University of Cadiz

UniBo – University of Bologna

WACOMA – Erasmus Mundus in Water and Coastal Management

RS – Remote Sensing

HAB- Harmful Algal Blooms

HPLC-DAD- High-performance liquid chromatography with diode-array detection

GC-Gas chromatography

LC_ Liquid chromatography

SFC- Supercritical Fluid Chromatography

SIMBIOS-Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies

U-HPLC- Ultra-High-Performance Liquid Chromatography

DHI-Danish Hydraulic Institute

Std- Standard deviation

CV- Coefficient of variation

Rs- Resolution

Rt- Retention time

Rˇs -The minimum resolution determined from a critical pair for which one of the pigments is a primary pigment

wˆB1 - The corresponding peak width of tR1 at its base.

wˆB2 -The corresponding peak width of tR2 at its base

ξinj -The injector precision

Chapter 1. Introduction

1.1. Research Topic

This thesis is integrated in the general research topic chemical analysis of environmental samples, as a contribution to the overall research topic of Earth Observation. While Earth Observation (EO) data is an important tool for assessing phytoplankton distribution worldwide and have been developed for a wide and significant range of marine and coastal management applications, its accuracy requires ongoing calibration/validation efforts using in-situ reference measurements obtained with the help of modern methodology in laboratory environment.

1.2. Relevance to Water and Coastal Management and main management issue

EO data are important for evaluating the global distribution of phytoplankton and have been developed for a wide range of marine and coastal management applications, such as harmful algal bloom (HAB) allocation [1,2], eutrophication assessment [3] and in freshwater studies [4], marine aquaculture management [5].

In the last decades, a growing number of communities have started or increased the use of EO data, because of its synoptic scale and relatively low-cost way of assessing marine environment. End-users of EO data include scientific community, marine and coastal managers, environmental public authorities, fishing, and aquaculture industries, within others. Provide accurate in-situ data is hence of major importance, not only to feed EO algorithms' development and training, but also to check the quality of the data produced by the remote sensors in satellites, and to have an idea on the level of uncertainty that might arise from it.

HPLC-DAD methods are within the most accurate for in-situ chlorophyll*a* (Chl*a*) quantification (*proxy* of phytoplankton biomass retrieved by satellite), as well as for other phytoplankton pigments which can be used as phytoplankton biomarkers. The more accurate in situ data of phytoplankton pigments is, the more confident the end users of EO data can be on using the data provided by such technologies. Besides contributing directly to the development of this scientific field, this thesis also

contributes for the determination of accurate and precise phytoplankton pigment concentrations (other than Chl*a*), necessary for the development of chemotaxonomic techniques for assessing phytoplankton, and which uses pigment concentrations as phytoplankton biomarkers.

This thesis seeks to assess the level of accuracy of a recent methodology for determining phytoplankton pigments concentration, and contributes, in this way for the development of the above enumerated management topics.

1.3. Hypothesis

The main hypothesis of this thesis is that the High-performance liquid chromatography with Diode array detection (HPLC-DAD), method presented by Sanz et al. [6] is suitable for the analysis of phytoplankton pigments in samples from the South Coast of Portugal, with an adequate level of accuracy and precision to be used for EO data validation (Chl*a*).

1.4. Overarching Aim

The main goal of this thesis was to critically evaluate the implementation of a recent published methodology [6] into a new High performance liquid chromatography (HPLC) equipment in University of Algarve, and to evaluate its effectiveness for the determination of Chl*a* and its derivatives, as well as other relevant phytoplankton pigments, for the end purpose of validating EO data such as satellite phytoplankton pigment concentration.

1.5. Specific Objectives

For the fulfillment of the main objective of the thesis, several specific objectives were to be met, as detailed below:

1. Implementation of the HPLC-DAD method for phytoplankton pigments determination in a new HPLC equipment in University of Algarve. For such purpose, several parameters describing the HPLC equipment were studied and assessed, namely retention time repeatability and reproducibility, retention time precision, injection precision, and resolution. Such parameters are defined as performance

metrics. Within this, defining the optimal volume and concentration for improving chromatographic peak shapes was also an objective in this part.

2. Assess and evaluation the main advantages and limitations of the selected chromatographic method and compare with other methods.

3. Quantification of phytoplankton pigments concentration using the implemented HPLC technique [6] in coastal water samples collected in an area where small exercises of validation of EO data projects have already been accomplished using spectrophotometric data as the in-situ reference data [7].

1.6. Research questions

These are the main research questions that were sought to be answered with the accomplishment of the present thesis:

1. What are the main advantages and limitations of Sanz et al. [6] chromatographic method for determining phytoplankton pigments?

2. How does Sanz et al. [6] method performs when implemented in CIMA-Lab**,** compared with reference performance metrics listed in Hooker et al. [8] and subsequent SeaHARRE reports?

3. How effective and efficient is the Sanz et al. [6]**,** HPLC methodology when implemented in CIMA-Lab for analyzing phytoplankton pigments in the South Coast of Portugal?

Chapter 2. Literature Review

2.1 Phytoplankton and its role in the ocean

Phytoplankton is microscopic marine algae that habitat in salty waters, as well as in freshwaters. In other words, phytoplankton is microalgae, which are similar to land plants. They contain pigments such as chlorophylls and carotenoids and require sunlight in order to live and grow [9,10].

Similar to terrestrial plants, phytoplankton absorbs the CO_2 and, with the help of the light energy from the sun, it synthesizes it into carbohydrates and produces oxygen. They are considered the basis of the marine and freshwater food chain and are present mostly in the surface layers of the water column, in order to harvest sunlight for the photosynthesis process. Phytoplankton is an important producer of atmospheric oxygen [9, 10, 11].

Phytoplankton can be either bacteria or protists, whereas the majority of phytoplankton are unicellular plants. These are some of the most common types of phytoplankton: cyanobacteria, silica-coated diatoms, dinoflagellates, green algae, and chalk-coated coccolithophores [9, 10, 12]. Regarding cell size, phytoplankton is considered to have three different classes: picophytoplankton which has a size of 0.2–2 µm, nano-phytoplankton when the size range is 2–20 µm, and micro-phytoplankton with the size varies from 20 to 200 µm [12]. Phytoplankton cell size is considered a good indicator of the functional role in many ecological and biogeochemical processes.

2.2. Phytoplankton size classes or functional types: the use of phytoplankton pigments as biomarkers.

Phytoplankton cells contain a vast range of pigments which are directly linked with photosynthetic activity of those cells but can also be involved in the photoprotection of the phytoplankton cell, and its concentration differ depending on the species, life stage and size of the cell. While Chl*a* is present in all phytoplankton cells, other pigments are exclusive or more typical from specific phytoplankton size classes and phytoplankton groups, and for that reason can be used as biomarkers [13]. Thus, several methods have been developed to access size structure and also phytoplankton community using the concentration of pigments in samples [13, 14, 15].

2.3. In vitro laboratory measurement methods of phytoplankton

Phytoplankton measurement in laboratory conditions is rather time-consuming, but the obtained results are quite valuable, since it gives the ratio of the quantity and volume of phytoplankton cells per volume of water [16].

One of the most applied methods for phytoplankton measurement is cell counting in a certain volume of solvent (such as the water). Flow cytometry is another technique to assess the number of phytoplankton cells in samples. Also, for the photosynthetic pigment concentration analysis, various laboratory procedures and instrumentation are used [16].

Chromatographic methods such as HPLC-DAD, as well as some optical methods such as spectrophotometric and fluorometric, are commonly used to measure phytoplankton pigments concentration. For such measurements, the first step is to extract pigments from the phytoplankton and then measure them by the above-mentioned techniques due to their optical characteristics (for example absorption of light in a specific wavelength) of pigments [16].

2.4. Chromatography for phytoplankton pigments determination

Phytoplankton pigments, including Chla and other phytoplankton pigments, can be measured via HPLC, a convenient measurement technique for several kinds of environmental samples.

Chromatography is able to separate, identify and quantify between several different components within the same complex sample, which is especially useful for environmental analysis [17].

Chromatographic discrimination of algal pigments, however, might be difficult due to the fact that these consist in a large number of molecules covering a wide range of polarities, many of which have extremely identical chemical structures [18]. Notwithstanding, it is important to distinguish between them, because of their usefulness as chemotaxonomic indicators, within other factors [19]. For example, the separation of mono and divinyl pairs of chlorophylls *a* and *b* is particularly important, since Chl*a* is the primary phytoplankton pigment and is employed as a *proxy* for the phytoplankton biomass in ocean colour sensors.

2.4.1. Chromatographic Methods

A chromatographic separation process is classified as a group of techniques that involve dividing or distributing a sample (solvent) between a mobile phase and a fixed (or stationary) phase. Chromatography can be understood as a series of equilibrations between mobile and stationary phases. The partition or distribution coefficient (the ratio

between solute concentration in stationary phase and mobile phase) is used to describe the relative interaction between these phases [20, 21, 22].

Mobile phases vary according to the method employed. They tend to be either gases (Gas Chromatography - GC), liquids (Liquid Chromatography - LC) or supercritical fluids (Supercritical Fluid Chromatography - SFC) [20, 21, 22].

Chromatography is a preeminent technique for analyzing multicomponent mixtures. Organic compounds, such as volatile hydrocarbons and biological fluids, are analyzed using chromatographic procedures to identify their qualitative and quantitative composition in environmental samples. Chromatography is used in pharmaceutical industry, medicine, oil refining, chemical manufacture, and other industries to ensure the quality of raw materials and final products, as well as to comply with environmental safety regulations [20, 21, 22].

2.4.2. Main Chromatographic Concepts

2.4.2.1. Chromatographic Resolution

Chromatography's fundamental purpose is the separation of sample compounds into distinct bands or peaks as they pass through the column. Retention duration, peak width, and peak height are all factors that describe a chromatographic peak [23].

The retention volume, V_R, is the amount of mobile phase required to elute a chemical from an LC column. The retention time, or t_R, is the length of time that is associated with retention. Resolution refers to the ability to distinguish between two elution peaks in chromatographic separation. It is calculated by dividing the retention time difference between two peaks by the overall width of the elution peaks [20, 23].

Chromatographic resolution is greatly affected by changes in retention time and peak width. Temperature, stationary phase, and mobile phase are all factors that influence the separation of peaks in the chromatogram. Column efficiency, selectivity, and the

capacity factor all affect chromatographic resolution. The higher the resolution between two peaks, the better the concentration estimation, whereas lower resolution between two important peaks in a sample can induce higher errors when determining the concentration of the compounds of the neighbor peaks [21, 22].

2.4.2.2. Calibration

Calibration is a process of defining relation/correlation between the measuring device output (same as signal or response) and the measure of input property or amount. Several kinds of calibration methods can be used within laboratory methods of analysis, as detailed below [20, 21, 22].

2.4.2.3. Method of absolute calibration

Experimentally, the relationship between the peak parameters and the concentration of the of the various compounds is determined, which is generally reached by running a set of standards of known concentration in the HPLC system, and registering their signal (most of the times, the area of the peaks). After that, graphs and tables are created with which the peak parameters in the sample chromatogram is compared. The approach is the most used for identifying trace contaminants due to its simplicity and high accuracy [20, 21, 22].

2.4.2.4. Internal normalization method

The total of the chosen peak parameters (such as their height or area) is taken as 100%. A mass fraction of a particular component in a sample is then determined by dividing the height of each peak by the total value [20, 21, 22].

2.4.2.5. The internal method of standardization

A calibration curve for a standard material is known ahead of time and is inserted into the mixture [20, 21, 22]. The peaks of the investigated components are then compared to those of the standard [20, 21, 22]. This approach is used while examining compositions with a variable but known amount of studied components [20, 21, 22].

2.4.3. High Performance Liquid Chromatography

In liquid chromatography, a liquid compound is applied as a mobile phase. The mobile phase in this case has the function of transporting the sample molecules along the column and adjusting the equilibrium constants, and also assure that they interact with the stationary phase (column) and the molecules of the substances to be separated [20, 21, 22].

