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Regular paper

Column chromatography as a useful step in purification of diatom pigments*

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Fucoxanthin, diadinoxanthin and diatoxanthin are carotenoids found in brown algae and most other heterokonts. These pigments are involved in photosynthetic and photoprotective reactions, and they have many potential health benefits. They can be extracted from diatom *Phaeodactylum tricornutum* by sonication, extraction with chloroform : methanol and preparative thin layer chromatography. We assessed the utility of an additional column chromatography step in purification of these pigments. This novel addition to the isolation protocol increased the purity of fucoxanthin and allowed for concentration of diadinoxanthin and diatoxanthin before HPLC separation. The enhanced protocol is useful for obtaining high purity pigments for biochemical studies.

Key words: column chromatography, *Phaeodactylum tricornutum*, fucoxanthin, diadinoxanthin, diatoxanthin, thin layer chromatography

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INTRODUCTION

Fucoxanthin (Fx), diadinoxanthin (Ddx) and diatoxanthin (Dtx) (Fig. 1A) are carotenoids found in a large group of marine algae but are also present in some fresh-water photoautotrophs. Fx and Ddx are both synthesized from neoxanthin, which is at a branching point in the carotenoid biosynthetic pathway from β-carotene (Dambek *et al.*, 2012). Dtx is a result of an enzymatic de-epoxidation of Ddx (Latowski *et al.*, 2011).

Fx is the main accessory pigment of the brown-algae and most other heterokonts, such as sea- and fresh-water diatoms. It is present in the light-harvesting complex, called fucoxanthin-chlorophyll protein, absorbs visible light in the 480–560 nm region and transfers the excitation energy to chlorophyll (Chl) molecules with one of the highest efficiencies among known carotenoids (Papagiannakis *et al.*, 2005). Recently, a lot of research has been focused on Fx, as it exhibits many properties that can be beneficial to the human health. First of all, it is a potent antioxidant, able to efficiently quench the singlet oxygen and scavenge free radicals. These properties are attributed to the presence of an unusual allenic bond (C=C=C) (Sachindra *et al.*, 2007). Moreover, Fx can act as an antioxidant under anoxic conditions, which is very rare among carotenoids (Nomura *et al.*, 1997). The beneficial health properties of Fx include anti-cancer (induction of apoptosis and cell cycle arrest in cancer cells), anti-obesity (induction of uncoupling protein-1 in white adipose tissue), anti-photoageing and antidiabetic activities (summarized by D'Orazio *et al.*, 2012; Mikami & Hosokawa, 2013; Kumar *et al.*, 2013; Moghadamtousi *et al.*, 2014; Miyashita & Hosokawa, 2015). Furthermore, it is capable of inhibiting the inflammatory response (Choi *et al.*, 2015).

Ddx is involved in a photoprotective diadinoxanthin cycle, which is typical for some groups of photosynthetic algae (diatoms, phaeophytes, dinophytes, and haptophytes) and protects these organisms from an oxidative stress mainly generated by high light intensity (Latowski *et al.*, 2011). Under high light conditions, Ddx molecules are de-epoxidized to yield Dtx molecules, which have one more conjugated double bond than Ddx. This conversion is responsible for avoiding damage caused by intensive illumination (Lavaud *et al.*, 2002). Dtx has been shown to suppress the expression of pro-inflammatory cytokines in murine cells (Konishi *et al.*, 2008), and both of these pigments are considered as constituents of sunscreens (Johnsen *et al.*, 2008).

While there are numerous publications describing the beneficial effects of Fx, the Ddx and Dtx have not been studied extensively, especially in animal or human cells, mainly because they are present is the source material in low amounts (Carreto & Cataggio, 1976) and their purification can be problematic.

In this work, we introduced an additional column chromatography step to an already established method for extraction of diatom pigments (Sadura, 2014). We assessed the utility of this novel pre-separation step in small-scale isolation procedure of all mentioned pigments. We used diatoms, which we found as the only known source allowing the simultaneous preparative isolation of Fx, Ddx and Dtx (Cohen, 1999; Pfeil *et al.,* 2014).

