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Carotenoid determination in recent marine sediments - practical problems during sample preparation and HPLC analysis

Magdalena Krajewska*, Małgorzata Szymczak-Żyła and Grażyna Kowalewska

Marine Pollution Laboratory, Institute of Oceanology, Polish Academy of Sciences, Powstańców Warszawy 55, 81-712 Sopot, Poland

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A B S T R A C T

An analytical procedure for the analysis of carotenoids in marine sediments rich in organic matter has been developed. Analysis of these compounds is difficult; the application of methods used by other authors required optimization for the samples studied here. The analytical procedure involved multiple ultrasound-assisted extraction with acetone followed by liquid-liquid extraction (acetone extract:benzene:water - 15:1:10 v/v/v) and HPLC analysis. The influence of column temperature on pigment separation and the quantification method were investigated – a temperature of 5 °C was selected for the Lichrospher 100 RP-18e column. The pigments in the sediment extract were quantified using a method based on HPLC analysis (at 450 nm) and spectrophotometric measurements (at 450 nm), and extinction coefficients were determined for standard solutions at this wavelength. It is very important to use the value of the extinction coefficient appropriate to the wavelength at which the detection of carotenoids was carried out.

1. Introduction

Carotenoids are a large group of natural compounds (at present *ca* 700 are known), ubiquitous in the aquatic environment.[1] They occur mainly in phytoplankton, macroalgae and bacteria but also, as metabolites of parent carotenoids, in different organisms from other levels of the food chain like zooplankton and fish.[1,2] Aquatic basin sediments contain a large variety of carotenoids, which can be used as proxies of plankton and macrophyta occurring in the adjacent waters and of postdeposition conditions.[3,4] The analysis of carotenoids is difficult, especially in organic-rich sediments, because they contain not only parent carotenoids and their derivatives, but also a variety of low and high molecular organic compounds, including chloropigments. The most troublesome are separations of compounds with very similar chemical structures, like lutein and zeaxanthin or α-carotene and β-carotene, which differ only in the position of one double bond in the cyclohexyl ring (see **Fig. 1**). All the steps, i.e. sample collection, extraction, preservation and the chromatographic analysis itself, can affect the results.

* Corresponding author.
E-mail address: mkrajewska@iopan.gda.pl (M. Krajewska)

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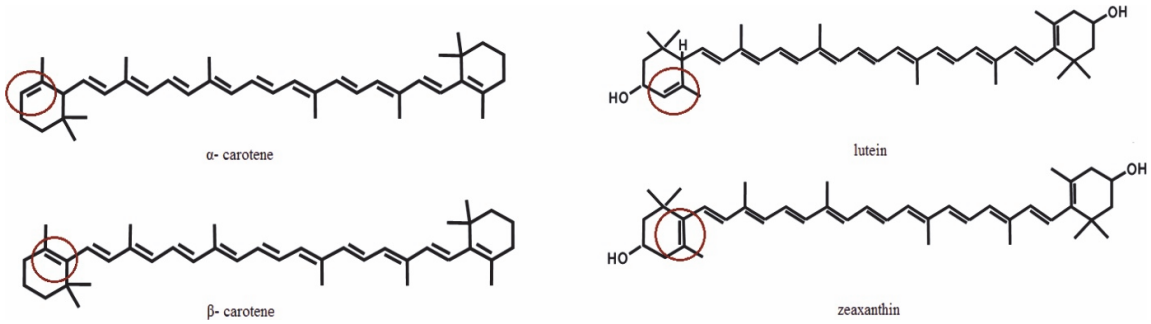

Fig. 1. Structures of α-carotene/β-carotene and lutein/zeaxanthin

Numerous extraction methods have been applied to sediments but most involve extraction by soaking in organic solvent.[4-6] The extraction efficiency depends on the type of solvent, its volume and time of extraction.[4,7-10] Pigments are extracted with different solvents, i.e. acetone, methanol, ethanol or their mixtures (see **Table 1**).

Table. 1. Methods of carotenoid extraction from sediments

Sediment	Extraction solvent	Additional information	References
freeze-dried	acetone	sonication, storage overnight at -20 °C, evaporation to dryness	5
		sonication in an ice bath for 40 s, extraction overnight at -20 °C	11
		sonication	12
		sonication (3x), evaporation to dryness	13
		sonication in an ice bath for 15 min, extraction at -20 °C for 20 h in the dark	14
		extraction at -20 °C for 20 h	15
	acetone:methanol:water (80:15:5 v/v/v)	storage at -10 °C to -20 °C for 12 h in the dark, evaporation to dryness	4
		extraction overnight at -4 °C, evaporation to dryness	16
	acetone:methanol (80:20 v/v)	sonication for 10 min, extraction at -20 °C for 24 h in the dark	17
	95 % buffered methanol (2% ammonium acetate)	sonication for 30 s, extraction at -20 °C for 15 min in the dark	6
wet	acetone	sonication at 0°C for 15 min (3x), evaporation to dryness	18
		sonication, evaporation to dryness	19
		sonication for 2-3 min, re-extraction to benzene, evaporation to dryness	this work

Sonication is applied to support the extraction process.[5,6,11] All these activities should be performed in dim light and as quickly as possible to prevent pigment decomposition. However, a balance between the best possible extraction efficiency and the risk of artefact formation should be maintained. To increase extraction efficiency, liquid-liquid extraction of pigments from an acetone extract to another solvent has sometimes been used.[9]

There are a number of publications on HPLC methods for determining carotenoids in sediments.[5,16,20-22] RP-HPLC (C18) has mostly been used for this purpose (see **Table 2**).