HPLC, the same as high-pressure liquid chromatography, is a more sophisticated type of LC compared with the traditional one. The main difference between the Gas Chromatography (where the movement of the mobile phase through the column is based on the force of gravity) and HPLC, is the high pressure applied, that in the case of HPLC, it reaches from 50 to 350 bars. In addition to the above-mentioned, column is full of adsorbent particles with quite a small size from 2 to 50 μm, that gives an opportunity for high resolution (makes easier the separation of components), hence making the HPLC method more preferable in comparison with GS [20, 21, 22].

HPLC has many applications, such as pharmaceuticals and food analysis. It shows quite effective results, especially for low-volatile or non-volatile organic substances, whereas gas chromatography encounters difficulties [24]. Gravity is the main factor that causes the solvent to move in HPLC (unlike the traditional LC method) [20, 21, 22].

2.4.3.1. HPLC equipment

The HPLC equipment consists of a mobile phase unity which contains the solvents used as the mobile phase for HPLC selectivity, a pumping system, a sample injector, the column (stationary phase) and the detector (**Figure 1**).

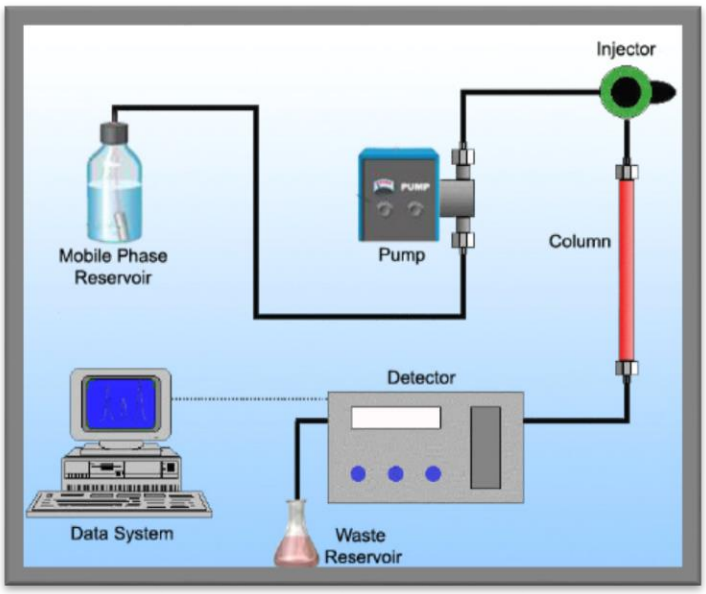

Figure 1. HPLC apparatus
(source:https://www.researchgate.net/publication/279957463_Strategies_for_Protein_Separation)

First of all, the injector delivers the sample into the mobile phase flow. Then the mobile phase takes it (sample or sample mixture) into the column. The role of the pump is to push the required flow and components of the mobile phase from the injector through the column, down to the detector, which then outputs/produces a signal proportional to the concentration of sample components exiting the column, allowing for the quantification of the sample components [25].

2.4.4. Sample detection and identification in HPLC

The graph termed "chromatogram" is used to identify compounds based on their retention period in the column. The chromatogram's x-axis commonly shows the retention time; the y-axis, on its turn, shows the signal of the detection method. The y axis can be, for example, displaying the intensity of absorbance of the sample molecules in the UV-Vis detectors.

Other types of detectors, such as fluorescence, refraction index, and mass spectrometry, can also be employed for applications that demand a higher level of sensitivity than the other detectors [20, 21, 22].

2.4.5 Advantages & Limitations of HPLC technique

There are several advantages of using HPLC for analysis. First of all, it can separate and measure a variety of compounds in a complex mixture; then, it only requires a small volume of sample size (only a few microliters of sample are required to inject into the system) and the results are exceptionally accurate.
On the other, dealing with small peaks is one of the most difficult elements of HPLC analysis, which tend to happen when the sample is of low concentration or when the sample injection volume is really low. In order to solve this common problem, it is required, for example, to increase the concentration of the sample or increase the volume of the injected sample.
Other challenges for HPLC analysis are the coelution of similar molecular structured compounds, adsorbed compounds, which will be described in detail below [20, 21, 22].

2.4.5.1 Coelution

Two compounds with the same structure and polarity can leave the chromatographic device at the same time or approximately at the same time and appear in the chromatogram as a sole peak. This is referred to as coelution. Coelution makes it difficult to determine which part of the mixture gets eluted at which stage [20, 21, 22], and makes it possible to isolate the contribution of each peak to the total peak area, and thus, determine the required individualized concentration of each of the components that are coeluting.

2.4.5.2 Adsorbed Compounds

Glass columns used in HPLC are filled with particles of different materials, which are designed to interact with different strengths to the substances of mixtures passed through these columns, and the strength of the bond between the particles and these chemicals depends on how similar their polarities are. In some cases, the chemicals bind very strongly to the beads and remain in the columns permanently bound to the beads and have no opportunity (cannot) to be measured, which might cause contamination in subsequent chromatograms [20, 21, 22].

2.5. Methods for Phytoplankton Pigments in Coastal Samples

The Wright et al. [26] method was one of the first methods developed to determine a wide range of pigment concentrations from waters sampled across the world's oceans. It was based on a C18 column, with ternary solvent systems, and each run was performed in 30 min. The higher challenge of the method is that it would not be allowed for chromatographic separation of the divinyl chlorophylls a and b from their respective monovinyl forms [27].

Van Heukelem and Thomas [28] determined chloropigments and carotenoids using sensitive reversed-phase HPLC after 28 minutes of run time. The method used pigment methanol extraction and Diode Array Detection (DAD) detection of various pigments, allowing automatic identification based on absorption spectra [27]. This method has the advantage of providing good resolution between most pigments, although some uncertainties may arise from the partial separation of monovinyl and divinyl forms of Chl b, for the separation of Chl c pigments, as well as for the separation of βε-Car [27]. Despite that, the method is effective in a wide range of types of waters, with different Chl*a* typical concentrations, and it is one of the most used methods presently, by state-of-the-art laboratories such as DHI Labs [27].

The method based on Zapata et al. [29] includes a C8 binary gradient reversed phase column, a temperature controlled column, and a DAD detector, with peak quantification at 440 nm. The method has been commonly used to separate most chlorophylls and carotenoids in coastal and oceanic Antarctic waters [30].

The HPLC method used by the Horn Point laboratory has been developed for use with various types of water. This method is based on a C8 HPLC column which is a methanol-based reversed phase gradient solvent system and separation of all quantifiable pigments using a simple linear gradient. The quantitative results for up to 25 pigments can be provided by the method [30].

A more recent method - Sanz et al. [6] - has been developed, with a different stationary phase material, and has several chromatographic and instrumental advantages when comparing to other methods, which are discussed below.

For example, the methods for the analysis of pigments developed by Wright et al. [26] and Jayaraman et al. [31] use triple gradients and thus cannot be implemented in high-pressure dual systems, whereas the method developed by Sanz et al. [6],

which is based on a binary elution gradient, can be replicated using instruments with high or low-pressure gradient mixing systems [6].

Since the eluents used do not absorb in the near-UV region, the detection of cis-carotenoids is also made possible [6].

While Van Heukelem and Thomas's [28] method uses tetrabutylammonium and in Zapata et al. [29] method pyridinium salts are applied, in the Sanz et al. [6], the application of the volatile ion-pairing reagent ammonium acetate makes the method more compatible with mass spectrometry detection [6].

Organic solvents, such as acetonitrile are used in the methods of Wright et al. (1991), Zapata et al. [29] Jayaraman et al. [31], as well as pyridine, in the methods of Zapata et al. [28] [6].

Those are highly toxic, whereas, in Sanz et al. [6], methanol and ethanol are applied. The latter (ethanol) is a highly desirable solvent because of it is less volatile, less toxic, and has low disposal costs [6].

2.5.1. The importance of Intercalibration exercises

The inter-calibration exercises are applied to set up the uncertainties in analytical methods quantifications, as well as for comparison of the efficiency of application of different methods for Chl*a* determination (for example in the case of comparison of spectrophotometric and HPLC methods). If the uncertainties in the various steps of the analytical method (such as separation, injection, etc.) are identified and quantified, this will be of great help for the quality assurance procedure and to reduce uncertainties. Moreover, such exercises are helpful in order to evaluate whether the average uncertainties for situ analyses of total chlorophyll-a are fulfilled the remote sensing requirements for in situ estimation/identification of Total Chl*a* were also examined. NASA SeaHARRE protocols (Hooker et al., [8] and subsequent reports) resulted from a series of intercalibration exercises between several reference laboratories that regularly determine phytoplankton pigments using HPLC-DAD methodologies. During these exercises a number of performance metrics were selected to be evaluated across all the participant laboratories, with the final objective of comparing between the different method’s performance.

2.5.1.1. HPLC-DAD Method Evaluation: Performance metrics

The procedures for the performance assessment of an HPLC-DAD method for the determination of phytoplankton pigments concentration the methods are important since it indicate the uncertainty level of measurement related to estimated pigment concentrations. In the case of SeaHARRE experiments [30], a Chl*a* intercalibration exercise has been sponsored by the he European Space Agency (ESA), under the sponsorship of the SeaWiFS and Sensor Intercomparison and Merger for Biological and Interdisciplinary Oceanic Studies (SIMBIOS) Projects [30, 32,33].

Such activities have provided enough information about HPLC methods to assess the progress of a fully developed method validation process, with the ultimate aim of supporting each laboratory to characterize uncertainties of measurement related to different trophic regimes independent of when filter samples were tested or data was reported [8]. The performance metrics defined in these exercises will, for that reason, be used in this thesis to assess the performance of the Sanz et al. [6] methodology, implemented in the University of Algarve during the accomplishment of this thesis, for the following method parameters: Injection precision, resolution and retention time precision.

2.6. Description of study site

Samplings were implemented from the eastern side of the Faro-Olhão inlet, off Ria Formosa in southern Portugal (**Figure 2**). The sampling has been done from July 2019 to November of 2021, and the seawater collection was carried out by a volunteer at about 7°51'55.67"W 36°57'52.88"N. This study site is in a coastal area that is influenced by the counter-current and by the upwelling [34;35], and in the 2021, a small study in this area was carried out to compare in situ concentration of Chl*a* determined with spectrophotometric data with EO data [7].

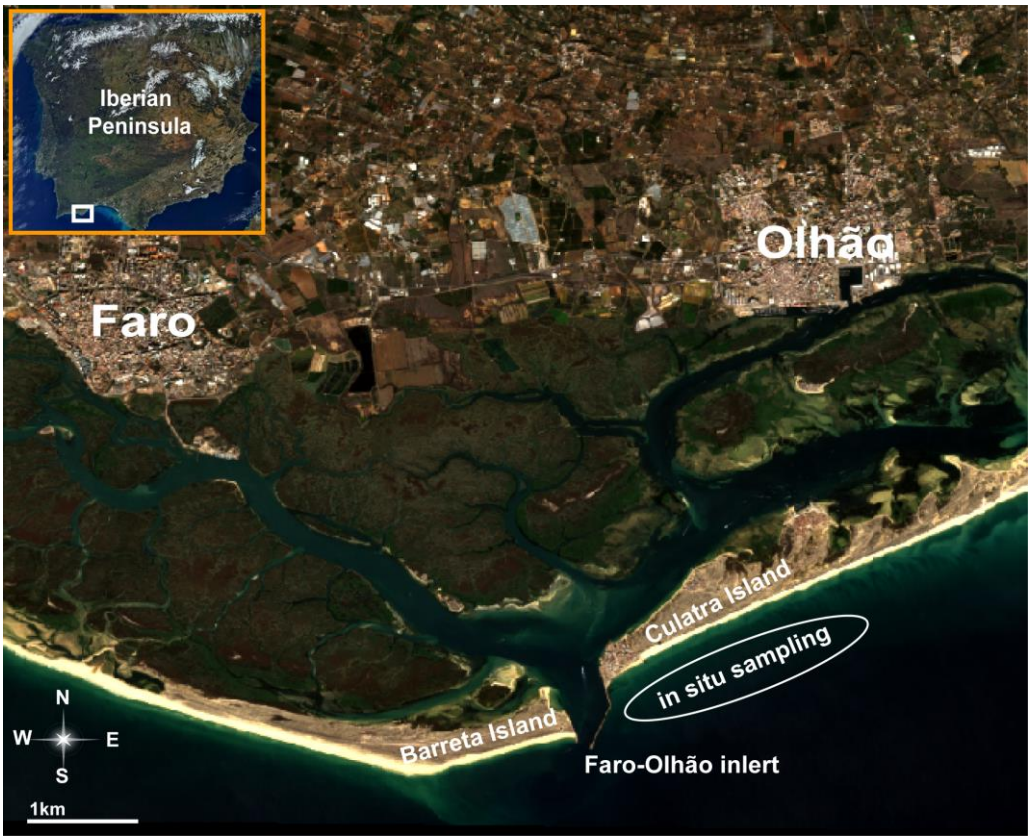

Figure 2. Geographic location of the in situ sampling off inlet of Faro-Olhão in the south coast of Portugal.

