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Novel protocol for lutein extraction from microalga *Chlorella vulgaris*

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Highlights

- A novel method for the extraction of lutein from microalgae was developed.
- Water was replaced by ethanol in the saponification step.
- Saponification and extraction were conducted simultaneously.
- Quantity of lutein extracted increased 3 folds compared to conventional methods.
- Final lutein purity increased from 73.6% to 93.7%.

ABSTRACT

Lutein is a pigment generally extracted from marigold flowers. However, lutein is also found in considerable amounts in microalgae. In this study a novel method was developed to improve the extraction efficiency of lutein from microalga *C. vulgaris*. Differently from conventional methods, ethanol was used instead of water in the saponification step, which was conducted simultaneously to the solvent extraction, performed using dichloromethane. The amount of lutein extracted from *C. vulgaris* dried biomass increased more than threefold, from 0.20 ± 0.00 mgLutein/gDM to 0.69 ± 0.08 mgLutein/gDM. Lutein purity was increased from 73.6% to 93.7% by decreasing the ethanol-water ratio from 85% to 50% in the resolubilization step. The novel method was also tested with tetrahydrofuran. The extraction proved to be again more effective than the conventional one; however

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dichloromethane outperformed tetrahydrofuran in terms of quantity and purity of the recovered lutein.

Keywords: Lutein; *Chlorella vulgaris;* extraction; saponification; dichloromethane

1. Introduction

Lutein is a yellow xanthophyll, member of the carotenoid group. It is one of the predominant carotenoids present in the macular region of the human eye, being involved in the protection against light-induced retinal damage and age-related macular degeneration (AMD)[1,2]. Lutein assumption also prevents some types of cancer [3] and cardiovascular disease [4]. Moreover, it can contribute to human bone health [5]. Lutein is also a fundamental and an established feed additive in the poultry industry to brighten the color of chicken skin and of egg yolk [6].

Lutein is synthesized only by photosynthetic organisms like land plants and microalgae [7]. The worldwide market for this pigment is steadily increasing, and marigold flowers are currently the main source for the extraction and production of lutein. Microalgae have several advantages over this flower for lutein production: first of all, a higher lutein content and a faster growth rate [8] and the fact that they can be grown all year on infertile land and therefore without competition with arable crops [9]. In spite of these advantages, there are still several limitations that hinder the exploitation of this biomass for lutein production. Besides improving microalgae cultivation technologies, to bring the production of lutein from this biomass into reality it is fundamental to improve and optimize the related downstream processes, in particular extraction.

Extraction protocols from microalgae generally involve the two following separated initial steps: 1) saponification, performed with an aqueous KOH solution to ensure a pH value of approximately 12, needed to convert lutein fatty acid esters into free lutein, 2) solvent extraction, performed using different organic solvents combined with a cell disruption technology [10–12].

The majority of the studies have so far focused on finding an optimal solvent for highest extraction efficiency; a solvent that at the same time would be compatible with the utilization of lutein for food and feed. Optimal solvents have so far being identified in dichloromethane (DCM) and tetrahydrofuran (THF) [11,13]. According to the Department of Health and Human Services, Food and Drug Administration (FDA) [14], DCM and THF should be limited for commercial production of carotenoids for human consumption, differently from ethanol. Further steps in the extraction protocols are in fact devoted to the elimination of the solvent by evaporation.

Lutein extraction protocols also aim to eventually remove impurities and other unwanted pigments to achieve the highest possible purity [11].

Aim of the present work was to develop an improved method for lutein extraction compared to the conventional ones, in order to achieve higher yield and purity and, at the same time, reduce the extraction time. In the new protocol we tested the replacement of water with ethanol in the saponification process. Moreover, we introduced the simultaneous saponification and extraction step. The two solvent tested were DCM and THF.

2. Materials and Methods

2.1. Chemical, reagents and biomass

All chemicals used in this work were of analytical grade and were purchased from Sigma Aldrich ApS (Brøndby, Denmark). Lutein standard (concentration 0.804 mg/L) was obtain by DHI Lab Products (Hørsholm,Denmark). Freeze-dried *C. vulgaris* biomass was supplied by Alpha-Biotech (France).