MATERIALS AND METHODS

Diatom cultures. *Phaeodactylum tricornutum* strain 1055/1 was obtained from the Culture Collection of Algae and Protozoa (CCAP; UK). The diatoms were grown for 5–8 days at 12°C in flasks containing a sterile Guillard's f/2 medium (Guillard, 1975) without added silicate. The light intensity was 48 μmol m^{-2} s^{-1} (as measured with Skye PAR Quantum) and the light/dark regime was 14/10 hours. Before pigment extraction,

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Abbreviations: Ddx, diadinoxanthin; Dtx, diatoxanthin; Chl, chlorophyll; Fx, fucoxanthin; HP-TLC, high-performance thin layer chromatography; RT, retention time

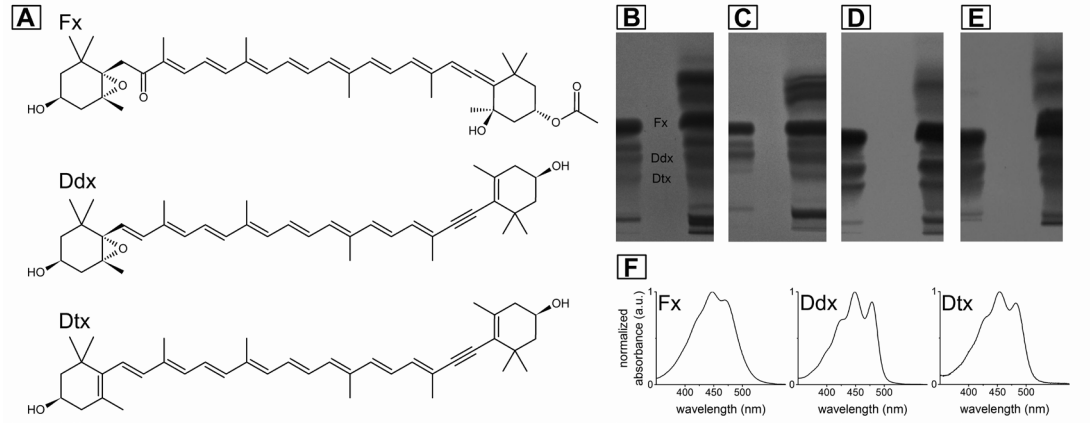

Figure 1. Pigment characteristics.
(**A**) chemical formulas of Fx, Ddx and Dtx. (**B–E**) fragments of representative HP-TLC plates after pigment separation. The left side of each plate shows the separation of pigments, when column pre-separation step was used and the mobile phase contained petroleum ether (**B and D**) or extraction gasoline (**C and E**). The right side of each plate shows the separation when no column pre-separation was used. The pigments were obtained from diatom cultures that were either kept in the darkness (**panels B and C**) or illuminated (**panels D and E**) before centrifugation. (**F**) representative spectra of Fx, Ddx and Dtx, recorded in acetone, using pigments isolated when column pre-separation with a solvent containing extraction gasoline was utilized. The R_f values are 0.44, 0.32 and 0.23 for Fx, Ddx and Dtx, respectively.

the flasks were kept in darkness for at least 1 hour (in the case of Fx and Ddx isolation) or were subjected to intense illumination (1550 μmol m^{-2} s^{-1}) for 1.5 hours, while stirring (in the case of Dtx isolation).

Pigment extraction. Grown cultures were centrifuged (4°C, 10 min, 4000 rcf), the pellet was suspended in one volume of the extraction buffer (50 mM phosphate buffer, pH 8.0, 300 mM NaCl) and sonicated (Sonics Vibra-Cell VC505, 10 minutes, 15s/10s on/off, amplitude 35%). This was followed by centrifugation (4°C, 10 min, 16900 rcf) and the supernatant was collected in a separate tube. The extraction solvent used was a mixture of chloroform:methanol 1:2. In the first extraction step, 1 mL and 0.7 mL of the extraction solvent was added to the pellet and supernatant, respectively. Both samples were mixed thoroughly and centrifuged, as described above. The organic phases from both samples were collected, and the aqueous phase from the supernatant sample was discarded. In the second extraction step, 1 mL of the extraction solvent was added to both, the pellet and supernatant sample. The samples were handled as described above (Sadura, 2014). For each purification procedure, a sample containing 1.5 μmol of Chl a was prepared.

HP-TLC separation. The collected organic phases, containing chlorophylls and carotenoids, were evaporated using nitrogen gas and dissolved in the HP-TLC solvent (methanol:water:NH_3 90:10:0.0012). The pigment solution was transferred to the HP-TLC plate (silica gel 60 RP-8 F_{254}s; Merck Millipore) using Linomat 5 (Camag, Switzerland). After separation, the pigments were scraped, extracted with acetone (Sadura, 2014), separated from silica by centrifugation, and stored at –20°C.