Chapter 3. Methods

For the purpose of this thesis, there have been developed two main parts – the first one features the implementation of an HPLC method for Phytopigment concentration and the evaluation of its performance, and in the second part, the implemented method was applied to analyze field samples from a coastal area in the South of Portugal.

3.1. Implementation of Sanz et al. (2015) method

In the first phase of the implementation of the Sanz et al. [6] method in CIMA-UAlg laboratory, pigment standard mixtures (acquired from DHI Labs ®, Denmark) with different concentrations - 3,317mg/ml (two different vials of this mixture concentration are identified DHI Mix A and DHI Mix B, and have been analyzed in September and November, respectively) and 0,2mg/ml, have been analyzed via HPLC-DAD with Sanz et al. [6] methodology, running each concentration with two different volumes of injection (250 µl and 125 µl), in order to choose the optimal concentration and volume for the calculation of the performance metrics and for the quality of chromatographic peak shape, respectively.

The pigments in the mixture were identified according to its retention time and observing each peak UV-Vis spectra and comparing those with Sanz et al. [6] data (Table 3, in Sanz et al. [6]). The differences in the retention time of the pigments were also observed over time and with different batches of solvents used in the eluent system, measuring the same standard pigment mixture after a three-month window (September and November).

On the second phase of the implementation of the Sanz et al. [6] method on the CIMA-UAlg lab, and with the purpose of evaluating the response of the method to a natural water sample, a seawater sample taken from coastal region near Faro, was also analyzed on the HPLC system. A duplicate sample of coastal water were filtered using glass-fiber filters (Whatman®, GF/F, 47mm diameter, 0.7µm porosity) and each of the duplicates was extracted in different volumes of 90% acetone: the first one was extracted in 3ml (Sample A), the second was extracted in 5ml (Sample B). After 4-5 hours of extraction time, the samples have been centrifuged (5000Rpm, for 15minutes), and after that the samples have been taken for the HPLC analyses. Before injection, Mili-Q water was added to the vial (0,2 ml water to 0,5 ml of sample)

to improve peak shape. Each of the sample extracts has been injected in 2 different injection volumes: 125 µl and 250 µl. Each run lasted 42 minutes. Identification pigment peaks have been implemented based on their absorption characteristics (absorption maximums and shapes), as well as base their elution order and the retention times have also analyzed.

3.2. Determination of Phytoplankton Pigment Concentration using HPLC

3.2.1. Phytoplankton Pigments Extraction

Seawater samples were collected on the south coast of Portugal off the inlet Faro-Olhão. The sampling has been done within 2 years (from July 2019 to November of 2021 and the seawater collection was done by a volunteer at about 7°51'55.67"W 36°57'52.88"N. The location is shown in **Figure 2**. The water samples were filtered, using glass-fiber filters (Whatman®, GF/F, 47mm diameter, 0.7µm porosity), wrapped in aluminum foil and kept frozen at -80ºC until further analysis by HPLC Sanz et al. [6] method.

The list of in-situ samples that have been measured by HPLC is shown in **Table 1**. In the table there are also shown the dates of sampling and the volume of water collected.

Table.1 The list of in-situ samples

Number	Date	The volume of filtered seawater (in L)
1	4/7/2019	2
2	16/10/2020	2
3	28/10/2020	1
4	19/11/2020	2
5	12/7/2021	2
6	7/4/2021	2
7	15/04/2021	2
8	4/2/2021	2
9	11/3/2021	2
10	5/12/2020	2

11	17/12/2020	2
12	9/12/2020	2

3.3. HPLC apparatus

For analytical quantification and identification of pigments, a U-HPLC system (Thermo Scientific UltiMate 3000 Rapid Separation (RS) Quaternary System) was used. The U-HPLC was equipped with a quaternary pump, a solvent degasser, an autosampler with

control temperature, a column compartment with control temperature and a Diode Array Detector (DAD).

Milli-Q water has been added to each standard and sample (0.2 ml of water to 0.5 ml of standard/sample) to avoid peak distortion right before the injection [6].

The injection volume was set to 125 µl. DAD (300–720 nm) detector was used to detect carotenoids and chlorophylls, and chromatograms were analysed at 440 nm for the identification and quantification the pigments. Identification of the pigment peaks was made via comparison of the retention times with the pure pigment standards injected into the column and according to the spectral characteristics of peaks (absorption maximums) with those of standards. Peak purity was checked by evaluating its spectral homogeneity.

3.3.1. Eluent System

In this relatively environmentally “friendly” method, the following solvents were used: solvent A was a solution of methanol and 225 mM ammonium acetate in a volume ratio of (82:18 v:v) and as solvent B, 99,99% ethanol was used. The gradient program used during all runs is given in the **Table 2**.

Таблица 1 **Table 2**. Gradient profile of the HPLC-DAD method (Source:Sanz et al.[6]).

Time (min)	SolventA(%)	Solvent B (%)
0	100	0
20	61.8	38.2
22	25	75
33	20	80
36	10	90
37	0	100
40	0	100

42	100	0

For the determination of phytoplankton pigment concentrations in field samples, individual pigment standards with known concentrations (acquired from DHI Labs ®, Denmark) were previously analysed in the HPLC system (each standard was injected 3 times for the calculation of the concentration response factors). The concentration of the same 21 pigment standards has been confirmed via spectrophotometric measurements that have been used as references for the HPLC peak identification.

According to Hooker et al. [30] the response factor for the pigment concentration determination in samples has been calculated according to Equation (1):

$$\boldsymbol{Rpi} = \frac{\boldsymbol{mpi}}{\boldsymbol{Api}} \quad (1)$$

where R_{pi} is the response factor, m_{pi} is the amount of injected pigment (given in nanograms), and A_{pi} is the area of the parent peak and associated isomers for pigment Pi (given either in milli absorbance units, or microvolts as a function of time). Obtained response factor for each pigment is meant to be used for the calculation of concentration for each pigment in the samples considering the areas of the peaks, extraction volume, water volume for filtration, sample injection volume, according to Equation (2):

$$\mathbf{Cpi} = \frac{\mathbf{V1\ mpi}}{\mathbf{V_3\ V_4}} \quad (2)$$

where V_1 is the extraction volume (in ml), V_3 is the volume (in ml) of sample extract injected onto the HPLC column, and V_4 is the volume of water filtered in the field to create the sample (measured in ml).

3.4. Determination of Performance Metrics of the implemented HPLC method

For the determination of the performance metrics, repeated runs of a mixture of pigment standards (DHI Mix-125, acquired from DHI Labs ®, Denmark), were performed in between samples injection. Enough volume of solvents for the eluent system were prepared to accomplish all runs (standards, pigment mixture and samples runs) and the

same column was used, to avoid changes in retention time related with other factors than the equipment itself.

The performance metrics were assessed for the Sanz et al. [6] methodology based on the following HPLC measurements: 1-2 replicates of the standard pigment mixture with the concentration of C=3.317mg/ml was run every 3-4hours time interval, with the injection volume of V=125µl, during 2 weeks of 2-sequence experiments (each sequence for 5 runs, in total 17 runs/replicates). During each run, 20 pigments in each standard pigment mixture (in total around 340 peaks) have been identified based on their retention time and spectra (shapes and absorption maxima).

3.4.1. Retention time precision

One of the main indicators for the quality of HPLC equipment is retention time precision, which gives an opportunity to assess the quality of the instrument starting time from the eluent part till the detector (when eluent system solvents and column is maintained). The retention times are also directly related to the eluent program. Therefore, it is important to assess the retention time repeatability and reproducibility, the same as retention time precision, which is very important for the correct identification of pigments [8]. For determining these parameters, the standard deviation (Std), mean and coefficient of variation (CV, absolute and in percentage) values of the retention time of the 20 identified pigments in the 17 replicate runs were calculated in order to determine the average retention precision ξRt. After that, the mean CV value for all the pigments has been calculated based on Equation (3):

$$\boldsymbol{\mu(\mathbf{CV(all)}) = \xi\mathbf{Rt}} \qquad (3)$$

where Rt is average retention time precision for all the pigments during all the 17 runs and the CV (all) of retention time is the same as average retention time precision ($\bar{\xi}$Rt)

3.4.2. Injection precision

Another important parameter to be accessed is injection precision, which gives an opportunity to evaluate the precision of the of HPLC apparatus injector, and to assess if the injector of the method is dispensing the same volume of solution that the analyst programs it to dispense in all the runs. For this reason, the coefficient of variance of the area of the peak is observed along several runs. Ideally, if the same amount is injected on the column by the injector, the area of the peak should be the same in all times, when the same mixture concentration is injected. The variance of the area gives us an idea on how the injector is working, once the solution is always the same.

Based on the peak area values of all the 2 sequences (1st and 2nd) of runs for Peridinin (as an early eluting pigment) and Chl*a* (as an late-eluting pigment), their std, mean, CV values and injection precision in percent (for the mid and long-term measurements) and given in the tables 4 and 5. Long-term precision describes the CV associated with the cumulative average of peak area or calibration factors when the same standard is analyzed on several occasions across many runs and/or sequences [30]. For the calculation of mid-term injection precision (for all the runs) for mentioned pigments following equations have been applied:

$$\bar{\xi}\mathbf{inj.Perid} = \mathbf{CVPerid} \quad (4)$$

$$\bar{\xi}\mathbf{inj.ChlA} = \mathbf{CVChla} \quad (5)$$

where $\bar{\xi}injperid$ is the average injection precision for Peridinin and $\bar{\xi}injC\square la$ is the average injection precision for Chl*a*, calculated with the coefficient of variation ($\mathbf{CV} = \frac{\mathbf{std}}{\mathbf{mean}}$) of the Peridinin and Chl*a* peak areas within the 17 replicate runs of the standard pigment mixture.

3.4.3. Resolution of critical pigment pair

One of the main limitations of phytoplankton pigment analysis by HPLC is the fact that several pigments might leave the column in very close retention times, due to their similar chemical structure, which might affect the correct integration of each individual peak and consequent quantification of each pigment concentration. In this way, it is important to evaluate the minimal resolution between critical pairs of pigments in each of the method, especially for pigments that might be important

biomarkers of specific phytoplankton species. In this case, the minimum resolution between lutein and zeaxanthin was accessed, once they are probably the two pair of important biomarkers that are visibly almost coeluting in this method. According to Hooker et al. [30], the column is suggested to be replaced if Rs < 1 for one of the critical pairs.

Resolution (Rs) of mentioned critical pair have been calculated in each chromatogram of each run by using the Equation (7). Min/ max values of the resolution, as well as mean values of resolution of critical pair for all the runs, are given in **Table 6**.

$$\boldsymbol{Rs} = \frac{(\boldsymbol{tR2} - \boldsymbol{tR1})}{\widehat{\mathbf{w}}\mathbf{B2} + \widehat{\mathbf{w}}\mathbf{B1}} \qquad (7)$$

where tR1 and *tR2* are the retention times of peaks 1 (in this certain case Zeaxanthin) and 2 (in this case Lutein), and $\widehat{\mathbf{w}}$B1 and $\widehat{\mathbf{w}}$B2 are the base widths of mentioned peaks, respectively.

Chapter 4. Results

4.1 Implementation of the Sanz et al. (2015) method in CIMA-UAlg laboratories

Taking into consideration the elution program and the HPLC parameters described in Sanz et al. [6] methodology, when concerning the column features, temperature and detection settings, standard mixtures were run in the CIMA-UAlg equipment, and the results were compared with those from the referred authors.

