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Improving the extraction and the purification of fucoxanthin from *Tisochrysis lutea* using centrifugal partition chromatography

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Abstract :

Fucoxanthin is the major carotenoid in oceans, found in golden-brown algae, and is considered as a bioactive molecule due to its numerous pharmaceutical and nutraceutical properties. This study aims to optimize the recovery and the purity of fucoxanthin from Tisochrysis lutea through an eco-extraction and eco-purification process, using the Centrifugal Partition Chromatography technique (CPC). CPC is based on the partition difference of fucoxanthin between two immiscible liquid phases, providing a complete sample recovery without fucoxanthin degradation, conducted at room temperature and being fast. Furthermore, CPC minimizes the amount of solvents to be effective. First, a solid-liquid US-assisted extraction was performed using dried T. lutea biomass with either acetone or ethanol. We obtained respectively 16.17 mg g−1DW and 13.54 mg g−1DW of fucoxanthin, and the specific consumption of extraction solvent was reduced by 20. Then, four experiments were conducted to optimize the organic solvent composition and the process operating conditions of the CPC. In the mobile phase, methanol used in the original method was replaced by ethanol, the amount of which has been reduced by 3 by adding H2O. The final CPC solvent system was cyclohexane:ethanol:H2O in 2:2:1 (v:v:v). Nine fractions with 100 % fucoxanthin (HPLC purity) were obtained, with 92 % of recovery yield.

Highlights

► Eco-extraction of fucoxanthin with 20 times less ethanol (green solvent): 16,37 $mg \cdot g^{-1}_{DW}$ ► Eco-purification of fucoxanthin using Centrifugal Partition Chromatography (CPC) ► Replacement of methanol by ethanol in the CPC solvent system ► Reduction of the CPC solvent amount: Cyclohexane:Ethanol:H_2O, 2:2:1 (v:v:v) ► Total separation of fucoxanthin (100 %) from other pigments in one purification step

Keywords : Fucoxanthin, extraction, purification, green chemistry, centrifugal partition chromatography

1. Introduction

Tisochrysis lutea, or *Isochrysis* affinis *galbana*, is a well-known golden-brown microalga belonging to the Chromista group and the division of haptophytes. This species was first isolated from Tahiti (French Polynesia), a tropical environment [1]. Because of its high content in polyunsaturated fatty acids, like the docosahexaenoic acid (DHA), and its high amounts of carotenoid pigments, *T. lutea* is of a great value in different application fields. In aquaculture, this species is commonly used to feed the bivalve hatcheries [2,3]. In the field of biotechnologies, the numerous properties of several *T. lutea* compounds are sought after in the nutrition and pharmacological industries [4,5]. Xanthophyll pigments in particular are highly valuable and their production ability is investigated [4,6,7]. These carotenoids possess a unique chemical structure distinct from carotenes as they contain oxygen, an epoxide group, and a conjugated carbonyl group, which confer them high anti-oxidant properties [8,9]. In 1990, the molecular structure of fucoxanthin, a xanthophyll, was fully described for the first time [10] and revealed the presence of an unusual allenic bond. This peculiarity is likely to be at the origin of the wide range of nutraceutical and pharmaceutical properties of fucoxanthin which are anti-cancerous, anti-inflammatory, anti-microbial, anti-obesity, anti-diabetes, anti-Alzheimer [11–16]. More recently, fucoxanthin protective effects against neurodegeneration, heart valve calcification, macular degeneration were also studied [17–19].

Currently, the major fucoxanthin-based products available on the market are brown-seaweed powder in capsules, they are used as nutraceuticals. However for pharmacological uses, it is necessary to provide a purified fucoxanthin molecule. Today, the scalable extraction and purification of fucoxanthin is an environmental and economical challenge. The use of conventional methods with hazardous and toxic organic solvents has been shown to be very effective, but is no longer in line with the current need for processes more respectful of green chemistry principles. For instance, a mixture of tetrahydrofuran and dichloromethane was reported to be the best solvent system for fucoxanthin separation from the chloroplast membrane [20], but if these solvents are efficient at lab-scale sample preparation for analysis, they are not recommended for process scale extraction or purification [21]. Medium polarity solvents like methanol (MeOH) or ethanol (EtOH) are usually preferred especially EtOH that is safer to use, more environmental-friendly and they were demonstrated to have high performance on fucoxanthin extraction [22,23]. Other environmental-friendly methods that efficiently extract fucoxanthin and other carotenoids were proposed in the literature [24]. Ionic liquids for example (solutions of salt with a melting point close to or under ambient

temperature), have demonstrated to efficiently extract astaxanthin [25,26], lutein [27] and fucoxanthin [28]. Promising studies also valorize the use of edible oils [22], natural deep-eutectic solvents [29–31], and subcritical or supercritical fluids [20,22,32,33].
Following extraction and according to further application, fucoxanthin needs to be purified from other pigments and some cell residues co-extracted.

Two methods emerge in the literature for fucoxanthin purification, both of them based on liquid-liquid partitioning, one on highly selective solvent systems and the other one on highly efficient extraction technology. The first one uses alcohol-aqueous two-phases systems for single stages extractions, implying the use of water and non-polluting solvents/salts, with high fucoxanthin recovery and purification yields [34]. The second one uses Centrifugal Partition Chromatography (CPC) column, which is a technique based on the partition difference of solutes such as fucoxanthin between two immiscible liquid phases [35,36]. This separation process is here convenient as it minimizes the amount of solvents to be effective. CPC is conducted at room temperature and provides a complete sample recovery without any degradation of the separated solutes [24,35]. Two studies reported the use of CPC for fucoxanthin separation so far [35,37]. Gonçalves de Oliveira-Junior *et al.* managed to isolate fucoxanthin from *T. lutea* with a purity superior to 65 % with a two steps process: one extraction of total pigments with EtOH and one CPC run with MeOH, H_2O and cyclohexane [35]. Coupled with flash chromatography, they managed to reach >99 % HPLC purity.
Through this paper, we aim to develop a green method of fucoxanthin extraction and purification from *T. lutea*, optimizing recovery and purity. We used solid-liquid extraction and CPC liquid-liquid fractionation for direct fucoxanthin enrichment. We worked especially on the solvent used for the total pigment extraction, the organic solvent composition of the two immiscible phases and the process operating conditions of the CPC.

2. Material and methods

2.1. Culture of Tisochrysis lutea and biomass harvesting

T. lutea strain CCAP 927/14 was cultivated in batch mode in three 300 L scobalites enriched with Walne's medium [38] at a continuous irradiance of 250 µmol photons m^2 s^{-1} at a temperature of 20 °C. Biomass was harvested when cultures reached $7x10^6$ cells mL^{-1} after seven days of culture, using a disc stack centrifuge (GEA Westfalia Separator Industry) at 10

000 rpm during approximately 300 L h^{-1} using a peristaltic pump. Biomass was then lyophilized and kept at -20 °C until further use.

2.2. US-assisted extraction of pigments

According to Gonçalves de Oliveira-Junior et al. [35], 1 g of lyophilized biomass was dispersed into 250 mL of (i) acetone (95 % acetone, 5 % absolute ethanol) or (ii) ethanol (absolute ethanol, EtOH). Two acetone extractions and one ethanol extraction were performed for the four conducted experiments.

Usually, the sonication-assisted extraction is used for diatoms possessing a silica frustule [39]. Despite *T. lutea* does not possess one, this species is covered with thin calcified scales forming a dense layer [1]. Furthermore, because fucoxanthin is attached to proteins and other pigments such as chlorophylls, the complex being anchored inside the membrane, the pretreatment of algal biomass can improve the fucoxanthin extraction [22,40,41]. Thus, the sonication is helpful in the pigment extraction process of this species.

Under constant stirring with a magnetic bar, an ultrasonic (US) probe (BIOBLOCK SCIENTIFIC, Vibracell^{TM} 75022) was used at 12 MHz, 30 W, for 5 s pulse cycles during 30 min. The US generates heat that can engender the thermal degradation of pigments within the solution. Then, the extraction was entirely performed with ice around the glass-beaker used for extraction. Furthermore, to avoid the light degradation of pigments, the laboratory light was switched off during the entire process. After US extraction, the extract was centrifuged 5 min at 8000 g to remove cellular debris (membranes, proteins, etc). The supernatant, containing the pigments and most of the lipids, was filtered using a 0.2 mm polytetrafluoroethylene (PTFE) filter before being transferred into a 500 mL distillation flask. The flask was partially immersed into a water bath at 40 °C, to favour the evaporation without thermally degrading pigments. The solvent was evaporated using an evaporator (BUCHI Rotavapor RII) during 45 to 120 min at 70 mbar for the absolute EtOH and 240 mbar for acetone. At the end of the evaporation, 4-5 mL of solvent (acetone or EtOH) were used to re-dilute the extract. 5 mL of highly concentrated extract were then obtained and transferred into an amber vial to evaporate the remaining solvent under nitrogen during a few hours and then kept at -20 °C until further use.