Column chromatography. An additional separation step was added between pigment extraction and HP-TLC separation. Two solvents were used in this step – petroleum ether (b.p. 60–80°C):acetone:n-propanol 60:40:3 (Aitzetmüller *et al.*, 1968) and extraction gasoline (n=7-8, b.p. 90°C):acetone:n-propanol 60:40:3. Glass column (length – 20 cm, internal diameter – 10 mm) was filled with neutral calcinated Al_2O_3 in a given solvent (Aitzetmüller *et al.*, 1968). The extracted pigments were evaporated, dissolved in the smallest amount of a given solvent and loaded onto the column. The separation was light protected and conducted at room temperature. The orange, carotenoid-containing fraction was collected, evaporated, dissolved in the HP-TLC solvent and separated on a HP-TLC plate, as described above.

Spectrophotometric analysis. Pigment spectra were measured in acetone, using the V-650 spectrophotometer (Jasco, Japan), and compared to the representative spectra presented by Jeffrey and coworkers (1997). Fx, Ddx and Dtx concentrations were assessed using a molar extinction coefficients equal to 109 000 mol^{-1} cm^{-1} (Haugan & Liaaen-Jensen, 1989), 130 000 mol^{-1} cm^{-1} (Johansen *et al.*, 1974) and 119 000 mol^{-1} cm^{-1} (Johansen *et al.*, 1974), respectively. Chl a's concentration was measured in 90% acetone according to Ritchie (2006).

HPLC analysis. HPLC setup consisted of an Agilent Technologies (USA) 1260 Quaternary Pump VL, 1260 Diode Array Detector VL and Nucleosil 300-5 C18 250 × 4 mm analytical column (Chromatographie-Service GmbH, Germany). The following solvents were used: extraction (90%, v/v [9:1 methanol:ammonium acetate 0.2 M], 10%, v/v ethyl acetate), A (85:15 methanol:ammonium acetate 0.5 M), B (9:1 acetonitrile:water), C (ethyl acetate). Ammonium acetate solution was prepared and filtered right before use. All other solvents were of an HPLC grade. Before separation, the acetone sample was evaporated, dissolved in the extraction solvent and loaded onto a column. The separation was carried out

Table 1. Solvent gradients used in HPLC analysis (Kraay *et al.*, 1992)

Time (min)	Solvent A (%)	Solvent B (%)	Solvent C (%)
0	60	40	0
2	0	100	0
7	0	80	20
17	0	50	50
21	0	30	70
28.5	0	30	70
29.5	0	100	0
30.5	60	40	0
35	60	40	0

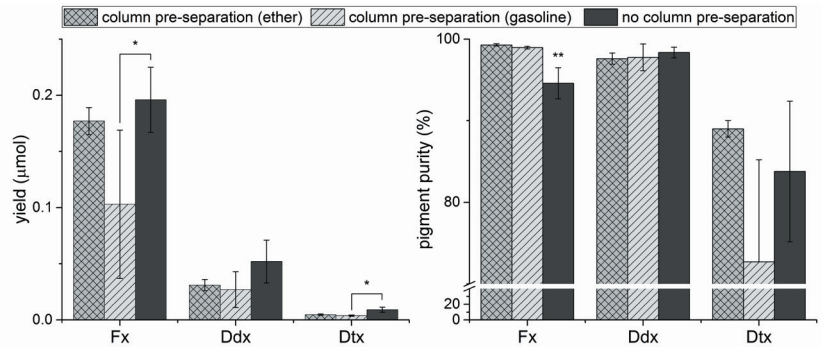

Figure 2. Yield and purity.
Purification procedure yield (from half of an HP-TLC plate; **left**) and the purity of obtained pigments (**right**). The column pre-separation step was conducted using a solvent containing either petroleum ether ("ether") or extraction gasoline ("gasoline"). *p<0.04 by ANOVA. **Kruskall-Wallis rank sum test determined that there is a significant difference between results in this group (p=0.0127); Wilcoxon rank sum test was used to determine that there is no significant difference between results obtained using column pre-separation step (p=0.1) in the said group.

as described by Kraay *et al.* (1992), using the flow rate of 0.8 mL min^{-1} and the elution profile presented in Table 1. The wavelengths used for detection were 430, 440 and 480 nm. The peaks were identified by their spectra (Jeffrey *et al.*, 1997) and retention times (Kraay *et al.*, 1992).