The identified pigments of the standard DHI mixture over time and difference of the retention time values in the DHI mixture /concentration of 3.17 mg/ml, volume of 125μl) between the runs of September (mixture A) and November (Mixture B) are presented in **Table 3**, and respective chromatograms are shown in **Fig.3 (a,b)**. In this table, the difference of retention time between September and November (Dif A-B) and the absorption maxima values are also mentioned, and compared to the reference, which in this case were the retention times and absorption maxima referred in Sanz et al. [6] methodology.

Based on these results, it is possible to see that the retention of the majority of pigments decreased between the mixture run in September and the mixture run in November, which is possibly due to the slight changes of the eluent system which resulted from different batches of solvents prepared for the two occasions. In theory, changes in retention times might be due either to changes of the columns, mechanical alterations in the detector and mainly due to differences in the eluent system, for that reason the preparation of the sufficient amount of eluent for a complete set of samples is important, so to maintain the stability of the retention times. Sometimes, even the change of the eluents brand might be sufficient to shift retention times.

The major co-elution identified in the case of Lutein and Zeaxanthin, which although not completely co-eluted (**Figure 3a**, peaks 14/15), the two peaks were not distinguished by the software as separate peaks for the purpose of peak area integration, and an overall peak area was given by the software for the two peaks. For that reason, the calculations of the resolution between the two peaks needed to be done manually.

In **Table 3**, absorption maximums of the peaks are either matched or shifted slightly to the left compared with the reference values based on the Sanz et al. [6] method (Sanz et al. [6], Table 3). Also, the maxima of absorption obtained in September where close to the ones observed in the November runs.

Table 3. Identified pigments of standard DHI mixture over a time and difference of retention time values with the DHI mixture concentration of 3.317 mg/ml with the volume of 125ul with the runs of September (mixture A) and November (Mixture B), and with the reference retention time absorbtion maximum values taken from Sanz et al. [6].

Pigment name		**Rt(min)**	**Absorption maxima (nm)**		
Chlorophyll c3	Reference	7.76	460	592	638
	Mix DHI (A)	**6.14**	**456**	**580**	-
	Mix DHI (B)	7.66	457	587	-
	Dif(A-B)	-1.52			
Peridinin	Reference	10.39	-	477	-
	Mix DHI (A)	**11.99**	-	**467**	-
	Mix DHI **(B)**	10.15	-	474	-
	Dif(A-B)	**1.84**			
Chlorophyll c2	Reference	11.09	449	583	633
	Mix DHI (A)	11.20	446	583	633
	Mix DHI **(B)**	11.21	447	584	633
	Dif(A-B)	0.01			
19´-Butanoyloxyfucoxanthin	Reference	13.24	-	454	471
	Mix DHI (A)	**15.19**	-	**448**	**468**
	Mix DHI **(B)**	13.05	-	443	468
	Dif(A-B)	2.137			
Fucoxanthin	Reference	14.18	-	454	-
	Mix DHI (A)	**16.21**	-	**452**	-
	Mix DHI (B)	13.90	-	451	-
	Dif(A-B)	**2.31**			
9´-cis-neoxanthin	Reference	14.75	414	438	467
	Mix DHI (A)	**16.88**	**413**	**436**	**465**
	Mix DHI (B)	**15.96**	414	435	463
	Dif(A-B)	**0.92**			
19´-Hexanoyloxyfucoxanthin	Reference	15.77	-	449	471
	Mix DHI (A)	**17.893**	-	**447**	**467**
	Mix DHI (B)	**16.953**	-	444	468
	Dif(A-B)	**0.94**			
Prasinoxanthin	Reference	16.32	-	460	-
	Mix DHI (A)	**18.61**	-	**457**	-
	Mix DHI (B)	**17.66**	-	457	-

	Dif(A-B)	**0.95**			
Violaxanthin	Reference	17.22	417	442	471
	Mix DHI (A)	**19.54**	**417**	**440**	**469**

	Mix DHI (B)	**18.6**	415	439	468
	Dif(A-B)	**0.94**			
Diadinoxanthin	Reference	19.00	-	448	478
	Mix DHI (A)	**21,42**	**-**	**445**	**474**
	Mix DHI **(B)**	**20,47**	-	445	474
	Dif (A-B)	**0,95**			
Alloxanthin	Reference	20.45	-	454	483
	Mix DHI (A)	**22.99**	**-**	**452**	**474**
	Mix DHI (B)	**22.06**	-	451	479
	Dif(A-B)	**0.924**			
Zeaxanthin	Reference	22.43	-	452	480
	Mix DHI (A)	**24.08**	-	**451**	**477**
	Mix DHI (B)	**23.70**	-	450	474
	Dif(A-B)	**0.38**			
Lutein	Reference	22.73	-	446	475
	Mix DHI (A)	22.26		**445**	**473**
	Mix DHI (B)	**23,81**	-	444	473
	Dif(A-B)	**0.36**			
DV Chlorophyll b+ Chlorophyll b	Reference	27.32	477	605	654
	Mix DHI (A)	**28.58**	**464**	**600**	**650**
	Mix DHI (B)	**28.17**	**464**	**600**	**650**
	Dif(A-B)	**0,407**			
DV Chlorophyll a	Reference	29.86	442	620	666
	Mix DHI (A)	**30,970**	**464**	**600**	**650**
	Mix DHI (B)	**30,440**	**432**	**620**	**665**
	Dif(A-B)	**0.53**			
Chlorophyll a	Reference	30.37	433	619	665
	Mix DHI (A)	**31,59**	**431**	**616**	**665**
	Mix DHI (B)	**31,03**	**431**	**619**	**665**
	Dif(A-B)	**0,56**			
DV Chlorophyll a epimer	Mix DHI (A)	**34,33**	**431**	**618**	**665**
	Mix DHI B)	**33,62**	**431**	**619**	**665**
	Dif(A-B)	**0.71**			
β-carotene	Reference	33.58	-	454	480
	Mix DHI (A)	**35.4**	**420**	**450**	**476**
	Sample(B)	**34.6**	**420**	**451**	**476**
	Dif(A-B)	**0.8**			

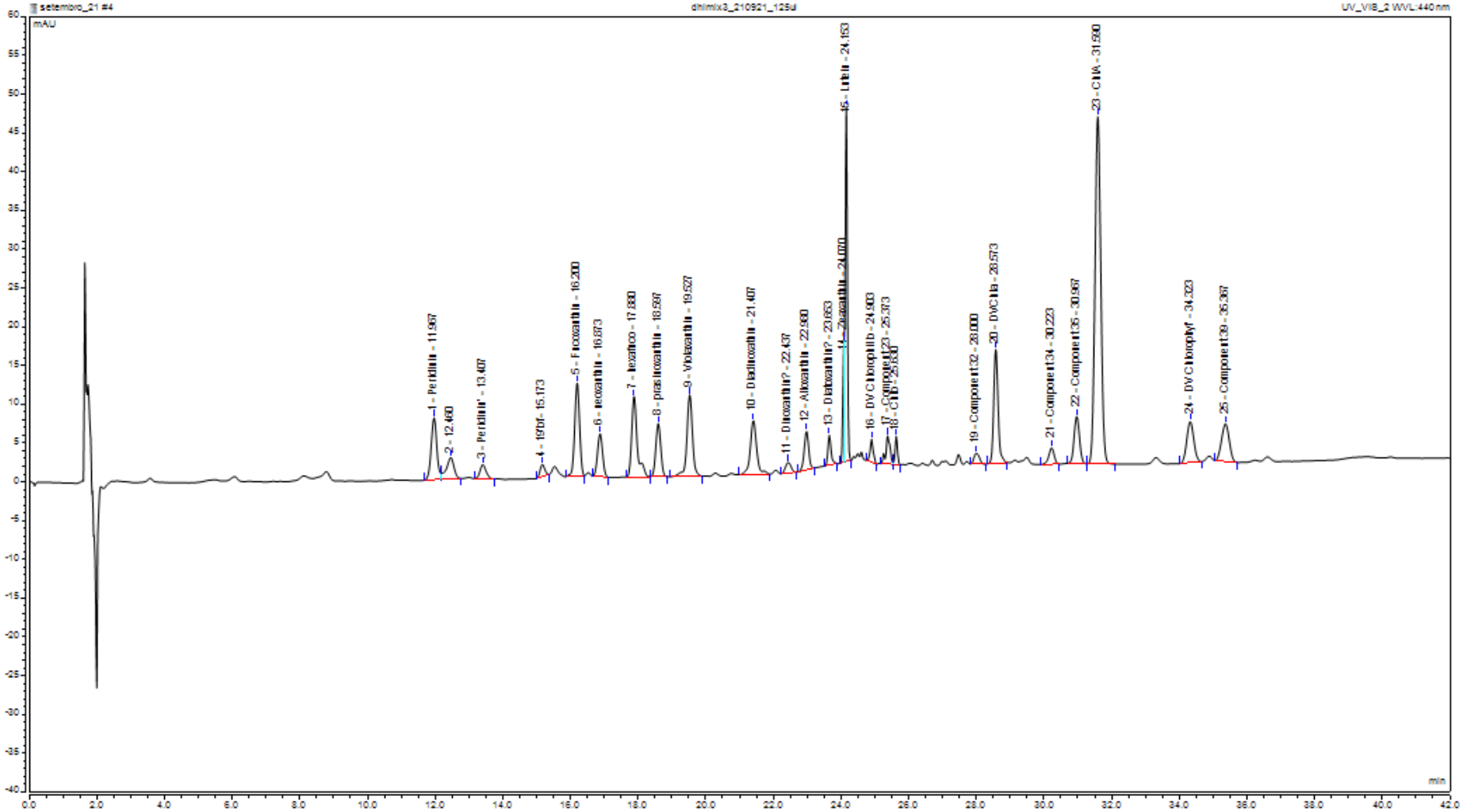

Figure 3a. DHI mix-125 with concentration of 3.317mg/l run in September (Mix DHI(A)), with an injection volume 125ml.

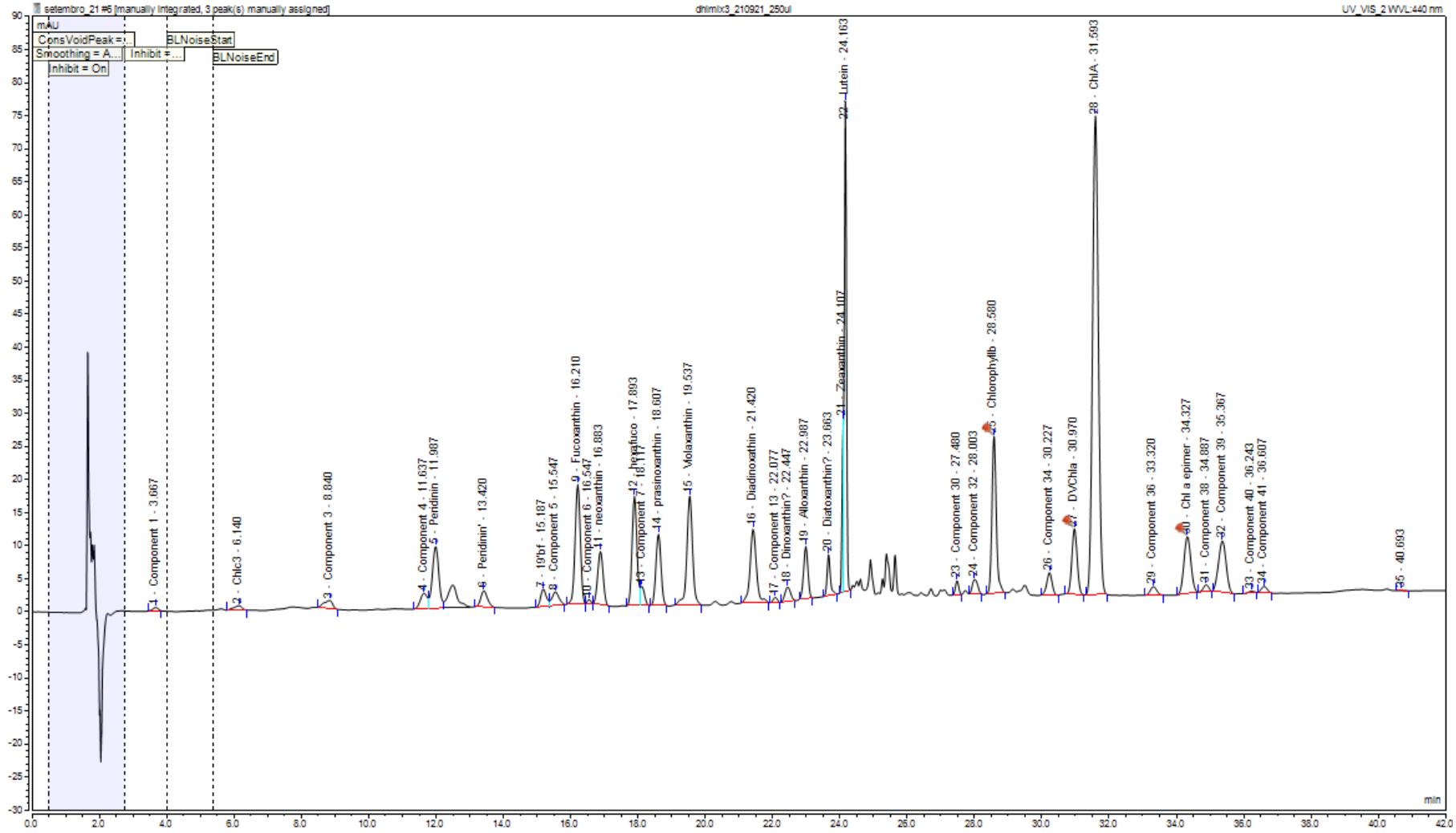

Figure 3b. DHI mix-125, with concentration of 3.317mg/l run in September (Mix DHI(A)), with an injection volume of 250ml.