Table. 2. HPLC methods for the determination of carotenoids in sediment extracts

Stationary phase	Mobile phase	Flow rate [ml/ min]	Ref.
Spherisorb ODS2 (250 x 4.6 mm, 5 µm)	A - 80:20 methanol:0.5 M ammonium acetate v/v B - 90:10 acetonitrile: water v/v C - ethyl acetate	1.0	2
Alltech Adsorbospher C_{18} (250 x 4.6 mm, 5 µm)	A - 80:20 methanol:0.5 M ammonium acetate v/v B - 90:10 acetonitrile:water v/v C - ethyl acetate	1.0	5,23
Navi C30- 5 (250 x 4.6 mm)	A - 90:10 acetonitrile:water v/v B - 100 % ethyl acetate	1.0	18
Supelcosil LC-18 (250 x 4.6 mm, 5 µm)	A - 80:20 methanol:0.5 M ammonium acetate v/v B - 90:10 acetonitrile:water v/v C- ethyl acetate	1.0	11
	A - 85:15 methanol: water (buffered with 0.5 M ammonium acetate) v/v B- 90:10 acetonitrile:water v/v C- ethyl acetate	0.6	6
	A - 75:25 acetone:water v/v B - 80:20 methanol:water v/v	0.85	14
Waters Spherisorb ODS2 (C18) (150 x 4.6 mm, 3 µm)	A - 80:20 methanol:ammonium acetate v/v B - 80:20 methanol:acetone v/v	0.8	17
COSMOSIL 5C18-AR	A - 63:7:30 acetonitrile:0.5 % triethylamine aqueous solution:methanol v/v/v B- ethyl acetate	1.0	12
Lichrospher 100Rp-18e (250 x 4 mm, 5 µm)	A - 85:15 methanol:0.5 M ammonium acetate v/v B - 90:10 acetonitrile:water v/v C - ethyl acetate	1.0	this work

The mobile phase used by different authors was usually the same, but the gradient system was sometimes changed slightly.[5,22] Other factors, such as the mobile phase composition or flow rate, were frequently modified.[6,11] The application of methods used by other authors always requires them to be optimized to one's own needs and the available equipment. The choice of appropriate temperature for carotenoid analysis is very important, but column thermostatting is required. Column temperature problems were studied by some authors in the case of phytoplankton,[24,25] and surficial riverine sediment carotenoids[26], but the results were contradictory. Another important issue is quantification. Some authors calculated pigment concentrations from a calibration curve using HPLC analyses of standards,[6,24,27] others used a method based on additional spectrophotometric measurements of the same but diluted solution as that analysed by HPLC and literature extinction coefficients of particular pigments.[9,11,28]

The aim of this paper was to develop an analytical procedure (sample preparation, HPLC analysis and quantification method) for determining carotenoids that could be applied to organic-rich sediments, and to indicate the problems to be overcome during the development of this method. The influence of liquid-liquid extraction from an acetone extract to benzene on carotenoid extraction efficiencies was checked. The effect of column temperature on pigment separation was investigated and the different quantification methods were compared.

2. Results and Discussion

Both wet and freeze-dried sediments were subjected to extraction by different authors (see **Table 1**). The time of extraction varied from several minutes[6,18,29] to several hours[4,7,26] and even overnight.[5,11,16,17] However, the influence on extraction efficiency of freeze-drying prior to extraction is not clear. In the case of freeze-dried sediments a 5-10 % content of water was recommended to improve extraction of polar compounds,[4,30] but some authors used acetone for extracting freeze-dried material.[11,13,14]

Ultrasonication is still in routine use in many laboratories and in this work was selected for extracting carotenoids from sediments. We used ultrasound-assisted extraction of wet sediments with acetone for the analysis of carotenoids in Gulf of Gdańsk sediments. This extraction method is used in our Marine Pollution Laboratory (MPL-IO) for chloropigments and has been intercalibrated with other traditional methods of pigment extraction.[9] Acetone has been shown to be an effective extraction solvent for wet sediment samples.[30] In many methods a small volume of solvent was used (up to 10 ml), after which the extract was analysed by HPLC.[5,11,29] In the case of organic-rich sediments a small amount of solvent might be insufficient to extract the whole amount of pigments. To ensure complete extractability in our method, multiple acetone extraction and total volume of solvent (15 – 45 ml), larger than that used by other authors, were applied. To reduce the sample volume, liquid-liquid extraction of carotenoids from an acetone extract to benzene was performed (acetone extract:benzene:water - 15:1:10 v/v/v). This extraction step speeds up the further evaporation of the solvent and concentrates the analytes, which increases the sensitivity of the method (see **Fig. 2**).

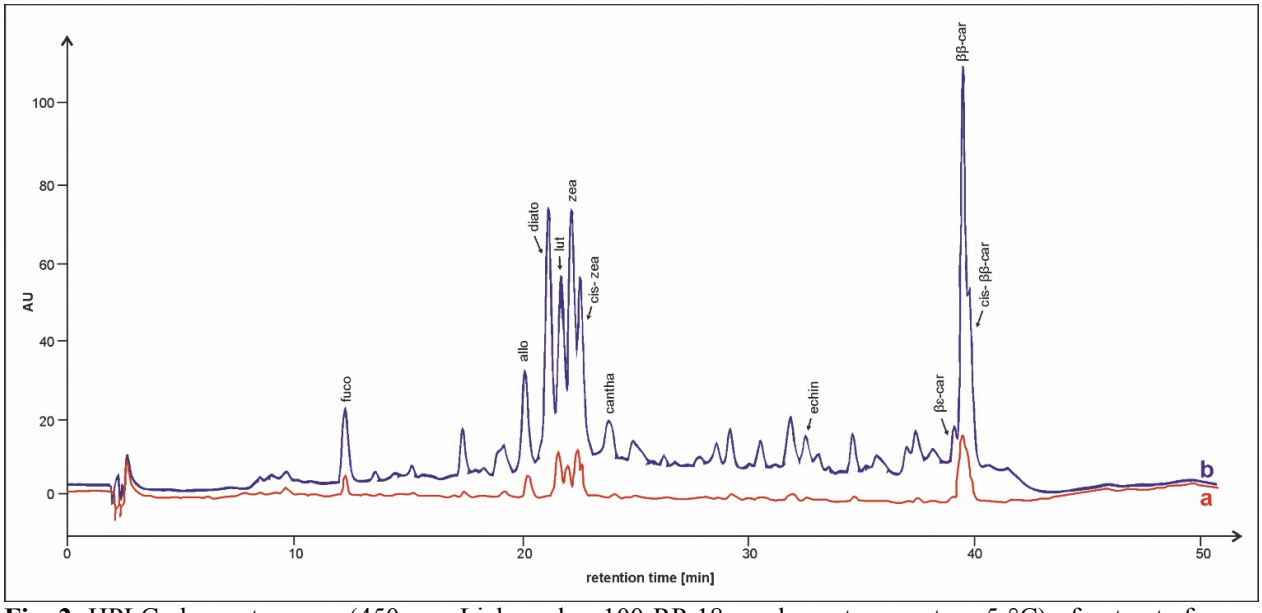

Fig. 2. HPLC chromatograms (450 nm, Lichrospher 100 RP-18e, column temperature 5 °C) of extracts from recent sediments (station M, 1-5 cm, Gdańsk Deep, Baltic Sea) (a) without and (b) with a liquid-liquid extraction step; for abbreviations - see **Table 3**.