Statistical analysis. The experiments were repeated at least three times (with or without the column pre-separation step). The data were analyzed with the R software, version 3.2.4 (R Core Team, 2016). The data concerning the purity of pigments and procedure yield were grouped in three categories, reflecting the employed procedures (no column pre-separation and pre-separation using solvent containing either petroleum ether or extraction gasoline). If the data in groups passed the Barlett test of homogeneity of variances, ANOVA and Tukey multiple comparisons of means were used to determine which means are significantly different from each other. If the data failed the Barlett test, then Kruskal-Wallis rank sum test was used to check if there is a difference between groups. Wilcoxon rank sum test was then used to determine which groups are not significantly different from each other.

RESULTS AND DISCUSSION

Pigment pre-separation

As a result of the additional column chromatography step, the separation of carotenoids from Chls was obtained. This may be especially important, as free Chl is a photosensitizer and can, upon illumination, lead to the generation of reactive oxygen species (Marder *et al.*, 1998; Krieger-Liszkay, 2005). This in turn may contribute to the lower pigment quality and purification yield. By introducing this purification step, we decreased the time when carotenoids are mixed with Chls and hence lowered the possibility of carotenoids being damaged by the photodynamic effect caused by free Chls. The separations where petroleum ether was used were completed slightly faster than those where the extraction gasoline was used. This may be attributed to the lower density of petroleum ether, as it consists of shorter hydrocarbon chains.

Spectral quality of purified pigments

The representative spectra of Fx, Ddx and Dtx are presented in Fig. 1F. The obtained spectra were consistent with those presented by Jeffrey and coworkers (1997). The additional column separation step did not cause a significant improvement in respect to spectral quality (general shape, peak positions and ratios), no matter which solvent was used. Furthermore, no isomerization was detected by this analysis. In some spectra, minuscule peaks originating from Chl were present (not shown). They were present more frequently when no column pre-separation step was employed. This supports the idea that additional column chromatography step increases the purity of the isolated pigments, while the spectral quality of the main constituent remains comparable.

Purification procedure yield

In the case of Fx and Ddx, when column pre-separation step was utilized, the solvent containing petroleum ether gave better results than the one containing the extraction gasoline. In these instances, the yield was higher in comparison to the other solvent and the yield variability was much lower. When Dtx is considered, the results were similar, as the yield was higher in the case of the solvent containing petroleum ether (Fig. 2). The highest overall yield was obtained for Fx, which is present in *P. tricornutum* cells in the largest amounts (Carreto & Cataggio, 1976). Pre-separation with column chromatography did not lead to the increase of the purification yield. It can be attributed to the fact that the additional purification procedure causes greater material losses. On the other hand, column pre-separation step eliminates the Chls from the extracts and allows for the separation of more concentrated samples on HP-TLC.

Pigment purity

The highest pigment purities obtained were above 99%, 99% and 93% for Fx, Ddx and Dtx, respectively. In the case of all tested pigments, the pigment purity (assessed by HPLC analysis) did not differ significantly between the two solvents used in the column chromatography step (Fig. 2). When this additional step was omitted, the purity of Fx was significantly lower, while the purity of Ddx and Dtx remained comparable to the purity obtained using the pre-separation step. This can be explained by the fact that Chl bands are closely located to the Fx band on the HP-TLC plate (Fig. 1B–E). This hinders the obtaining of Fx. Moreover, while the HP-TLC separation of the sample is still developing, Fx remains in contact with Chls for a longer time than Ddx and Dtx in the sample that has not been pre-separated, which are characterized by a lower retardation factor in the system used in this study. It can lead to photodamage of the Fx molecules when the HP-TLC plate is accidentally illuminated. This can explain the contaminations detected by the HPLC analysis with Chls and carotenoid isomers (Fig. 3). In the case of Ddx, we observed the