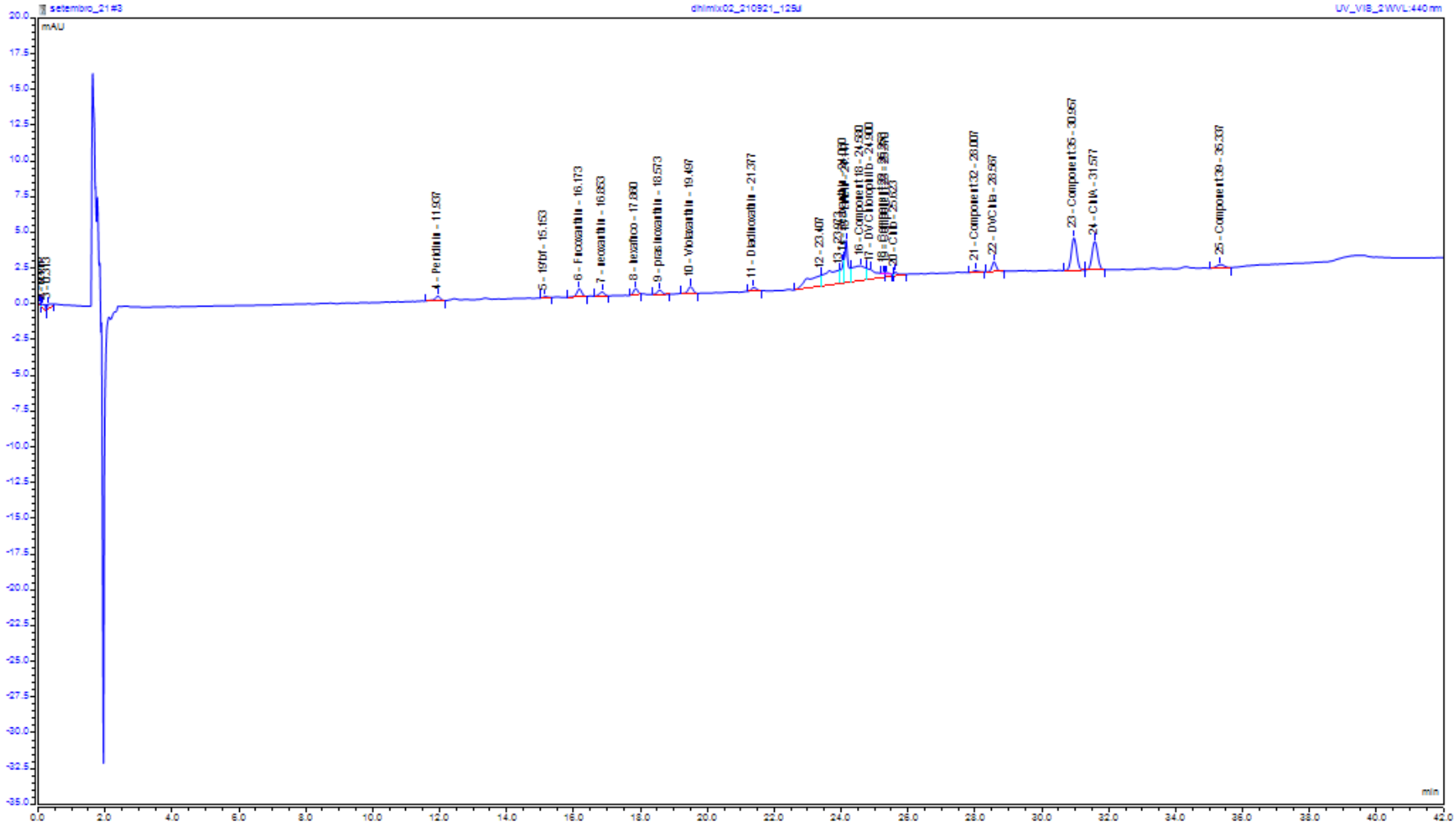

Figure 3c. DHI mix with concentration of 0.2mg/l run in September, with an injection volume of 125ml.

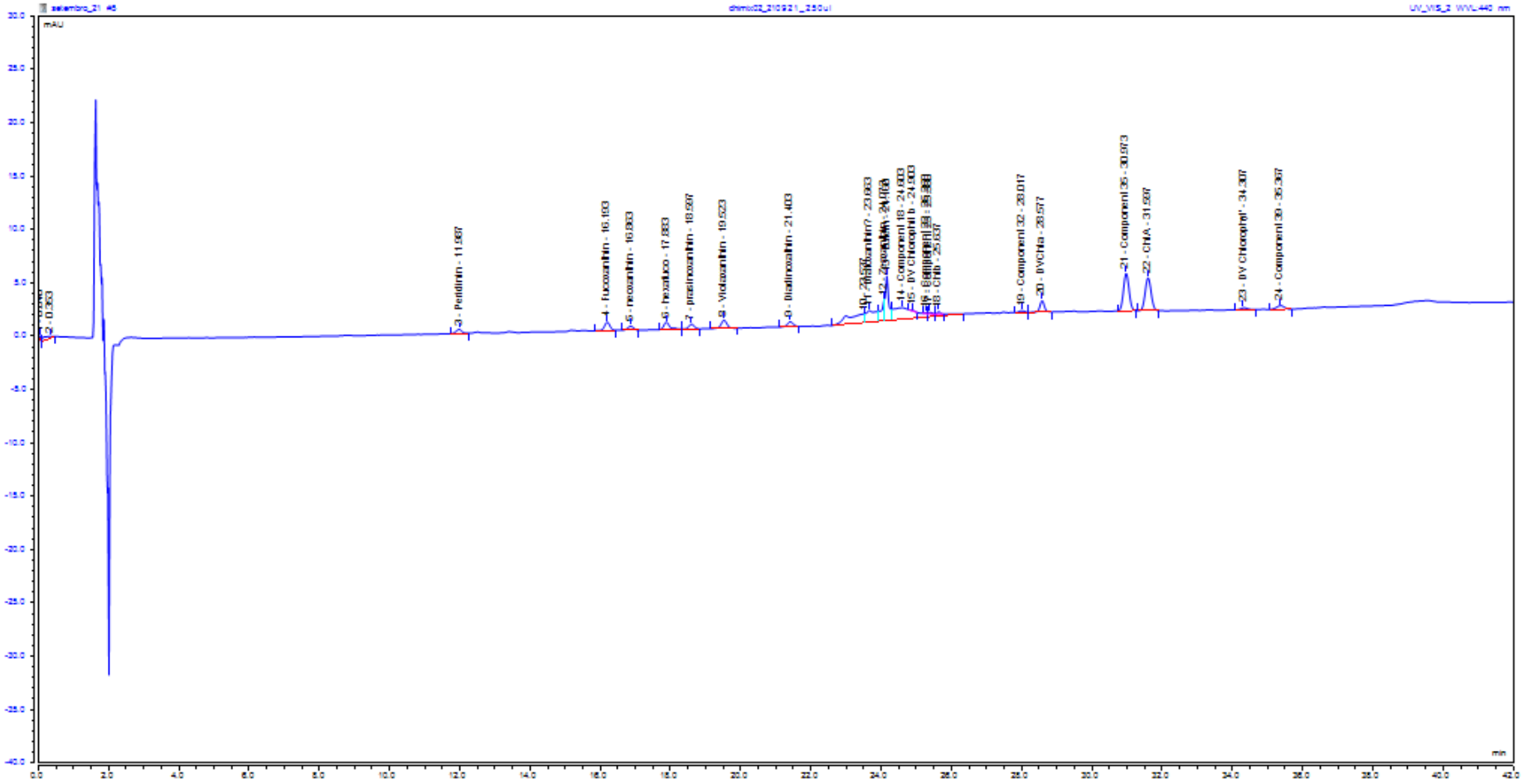

Figure 3d. DHI mix with concentration of 0.2mg/l run in September, with an injection volume of 250ml

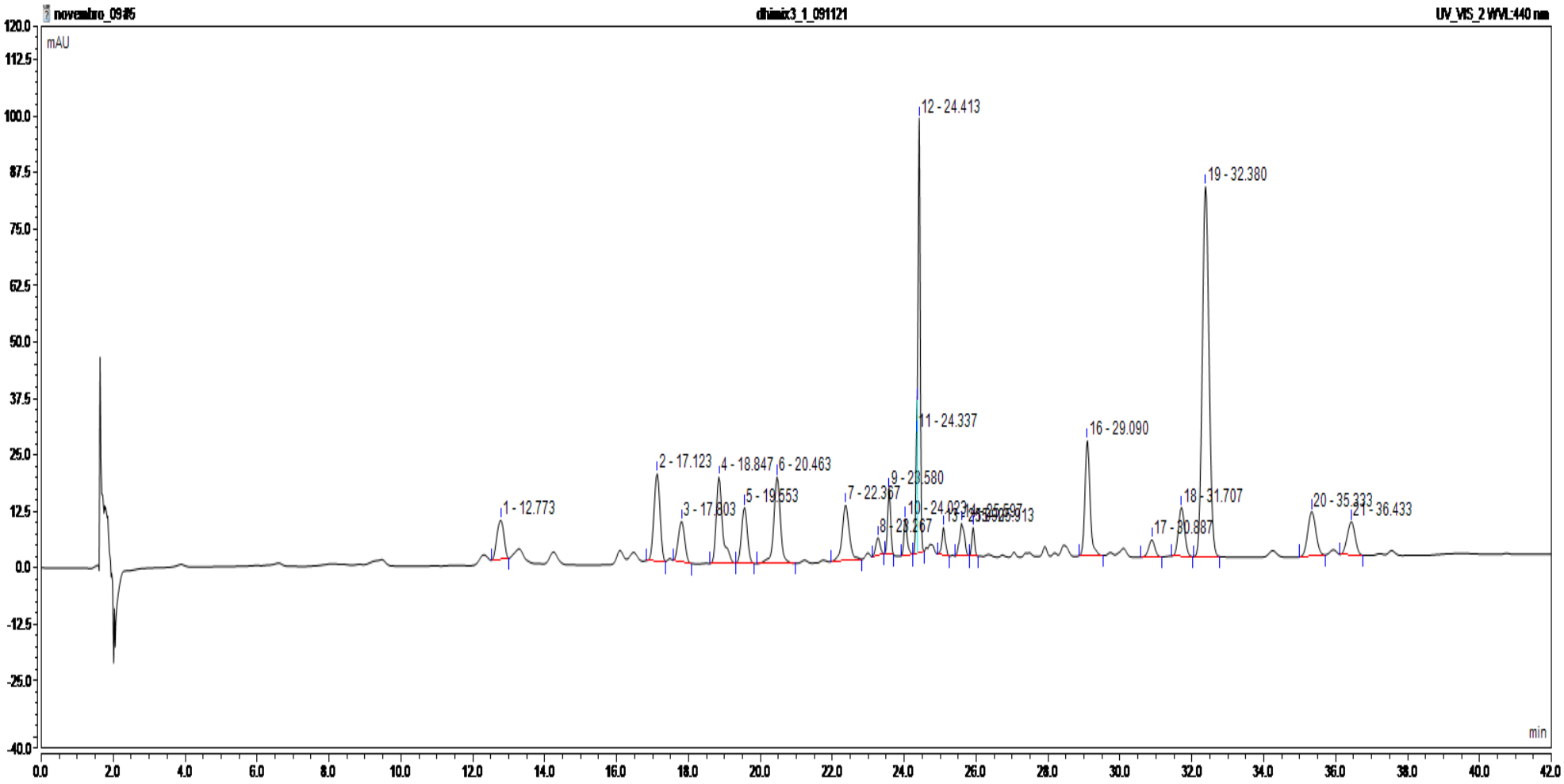

Figure 3e. DHI mix with concentration of 3.317mg/l run in November (Mix DHI(B)), with an injection volume 250 ml.