We finally obtained three final extracts, named Ac-extract1, Ac-extract2 and EtOH-extract (acetone or EtOH solvent), representing 30 to 40 % of the initial biomass.

2.3. Centrifugal partition chromatography

Pigments separation was performed with centrifugal partition chromatography [42] on a FCPCA 200 (Kromaton, Annonay, France, Figure 1).

Figure 1: Centrifugal partition chromatography FCPCA 200 (Kromaton, Annonay, France) in the GEPEA laboratory, Saint Nazaire, France.

The 242 mL column consists in 840 twin-cells in series, coupled with a Puriflash 5.250 (Interchim). The CPC method was operated in Descending Mode, *i.e.* the upper phase of the liquid-liquid biphasic system was the stationary phase (SP) and the lower phase was the mobile phase (MP).

The biphasic system was a cyclohexane / alcohol / water one. We compared methanol (MeOH) and ethanol (EtOH) in different volume proportions with water. Table 1 summarizes the four experiments with the three solvent systems tested in our work.

Table 1: Type of extract and volume proportions of the three solvent systems used for CPC. MeOH: methanol; EtOH: ethanol; Ac: acetone.

Experiment	**Extract**	**Solvent proportions**	Cyclohexane: (stationary)	MeOH:H_2O (mobile)	EtOH:H_2O (mobile)
A	Ac-extract1	6:6:1 v:v:v	6:	6:1	-
B	Ac-	6:6:1 v:v:v	6:	-	6:1

	extract2				
C	Ac-extract2	2:2:1 v:v:v	2:	-	2:1
D	EtOH-extract	2:2:1 v:v:v	2:	-	2:1

The CPC column was filled with stationary phase first, and then equilibrated with mobile phase at 1200 rpm with a constant flow rate at 10mL.min^{-1}. The retention (S_f) of the stationary phase (SP) in the column volume (Vc) was different for each solvent system and each extract, so it was calculated as follow:

$$S_f\ \% = \frac{V_{SP}}{V_C}$$

With:

$$V_{SP} = V_C - V_{MP}$$

V_{MP} was directly measured as the flushed volume of stationary phase during equilibrium, minus the dead volume in pipes and pump.

The S_f of each experiment were:

A. S_f = 81 % (V_{SP} = 242-45 = 197±2 mL)
B. Sf = 71 % (V_{SP} = 242-70 = 172±2 mL)
C. Sf = 65 % (V_{SP} = 242-85 = 157±2 mL)
D. S_f = 76 % (V_R = 242-59 = 183±2 mL)

100 mg of extract were solubilized into 10mL composed of equal volumes of upper stationary phase and lower mobile phase (Figure 2). The solution was manually injected through a 10 mL injection loop. At the column outlet, fractions of 5 mL were collected during the DM elution until reaching V_C, *i.e.* approximately 48 fractions. They were numbered from the first fraction containing pigments. Then, the CPC was set up in AM at the same flow rate for the SP extrusion (the stationary phase volume is then recovered without mixing and can be fractionated), fractions were also collected every 5 mL (Figure 2).

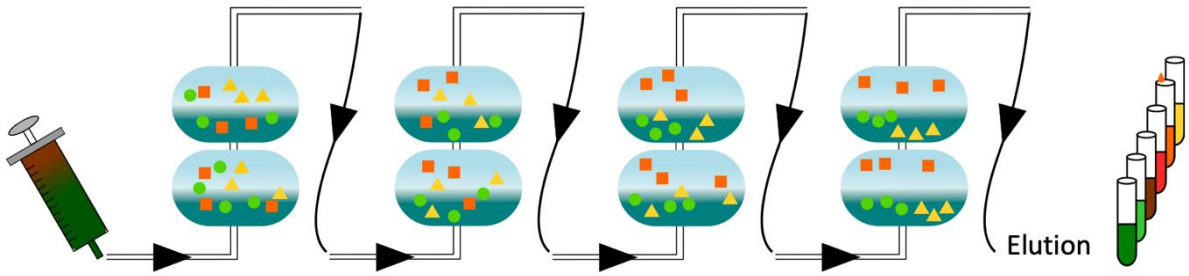

Figure 2: Details of twin cells of a centrifugal partition chromatography, containing two immiscible phases in which solutes (in color) are separated throughout the circulation of the mobile phase and eluted in 5 mL fractions.

The equilibrium partitioning coefficient of fucoxanthin ($K_{D,Fx}$) was calculated as follows:

$$V_{R,Fx} = V_M + K_{D,Fx} * V_{SP}$$

$$i.e.\ K_{D,Fx} = \frac{V_{R,Fx} - V_M}{V_{SP}}$$

With $V_{R,Fx}$ the retention volume of Fx, V_M the volume of the mobile phase eluted before the first fraction was collected, V_{SP} the volume of the stationary phase within the column.

2.4.HPLC analyses

Pigments were analyzed by HPLC-UV-DAD (Agilent Technologies series 1200 HPLC-UV-DAD) using an Eclipse XDB-C8 reverse phase column (150 x 4.6 mm, 3.5 µm particle size, Agilent) following the method described by Van Heukelem & Thomas (2001) [43]. Briefly, solvent A was 70:30 MeOH: H_2O 28 mM ammonium acetate and solvent B was pure MeOH. Gradient elution was the same as described in Van Heukelem & Thomas (2001). Quantification was carried out using external calibration against pigments standard provided by DHI, Denmark.

Fx content was calculated from HPLC chromatogram after normalization with a calibration curve obtained with a commercial standard of Fx. The purity of Fx in each fraction was calculated considering the other pigments analyzed by HPLC and did not include the lipids.

2.5. HPTLC analyses

Lipids classes were analyzed by HPTLC with the protocol describe by [44]. Two methods were used, one for the Neutral Lipids (NL) and one for the Polar lipids (PL). The samples were spotted on the plates by the CAMAG automatic sampler (CAMAG, Switzerland) for each method.

For the Neutral Lipid (NL), a double development with two distinct solvent systems was performed. The first solvent system contained hexane:diethyl ether:acetic acid (20:5:0.5, v:v:v), whereas the second was com-posed of hexane:diethyl ether (97:3, v:v).
For Polar Lipid, the development was performed with the solvent system methyl acetate : isopropanol : Chloroform : Methanol : KCl aqueous solution of 0.25 % (10:10:10:4:3.6 v/v). After dipping plates in a copper sulfate–phosphoric acid solution and heating for 30 min at 180 °C, the identification and quantification of the lipids were performed by scanning densitometry using winCATS software for data treatment (CAMAG, Switzerland). Available lipids standards were monogalactosyl diacylglycerol (MGDG) and digalactosyl diacylglycerol (DGDG).

3. Results and Discussion

3.1. Characterization of the total pigments extract

3.1.1. Extraction yield

First, pigments were directly extracted from the lyophilized biomass with acetone. This solvent was tested as a reference one as it is widely used in our laboratory and known to be very efficient in extracting all pigments. The pigment extraction yield of the first acetone extract (Ac-extract1) was 31 %, *i.e.* 307 mg g^{-1}_{DW}. The pigment extraction yield of the second acetone extract (Ac-extract2) was 30 %, *i.e.* 295 mg g^{-1}_{DW} showing the reproducibility of the extraction method.

EtOH is evaluated as an environmental-friendly solvent and then a greener substitute to acetone. The pigment extraction yield of the ethanolic extract was 39 %, *i.e.* 387 mg g^{-1}_{DW}. The global extraction yield was higher with absolute EtOH.
It is known that acetone and ethanol extracts of both *T. lutea* and *P. tricornutum* contain many pigments and lipids, that they are not very selective solvents and that another step would be required to purify the pigments [5,40,45,46].