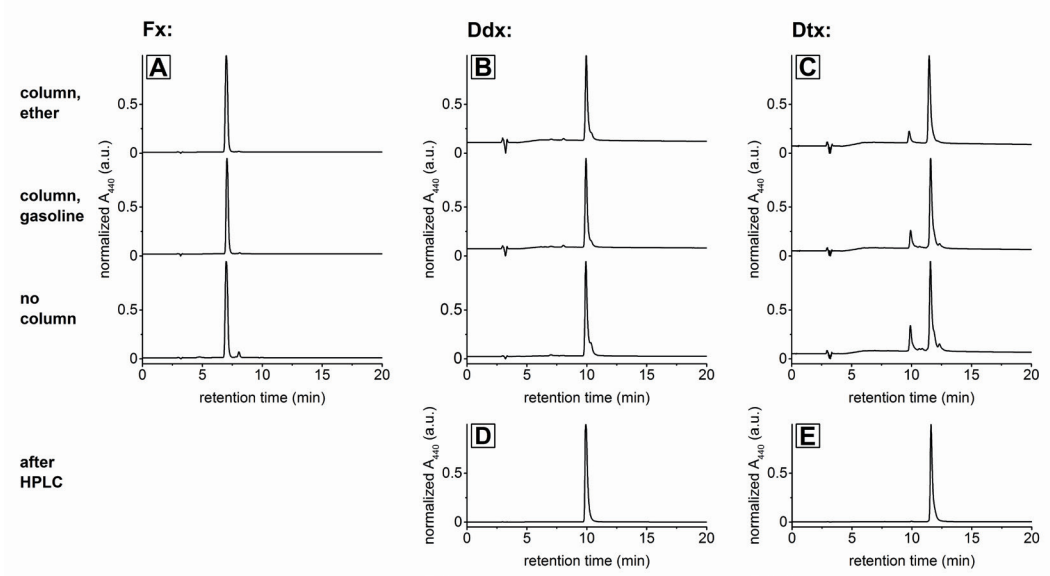

Figure 3. Representative chromatograms of Fx, Ddx and Dtx purified using different methods.
(**A**) Fx (RT 7.0 min). (**B**) Ddx (RT 9.9 min). (**C**) Dtx (RT 11.5 min). The pigments were isolated using a column chromatography step with mobile phase containing petroleum ether (top) or extraction gasoline (middle), or without this additional step (bottom). (**D–E**) fractions collected after the preparative HPLC separation of Ddx (**D**) and Dtx (**E**) samples that were pre-concentrated using the column chromatography step. Presented chromatograms show the first 20 out of 35 minutes of separation, according to gradient presented in Table 1. The detected contaminations are Chls (RT 4.84 min) and other carotenoids (RT 8.02, 10.35 and 12.34 min). Dtx samples are contaminated with Ddx, which can be eliminated by preparative HPLC separation. Chromatograms were normalized to 1 at the peak maximum.

co-elution of another compound, most probably a diadinochrome (Jeffrey *et al.*, 1997). These compounds are formed when Ddx epoxide groups are converted to furanoid groups during a purification procedure (Liaaen-Jensen, 1989). This co-eluting compound was consistently present in all analyzed samples of Ddx, which indicates that the group rearrangement is not caused by the reagents used in the column chromatography (Al_2O_3, solvents).

The Dtx samples were almost always contaminated with small amounts of Ddx, which can be attributed to their close proximity on a HP-TLC plate.

Further purification using HPLC

In order to remove contaminations, the samples of Ddx and Dtx were subjected to further purification, using HPLC (the same method as described above). The collected fractions of Ddx (RT 9.6–10.2 min) and Dtx (RT 11.3–12 min) were analyzed once again by HPLC. The results demonstrated that the purity of Ddx exceeded 99.7% and that of Dtx reached 99.5%, with room for improvement.

We also developed a shorter version of the protocol for preparative HPLC separation of Fx, Ddx and Dtx, which comprises of the same solvents and takes only 20 minutes (Fig. 3).

CONCLUSIONS

The additional pre-separation step, utilizing the column chromatography, significantly enhances the purity of Fx and lowers its variability. The pre-separation step does not influence the purity of Ddx or Dtx, which can be further purified, employing preparative HPLC, to the purity of at least 99.5%. The column pre-separation step is an easy and cheap way to concentrate the samples before HPLC separation, which lowers the overall cost of the isolation procedure. While this step decreases the overall yield, it can limit the potential photodynamic effect from Chl, as the carotenoids and Chls are separated earlier in the process. We conclude that adding a column pre-separation step can be useful for small-scale laboratory preparations of Fx and it allows the inexpensive concentration of Ddx and Dtx before further purification and analysis. The improved purification protocol can be used for obtaining material for further studies, concerning the biochemical and biophysical properties of Fx, Ddx and Dtx, as well as the elucidation of molecular mechanisms underlying the beneficial health effects of Fx, Ddx and Dtx. It is especially important for Ddx and Dtx, which are not well studied. We trust that the method developed in this work will advance the research focused on these pigments.

Conflict of interests

The Authors declare no conflict of interests.

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