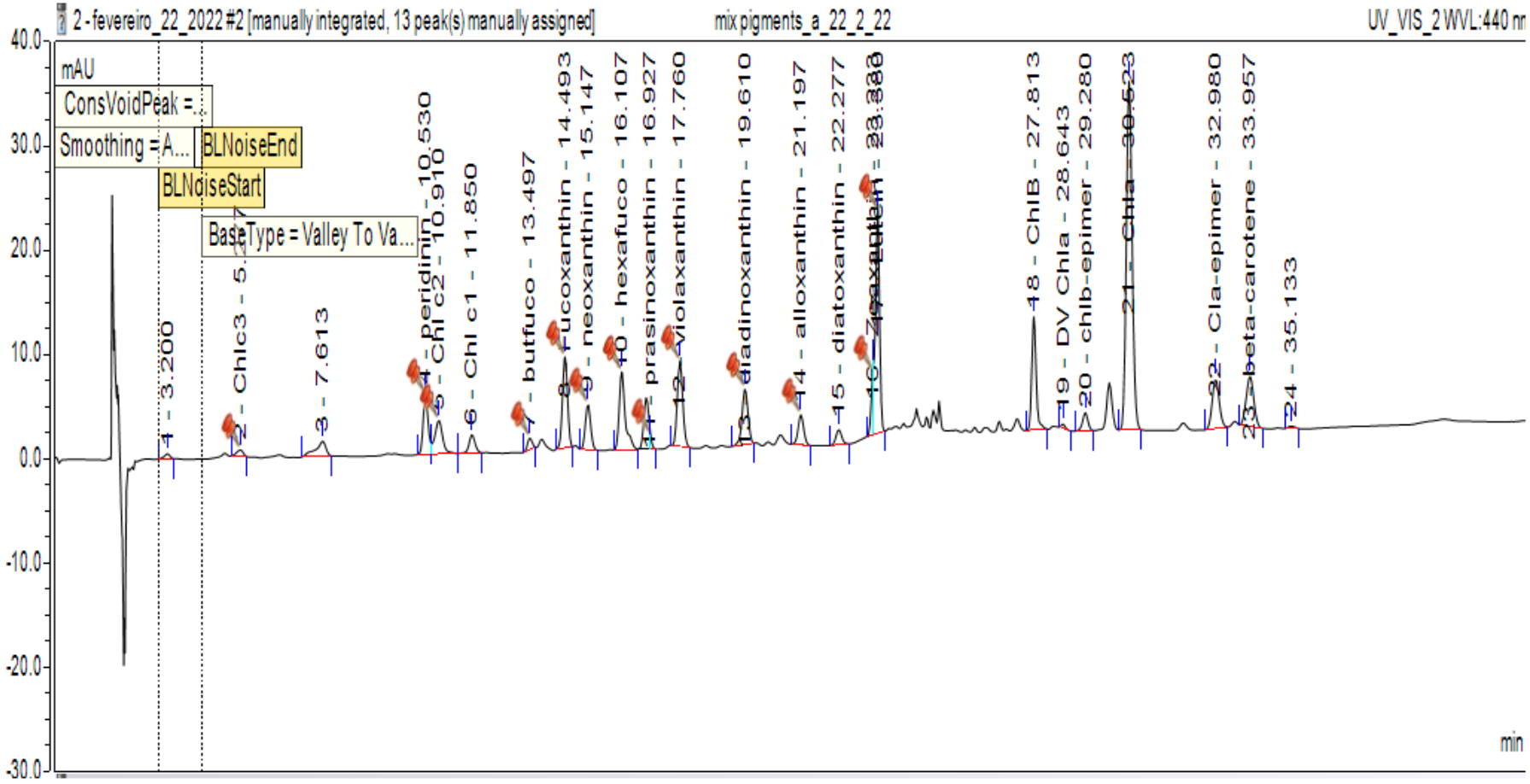

Figure 3f. DHI standard pigment mixture with optimal concentration (C=3.317mg/l) and volume (V=125 ml), which were then selected for further measurements.

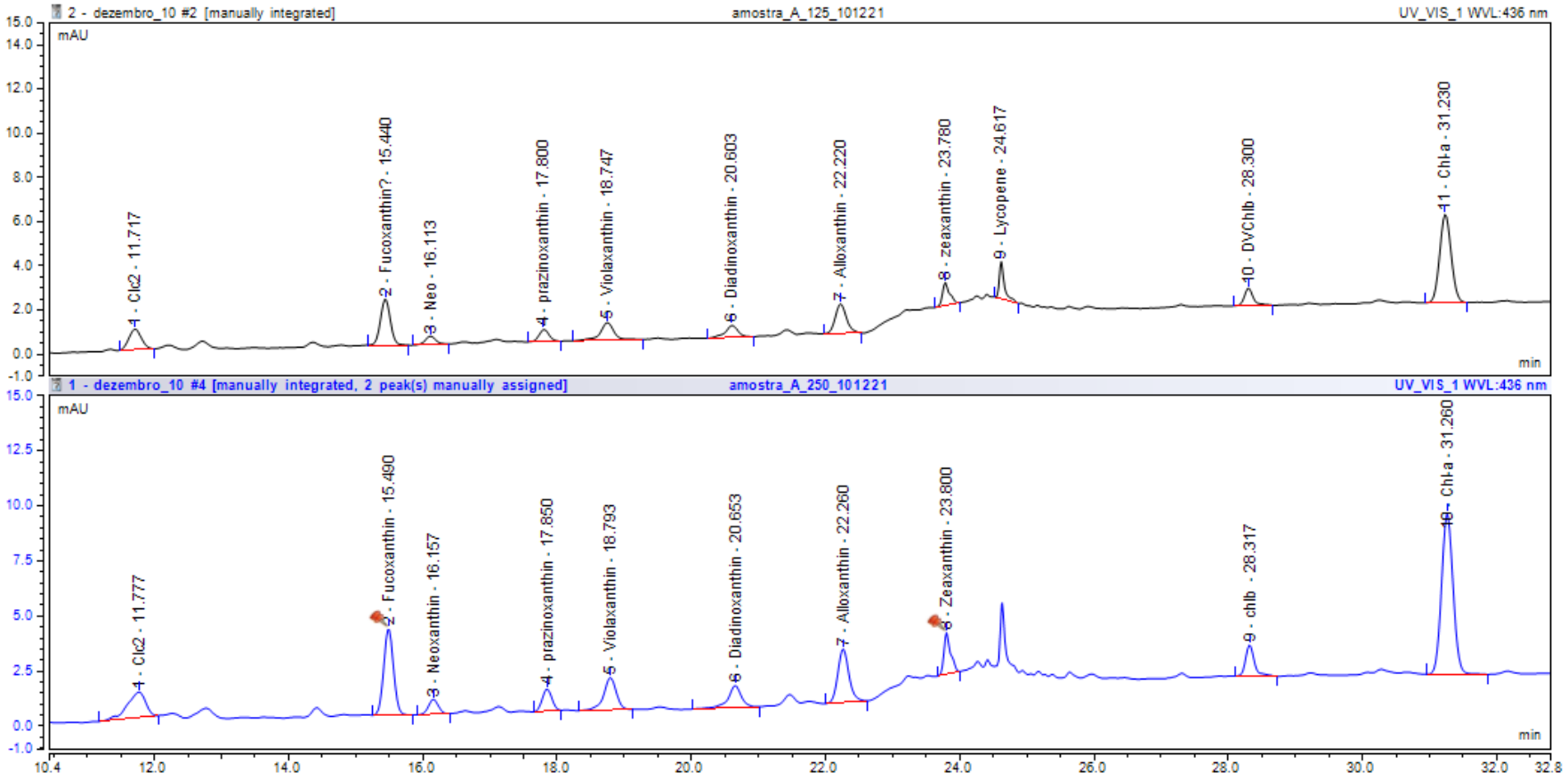

Figure 3g. In situ sample extracted in 3 ml of 90% of acetone, both with injection volumes of 125ml (upper panel) and injection volume of 250ml (lower panel).

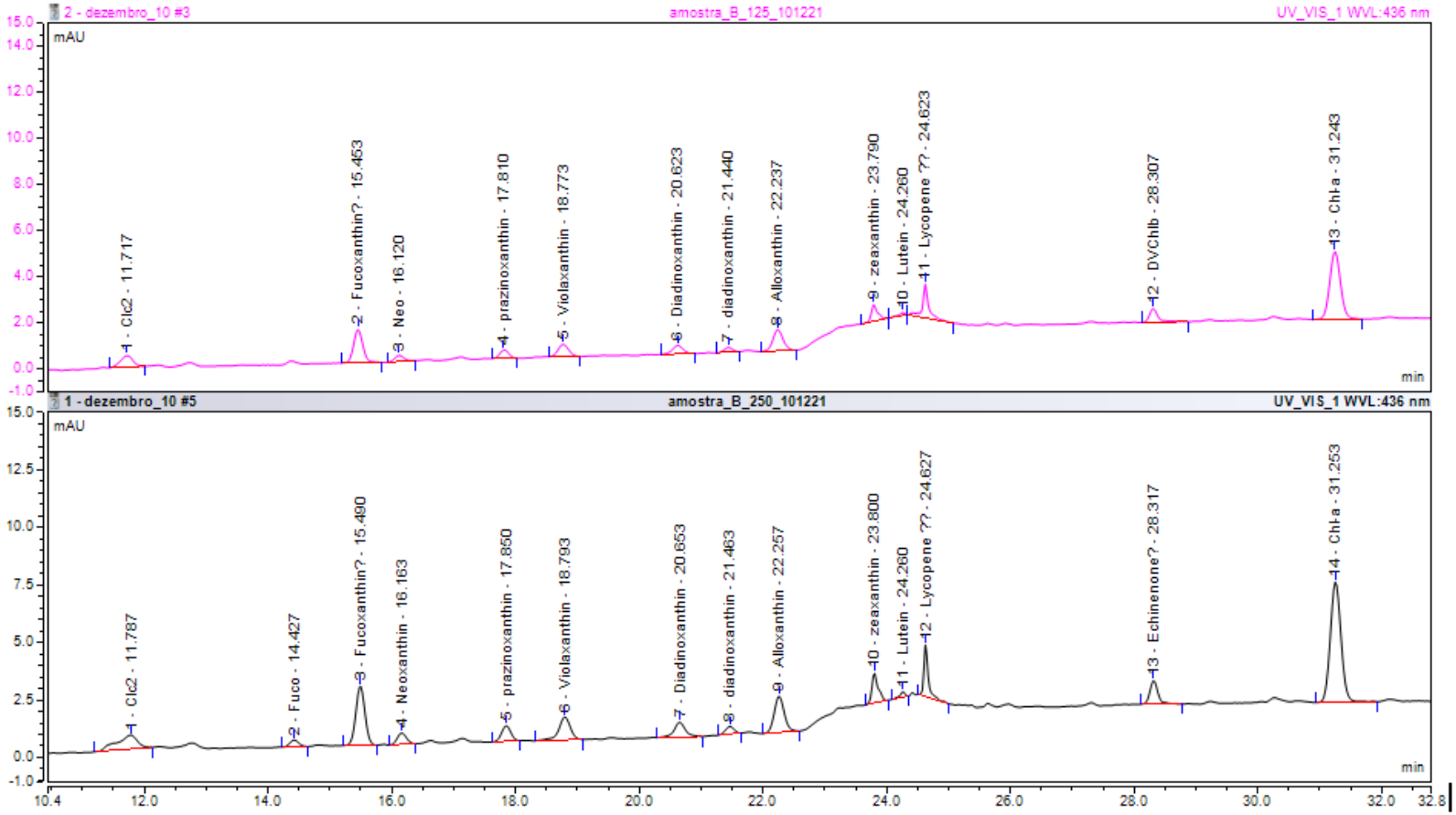

Figure 3h. In situ sample filter dissolved/extracted in 5 ml of 90% acetone, both with injection volumes of 125ml (upper panel) and injection volume of 250ml (lower panel).