3.1.2. Pigment concentration of the extract

HPLC analyses were performed to determine the pigments concentration in each extract (Table 2). In total, pigments represent 4,41 % of the biomass in Ac-extract1, 4,21 % in Ac-extract2, and 4,36 % in EtOH-extract. Results include the Chl *c* MGDG, which represents Chl *c* residues linked to the sugar moiety of a monohexosyldiacylglycerol (MGDG), as it was already observed in *Emiliania huxleyi*, a haptophyte and close species to *T. lutea* [47]. The

EtOH-extract contains the same quantity of pigments than acetone ones (40 mg g^{-1} approx). The Fx content is 13.5 mg g^{-1}_{DW} (13.8 mg g^{-1}_{DW} in the duplicate), slightly lower than Ac-extracts (14.4 mg g^{-1}_{DW} and 16.2 mg g^{-1}_{DW}). EtOH is as efficient as acetone for pigment extraction but it seems to be less selective (pigments represents 11 % of extracted DW, vs 14 % for acetone), containing then more impurities.

Table 2: Pigment concentration in the three extracts used for the separation experiments. EtOH: ethanol; Ac: acetone; Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Extract	Chl *c*	FxOH	Fx	Vx	Ddx	Dtx	Chl *c* MGDG	Chl *a*	βcar
	(mg g^{-1} DW)								
Ac-extract1	3.45	0.04	14.38	0.02	2.06	0.53	0.72	22.04	0.87
Ac-extract2	3.35	0.05	16.17	0.02	2.10	0.57	0.74	18.26	0.79
EtOH-extract	4.82	0.04	13.54	0.03	2.08	0.52	0.75	21.05	0.76

3.2. CPC fractionation of acetone extract

Experiment A

The first CPC experiment was carried out with cyclohexane:MeOH:H_2O after acetone extraction (experiment A, Table 1). After 100 mL elution of mobile phase (V_{MP}), fractions were collected. On the Fig 1.B, fractions from n°1 to 40 correspond to the elution of the mobile phase. Fractions from 1 to 11 contain most of the pigments: Chl *c*, Fx, Ddx, Dtx, FxOH, Vx, Chl *c* MGDG. Among them, fractions n°2, 3 and 4 (bold rectangular on Fig 1.A) are the most concentrated in Fx, with the highest purity of 79 % for fraction n°3 and 4 (Table 3). They contained impurities such as Chl *c1*, Chl *c2* and Ddx (Figure 4). The process did not allow to separate Chl *c1* and *c2* (pooled as Chl *c*) from Fx as the main Chl *c* fractions were also the n°2, 3 and 4 (Figure 3 B). Fraction n°3 and 4 also contained Ddx (Figure 3 A,B). In comparison, Gonçalves de Oliveira-Júnior et al. (2020) obtained Fx-enriched fractions with a maximum purity of 65.7 % after a single CPC run, with Chl *c1* and Chl *c2* being also the major impurities. In our results, the retention volume of Fx, according to Figure 3 B was 115 mL ($V_{R,Fx}$). The next fractions from n°5 to 40 were dark yellow to light yellow and were mainly composed of Ddx, Chl *c* MGDG, and traces of Fx. We noticed a very little loss of stationary phase in these fractions n°1 to 40 (visually corresponding to a thin layer of

cyclohexane at the surface), and no surface emulsion. The solvent system was thus relatively stable. Fractions n°42 to 51 were collected during the extrusion of the stationary phase and contained β-carotene and Chl *a*. Overall, this first CPC conditions allowed the separation of the most polar pigments from the non-polar ones.

$K_{D,Fx,A}$ was 0.11 (calculated with $V_{R,Fx}$ = 115 mL, V_M = 100 mL, V_S = 142 mL, see 2.3.), which indicated that Fx was predominantly present in the mobile phase. As all xanthophyll pigments, and contrary to other carotenoids such as β-carotene, Fx is composed of hydroxyl groups (OH). Even if Fx is a relatively non-polar metabolite, this characteristic is however responsible for a higher polarity of this molecule compared with β-carotene and Chl *a*, which are completely non-polar. Thus, Fx was logically eluted in the most polar phase, *i.e.* the mobile phase, containing MeOH.

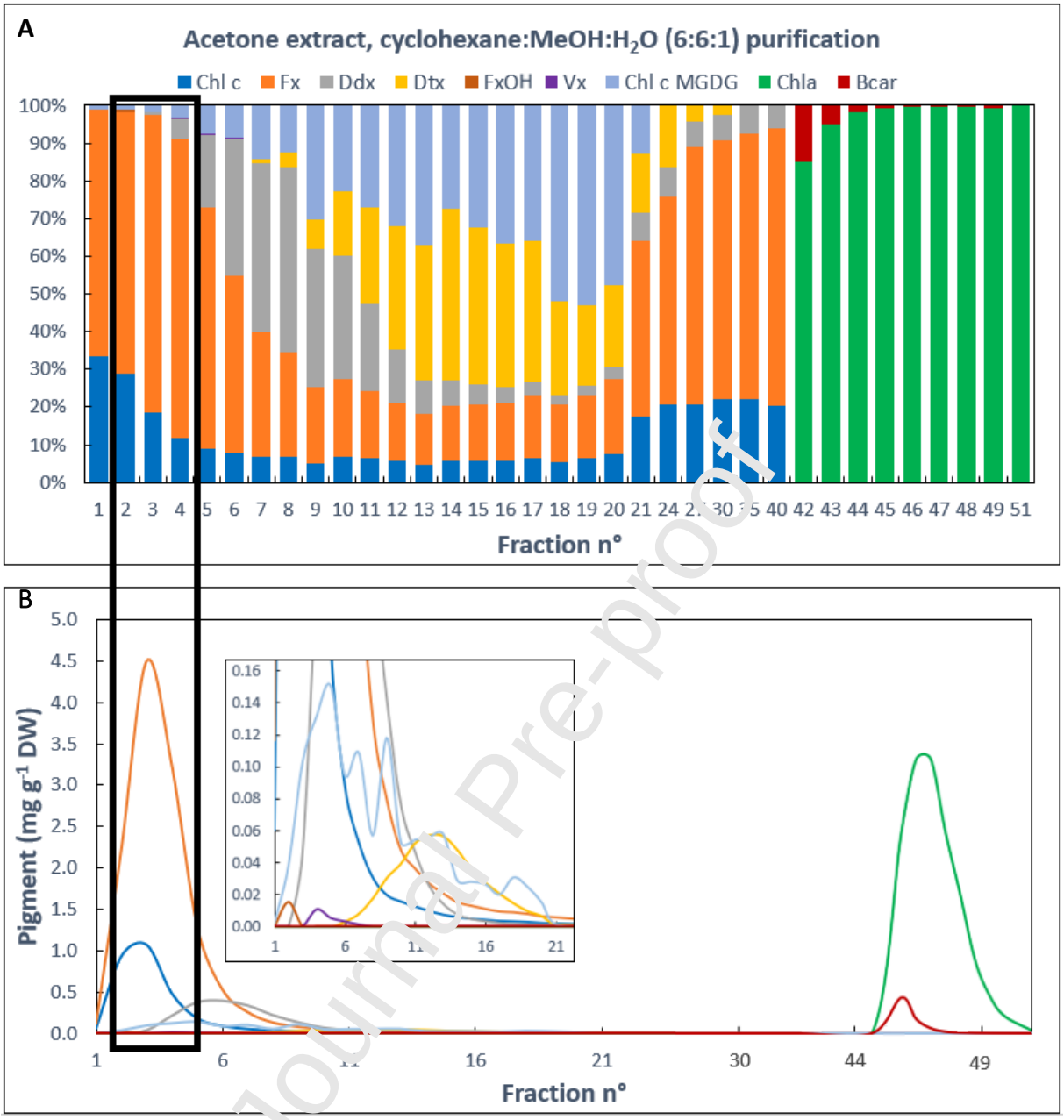

Figure 3: Characterization of the fractions collected from an acetone extract of *T. lutea* (Ac-extract1), using the cyclohexane:MeOH:H_2O CPC solvent system in 6:6:1 v:v:v proportions (experiment A). (A) Cumulated percentages of pigments in each fraction, (B) Retention of the pigments in the CPC column, represented by their concentration in each fraction (mg g^{-1} DW). The black frame highlights the most concentrated and purest fractions in fucoxanthin. Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