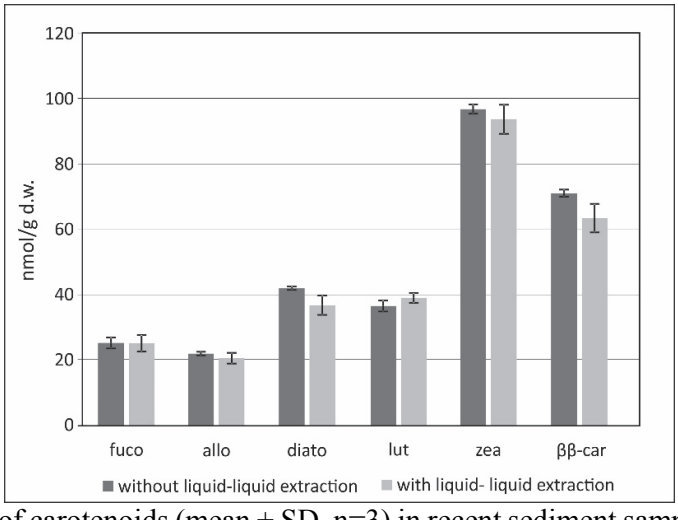

Fig. 3. Concentrations of carotenoids (mean ± SD, n=3) in recent sediment samples (station M, 1-5 cm, Gdańsk Deep, Baltic Sea) (a) without and (b) with liquid-liquid extraction step; for abbreviations - see **Table 3**.

The chromatogram peaks were taller, which means that compounds present in low concentrations (e.g. echinenone, α-carotene) could also be determined. This extraction step did not affect the composition of pigments in the sediment sample. The differences in carotenoid concentrations in a sample before and after liquid-liquid extraction were not statistically significant; this was demonstrated for the most abundant carotenoids (see **Fig. 3)**. Only the major peaks were compared because the values for echinenone and α-carotene in the samples not subjected to liquid-liquid extraction were below the limit of quantification.

It is difficult to analyse all the carotenoid compounds present in a sample in one HPLC run[6,31], especially in a mixture with chloropigments (which also absorb at 450 nm). Sediment samples contain not only parent carotenoids but also their derivatives (see **Fig. 2b**), such as cis-zeaxanthin, which is formed by geochemical reduction from diatoxanthin in warm-anoxic conditions,[32] making HPLC separation even more complicated. Therefore, it is important to decide which compounds we wish to quantify first of all; the HPLC method should then be selected accordingly. In this work, the greatest stress was placed on parent pigments, markers of the main phytoplankton groups occurring in the Baltic. These were peridinin and dinoxanthin as markers of dinoflagellates, fucoxanthin (diatoms), diatoxanthin and diadinoxanthin (diatoms and dinoflagellates), alloxanthin (cryptomonads), lutein (green algae), zeaxanthin (cyanobacteria and green algae), canthaxanthin and echinenone (cyanobacteria), α-carotene (cryptomonads) and β-carotene (most algae).[2] Methods developed for seawater are not satisfactorily applicable to sediment extracts. Moreover, the methods used for sediments by other authors (see **Table 2**) require certain modifications – these may apply to the column (length, diameter or stationary phase), gradient system or column temperature.

The separation of lutein (a pigment of green algae and higher plants) from zeaxanthin (cyanobacteria and green algae) is very important for aquatic environmental studies, as these pigments are useful for analysing taxonomy. The problem of separating lutein from zeaxanthin was noticed already some time ago:[33] these two compounds have very similar retention times and sometimes coelute. Their good separation is evidence that the appropriate analytical method was chosen. The influence of the Lichrospher 100 RP-18e column temperature on the resolution of compounds very similar in chemical structure (lutein/zeaxanthin and α-carotene/β-carotene) was assessed. It was found that the lower the column temperature, the longer the time of analysis, which enhances separation of the mixture's components. Better separation of the lutein/zeaxanthin was achieved using a column thermostatted at 5 °C rather than at 25 °C (see **Fig. 4**). The resolution of separation at 5 °C between lutein/zeaxanthin ($R_s = 1 \pm 0.07$ for sediment, $R_s = 0.79 \pm 0.04$ for pigment standards) and α-carotene/β-carotene ($R_s = 0.96 \pm 0.06$ for sediment, $R_s = 0.65 \pm 0.06$) was significantly higher ($p < 0.05$) than at other temperatures. The resolution specifies the selectivity of the stationary phase and column efficiency. The lower resolution of the pigment pair in the standard mixture than in the sediment extract may be because the concentrations of available pigment standards are very low.

For the chromatographic separation of chlorophylls and carotenoids in a phytoplankton extract of seawater Zapata et al. [25] used a column thermostatted at 25 °C, and Jun et al.[26] did likewise for a riverine sediment extract; neither paper gave any explanation why this particular column temperature was applied. Van Heukelem et al.[24] studied different column temperatures to improve separations of phytoplankton pigments from lake water using HPLC; they recommended a higher temperature (60 °C) as optimal for carotenoid separations. These observations do not agree either among themselves or with our results. We chose a column temperature of 5 °C as the most suitable for our purposes. Before injection, the samples were stored in an auto-sampler at the same temperature (5 °C). The lower temperature and darkness prevented pigment decomposition. Reuss and Conley recommended storage of samples in an auto-sampler at an even lower temperature (4 °C).[11] It should be emphasized that we chose the column temperature of 5 °C as optimal for the Lichrospher 100 RP-18e column; for other columns, the best temperature may be different. When optimizing a literature method, it is very important to do so with respect to one's own equipment and working conditions.