2.2. Total pigments extraction

The extraction of the hydrophobic pigments was performed with 2 mg of freeze-dried biomass using 3 ml of 95% acetone followed by 10 min of sonication (Branson 3510MT) in ice bath. This solvent was employed due to its low specificity and therefore to obtain a qualitative profile of the pigments present in the chosen microalgal biomass. The supernatant was separated from the residual biomass by centrifugation at 13,000 rpm for 10 min and analyzed using a high performance liquid chromatography (HPLC) system. The separation was achieved at 60 °C with a HPLC (Dionex UltiMate 3000, Thermo Scientific, USA) equipped with a Zorbax Eclipse plus C8 RRHD 1.8 μm 3.0×150 mm column (Spherisorb-ODS1 Waters, Milford, MA, USA). The mobile phase consisted of (A) methanol/28 mM TBAA (tert-Butyl acetoacetate), pH 6.5 (70/30) and (B) methanol and the flow rate was 0.3 ml min^{-1}. Detection utilized UV–VIS at 450 nm. Samples and standards were dissolved in 100% ethanol and filtered by 0.22 μm filters. After filtering a buffer was added in 1:3 sample/buffer ratio, using 28 mM TBAA pH 6.5.

2.3. Conventional lutein extraction protocol (A)

The main steps of the conventional extraction protocol (A) are summarized in Figure 1.

For the saponification process to 1g of microalgal biomass were added 2.5 ml of 10 M KOH containing 2.5% (w/v) ascorbic acid. The mixture was incubated for 10 min at 60 °C.

In the second step, 10 ml of DCM were added. The mixture was placed in a sonicator bath for 1 hour and afterwards agitated at 300 rpm for 2 hours in an orbital shaker. After brief vortexing the mixture was centrifuged for 5 min at 4000 rpm and the supernatant was collected. This step was repeated until the extract was almost colorless and all extracts were combined (in total approximately 60 mL of DCM were added). Afterwards distilled water of the same volume as dichloromethane was added and stirred in a beaker for 4-5 minutes. This step was performed in order to separate the lutein containing fraction from the hydrosoluble pigments. The organic phase was then dried using a rotary evaporator, set at 40°C. The residue was redissolved in 7 ml of 85% aqueous ethanol (v/v). Hexane was added to the mixture in the ratio 1:4 (v/v) and, after brief vortexing, was removed to eliminate the fat soluble contaminants. Water was then added to bring the final concentration to 8.5% ethanol (v/v) and the mixture was then centrifuged at 13000 rpm for 10 minutes. The lutein precipitated was then resuspended in 100% ethanol.
For each extraction process samples for HPLC analyses were taken from all the extracts combined (60mL DCM); after the rotary evaporation step, following the addition of 85% ethanol (Figure 1). The entire procedure was performed in triplicates.

2.4. Novel saponification and extraction protocol (B)

The main steps of the novel extraction protocol (B) are summarized in Figure 1.
For this novel protocol the saponification and the extraction steps were conducted simultaneously. To 1g of microalgal biomass 2.5 mL of ethanolic KOH 2M and 10 ml of

DCM were added. The mixture was placed in a sonicator bath for 1 hour and afterwards agitated at 300 rpm for 2 hours in an orbital shaker. At this point another 2.5 mL of ethanolic KOH 2M were added and, after brief vortexing, the mixture was centrifuged for 10 min at 4000 rpm and the supernatant was collected. At this point the extraction was repeated by adding only DCM (in total 60 ml of DCM were used, in order to match the quantity used in the previous protocol). The organic phase was then dried using a rotary evaporator, set to 40°C. The residue was redissolved in 7 ml of 50% aqueous ethanol (v/v). This solution was stirred for 10 minutes and centrifuged at 13000 g. The supernatant was collected and the pellet resuspended in 100% ethanol (7 mL).