For the purpose of comparing and finding optimal injection volumes for the implementation of the method in the laboratory, the panels c and d from **Figure 3** show the chromatograms of the measurements of DHI mix-125 (run in September) with concentrations of 0.2mg/l and volume 125μL (**Figure 3c**) and with 250μl (**Figure 3d**), respectively. When these chromatograms are compared, it might be observed that the peaks with the lower volume (125μl) are less sharp in comparison to the ones with same concentration, but higher volume (250μl). As an optimal concentration and volume combination has been chosen standard pigment mixture with C=3.17mg/l and a volume of 125ul, since this was the one where more peaks could be identified, with better peak shape (**Figure 3f**). This was then the chosen mixture concentration and volume for the standard pigment mixture runs between samples required for the calculation of the performance metrics of the method.

The next step was to apply the same tests in real (natural) samples of coastal water. The resulting chromatograms are presented in **Figure 3g** and **Figure 3h**. In this case, it was observed that the samples with the higher volume of injection has sharper peaks in comparison with the same sample injected with a lower volume of injection. **Figure 3h** shows also the same test applied to a sample filter dissolved/extracted in 5 ml of 90% of acetone, with injection volumes of 125 μl and 250 μl. When comparing the extraction volumes, it was possible to observe slightly better shaped peaks in the chromatograms from the lower volume of extraction sample, which means that the results might be more accurate in more concentrated extracts of phytoplankton pigments.

4.2. Retention time precision

The std, mean and CV values of the identified pigments of all 17 runs and average retention precision of all the mentioned pigments (which is also presented in percent value) were calculated and presented as the ξRt (mean CV, in percentage) and the results are shown in **Table 4**.

Table 4. Standard deviation (Std), Mean and Coefficient of variation (CV) values of the retention time for the identified pigments and average retention precision (mean CV) of all the pigments in percentage.

	Std	Mean	CV
Chlorophyll c3	0.07	5.4	0.01
Peridinin	0.11	10.7	0.01
Chlorophyll c2	0.12	11.1	0.01
Chlorophyll c1	0.12	11.99	0.01
19'Butanoyloxi-fucoxanthin (Butfuc)	0.13	13.64	0.01
Fucoxanthin	0.13	14.7	0.01
Neoxanthin	0.132	15.3	0.01
19'Hexanoyloxifucoxanthin (Hexafuco)	0.132	16.3	0.01
Prasinoxanthin	0.134	16.98	0.01
Violaxanthin	0.15	17.94	0.01
Diadinoxanthin	0.22	19.8	0.01
Alloxanthin	0.15	21.4	0.01
Diatoxanthin	0.15	22.5	0.01
Zeaxanthin	0.13	23.34	0.01
Lutein	0.39	23.63	0.02
Chlorophyll b	0.23	27.96	0.01
Chll b-epimer	0.18	29.4	0.01
Chlorophyll A	0.12	30.7	0.004
Chl a epimer	0.14	33.14	0.004
β-carotene	0.15	34.12	0.004
Mean CV	0.0084		
ξtR in percent	0.84 %		

As it is shown in the **Table 4**, the CV for almost all of the identified pigments CV value is equal to 0.01 (the same as 1%), with an exception of Lutein- with the CV value of 0.02 and other 3 pigments(Chll b –epimer, Chl a epimer, β-carotene) that elute at the end part of chromatogram (at the end of the run) and have a CV value equal to 0.004 (0.4%).

4.3. Injection precision

Based on the peak area values of all the 2 sequences (1st and 2nd) of runs, using as reference the peaks for Peridinin and Chl*a*, their std, mean values, CV values and injection precision in percent (for the mid/long-term measurements) are given in the **Tables 5** and **6**. Time interval between the measurements of pigment standard mixture within one day varies from 4 to 7 hours, and between those an approximate number of 5-10 samples were run in the system.

Table 5. Peak area values of all the 1st sequence runs for Peridinin and Chl*a*, their standard deviation (std), mean, coefficient of variation (CV) values and injection precision in percent (for the mid/long-term measurements)**.**

Date of the run	**Mid/long term injection precision ξinj (1st sequence)**	
	Peridinin Peak area in DHImix	**Chl*a* Peak area in DHImix**
21.02.2022	0.9	7.1
21.02.2022	0.73	6.5
22.02.2022	0.95	6.4
22.02.2022	0.96	6.8
23.02.2022	1	7.7
23.02.2022	1	7.7
24.02.2022	0.94	7.5
24.02.2022	1.1	8.6
std(σ)	**0.11**	**0.74**
mean(μ)	**0.95**	**7.3**
CV=σ/μ	**0.113**	**0.1014**
ξinj in percent	11.3%	10.14%

Table 6. Peak area values of all the 2nd sequence runs for Peridinin and Chl*a* and their std, mean, CV values and injection precision, absolute and in percent (for the mid/long-term precision measurements).

	Mid/long term injection precision **ξinj(2nd sequence)**	
Date of the run	Peridinin Peak area in DHImix	Chl*a* Peak area in DHImix
2.03.2022	1.0853	8.7004
2.03.2022	1.0947	8.8225
3.03.2022	1.0398	8.3728
3.03.2022	1.0466	8.4805
4.03.2022	0.9793	8.2478
4.03.2022	0.8932	8.2478
std(σ)	0.0757	0.24
mean(μ)	1.023	8.48
CV=σ/μ	0.074	0.028
ξinj in percent	7.4	2.8

As it can be inferred from the data shown in **Table 5**, the peridinin peak areas for almost all the 1st sequence runs are around 1, while standard deviation is equal to 0.11 and mid/long term injection precision (ξinj) is 11.3%. At the same time during the 1st sequence run experiments for Chl*a* peak area were registered values that varies from 6.4 to 8.6, with the standadrd deviation value of 0.74 and mid/long term injection precision value of 10.14%. In the second run (**Table 6**), results show that the peridinin peak areas for almost all of the 2nd sequence runs are also around 1, but the CV decreased significantly to 7.4%, In the case of Chl*a*, the peak areas were around 8.5, and the std decreased to 0.028, resulting in an average Chl*a* CV value of 2.8%.

4.4. Resolution between critical pigment pairs

The *minima* and *maxima* resolution between the selected critical pair of pigments - Lutein and Zeaxanthin are given in **Table 7**, as well as mean values.

According to these results, the resolution between these two pigments was rather constant between runs along all the experiments, with maximum resolution value for all the runs achieved of 0.58, whereas the minimum resolution is 0.36. The mean resolution between those two peaks value was approximately 0.5, half of the desired resolution of 1. Despite that, the variations were random between runs, which means that the column condition was at least stable.

Table 7. Min/ max and mean values of resolution of critical pair (Lutein /Zea) for all the runs.

Date	**Rs Lutein/Zea**
21.02.2022/Rs1st	0.55
21.02.2022/Rs 2nd	0.56
22.02.2022/Rs 1st	0.51
22.02.2022/Rs 2nd	0.54
23.02.2022/Rs 1st	0.56
23.02.2022/Rs 2nd	0.40
24.02.2022/Rs1nd	0.48
24.02.2022/Rs2nd Rs max	0.58
25.02.2022/Rs1nd	0.57
2.03.2022/Rs1nd	0.40
2.03.2022/Rs2nd	0.40
3.03.2022/Rs1nd	0.40
3.03.2022/Rs2nd	0.37
4.03.2022/Rs1nd	0.40
4.03.2022/Rs2nd Rs min	0.36
7.03.2022/Rs	0.51
8.03.2022	0.4
Mean	0.5

4.5. Summary of the performance metrics results

Table 8 summarizes all the results for the calculations of the performance metrics of the implemented Sanz et al. [6] methodology in CIMA-UAlg laboratory. It shows that for minimum and maximum values of resolution we obtained 0.36 and 0.58 respectively, for average retention time precision 0.843 % and for average injection precision value 7.4-11.3% for peridinin and 2.8-10.14 % for Chl*a*.

Table 8. Performance metrics results based on our experiments.

Performance Category	Separation		Injection ($\bar{\xi}_{inj}$) mid-term	
	$Rs_{min}(\check{R}_S)$ /Rs_{max}	$\bar{\xi}_{tR}$	Perid	Chl*a*
	0.36/0.58	0.843 %	7.4-11.3%	2.8-10.14 %

Table 9 shows the performance metrics categories as defined by Hooker et al. [8] eaHARRE-2, and which will serve as the reference for comparison of this work. In Hooker et al. [8], several ranks were defined, according to the performance in each of the studied parameters, and are organized from the *Routine* to the *State-of-the-Art* level, ordered by increasing level of method performance.

Table 9. Performance metrics ranks mentioned as reference purposes, according to Hooker et al. [30].

Performance Weight, Category, score	Separation		Injection ($\bar{\xi}_{inj}$)	
	$\check{R}_S$	$\bar{\xi}_{tR}$	Perid	Chl a
1. Routine	0.8	0.18%	10%	6%
2.Semiquantitative1.5	1.0	0.11	6	4
3. Quantitative 2.5	1.2	0.07	4	2
4. State-of-the-Art 3.5	≥1.5	≤0.04	≤2	≤1

4.6. Determination of Pigment Concentrations in natural water samples

After running the 12 samples on the HPLC-DAD system with the reference methodology [6], all the detected peaks corresponding to different phytoplankton pigments were identified, based on their retention time, elution order and absorption spectra, and respective peak areas were determined, for the final purpose of calculation of concentration. **Table 10** summarizes all the obtained results for the analyzed samples.

Table 10. Concentrations of determined pigments in 12 in situ samples given in ng/ml.