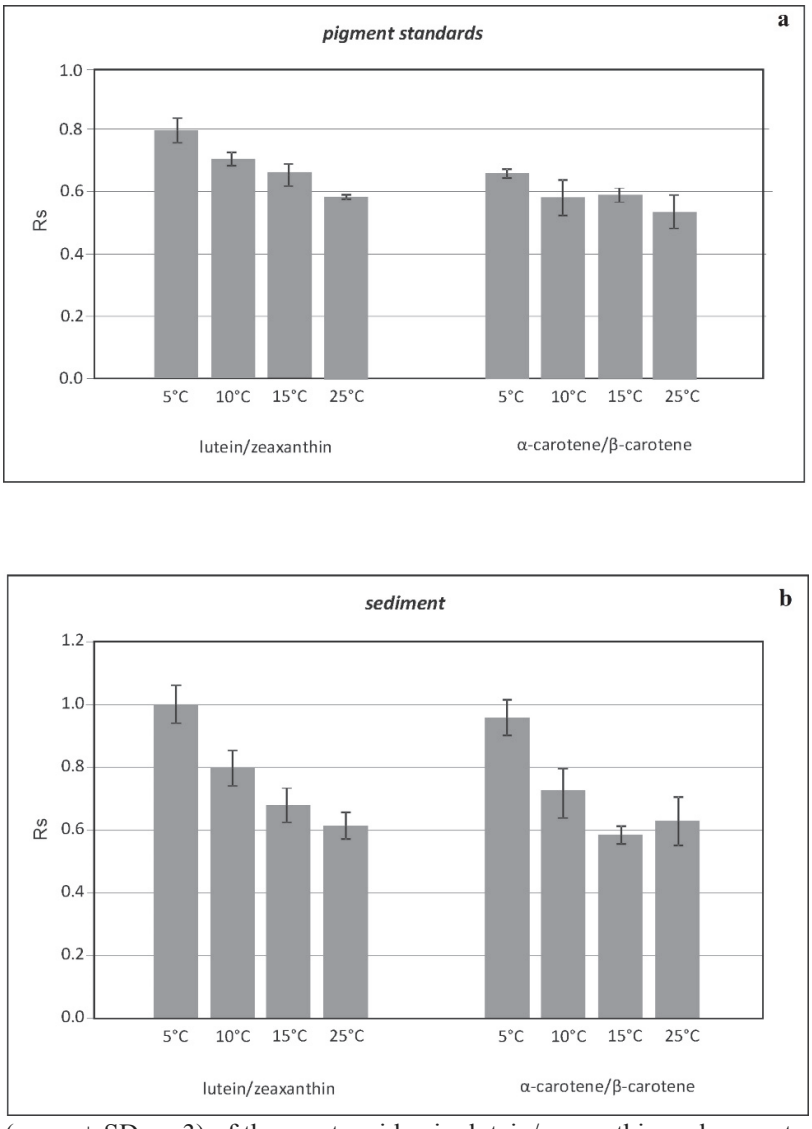

Fig. 4. Resolution (mean ± SD, n=3) of the carotenoid pairs lutein/zeaxanthin and α-carotene/ β-carotene from (a) a mixture of pigment standards (DHI, Denmark) and (b) recent sediment (station M, 1-5 cm, Gdańsk Deep, Baltic Sea) using different column temperatures; for abbreviations - see **Table 3**.

Another problem that arose during the analysis of carotenoids in the sediment extracts was quantification. Some authors have based their calculations on HPLC standard calibration curves,[6,14,27] others have used a method based on an additional spectrophotometric measurement of the same solution as that analysed by HPLC and literature extinction coefficients of particular pigments.[9,11,28] The different calculation methods are compared in **Fig. 5**.

In the case of the spectrophotometric method it is very important to use the appropriate extinction coefficients, i.e. those determined for wavelength at which the detection of carotenoids was carried out. In this work, the chromatograms were recorded and integrated at 450 nm. **Table 3** lists the extinction coefficients calculated from absorption measurements of carotenoid standards at 450 nm using the Lambert-Beer law.