For each extraction process samples for HPLC analyses were taken from all the extracts combined (60mL DCM); from the organic fraction after the cleaning water step; after the rotary evaporation step, following the addition of 50% ethanol (Figure 1). The entire procedure was performed in triplicates.

Once determined that this protocol resulted in a higher extraction efficiency compared to the conventional one and also for being less time consuming, the same procedure was repeated replacing DCM with THF (method C) in order to compare the respective lutein quantities.

2.5. Quantification of lutein

The lutein extracts were analyzed by HPLC as described in Section 2.2. The lutein content was detected by measuring absorbance at the wavelength range of 350–700 nm. Two absorbance wavelengths were used (450 and 665 nm) and the maximal absorbance (450 nm) was chosen for quantification of lutein extracts.

Lutein was identified using Chromeleon 7.2 Chromatography Data System (CDS) software by comparison with a library of pigments spectra. Lutein was quantified using a calibration curve generated with different quantities of lutein standard.

Purity of lutein was determined comparing the peak area of the lutein with the area of the other peaks in the chromatogram.

The lutein quantity for each sample was calculated by using the following equation:

$$Lutein\ quantity\ \left(\frac{mg}{gDM}\right) = \frac{Lutein\ concentration\ \left(\frac{mg}{L}\right) \times Volume\ of\ solution\ (L)}{Dry\ cell\ weight\ (g)}$$

2.6. Statistical analysis

Data was analyzed with a one-way Analysis of Variance (ANOVA) followed by Tukey's test ($p < 0.05$) to evaluate if there were significant differences among the results obtained for different extracts. The software used to carry out the statistical analyses was OriginPro2016 (OriginLab Corporation, USA).

3. Results and Discussion

3.1 Pigments composition

Figure 2 shows the pigment composition profile of the *C. vulgaris* biomass tested in this study. The chromatogram shows high heterogeneity of the hydrophobic pigments, with the most abundant ones being lutein (Figure 2, peak 3), chlorophyll a (Figure 2, peak 4) and chlorophyll b (Figure 2, peak 5). Therefore, a procedure to selectively extract and purify lutein is needed.

3.2 Summary of the differences between the conventional and the novel methods

A novel protocol was developed and tested on *C. vulgaris* biomass. In this protocol, water was replaced with ethanol in the saponification process, based on the assumption that, being lutein insoluble in water and 100% soluble in ethanol, employing this solvent would minimize partial precipitation of the pigment in this specific step. The saponification and the extraction steps were also combined in order to simplify the entire process as it was done for marigold flowers [15,16]. The validity of this improved protocol was assessed by performing the extraction both with the conventional extraction method (saponification with aqueous KOH and separated from the solvent extraction) and the one developed in this work, and eventually comparing the results. DCM was employed as solvent for this comparison as it was the one most widely used for to extraction of lutein from microalgae. After having proved that these modifications hugely improved the conventional protocol as hypothesized, THF was also tested in the same optimized conditions in order to determine whether this solvent could outcompete DCM as the elective organic solvent for the extraction of this pigment.

3.3 Saponification and extraction

The differences in the initial steps of the two extraction protocols (i.e., conventional method and novel method) are summarized in Figure 1. The chromatographic analyses (Figure 3) showed that, due to the introduced modifications but most probably mainly for the replacement of water with ethanol, the quantity of lutein extracted increased of a remarkable 3 folds, from 0.31 ± 0.06 mgLutein/gDM (conventional method) to 1.00 ± 0.15

(Table 1, step 1). Most probably the reason of such an increase is imputable to the different solubility of lutein in water and ethanol (0 and 100% respectively). The utilization of ethanol instead of water in this critical step has probably resulted into a higher conversion of lutein fatty acid esters into free lutein and this despite the much lower concentration of KOH that guaranteed anyway the maintenance of pH at 12 for the entire process. In both cases the purity of lutein amounted to approximately 70% due to the presence in the extract of other pigments, mainly neoxanthin and violaxanthin, Figure 3.