[Pigment] (ng/ml) / Sample	1	2	3	4	5	6	7	8	9	10	11	12
Chlorophyll *a*	1.94	0.49	0.54	0.66	140	0.4	0.3	0.81	1.5	0.7	0.84	0.5
Chlorophyllide *a*	-	-	-	-	-	-	-	-	-	-	-	-
Chlorophyll c1	-	0.011	0.008	0.012	-	-	-	0.01	0.03	0.01	0.01	0.006
Chlorophyll c2	1.78	0.009	0.044	0.103	0.008	0.07	0.05	0.1	0.17	0.1	0.09	0.06
Chlorophyll c3	0.16	0.06	0.019	0.05	0.004	-	-	-	-	-	-	-
DVChl *a*	-	-	-	-	-		0.01	0.6	-	-	-	-
Pheophytin *a*	-	-	-	-	-	-	-	-	-	2.4	-	4.1
Pheophorbide *a*	-	-	-	-	-	-	-	-	-	-	-	-
Alfa-carotene	-	-	-	-	-	-	-	-	-	-	-	-
Beta-carotene	-	0.01	0.01	0.021	0.001	0.01	0.01	0.02	0.03	0	0.02	0.013
Chlorophyll *b*	-	0.025	0.029	0.13	0.001	0.06	-	0.1	0.04	0.13	0.07	0.08
Peridinin	3.5	0.007	0.03	0.023	-	0.15	0.003	0.004	0.005	0.01	0.002	0.06
19'butanoyloxifucoxanthin	-	0.01		0.025	-	0.02	0.03	0.01	0.04	0.022	0.013	0.01
Fucoxanthin	0.6	0.3	0.07	0.163	0.018	0.09	0.07	0.34	0.62	0.16	0.36	0.16
Neoxanthin	-	-	-	0.012	-	0.005	0.01	0.009	0.002	0.00998022	0.005	0.008
19'hexanoyloxifucoxanthin	-	0.019	-	0.029	0.001	0.016	0.04	0.012	0.012	0.01940648	0.02	0.012
Prasinoxanthin	-	-	-	-	-	0.02	0.01	0.016	0.005	0.02	0.01	0.014
Violaxanthin	-	-	-	-	-	-		-				
Diadinoxanthin	-	-	-	-	0.001	-		-				
Alloxanthin	0.6	-	0.02	0.04	-	-	0.02	0.0134	0.011	0.04	0.01	0.3
Diatoxanthin	0.5	-	-	-	-	-	-	-	-	-	-	-
Zeaxanthin	-	-	-	-	-	-	-	-	-	-	-	-
Lutein	-	-	-	-	-	-	-	-	-	-	-	-
Chl *a*-epimer	-	12.5	-	-	-	-	-	-	-	-	-	-

The concentrations of identified pigments in 12 in situ samples sampled were detected in the eastern side of the Faro-Olhão inlet, off Ria Formosa in southern Portugal.

It was possible to detect Chl*a*, Chlorophyllc2 and fucoxanthin in all the samples, beta-carotene, Chlorophyll*b*, peridinin, 19'butanoyloxifucoxanthin, 19'hexanoyloxifucoxanthin have been detected in the majority of the samples, with the exception of 1 or 2 samples. Some of the pigments such as phaeophorbide a, chlorophyllide a, zeaxanthin and lutein and alfa-carotene have not been at all detected in these samples.

It is important to refer in this context that the total Chl*a* concentration is equal to the sum of the concentrations of monovinyl chlorophyll *a*, divinyl chlorophyll *a*, chlorophyllide *a*, chlorophyll *a* epimer, phaeophorbide *a*, pheophytin *a*, and it is this sum of Chl*a* derivates which should in a future stage be compared with EO data of Chl*a*.

When results are observed, it was clear that Chl*a* epimer have been detected only in the sample 2 one, for the same sample the total Chl*a* is calculated as 12.94 ng/ml. Pheophytin*a* was only detected in samples 10 and 12. For these samples, Total Chl*a* values are 3.1 ng/ml and 4.6 ng/ml. So it can be said that relatively high value of total Chl*a* was registered in October- December (12.94 ng/ml and 4.6 ng/ml respectively) period of 2020 and quite high value was registered in July 2021 (140 ng/ml), which seems to be an aberrant value for the area, and, in the absence of duplicates samples to check this result, it should be perhaps discarded. During the rest of the sampling period the concentration of Chl*a* varied from 0.3 ng/ml to 0.84 ng/ml.

Chapter 5. Discussion

The experiments accomplished in this thesis allowed for interesting findings regarding the implementation of a different methodology in a recent equipment acquired in CIMA-UAlg laboratories, and to infer on the performance of such methodology in the context of the analysis of coastal water samples from the south of Portugal.
In the first phase of the implementation of the method, the various tests which consisted in changing volumes of injection and the concentration of the injected standards and samples allowed to decide on an adequate concentration and injection volume which was then chosen for the following experiments, which was the standard pigment mixture with C=3.17mg/l and a volume of 125ul. With this combination of volume and concentration, it was possible to identify more pigments, and with better shaped peaks (**Figure 3f**), which will translate later, with this combination of volume and concentration, it was possible to identify more pigments, and with better shaped peaks (**Figure 3f**), which will translate later in more adequate chromatograms to evaluate performance metrics of the equipment.

Based on the result of **Table 3**, it is easy to see that the Rt of the majority of pigments decreased over time which is possibly may be a result of a slight change of an eluent system, since the retention times are affected by differences in the composition of the eluents. In theory, changes in retention times may be either due to alterations in the column (different columns have different retention times), mechanical alterations in the detector or mainly due to differences in the eluent system. Hence, the preparation of the sufficient amount of eluent for a complete set of samples is important, so to be more confident on the stability of the retention times. Sometimes, even changing one of the eluents brand is sufficient to shift retention times.

The coelutions problem identified in the case of Lutein and Zeaxanthin, which although not completely co-eluted (**Figure 3a**, peak 14/15), cannot be distinguished by the software as separate peaks, even showing that the column should probably be changed, this factor for itself is not a major limitation of the implemented method. In fact, the authors of Sanz et al. [6], in their work suggest that it is possible to enlarge the separation of Zeaxanthin and Lutein critical pair of pigments by increasing the ethanol content; however this may cause a decrease of resolution of acidic chlorophylls.

The fact that the absorption maxima were observed to be quite constant over the study period points out that the diode array detector of the system shows an adequate level of stability, which is a major advantage for pigment peaks identification.
When concerning the determination of the method performance metrics, and while comparing **Table 8** (with the performance metrics of the implemented Sanz et al. [6] methodology in our laboratory) and **Table 9** (from Hooker et al.[8]), it is possible to see that the average retention precision time in our studies is 0.843%, which is much higher (around 4.7 times) than the routine category of retention time precision in Hooker et al. [8] – SeaHARRE-2 protocol, which was as low as 0.18% for this routine category. As it is shown in the table 4, for almost all the identified pigments CV value is equal to 0.01 (the same as 1%), with the exception of Lutein- with the CV value of 0.02, and other 3 pigments (Chlorophyll A, Chlorophyll a epimer and β-carotene).

Since only last 3 identified pigments have low CV values, it is possible to presume that during the initial phase of the chromatogram, when A solvent (Methanol: Ammonium Acetate solvent) is in major percentage passing through the column and during the mid phase of chromatogram when solvent A and B (ethanol) are passing through the column together, there is a lower retention time stability in comparison to the final part of the chromatograms, where the majority of the percentage of eluent passing the column is with solvent B (ethanol).

Although this performance metric (average retention time precision) has not shown a “State-of-the-art” result, this not a major limitation of the method in CIMA-UAlg, since the identification of the peaks in the samples have been done manually, one by one, by taking into account the shapes and the absorption maximum of their spectra, and considering the retention time of each peak also as well as elution order of all the identified peaks. Also, the eluent system was the same, because the eluents have been prepared in the beginning once and not been changed during the all the runs of sequential experiment. Based on the abovementioned observations, it could be advised that manual identification should always be performed with visual observation of the absorption spectra and confirming the elution order of the peaks, rather than trusting the automatic identification of the peaks that the software does, in order to overcome the impacts of a lower retention time precision in the analysis of phytoplankton pigments.

Comparison of the long-term injection precision values show that the performance metrics result of our studies almost corresponds to the injection precision in Hooker et

al. [8, 30] - SeaHARRE protocols for the routine Category (both for Peridinin and Chla). Injection precision value for peridinin in our studies ranges from 7.4 to 11.3%, whereas in SeaHARRE-2 protocol the same parameter in routine category is 10% for Peridinin. Injection precision in our studies for Chl*a* ranges from 2.8% to 10.14%, while in Hooker et al. [30] for the routine category it is 6%. It can be assumed that the injector works precise enough for routine work, however, a bad performance in this metric is a more serious limitation, once the uncertainties in the peak areas can originate relevant errors in the quantification of the concentration of the pigments, once this metric is directly related with the variation on the area of the pigments. So, is that in this case, this is performance metric that the analyst should be more careful and attentive to possible deterioration in these values in future analysis, when compared with the other performance metrics, for the purpose of measuring chlorophyll a and compare with satellite values. A suggestion for limiting the impacts of such limitation in the pigment concentration determination with this methodology is, once more, to assure the manual integration the peak areas, to avoid the equipment software to introduce a higher level of uncertainty in this parameter.

The minimum resolution value obtained in our performance metrics studies is 0.36, whereas in the Seaharre protocol [30], the minimum resolution (routine level) should be 0.8. In the case of the implemented method in CIMA-UAlg laboratory. In the case of Sanz et al. [6] they were able to obtain higher values of resolution (~0.98), which matches to semi-quantitative category of Hooker et al. [8,30]. In this work, the Zeaxanthin/Lutein critical/ pair is not fully resolved. One of the recommendations for improvement of this value in the future are the one suggested by Sanz et al. [6], which refer that it is possible to enlarge the separation of Zeaxanthin and Lutein critical pair of pigments by increasing the ethanol content. However, this should be only applied if the accurate determination of these two pigments (Lutein/Zeaxanthin) is relevant for the specific studies, once this change may also cause a decrease of resolution of acidic chlorophylls as a side effect [6]. Hooker et al. [8] protocols also suggest the replacement of the column if the resolution is lower than 1 for these critical pairs [8]. Since, based on this thesis experiments, the minimum resolution of critical pair Zeaxanthin/ Lutein is 0.36, it is advised that the column should be replaced for a new one.

Regarding the application of the implemented methodology for the determination of phytoplankton pigment concentration in natural samples, it was possible to

observe that this methodology allowed the quantification of approximately 15 different phytoplankton pigments with adequate resolution and peak shape. When the determined performance metrics are observed together with these results for natural samples, it is possible to observe that the most important pigment in terms of EO data validation – Chl*a* – and despite the method not showing high levels of performance metrics ranks, the results can still be trustworthy, at least for routine work. The facts that the method is relatively easy to implement and uses a relatively low toxicity eluent system allows to infer that, having in mind the abovementioned caveats are added values of the methodology which should not be neglected.

Chapter 6. Conclusion

The main conclusions to be withdrawn for the accomplishment of this study will now be presented in the format of answers to the initially presented research questions of this thesis, as follows:

1. What are the main advantages and limitations of Sanz et al. (2015) chromatographic method for determining phytoplankton pigments?

One of the advantages of the method was the application of less toxic eluents (methanol, ammonium acetate, and ethanol) [6], whereas most of the current HPLC methods for the determination of phytoplankton pigments contain relatively toxic solvents. So we can assume that the method is environmentally friendly. Besides that, simple binary elution system, and not an extremely requiring instrumental setup is also an added value. On the other side, the main limitations are related with the performance of the metrics which have been discussed and are summarized in the following research questions identified.

2. How does Sanz et al. (2015) method performs when implemented in CIMA-Lab compared with reference performance metrics listed in Hooker et al. (2005) (20) and subsequent SeaHARRE reports)?

The performance metrics parameters studied and determined during this thesis correspond to those in routine category in Hooker et al. [8] –SeaHARRE-2, in case of average injection precision of Peridinin as well as for Chl*a*. This means that injector works precise enough for routine work, although the analyst should be more careful and attentive to possible deterioration in these values in future analysis, when compared with the other performance metrics, for the purpose of measuring chlorophyll a and compare with satellite values. For the other performance metrics, lower than routine rank of performance metrics was obtained. Despite that, several suggestions to assure that these limitations do not have a heavy impact of the uncertainty of the phytoplankton pigment concentrations were also discussed and presented, such as the replacement of the current chromatographic column, the manual integration of the peak area and the identification of the peaks based not only on the retention time of the pigments, but also based on the elution order and on the UV-VIS spectra.

3. How effective and efficient is the Sanz et al. (2015) HPLC methodology when implemented in CIMA-Lab for analyzing phytoplankton pigments in the South Coast of Portugal?

By using Sanz et al. [6] method an optimal concentration and volume combination has been chosen for the further application for identification of pigments in the pigment mixtures as well as in in-situ samples. 12 in-situ samples have been measured and concentrations of Chl*a*, as well as other pigments have been calculated based on the SeaHARRE-2 protocol equations. The obtained concentrations might in the future be used to compare with the Chl*a* concentrations retrieved from through Earth Observation technology. Notwithstanding, such comparison should have in account the uncertainties that can also come from the HPLC results in this implemented methodology.

An extra achievement of this work was the creation a library based on the pigments' absorption maximums of spectra, and their retention time. With this, during future measurement achieved with Sanz et al. [6] method it will be easier to identify peaks comparing with the individual standard pigment characteristics.

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