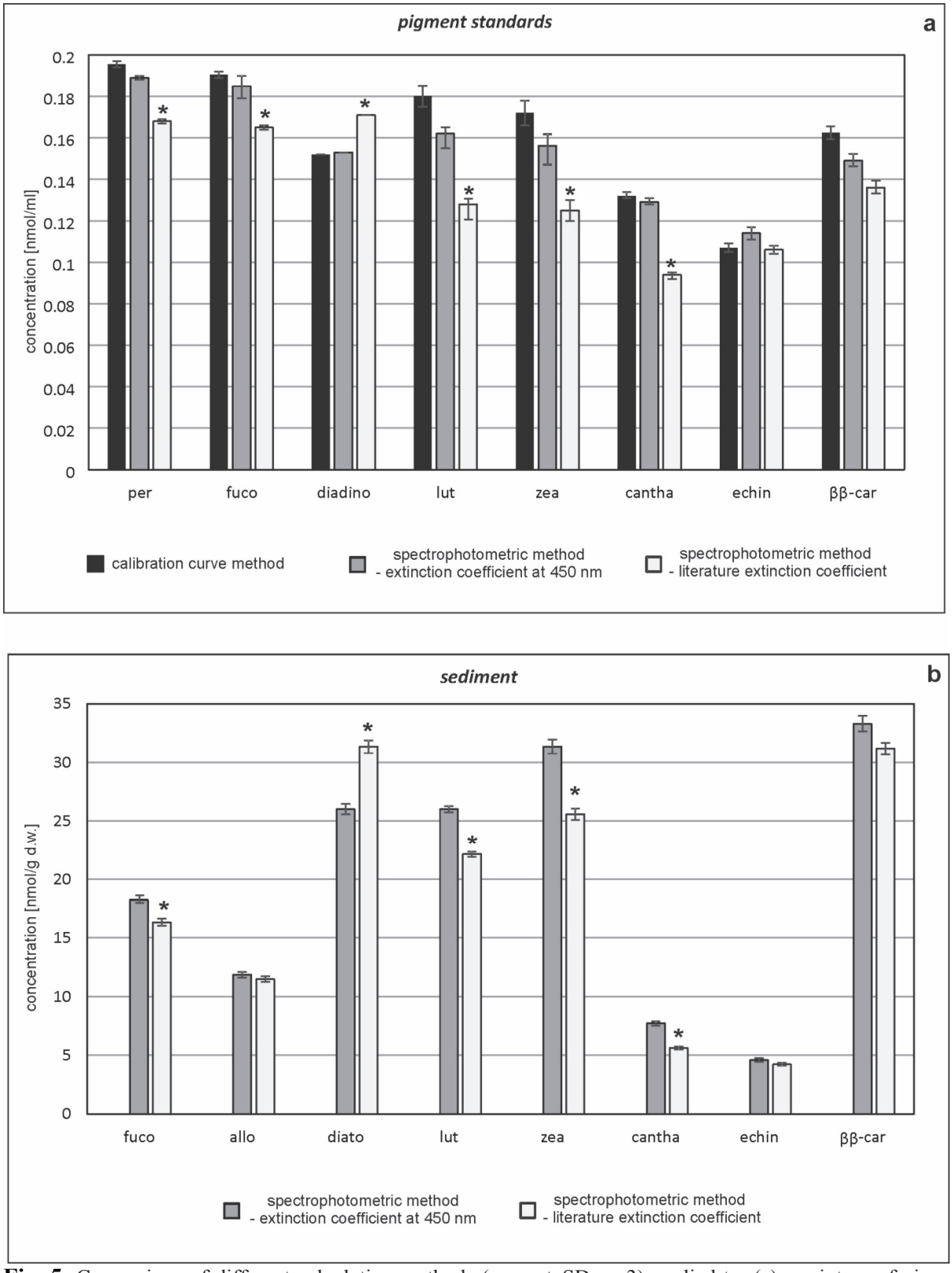

Fig. 5. Comparison of different calculation methods (mean ± SD, n=3) applied to: (a) a mixture of pigment standards (DHI, Denmark) and (b) recent sediment (station M, 1-5 cm, Gdańsk Deep, Baltic Sea); * - statistically significant difference; for abbreviations – see **Table 3**.

Table 3. Absorption maxima, extinction coefficients and molecular masses of individual parent carotenoids

Carotenoid	Abbreviation	Absorption maxima (nm)	Extinction coefficient at 450 nm* ($ml \cdot mg^{-1} \cdot cm^{-1}$)	Extinction coefficient ** ($ml \cdot mg^{-1} \cdot cm^{-1}$)	Molecular mass ($g \cdot mol^{-1}$)
Peridinin	Perid	475	119 (in ethanol)	134 (at 466 nm in acetone)	631
Fucoxanthin	Fuco	450,470	152 (in ethanol)	166 (at 443 nm in acetone)	659
Dinoxanthin	Dino	418, 442, 471	160 (in ethanol)	210 (at 442 nm in acetone)	643
Diadinoxanthin	Diadino	424, 447, 478	245 (in ethanol)	223 (at 447.5 nm in acetone)	583
Alloxanthin	Allo	428, 453, 483	242 (in ethanol)	250 (at 454 nm in acetone)	565
Diatoxanthin	Diato	428, 453, 481	245 (in ethanol)	210 (at 452 nm in acetone)	567
Lutein	Lut	423, 446, 474	215 (in ethanol)	255 (at 445 nm in ethanol)	569
Zeaxanthin	Zea	428, 452, 479	192 (in ethanol)	234 (at 452 nm in acetone)	569
Canthaxanthin	Cantha	472	160 (in ethanol)	220 (at 466 nm in petroleum ether)	565
Echinenone	Echin	461	198 (in ethanol)	216 (at 458 nm in petroleum ether)	551
α-carotene	βε-car	425, 448, 476	260 (in acetone)	270 (at 448 nm in acetone)	537
β-carotene	ββ-car	429, 454, 480	233 (in acetone)	250 (at 454 nm in acetone)	537

* calculated from absorption measurements of carotenoid standards at 450 nm using the Lambert-Beer law **(this work)**
*** according to literature data*[2]

Particular carotenoids have absorption maxima at different wavelengths, whereas the maximum wavelength depends, for example, on the solvent in which it is determined. Also, the solvent composition during HPLC analysis at the retention time of an analyte is usually different than the solvent for which literature extinction coefficients are available. For example, canthaxanthin has an absorption maximum at 472 nm (in ethanol)[2], while the extinction coefficient equal to 220 $ml \cdot mg^{-1} \cdot cm^{-1}$was determined at 466 nm (in petroleum ether)[2] (see **Table 3**); in this work, the extinction coefficient calculated at 450 nm (in ethanol) was 160 $ml \cdot mg^{-1} \cdot cm^{-1}$. In the spectrophotometric method used in this work both the HPLC chromatogram and absorption spectrum were measured at the same wavelength (450 nm) to minimize errors.

In the case of pigment standards, the concentrations calculated in this way are comparable to the values obtained from calibration curves, while the use of literature extinction coefficients (see **Table 3**), especially those set for other wavelengths, significantly affects the results **(see Fig. 5a**). Pigment concentrations in sediments cannot be quantified using HPLC calibration curves. The concentrations of carotenoids in commercial standard mixtures are usually low. Indeed, they were very low (0.77- 1.36 mg/l) in the commonly used individual standards that we purchased, whereas the concentrations of these carotenoids in sediment samples were several or even several dozen times higher. Peak areas of carotenoids in sediment extracts were beyond the range of calibration curves. One solution would be to dilute the sediment extract; unfortunately, however, the concentrations of individual pigments in the sediment extracts differ significantly from each other. Diluting a sample could place carotenoids like canthaxanthin, echinenone and α-carotene, present in sediments in low concentrations (see **Fig. 2b**), beyond the limits of detection. There was no single suitable concentration of sediment extracts for accurately determining all the analytes in a sample, thus enabling the calibration curves of available standards to be used. The analysis of one sample at different concentrations would be time-consuming, especially when a lot of samples have to be analysed. That is why we used the spectrophotometric method of quantification. In this case, it is also important to use the appropriate extinction coefficients,

determined for the wavelength at which the detection of carotenoids was carried out (see **Figure 5b**), as indicated above; the differences are often significant ($p < 0.05$).

3. Conclusions

The sediments from the Gulf of Gdańsk contained a variety of carotenoids, typical of both marine and freshwater organisms, and their derivatives. A Lichrospher 100 RP-18e column temperature of 5 °C was selected for the parent pigments, which are markers of the phytoplankton groups abundant in the Baltic. Resolution at this temperature was better than at the other temperatures studied. Liquid-liquid extraction from an acetone extract to benzene during sample preparation concentrates the analytes, which increases the method's sensitivity.