3.4 Effect of different ethanol concentration on the final purity

The final quantity of lutein achieved using protocol B was 0.69 ± 0.08 mgLutein/gDM, more than 3 times the amount achieved with protocol A (0.20± 0.00 mgLutein/gDM). All the results are summarized in Table 1 and can be visualized in Figure 5. It is also noteworthy that in the conventional protocol more lutein is lost through the different purification steps compared to protocol B (Table 1). The rotary evaporator step was used to completely remove the DCM before resolubilizing the dried pigments mixture, mainly constituted by lutein, in a water-ethanol solution. The importance of completely removing DCM is due to its toxic nature even in remote traces, which is inappropriate in drug and food products [14]. This step was also necessary for increasing the purity of lutein. Indeed, by choosing an appropriate ethanol-water ratio, it is possible to exploit the physico-chemical properties of lutein to selectively precipitate this pigment while keeping the others in solution. Lutein being completely insoluble in water, maximizing the ethanol in the resolubilization step enables a higher lutein recovery. Our results show (Figure 4) that a

50% ethanol-water ratio translates into a much higher lutein purity compared to 8.5% ratio utilized in the conventional protocol, with a purity exceeding 90%.

In Figure 4 is possible to notice that neoxanthin and violaxanthin were almost completely removed from the mixture. Another important alteration in comparison with the conventional extraction method is the removal of the hexane step from the protocol. This is an additional advantage of the novel protocol as hexane is also considered toxic for food and drugs.

3.5 Effect of different solvents on the novel extraction protocol

Once determined that protocol B is strongly preferable compared to A both in terms of quantity and quality of lutein recovered, the same protocol was tested using THF instead of DCM. THF was chosen because this solvent was proven to be compatible for the solubilization and purification of lutein from microalgae in previous works [13]. The results showed that DCM was still the solvent of election for this process as protocol B corresponds a quantity of lutein equal to 0.69 ± 0.08 mgLutein/gDM compared to 0.41± 0.00 mgLutein/gDM of THF (Table 1, step 3 and Figure 5). Moreover protocol B achieves a higher purity than protocol C (93.7 % and 87.4% respectively) (Table 1).

3.6 Lutein yield

In literature, the total lutein content of microalgae biomasses are calculated using extraction protocols whit diethyl ether or DCM as solvent [10,13]. This specific value is necessary to calculate the final extraction yield. In Chan et al. the quantity is extracted with DCM and diethyl ether is not significantly different. In Chen et al. THF is found to outperform diethyl ether. None of these methods utilizes ethanol in the saponification step which, according to

our results and to a vast number of papers in which lutein is extracted from Marigold flowers [17,18], increases remarkably the solvent performance. Indeed, the higher lutein solubility in ethanol enables a lower lutein precipitation in the saponification step, with a consequent higher yield. Furthermore, always according to our results, DCM outperforms THF significantly. Based on all these considerations it was concluded that the amount of lutein extracted from the first step of protocol B (DCM as extraction solvent and ethanol in the saponification process) is the most accurate and therefore the one to be considered as total lutein content of this specific biomass (1.00 ± 0.15 mg/gDM). Based on this assumption the final yields resulted to be 20, 69 and 41% for protocol A, B and C, respectively. Moreover, considering a microalgal biomass productivity of 0.54 g/L/d as reported in the study of Lin et al. (2015), a lutein productivity of 0.11, 0.37 and 0.22 g/L/d could be achieved in our work for protocol A, B and C, respectively.

Conclusion

In this work a novel protocol for the extraction of lutein from microalgae was developed. In the saponification step water was replaced with ethanol to minimize loss due to precipitation. Also, saponification and solvent extraction were performed simultaneously. Lutein yield, quantitatively and qualitatively, increased significantly in respect to conventional protocols, both when using DCM or THF as extraction solvent. The use of DCM translated into a better performance compared to THF.