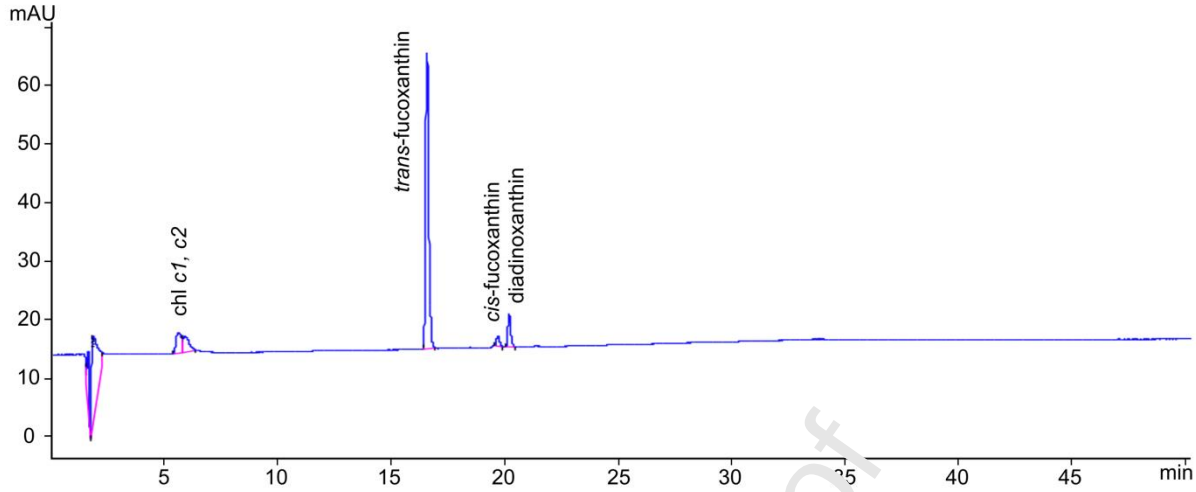

Figure 4: Chromatogram of fraction n°4, showing peak area (in mAU = milli arbitrary unit) relative to elution time (min). First peak at 2 min is an analysis artefact present in all chromatograms. Second peaks between 5.5 and 5.9 min are chl *c1* and *c2* respectively. Third peak at 16.4 min is *trans*-fucoxanthin, third peak at 19.8 min is *cis*-fucoxanthin. Fourth peak at 20.05 min is diadinoxanthin.

Table 3: Concentration of pigments in the ten fractions containing fucoxanthin (mg g^{-1} DW), from an acetone extract of *T. lutea* (Ac-extract1), using the cyclohexane:MeOH:H_2O CPC solvent system in 6:6:1 v:v:v proportions (experiment A). The black frame highlights the most concentrated and purest fractions in fucoxanthin.

Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Fraction n°	Chl *c*	FxOH	Fx	Vx	Ddx	Dtx	Chl *c* MGDG	Chl *a*	βcar	% Fx
	(mg g^{-1} DW)									
1	0.06	0.00	0.12	0.00	0.00	0.00	0.00	0.00	0.00	65 %
2	0.93	0.02	2.22	0.00	0.00	0.00	0.04	0.00	0.00	69 %
3	1.07	0.00	4.49	0.00	0.05	0.00	0.10	0.00	0.00	79 %
4	0.48	0.00	3.23	0.01	0.22	0.00	0.13	0.00	0.00	79 %
5	0.18	0.00	1.28	0.01	0.38	0.00	0.15	0.00	0.00	64 %
6	0.09	0.00	0.53	0.00	0.40	0.00	0.09	0.00	0.00	47 %

7	0.05	0.00	0.25	0.00	0.34	0.01	0.11	0.00	0.00	33 %
8	0.03	0.00	0.13	0.00	0.23	0.02	0.06	0.00	0.00	28 %
9	0.02	0.00	0.08	0.00	0.14	0.03	0.12	0.00	0.00	20 %
10	0.02	0.00	0.05	0.00	0.08	0.04	0.05	0.00	0.00	21

Experiment B

Results obtained in experiment A were encouraging, and the next objective was multiple: separate the major impurities Chl *c1* and *c2* in Fx-enriched fractions and develop a greener process (EtOH in spite of MeOH and less solvent consumption).

Several operating conditions were sequentially tested such as modifying the flow rate from 10 to 20 mL min^{-1}, or the ascending mode instead of descending mode. These tests resulted in the destabilization of the system with the appearance of emulsion, the loss of stationary phase, and above all less purity in Fx-enriched fractions.

Solvent substitution: Cyclohexane has a high $logP_{oct}$ of 3.44. This property is essential for fractionation as it allowed to efficiently separate the non-polar pigments Chl *a* and β-carotene. However, we did not manage to find a solvent which was more environment-friendly with a close $logP_{oct}$. EtOH replaced MeOH in the mobile phase as it is an environmental-friendly solvent which is bio-sourced. Despite EtOH is slightly less polar than MeOH, its partitioning between cyclohexane and water is lower ($logP_{oct}$ EtOH = 0.32, $logP_{oct}$ MeOH = 0.70), leading to a different selectivity of the ternary system. The system was cyclohexane:EtOH:H_2O in the same 6:6:1 v:v:v proportions than experiment A.

After 100 mL elution of mobile phase only (V_{MP}), fractions were collected from n°1 to 45. Fractions n°1 to 20 corresponded to the elution of the mobile phase. Despite the replacement of MeOH by EtOH, the whole solvent system seemed stable as no significant loss of stationary phase nor emulsion were observed in these fractions. As for experiment A, mobile phase fractions between 1 and 11 contained most of the pigments (Chl *c*, Fx, Ddx, Dtx, FxOH, Vx, Chl *c* MGDG). Three interesting Fx-enriched fractions were obtained, n°3, 4 and 5 (Figure 5 A, B). The purity in Fx reached 87 % in fraction n°4 (Table 4), which was higher than 79 % purity in fractions n°3 and 4 of experiment A. Chl *c1* and *c2* were still the major impurities of these fractions. However, we observed that the retention time of the Chl *c* pool was slightly shorter than the one of Fx in experiment B compared with experiment A (Figure

5 B). Rare traces of Chl *a* were found in some fractions of the mobile phase (n°1, 2, 7, 8, 9) but not in the Fx-enriched fractions. Fractions n°21 to 45 corresponded to the elution of the stationary phase (cyclohexane). Contrary to experiment A, β-carotene was eluted first and distinctly from Chl *a*.

$K_{D,Fx,B}$ was 0.19 which indicated that Fx was still predominantly present in the mobile phase but was slightly more partitioned between the two phases than for experiment A ($K_{D,Fx,A}$ = 0.11). However, as observed in the fractions, the selectivity gain was not sufficient to separate the major pigment impurities, Chl *c1* and *c2*.