The pigments in the sediment extract were quantified using a method based on spectrophotometric measurements and extinction coefficients. It is very important to use the appropriate extinction coefficients values, i.e. determined for the wavelength at which the detection of carotenoids was carried out.

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4. Experimental

4.1. Materials

4.1.1. Samples

Muddy sediment (1-5 cm layer), rich in organic carbon (C_{org} ~6 %) and the fine grain size fraction (<63 μm ~100%), sampled at station M (54°44.912'N; 19°17.662'E, Gdańsk Deep (southern Baltic), water depth ~100 m), was used in this work for optimizing the analytical procedure for carotenoid determination.

4.1.2. Standards and solvents

The pigment standards used, the ones usually used for carotenoid analyses, were from DHI Water & Environment, Denmark. They included a pigment mix (PPS-MIX-1) and the following individual pigments: peridinin, fucoxanthin, dinoxanthin, diadinoxanthin, alloxanthin, diatoxanthin, lutein, zeaxanthin, canthaxanthin, echinenone, α-carotene and β-carotene.

The solvents (HPLC grade, VWR International Sp. z o.o.) were filtered and degassed with helium (5.0, Linde) before analysis.

4.2. Pigment extraction

The carotenoids from a sediment sample were extracted according to the procedure used for chloropigments by the Marine Pollution Laboratory - Institute of Oceanology, Polish Academy of Sciences (MPL-IO).[34] A frozen sediment sample (~3 g) was placed in a glass centrifuge tube and left in the dark to thaw (~5 min). The water was removed by centrifugation (6 min, 2500 rpm). The sample

was flushed with 15 ml acetone, mixed, sonicated (2 – 3 min), centrifuged again and the extract decanted. The extraction was repeated until the supernatant was colourless (max. 3 times). The acetone extracts were transferred to a separating funnel for liquid-liquid extraction in an acetone extract:benzene:water system (15:1:10 v/v/v). The benzene layer was transferred to a glass vial, evaporated to dryness in a stream of argon and kept frozen (–20°C) until HPLC analysis. The extracted sediment was dried at 60°C and weighed. The pigment content was calculated per dry sediment weight.

To assess the influence of liquid-liquid extraction on carotenoid analysis, the following experiment was performed (in triplicate). The acetone extract of the sediment sample (30 ml) was divided into two portions. One (15 ml) was extracted in an acetone extract:benzene:water system (15:1:10 v/v/v), then evaporated and dissolved in a smaller volume of acetone (1 ml) and analysed using HPLC, whereas the other (15 ml) was analysed without the re-extraction step. The carotenoid concentrations of both sub-samples were determined and the results compared (**Fig. 3**). The separations were carried out according to the procedure described in the *HPLC analysis* section (Lichrospher 100RP-18e column, column temperature 5°C).

4.3. HPLC analysis

4.3.1. HPLC set, mobile phase and detection

A sediment sample, previously evaporated to dryness, was dissolved in acetone and injected (50 µl) into the column through a guard column. The HPLC system (Knauer, Germany) consisted of an autosampler (Optimas), three pumps (Smartline 100), a degasser (Smartline), a column thermostat (Smartline) and a detector (DAD K-2800).

The column was a Lichrospher 100RP-18e (250 x 4 mm, 5 µm, 100 Å; Merck, Germany) with a guard column (Lichrospher 100RP-18e, 4 mm x 4 mm; Merck, Germany); this same system had previously been used for chloropigment analysis by Szymczak-Żyła and Kowalewska.[34] The mobile phase and gradient system was a modified version of that used by Chen et al.[5] The gradient programme (1ml/min) began isocratically with mobile phase A (85:15 methanol:0.5 M ammonium acetate, aq. v/v), which was then ramped to 100% B (90:10 acetonitrile:water v/v) for 4 min. and to 25% B and 75% C (100% ethyl acetate) over the next 34 min. The gradient was returned to 100% B during 4 min, and ramped to 100 % A for another 4 min. Finally, the solvent was run isocratically for 4 minutes with 100% A. The whole analysis lasted 50 minutes. During the HPLC analysis, absorption spectra were collected from the 360-700 nm range.

4.3.2. The influence of column temperature

The carotenoid separations obtained for four different column temperatures (5 °C, 10 °C, 15 °C and 25 °C) for the mixture of pigment standards and sediment extracts were compared (**Fig. 4**). The analyses were carried out according to the procedure described in the *HPLC analysis* section (in triplicate; Lichrospher 100RP-18e column).

4.4. Pigment identification and quantification

The chromatograms (recorded at 450 nm) were integrated using ClarityChrom Software ver. 5.0.2. Individual carotenoids were identified on the basis of retention times and absorbance spectra compared with individual pigment standards. The carotenoid concentrations in a sample were calculated in the same way as those of chloropigments[35] using the following equation:

$c_1 = E_{450} \cdot \%A_{450} \cdot v \cdot D \cdot 1000 / (\varepsilon \cdot l \cdot w)$ [µg/g]
$c_2 = c_1 \cdot 1000 / M_m$ [nmol/g]
where: c_1, c_2 – carotenoid concentrations in µg/g and nmol/g, respectively; E_{450} – extinction measured spectrophotometrically at 450 nm (UV 1800 Shimadzu, Japan); $\%A_{450}$ – % of the pigment peak area

in the total area of all integrated peaks of the $HPLC_{DAD}$ chromatogram recorded at 450 nm; v – volume of acetone solution prepared for HPLC analysis (ml); D – dilution factor (dilution of the solution prepared for HPLC, for spectrophotometric measurement); ε – extinction coefficient for the carotenoids ($ml \cdot mg^{-1} \cdot cm^{-1}$); l – optical path length (cm); w – weight of dried sediment (g); M_m – molecular mass.

The extinction coefficients of the carotenoids were calculated from absorption measurements at 450 nm of carotenoid standards using the Lambert-Beer law (**Table 3**) and were compared with the values gleaned from the literature.[2] The extinction coefficients from the literature and calculated for the individual standards used in this work were used to calculate carotenoid concentrations. The results are presented in Table 3.