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Figure captions

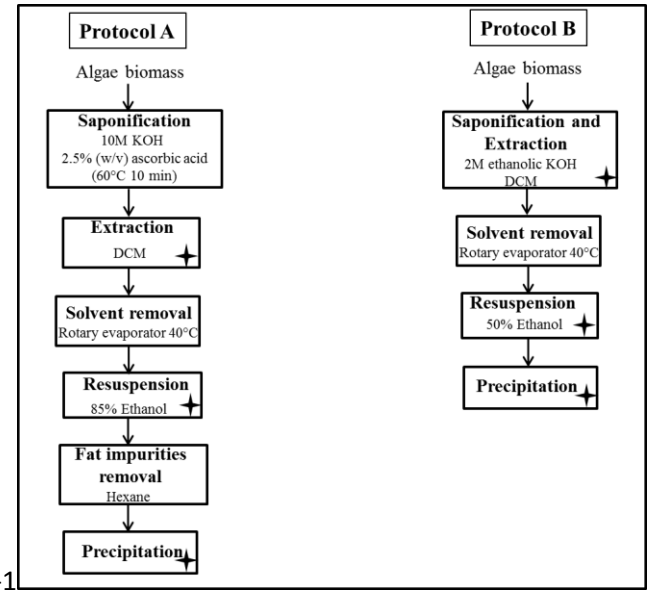
Figure 1: Schematic representation of the conventional (Protocol A) and novel (Protocol B) methods. Only the main steps are included. Samples for HPLC analyses were taken from the steps marked with an asterisk.

Figure 2: Chromatogram of the pigments extracted from *C. vulgaris*. Peaks: 1, neoxanthin; 2, violaxanthin; 3, lutein; 4, chlorophyll b; 5, chlorophyll a.

Figure 3: Chromatogram of crude lutein obtained by extraction with DCM with the conventional method (a) and with the novel one (b). Peaks: 1, neoxanthin; 2, violaxanthin; 3, lutein.

Figure 4: Chromatogram of purified lutein obtained with DCM with the conventional method (a) and with the novel one (b). Peaks: 1, neoxanthin; 2, violaxanthin; 3, lutein.

Figure 5: Chromatogram of purified lutein obtained with the novel method using DCM (a) or THF (b) as solvent. Peaks: 1, neoxanthin; 2, violaxanthin is not present; 3, lutein.



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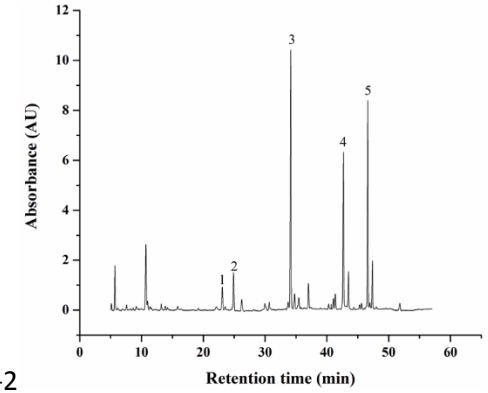

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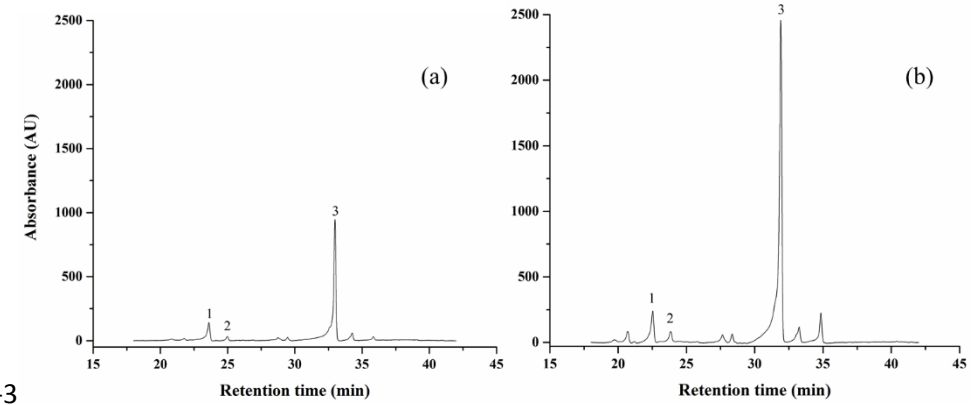