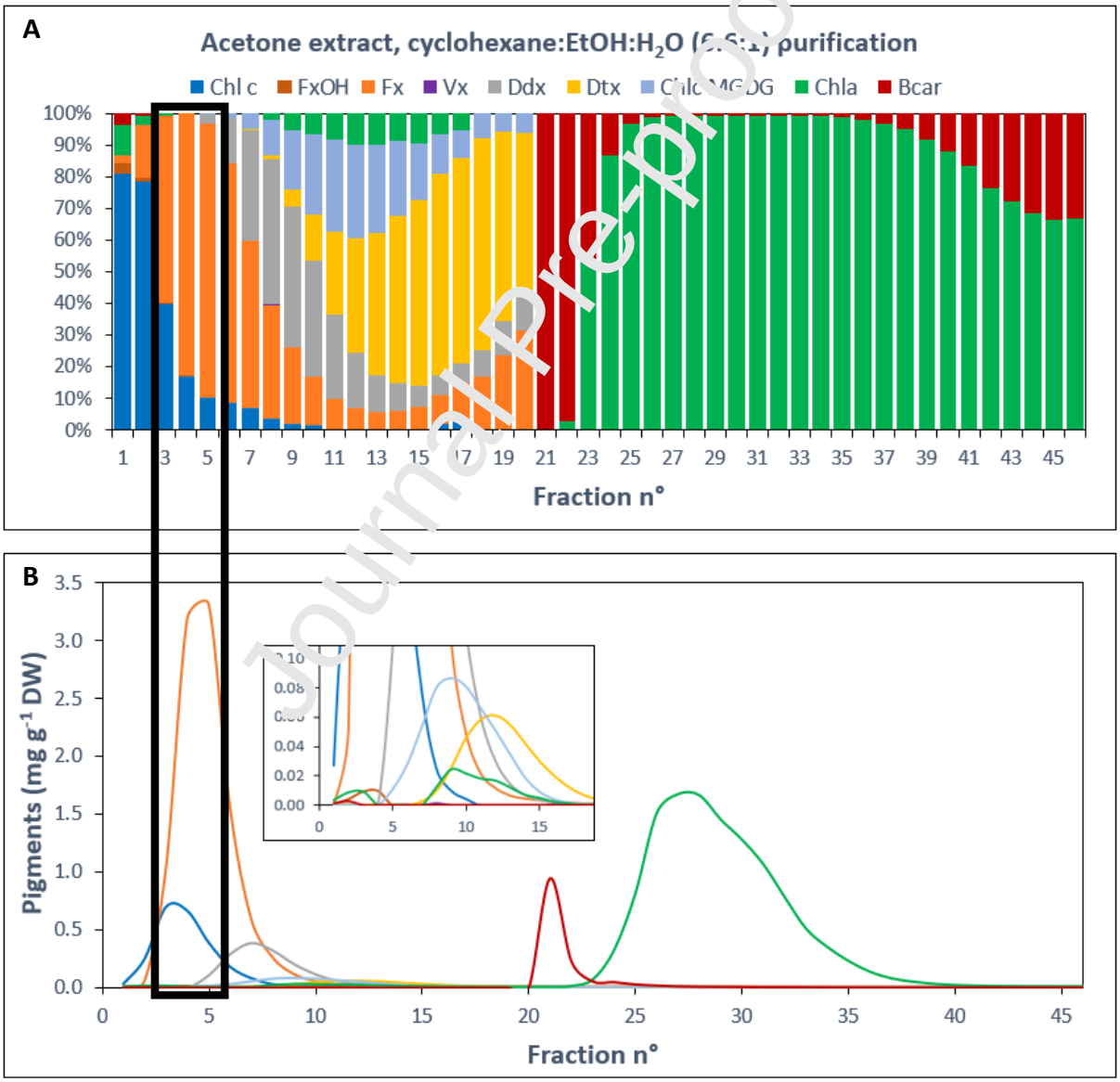

Figure 5: Characterization of the fractions collected from an acetone extract of *T. lutea* (Ac-extract2), using the cyclohexane:EtOH:H_2O CPC solvent system in 6:6:1 v:v:v proportions

(experiment B). (A) Cumulated percentages of pigments in each fraction, (B) Retention of the pigments in the CPC column, represented by their concentration in each fraction (mg g^{-1} DW). The black frame highlights the most concentrated and purest fractions in fucoxanthin. Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Table 4: Concentration of pigments in the nine fractions containing fucoxanthin (mg g^{-1} DW), from an acetone extract of *T. lutea* (Ac-extract2), using the cyclohexane:EtOH:H_2O CPC solvent system in 6:6:1 v:v:v proportions (experiment B). The black frame highlights the most concentrated and purest fractions in fucoxanthin.

Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Fraction n°	**Chl *c***	**FxOH**	**Fx**	**Vx**	**Ddx**	**Dtx**	**Chl *c* MGDG**	**Chl *a***	**βcar**	**% Fx**
					(mg g^{-1} DW)					
1	0.24	0.00	0.05	0.00	0.00	0.00	0.00	0.01	0.00	**17 %**
2	0.69	0.01	1.03	0.00	0.00	0.00	0.00	0.01	0.00	**59 %**
3	0.66	0.01	3.19	0.00	0.00	0.00	0.00	0.00	0.00	**83 %**
4	0.39	0.00	3.21	0.00	0.11	0.00	0.01	0.00	0.00	**87 %**
5	0.18	0.00	1.56	0.00	0.29	0.00	0.03	0.00	0.00	**76 %**
6	0.08	0.00	0.58	0.00	0.39	0.00	0.05	0.00	0.00	**53 %**
7	0.02	0.00	0.25	0.00	0.32	0.01	0.08	0.01	0.00	**36 %**
8	0.01	0.00	0.11	0.00	0.21	0.03	0.09	0.02	0.00	**24 %**
9	0.00	0.00	0.05	0.00	0.12	0.05	0.08	0.02	0.00	**15 %**

Experiment C

The solvent combination of cyclohexane, EtOH and H_2O provided a better synergistic effect on the Fx separation than the combination of cyclohexane, MeOH and H_2O. To optimize the separation of Fx from other pigments in Ac-extract2, within this new solvent system, the objective was to adjust the polarity while making the whole process even more environmental-friendly. Indeed, Fx and Chl *c1* and *c2* were eluted at the same time in

experiment B, whereas Chl *c1* and Chl *c2* are more polar than Fx [48]. With the previous two experiments, we observed that increasing the polarity of the mobile phase resulted in a better Fx separation. Thus, we tested an increases polarity of the mobile phase by increasing H_2O proportion from 1:12 (cyclohexane:EtOH:H_2O, 6:6:1 v:v:v) to 1:4 (cyclohexane:EtOH:H_2O, 2:2:1 v:v:v). The most polar molecules such as Chl *c1* and Chl *c2* might have a better affinity for the mobile phase which will be more polar, while the less polar molecules such as Fx will probably partition more with the non-polar phase. Furthermore, adding this proportion of water would reduce by three the consumption of EtOH in the mobile phase.

After 85 mL elution of mobile phase only (V_{MP}), fractions were collected from n°1 to 46. Fractions n°1 to 24 corresponded to the elution of the mobile phase and the pigments are present from fraction 1 to 19. In these fractions, an important emulsion was observed with approximately 1mL of stationary phase lost in each 5mL fraction. The solvent system seemed therefore less stable. However, the emulsion was not permanent and dissipated after decantation and the two phases present in each fraction became distinct. The stationary phase above was almost colourless, therefore did not contain a high concentration of pigments. Only the mobile phase of each fraction was collected for further analyses. Mobile phase fractions contained less pigments than in experiments A and B. We obtained 9 interesting Fx-enriched fractions, from n°10 to 18 (Figure 6 A, B). The Fx purity reached 100 % in fractions n°11 to 14, above 97 % in fractions n°10, 15, 16 and 17, and above 90 % in fractions n°9 and 18 (Table 5). Chl *c1* and *c2* were eluted before and distinctly from Fx, within fractions n°4 to 9 (Figure 6 A). Their retention time was thus lower than Fx, corresponding to the greater affinity of Chl *c1* and *c2* with the mobile phase. The peak of elution of Fx was broader than the ones of experiment A and B (Figure 6 B), which indicated that Fx partitioned more between the two phases as expected. The partition of solutes was, in this solvent system, precise enough to allow the distinction between the two Chl *c1* and *c2*, with two distinct elution peaks (Figure 6 B).

The first fractions from n°1 to 5 contained a combination of all polar and non-polar pigments. This result was characteristic of the instability of the whole system involving the injected extract and the solvent system. The injected extract formed a blockage into the CPC column at the beginning of the mobile phase elution, resulted into a few fractions containing all pigments. However, the amount of pigments within these fractions was low compared with all other fractions.

Fractions n°25 to 46 corresponded to the elution of the stationary phase. Contrary to experiment B and similar to experiment A, β-carotene was eluted at the same time than Chl *a*. These fractions were also composed of Dtx and Chl *c* MGDG contrary to experiments A and B, where these pigments were eluted in the middle and last fractions of the mobile phase respectively. This result illustrated even more the influence of the polarity of our solvent system on the separation of pigments. Indeed, Dtx is less polar than Fx [49], and did not partition between the two phases as it was entirely eluted with the stationary non-polar phase.

$K_{D,Fx,C}$ was 0.50, which indicated that Fx was still predominantly present in the mobile phase but was more partitioned between the two phases than for experiments A and B ($K_{D,Fx,A}$ = 0.11, $K_{D,Fx,B}$ = 0.19). These results were consistent with the Fx being mainly present in nine mobile phase fractions contrary to three fractions for experiments A and B. Furthermore, $K_{D,Chlc,C}$ was 0.29. Chl *c1* and *c2* partitioned less than Fx between the two phases, the ratio $K_{D,Fx,C}$ / $K_{D,Chlc,C}$ being 1.7 and the method much more selective. The polarity increase of the mobile phase by increasing H_2O proportion was a successful strategy to separate Fx from Chl *c1* and *c2*. Moreover, the water addition allowed the process to be more aligned with green chemistry principles as we reduced the proportion of EtOH.