This quantification method was compared with the one based on calibration curves. The calibration curves (peak area as a function of concentration) were determined for particular carotenoids: peridinin, fucoxanthin, diadinoxanthin, diatoxanthin, lutein, zeaxanthin, canthaxanthin, echinenone and β-carotene. The calibration curves were prepared for five different concentrations; the calibration curve for β- carotene is exemplified in **Fig. 6**.

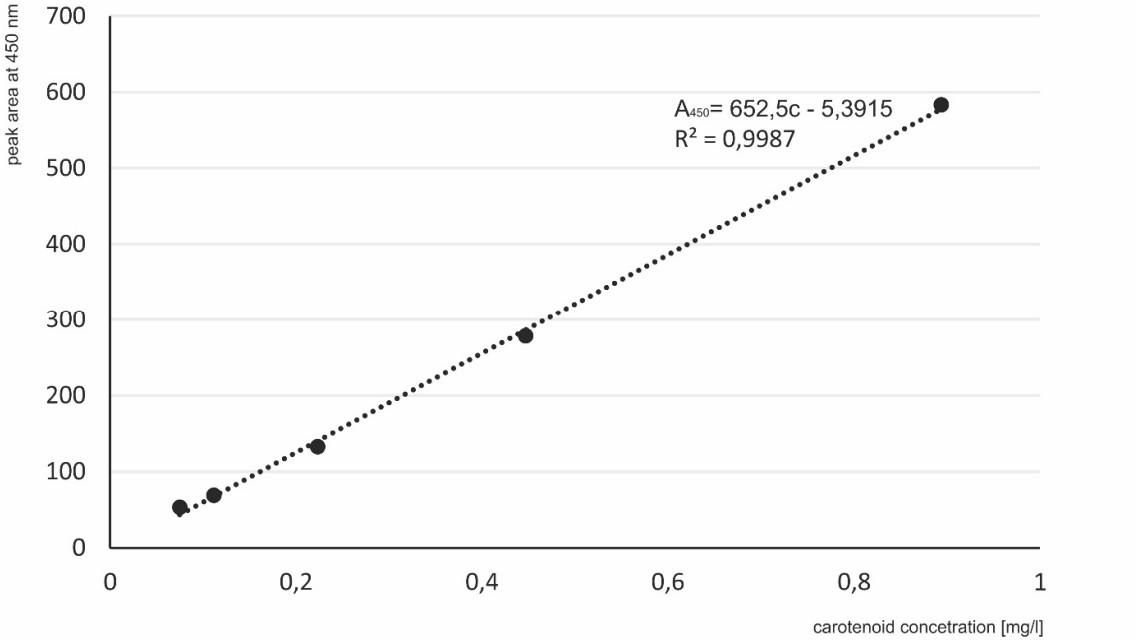

Fig. 6. Calibration curve for β- carotene (1 $mg \cdot l^{-1} \sim 2\ nmol \cdot ml^{-1}$)

4.5. Statistical analysis

The results were statistically processed using STATISTICA 12.5 software (StatSoft, Poland): Student's t-test and one-way analysis of variance (ANOVA) were used.

References

1 Lohr M. (**2011**) Carotenoid metabolism in phytoplankton, in: Roy S., Llewellyn C. A., Skorstad Egeland E., and Johnson G.(Eds.) *Phytoplankton Pigments*. Cambridge University Press, New York, 113- 161.

2 Porra R. J., Pfündel E. E, and Engel N. (**1997**) Metabolism and function of photosynthetic pigments, in: Jeffrey S. W., Manoura R. F. C., and Wright S. W. (Eds.) *Phytoplankton pigments in oceanography*. SCOR-UNESCO Publishing, France, 85- 126. Jeffrey S. W., Mantoura R. F. C., and Bjørnland T. Data for the identification of 47 key phytoplankton pigments. ibid., 447- 559.; Wright S.W. High- resolution HPLC system for chlorophylls and carotenoids of marine phytoplankton. ibid., 327- 341.

3 Bianchi T. S., and Canuel E. (**2011**) *Chemical Biomarkers in Aquatic Ecosystem*. Princeton University Press, Princeton, New Yersey.

4 Leavitt P. R., and Hodgson D. A. (**2001**) Sedimentary pigments, in: Smol J. P., Birks H. J., and Last W. M. (Eds.) *Tracking Environmental Change Using Lake Sediments*. Kluwer Academic Publisher, Dordreht, 295- 325.

5 Chen N., Bianchi T. S., Mckee B. A., and Bland, J. M. (**2001**) Historical trends of hypoxia on the Louisiana shelf: application of pigments as biomarkers. *Org. Geochem*., 32 (4) 543- 561.

6 Mendes C. R., Cartaxana P., and Brotas V. (**2007**) HPLC determination of phytoplankton and microphytobenthos pigments: comparing resolution and sensitivity of a C18 and C8 method. *Limnol. Oceanogr.- Meth.,* 5 (10) 363- 370.

7 Buffan- Dubau E., and Carman, K. R. (**2000)** Extraction of benthic microalgal pigments for HPLC analyses. *Mar. Ecol.- Prog. Ser*., 204 293- 297.

8 Louda J. W., Loitz J. W., Rudnick D. T., and Baker E. W. (**2000**) Early diagenetic alteration of chlorophyll-a and bacteriochlorophyll-a in a contemporaneous marl ecosystem; Florida Bay. *Org. Geochem.,* 31 (12) 1561- 1580.

9 Szymczak- Żyła M., Louda J. W., and Kowalewska, G. (**2008**) Comparison of extraction and HPLC methods for marine sedimentary chloropigment determinations. *J. Liq. Chromatogr. R. T.,* 31 (8) 1162- 1180.

10 Leruste A., Hatey E., Bec B., and De Wit R. (**2015**) Selecting an HPLC method chemotaxonomic analysis of phytoplankton community in Mediterranean coastal lagoons. *Transitional Waters Bulletin,* 9 (1) 20- 41.

11 Reuss N., and Conley, D.J. (**2005**) Effects of sediment storage conditions on pigment analyses. *Limnol. Oceanogr- Meth.,* 3 (10) 477- 487.

12 Watanabe K., Park H. D., and Kumon F. (**2012**) Historical change of phytoplankton in a eutrophic lake in Japan as determined by analysis of photosynthetic pigments in a lakebed sediment core. *Environ. Earth Sci.,* 66 (8) 2293- 2300.