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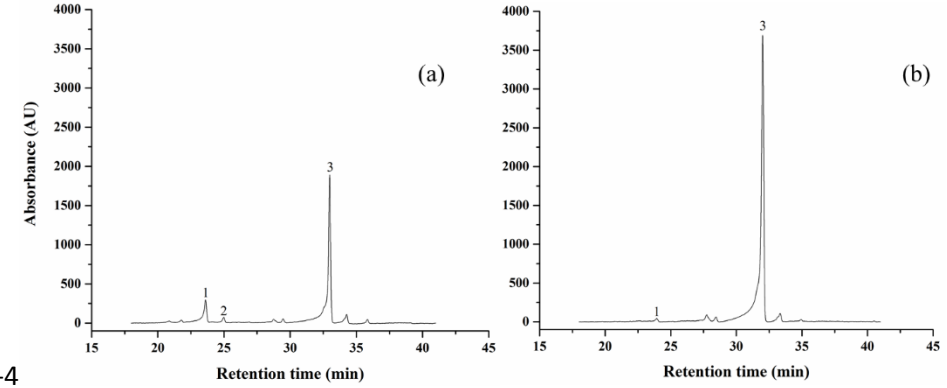

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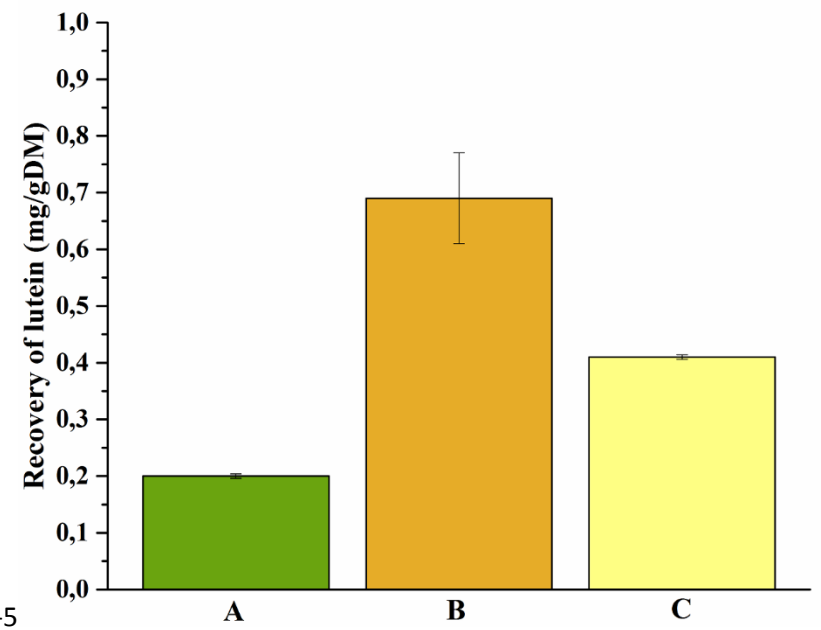

Figr-5

Table 1: Lutein extracted (mggDM^{-1}) and purity through the different purification steps in protocol A, B and C.

	Step 1			Step 2			Step 3		
	Protocol A	**Protocol B**	**Protocol C**	**Protocol A**	**Protocol B**	**Protocol C**	**Protocol A**	**Protocol B**	**Protocol C**
Lutein (mg/gDM)	0.31 ± 0.06	1.00 ± 0.15	0.57 ± 0.04	0.33 ± 0.04	0.88 ± 0.04	0.53 ± 0.02	0.20 ± 0.00	0.69 ± 0.08	0.41 ± 0.00
Lutein purity (%)	70.3	70.1	71.4	73.2	71.6	72.1	73.6	93.7	87.4