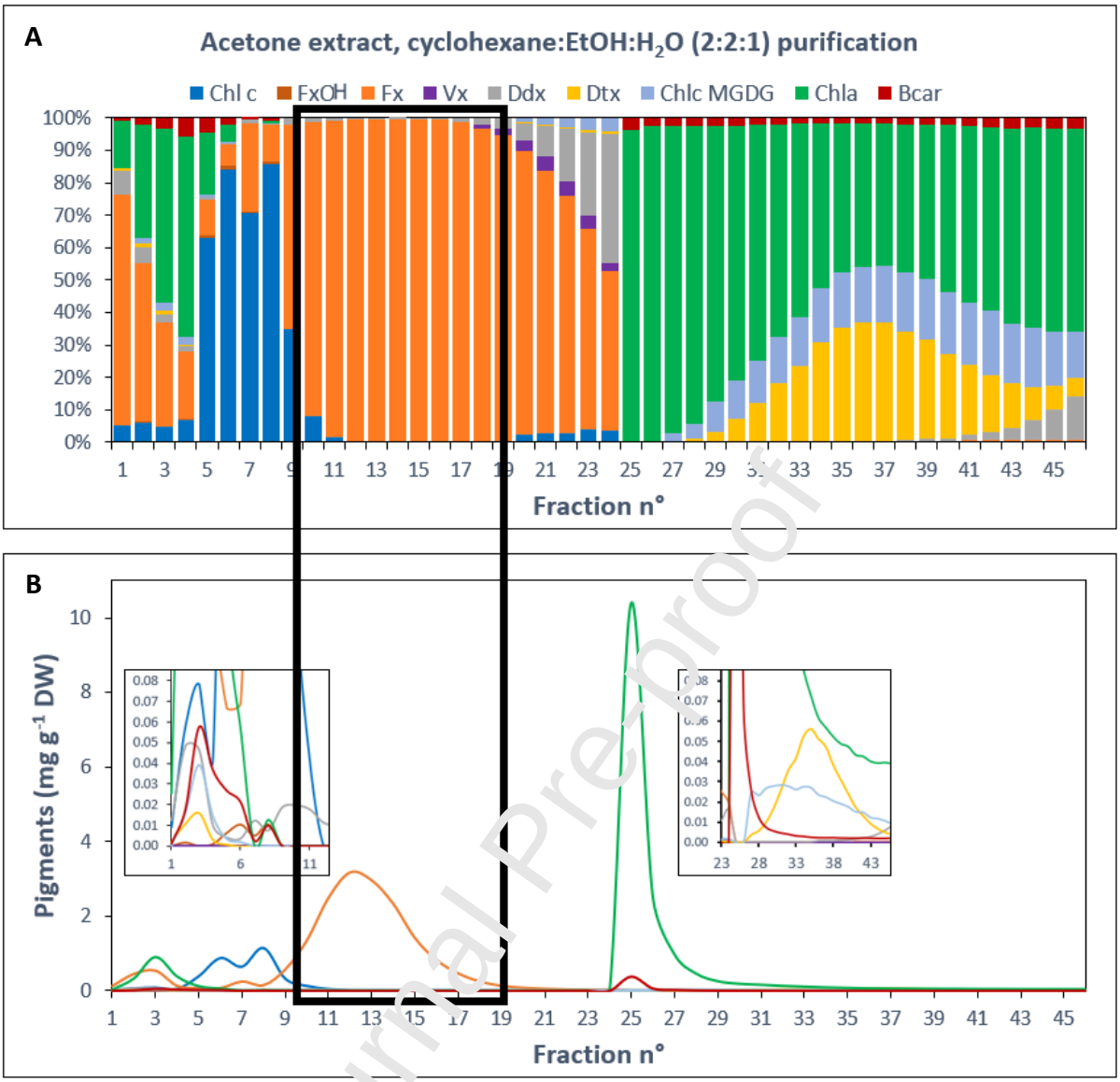

Figure 6: Characterization of the fractions collected from an acetone extract of *T. lutea* (Ac-extract2), using the cyclohexane:EtOH:H_2O CPC solvent system in 2:2:1 v:v:v proportions (experiment C). (A) Cumulated percentages of pigments in each fraction, (B) Retention of the pigments in the CPC column, represented by their concentration in each fraction (mg g^{-1} DW). The black frame highlights the most concentrated and purest fractions in fucoxanthin. Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Table 5: Concentration of pigments in the ten fractions containing fucoxanthin (mg g^{-1} DW), from an acetone extract of *T. lutea* (Ac-extract2), using the cyclohexane:EtOH:H_2O CPC

solvent system in 2:2:1 v:v:v proportions (experiment C). The black frame highlights the most concentrated and purest fractions in fucoxanthin.

Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Fraction n°	Chl *c*	FxOH	Fx	Vx	Ddx	Dtx	Chl *c* MGDG	Chl *a*	βcar	% Fx
	(mg g^{-1} DW)									
10	0.12	0.00	1.35	0.00	0.02	0.00	0.00	0.00	0.00	**90 %**
11	0.04	0.00	2.50	0.00	0.02	0.00	0.00	0.00	0.00	**98 %**
12	0.00	0.00	3.19	0.00	0.01	0.00	0.00	0.00	0.00	**~100 %**
13	0.00	0.00	2.97	0.00	0.01	0.00	0.00	0.00	0.00	**~100 %**
14	0.00	0.00	2.34	0.00	0.01	0.00	0.00	0.00	0.00	**~100 %**
15	0.00	0.00	1.42	0.00	0.01	0.00	0.00	0.00	0.00	**~100 %**
16	0.00	0.00	0.82	0.00	0.01	0.00	0.00	0.00	0.00	**99 %**
17	0.00	0.00	0.46	0.00	0.01	0.00	0.00	0.00	0.00	**99 %**
18	0.00	0.00	0.25	0.00	0.01	0.00	0.00	0.00	0.00	**97 %**
19	0.00	0.00	0.13	0.00	0.00	0.00	0.00	0.00	0.00	**94 %**

3.3. CPC fractionation of ethanolic extract

Experiment D

The solvent system of experiment C allowed the best separation of Fx. It was then evaluated for ethanolic extract (EtOH-extract) fractionation (experiment D). The use of EtOH instead of acetone would indeed be an improvement in making the whole process more environmental-friendly.

After 80 mL elution of mobile phase only (V_{MP}), fractions were collected from n°1 to 45. Fractions n°1 to 33 corresponded to the elution of the mobile phase. As for experiment C, an important emulsion was observed at the surface of these fractions. Between 0.5 and 1mL of stationary phase lost in each fraction. As for experiment C, mobile phase fractions contained

Chl *c*, Fx, Ddx, FxOH and Vx. We obtained 16 interesting Fx-enriched fractions, n°9 to 24 (Figure 7 A, B).

All these Fx-enriched fractions were even more pure than in experiment C. The purity in Fx was 100 % in nine fractions, n°10 to 18 (Figure 8), which were also the most concentrated in Fx (Table 6). In fractions n°19 to 21, purity was 99 % and above 92 % in fractions n°9, and 22 to 24 (Table 6). The few impurities in these fractions were Vx traces (under quantification limits in Table 6). Chl *c1* and *c2* were eluted before and distinctly from Fx, within fractions n°2 to 8 (Figure 7 A). As for experiment C, their retention time was lower than Fx, which validated the reproducibility of the CPC solvent system. As for all previous experiments, the main non-pigment impurities of the Fx-enriched fractions were polar lipids (PL, 75 % of all lipids in each fraction). 79 % of these PL were the monogalactosyl diacylglycerol (MGDG) and the digalactosyl diacylglycerol (DGDG), which are the main lipids of the chloroplast membrane. The majority of neutral lipids was found in the stationary phase fractions. Contrary to experiment C, the first mobile phase fractions were not composed of a combination of all pigments, there was no blockage into the CPC column. Thus, the use of an EtOH-extract improved the stability of the whole system at this level. Fractions n°33 to 45 corresponded to the elution of the stationary phase (cyclohexane), and were similar to the ones of experiment C.

$K_{D,Fx,D}$ was 0.40, which confirmed that Fx was partitioned between the two phases, more than experiments A and B, and less than experiment C. However, even with a lower K_D, Fx-enriched fractions were purer in experiment D than in experiment C. Indeed, $K_{D,Chlc,D}$ was 0.09 which was lower than in experiment C ($K_{D,Chlc,C}$ = 0.29). and the ratio $K_{D,Fx,D}$ / $K_{D,Chlc,D}$ was therefore 4.4, showing an selectivity increase. Then, experiment D was the best extraction and separation process of Fx from *T. lutea* as we obtained the highest purity of fucoxanthin and a very high final separation efficiency (Table 6). Furthermore, this process was the greenest that we tested.