13 Meyer K. M., Macalady J. M., Fulton J. M., Kump L. R., Schaperdoth I., and Freeman K. H. (**2011**) Carotenoid biomarkers as an imperfect reflection of the anoxygenic phototrophic community in meromictic Fayettevile Grren Lake. *Geobiology,* 9 (4) 321- 329.

14 Josefson A. B., Norkko J., and Norkko A. (**2012**) Burial and decomposition of plant pigments in Surface sediments of the Baltic Sea: role of oxygen and benthic fauna. *Mar. Ecol.- Prog. Ser.,* 455 33- 49.

15 Deshpande B. N., Tremblay R., Pienitz R., and Vincent W. F. (**2014**) Sedimentary pigments as indicators of cyanobacterial dynamics in hypereutrophic lake. *J. Paleolimnol.,* 52 (3) 171- 184.

16 Mc Gowan S., Barker P., Haworth,E. Y., Leavitt P. R., Maberly S. C., and Pates J. (**2012**) Humans and climate as drivers of algal community change in Windermere since 1850. *Freshwater Biol.,* 57 (2) 260- 277.

17 Freiberg R., Nõmm M., Tõnno I., Alliksaar T., Nõges T., and Kisand A. (**2011**) Dynamics of phytoplankton pigments in water and surface sediments of a large shallow lake. *Est. J. Earth Sci.,* 60 (2) 91-101.

18 Tani Y., Matsumoto G. I., Soma M., Soma,Y., Hashimoto S., and Kawai T. (**2009**) Photosynthetic pigments in sediment core HDP- 04 from Lake Hovsgol, Mongolia, and their implication for changes in algal productivity and lake environment for the last 1 Ma. *Quatern. Int.,* 205 (1-2) 74- 83.
19 Romero- Viana L., Keely B. J., Camacho A., Vicente E., and Rosa Miracle M. (**2010**) Primary production in Lake La Cruz (Spain) over the last four centuries: reconstruction based on sedimentary signal of photosynthetic pigments. *J. Paleolimnol.,* 43 (4) 771- 786.
20 Leavitt P. R., Vinebrooke R. D., Donald D. B., Smol J. P., and Schindler, D. W. (**1997**) Past ultraviolet radiation environments in lakes derived from fossil pigments. *Nature,* 388 457- 459.
21 Bianchi T.S, Demetropoulos A., Hadjichristophorou M., Argyrou M., Baskaran M., and Lambert C. (**1996**) Plant Pigments as Biomarkers of Organic Matter Sources in Sediments and Coastal Waters of Cyprus (eastern Mediterranean). *Estuar. Coast. Shelf S.,* 42 (1) 103- 115.
22 Bianchi T. S., Johansson B., and Elmgren R. (**2000**) Breakdown of phytoplankton pigments in Baltic sediments: effects of anoxia and loss of deposit-feeding macrofauna. *J. Exp. Mar. Biol. Ecol.,* 251 (2) 161- 183.
23 Bianchi T. S., Johansson B., and Elmgren R. (**2000**) Breakdown of phytoplankton pigments in Baltic sediments: effects of anoxia and loss of deposit-feeding macrofauna. *J. Exp. Mar. Biol. Ecol.,* 251 (2) 161-183.
24 Van Heukelem L., Lewitus A. J., Kana T. M., and Craft N. E. (**1994**) Improved separations of phytoplankton pigments using temperature- controlled high performance liquid chromatography. *Mar. Ecol.- Prog. Ser.,* 114 303- 313.
25 Zapata M., Rodriguez F., and Garrido J. L. (**2000**) Separation of chlorophylls and carotenoids from marine phytoplankton: a new HPLC method using a reversed phase C_8 column and pyridine-containing mobile phases. *Mar. Ecol.- Prog. Ser*., 195 29- 45.
26 Jun Z., Peng Y., Zhigang Y. U., and Bianchi T. S. (**2011**) Orthogonal design for optimization of pigment extraction from surface sediments of the Changjiang River Estuary. *Acta Oceanol. Sin.,* 30 (4) 33- 42.
27 Hobbs W. O., Lalonde S. V., Vinebrooke R. D., Konhauser K. O., Weidman R. P., Graham M. D., and Wolfe A. P. (**2010**) Algal- silica cycling and pigment diagenesis in recent alpine lake sediments: mechanisms and paleoecological implications. *J. Paleolimnol.,* 44 (2) 613- 628.
28 Hagerthey S. E., Louda J. W., and Mongkronsri P. (**2006**) Evaluation of pigment extraction methods and a recommended protocol for periphyton chlorophyll a determination and chemotaxonomic assessment. *J. Phycol.,* 42 1125- 1136.
29 Brotas V., and Plante- Cuny M.-R. (**2003**) The use of HPLC pigment analysis to study microphytobenthos communities. *Acta Oecol.,* 24 (1) 109- 115.
30 Szymczak- Żyła M. (**2016**) Analysis of chloropigments in marine sediments using accelerated extraction (ASE). *Limnol. Oceanogra.- Meth.*, 14 (7) 477- 489.
31 Van Heukelem L., and Thomas, C. S. (**2001**) Computer- assisted high- performance liquid chromatography method development with applications to the isolation and analysis of phytoplankton pigments. *J. Chromatogr. A,* 910 31- 49.
32 Louda J. W., Liu L., and Baker E. W. (**2002**) Senescence- and death- related alteration of chlorophylls and carotenoids in marine phytoplankton. *Org. Geochem.,* 33 (12) 1635- 1653.
33 Vidussi F., Claustre H., Bustillos- Guzman J., Cailliau C., and Marty J -C. (**1996**) Determination of chlorophylls and carotenoids of marine phytoplankton: separation of chlorophyll *a* from divinylchlorophyll *a* and zeaxanthin from lutein. *J. Plankton. Res.,* 18 (12) 2377- 2382.
34 Szymczak-Żyła M., and Kowalewska G. (**2007**) Chloropigments a in the Gulf of Gdańsk (Baltic Sea) as markers of the state of this environment. *Mar. Pollut. Bull*., 55 (10-12) 512- 528.
35 Kowalewska G. (**1997**) Chlorophyll a and its derivatives in recent sediments of the southern Baltic Sea collected in the years 1992- 1996. *Oceanologia*, 39 (4) 413- 432.