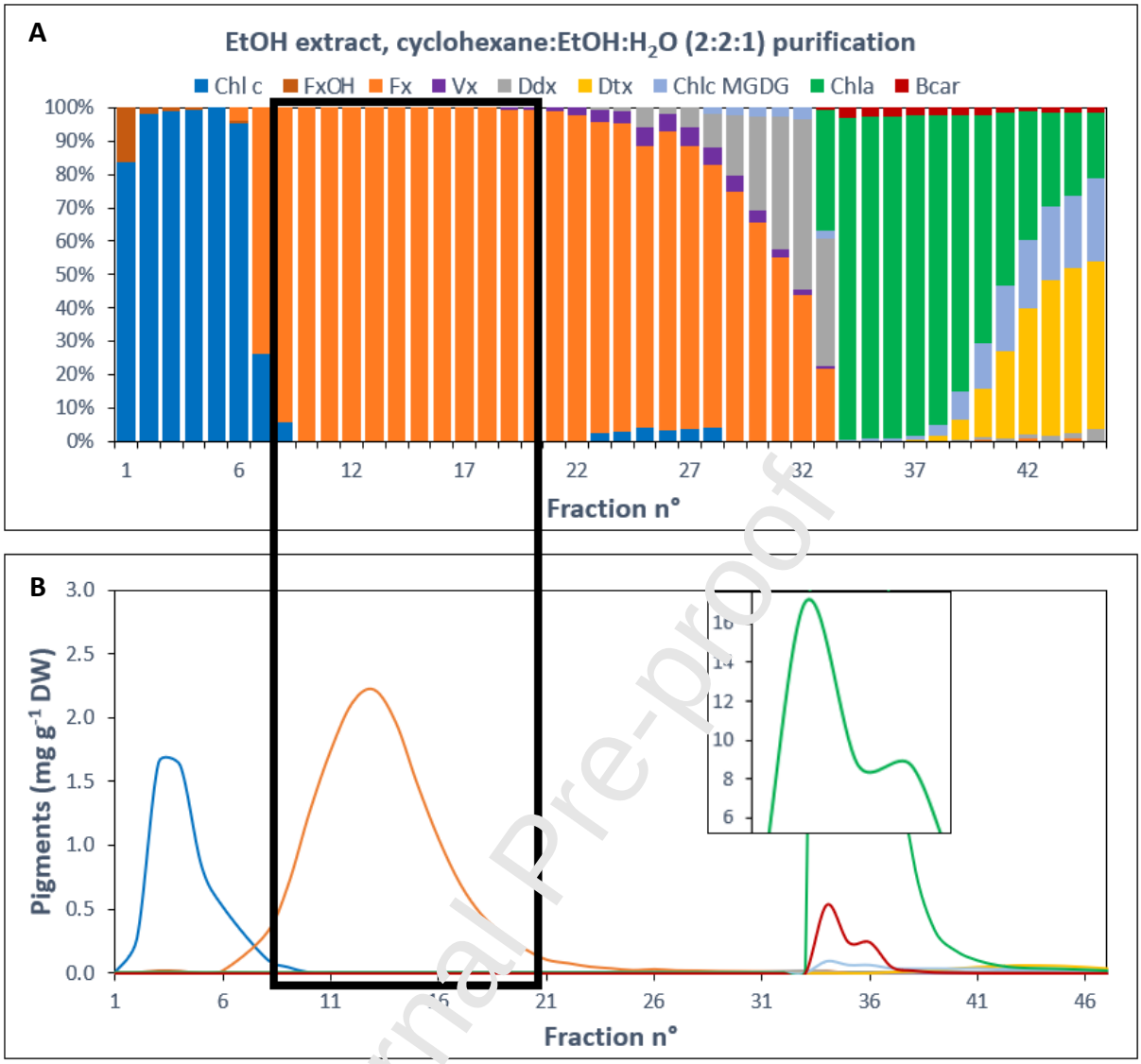

Figure 7: Characterization of the fractions collected from an ethanolic extract of *T. lutea* (EtOH-extract), using the cyclohexane:EtOH:H_2O CPC solvent system in 2:2:1 v:v:v proportions (experiment D). (A) Cumulated percentages of pigments in each fraction, (B) Retention of the pigments in the CPC column, represented by their concentration in each fraction (mg g^{-1} DW). The black frame highlights the most concentrated and purest fractions in fucoxanthin.

Table 6: Concentration of pigments in the ten fractions containing fucoxanthin (mg g^{-1} DW), from an ethanolic extract of *T. lutea* (EtOH-extract), using the cyclohexane:EtOH:H_2O CPC solvent system in 2:2:1 v:v:v proportions (experiment D). The black frame highlights the most concentrated and purest fractions in fucoxanthin.

Chl: chlorophyll; FxOH: fucoxanthinol; Fx: fucoxanthin; Vx: violaxanthin; Ddx: diadinoxanthin; Dtx: diatoxanthin; MGDG: monogalactosyldiacylglycerol; βcar: beta-carotene; DW: dry weight.

Fraction n°	**Chl *c***	**FxOH**	**Fx**	**Vx**	**Ddx**	**Dtx**	**Chl *c* MGDG**	**Chl *a***	**βcar**	**% Fx**
					(mg g^{-1} DW)					
9	0.04	0.00	0.68	0.00	0.00	0.00	0.00	0.00	0.00	**94 %**
10	0.00	0.00	1.26	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
11	0.00	0.00	1.75	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
12	0.00	0.00	2.12	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
13	0.00	0.00	2.22	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
14	0.00	0.00	1.96	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
15	0.00	0.00	1.48	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
16	0.00	0.00	1.05	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
17	0.00	0.00	0.69	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
18	0.00	0.00	0.45	0.00	0.00	0.00	0.00	0.00	0.00	**100 %**
19	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00	**99 %**
20	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00	0.00	**99 %**
21	0.00	0.00	0.10	0.00	0.00	0.00	0.00	0.00	0.00	**99 %**
22	0.00	0.00	0.08	0.00	0.00	0.00	0.00		0.00	**98 %**
23	0.00	0.00	0.05	0.00	0.00	0.00	0.00	0.00	0.00	**93 %**
24	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.00	**92 %**

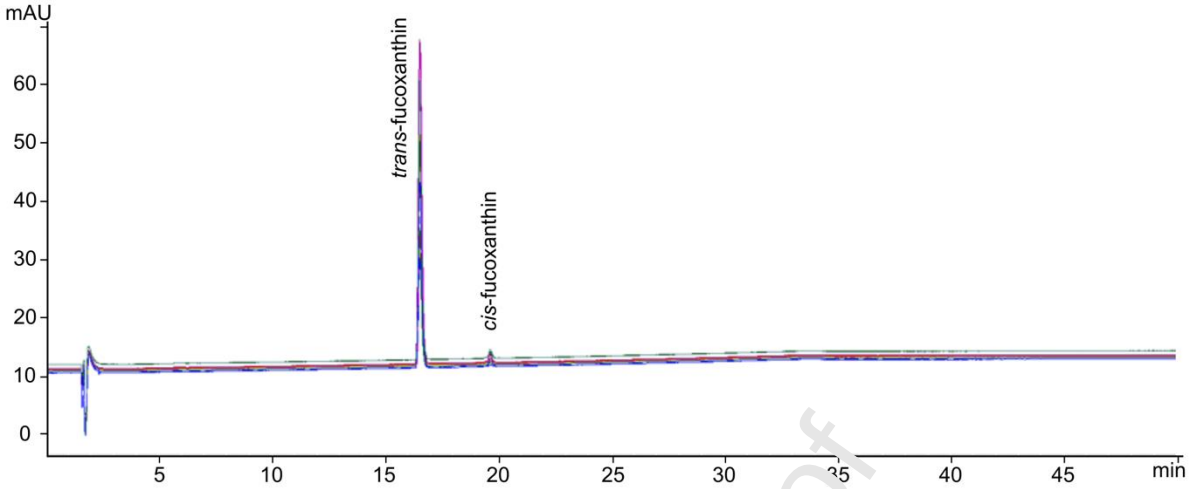

Figure 8: Combined chromatograms of fractions n°10 to n°18 showing peak area (in mAU = milli arbitrary unit) relative to elution time (min). First peak at 2 min is an analysis artefact present in all chromatograms. Second peak at 16.4 min is *trans*-fucoxanthin, third peak at 19.8 min is *cis*-fucoxanthin.

3.4. Is fucoxanthin separated from the Fucoxanthin Chlorophyll a,c binding Protein?

The light-harvesting system of *T. lutea* is composed of pigments, including Fx and chlorophylls, which are bound to the Fucoxanthin Chlorophyll *a,c* binding Protein (FCP) [50]. It is the combination of the FCP and the pigments which allows cells to capture photons and perform photosynthesis. FCP belongs to the superfamily of transmembrane light-harvesting complex proteins and is located in chloroplasts [51]. Then, it is possible that Fx was still attached to the FCP after the extraction and purification process in our experiments. To verify if we managed to isolate Fx, we analysed the absorption spectra of the EtOH-extract (containing all pigments) and of a Fx-enriched fraction highly concentrated in Fx. Proteins absorb at 280 nm, and we did detect a peak between 270 and 280 nm, both in the EtOH-extract and in the Fx-enriched fraction (Figure 9 A). However, this peak is also present in the absorption spectra of the Fx standard (Figure 9 B) in the same proportion relative to the main Fx peak (450 nm) than the one in the Fx-enriched fraction. Therefore, it is likely there was no FCP co-purified with the Fx in our Fx-enriched fractions.

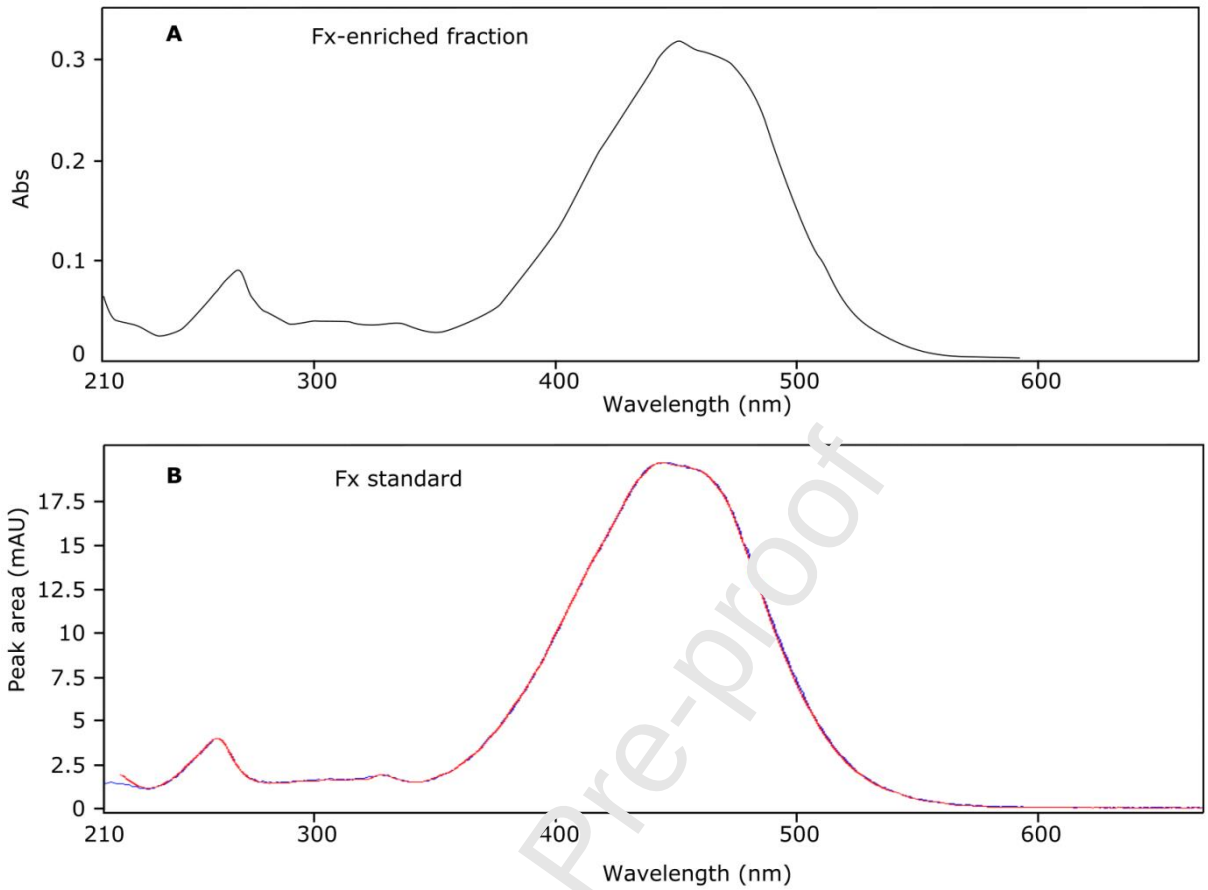

Figure 9: (A) Spectrum of a fucoxanthin enriched fraction in experiment D. (B) Spectrum of a fucoxanthin standard.

3.5. Reduction of the solvent ratio for ethanol extraction

The method used for pigments extraction from *T. lutea* biomass was based on huge ratio of extraction solvent per gram of biomass (250 mL to extract 1 g of lyophilized biomass). In order to reduce this amount and be more in line with green chemistry principles, extraction were performed with less EtOH, and monitored by pigment composition and concentration. The viscosity of the extract before evaporation was increased with less solvent, as the biomass concentration increased. Therefore, a different beaker was used with wider edges to prevent the solution from being sprayed and losing biomass during US extraction. Table 7 presents the different amounts of solvent tested (absolute EtOH) for 1 g of biomass, the amount of EtOH-extract obtained, and the concentration of Fx in each EtOH-extract. With less solvent, the quantity of EtOH-extract obtained tended to slightly decrease gradually. But the Fx extraction, in the contrary, slightly increased from 13.5 to 16.4 mg g^{-1}_{DW}, the extract was then more concentrated with less EtOH. The highest Fx recovery (16.4 mg g^{-1}_{DW}) and proportion

(4.9 %) was achieved with the lowest amount of absolute EtOH (9 g for 1 g of lyophilized biomass). We obtained 21 % more Fx per gram of dried biomass by extracting it with 20 times less solvent.

Table 7: Pigment extraction with five different amount of absolute EtOH, quantity of EtOH-extract obtained (mg g^{-1}_{DW}) and Fx concentration obtained (mg g^{-1}_{DW}).
EtOH: ethanol; Fx: fucoxanthin; DW: dry weight.

	for 1 g of lyophilized biomass				
Absolute EtOH (g):	**200 g**	**99 g**	**49 g**	**19 g**	**9 g**
EtOH-extract (mg g^{-1}_{DW})	387	410	341	306	331
Fx (mg g^{-1}_{DW})	13.54	15.69	12.53	14.85	16.37
Fx proportion in the extract	3.5 %	3.8 %	3.7 %	4.9 %	4.9 %

4. Conclusion

In this study, we managed to reduce by 20 the specific consumption of the extraction solvent (EtOH) to extract pigment from *Tisochrysis lutea*. Then, we managed to confirm the use of the centrifugal partition chromatography in one step to separate and purify fucoxanthin from other pigments. We optimized an existing protocol by replacing MeOH by EtOH in the mobile phase of the CPC solvent system and by increasing the proportion of water in the mobile phase. The final solvent system was cyclohexane:EtOH:H_2O in 2:2:1 proportions (v:v:v). Several fucoxanthin fractions at 100 % HPLC purity were obtained, for a total fucoxanthin recovery yield of 92 %.

By optimizing both the pigment extraction and the fucoxanthin purification, more green chemistry principles were respected: (1) with the reduction of the specific solvent consumption there was less waste during the whole process ("Prevention" principle); (2) we used EtOH, which is a less toxic solvent to human health and environment ("Less hazardous chemical syntheses & Safer solvents and auxiliaries" principle); (3) we managed to minimize toxicity, while simultaneously maintaining function and efficacy ("Designing safer chemicals" principle); (4) the whole process was at ambient temperature and pressure, and the CPC separation was performed in a short time ("Design for Energy Efficiency" principle); (5) finally, there were only a few numbers of steps until fucoxanthin separation and purification ("Reduce derivatives" principle).

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Declaration of Conflict of Interest

About the original research article entitled “Improving the extraction and the purification of fucoxanthin from *Tisochrysis lutea* using centrifugal partition chromatography”:

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

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Highlights

- Eco-extraction of fucoxanthin with 20 times less ethanol (green solvent): 16,37mg.g^{-1}_{DW}.
- Eco-purification of fucoxanthin using Centrifugal Partition Chromatography (CPC).
- Replacement of methanol by ethanol in the CPC solvent system.
- Reduction of the CPC solvent amount: Cyclohexane:Ethanol:H_2O, 2:2:1 (v:v:v).
- Total separation of fucoxanthin (100%) from other pigments in one purification step.