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Optimization of Lutein Extraction from *Scenedesmus almeriensis* using Pressurized Liquid Extraction

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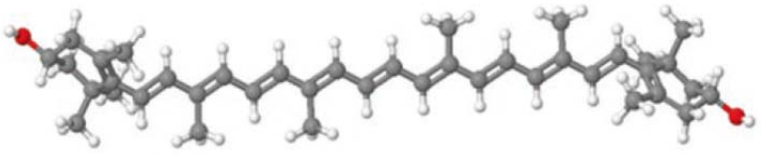
Lutein is a powerful carotenoid that is used as a feed additive for the colouring properties and as supplement in nutraceutical products. Its principal healthy properties are antioxidant, anti-inflammatory and protective against age-related macular degeneration. Lutein is indeed a macular pigment in human eyes between zeaxanthin and their function is very important for the eyes health. Lutein is found in some vegetables as spinach, parsley, and kale with a content of around 1-10 mg/100 g. The commercial and recognized source of lutein is the plant marigold (*Tagetes erecta L.*). Lutein is extracted from marigold petals as oleoresin and the lutein content can be around 0.03% (based on dry biomass weight). Since the costs for harvesting are expensive, land and water use are requested for marigold cultivation and due to the seasonality of the growth, an alternative source of lutein has been searched among microalgae. *Scenedesmus almeriensis* is considered a promising source for lutein production whose content is around 4.5 mg/g dry weight.
The aim of this paper is to improve lutein extraction from *Scenedesmus almeriensis* by using pressurized liquid extraction through accelerated solvent extractor (ASE 200©Dionex). Extractions were performed using different solvent as the mixture chloroform:methanol (1:1 v/v), ethanol, hexane, acetone. Several temperatures were tested from 20° to 80°C and biomass was mechanically pre-treated. Each extraction cycle was repeated until to complete biomass decolouring.

1. Introduction

Lutein ($C_{40}H_{56}O_2$) is a fat-soluble carotenoid belonging to the class of xanthophylls that contain hydroxyl groups or oxygen molecules in their molecular structure (figure 1). This carotenoid is widely distributed in plants, in foods such as vegetables, of which spinach and broccoli have the highest content at around 0.7-6 mg per 100 of product (Eisenhauer et al., 2017).



$C_{40}H_{56}O_2$

Figure 1: Lutein molecular structure (Spinola and Díaz-Santos 2020)

Lutein has important health properties for human eyes as it is one of the colouring compounds present in the macula of the eye together with zeaxanthin. The disease age-related macular degeneration (AMD) damaged more than 20% of the ageing worldwide population and lutein is recommended as a supplement to prevent the

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onset of this disease (Lim et al., 2012). The recommended daily uptake of lutein was established by JECFA around 0 – 2 mg/kg body weight (JEFCA 2005). It is used like other types of carotenoids as an additive in food and feed because of its colouring properties, which have also been recognized at European level (Marino et al., 2019).

Currently the recognized source of lutein is the plant *Tagetes erecta* from which lutein is extracted from the flower petals in the form of lutein oleoresin. Lutein esters from Tagetes erecta can be used as an additive in foods in quantity equal to 2.0 –330 mg/kg (Cantrill et al., 2016). The lutein content that can be extracted from the plant *Tagetes erecta* could be around 0.15 g/kg and around 0.03% (based on dry biomass weight) (Lin et al., 2015a). The production of lutein from the plant *Tagetes erecta* has limitations due to the seasonality of the crop that is limited from July to October, land use that could be used for food crops, and the need of water for irrigation (Lin et al., 2015b). Lin et al., 2015b estimated that an annual average production can be equal to 30–60 tons/hectare and water demand can be equal to 5250–6750 m^3/hectare for the production of *T. erecta*.

The lutein content was then investigated in some microalgae species such as *Scenedesmus almeriensis* (Céron et al., 2008), *Chlorella vulgaris* (Li et al., 2002), *Chlorella sorokiniana* (Cordero et al., 2011) as potential source respect to *Tagetes erecta*.

In this paper, pressurized liquid extraction was used for the extraction of lutein from the microalgae *Scenedesmus almeriensis*. Accelerated solvent extractor (ASE 200©Dionex) was used to perform extraction using different organic solvents and temperatures. Several extraction cycles were carried out till to perform the total extraction of lutein content.

2. Materials and methods

The microalgae *Scenedesmus almeriensis* was purchased as lyophilized biomass from AlgaRes Srl, (Rome, Italy). The starter inoculum was gently provided from the University of Almeria, Spain. The biomass was stored at -20 °C before the use to avoid degradation process due to the temperature and light.

The biomass (around 2 grams) was mechanically pre-treated using a planetary ball mill (Retsch PM200, Technology GmbH, Haan, Germany) adding as inert material an appropriate quantity of diatomaceous earth (DE). The mechanical pretreatment was carried out at the optimized operative conditions as reported by Mehariya et al., 2019. Pressurized fluid extraction was carried out to extract lutein from microalgae biomass using the technology Accelerator Solvent Extractor (ASE 200®Dionex, Salt Lake City, UT, USA). The extractions were performed testing different solvents and temperatures as reported in table 1.

Table 1: Extraction operative conditions

Solvent	Temperature °C
Chloroform/Methanol (1:1)	20
	40
	60
	80
Hexane	20
	40
	60
	80
Ethanol	20
	40
	60
	67
	75
	80
Acetone	20
	40
	60
	80

Each extraction cycle was performed for around 20 minutes and several (3-4 cycles) were carried out till to the discolouring of the biomass. The extracts obtained (~ 10-15 ml) were automatically collected in appropriate amber glass vials. The dry weight of extracts was quantified after solvent evaporation under nitrogen atmosphere using the instrument TurboVap®.

Lutein was quantified after the saponification of extracts using a methanolic solution of KOH 0.05M for 6 hours at 30°C. The saponification was stopped by adding a methanolic solution of NH_4Cl 0.05 M. After saponification, the samples were analysed using an Agilent 1290 Infinity II with a DAD (Diode array detector). A Zorbac plus C18 reversed phase column (Agilent®) was used and lutein was eluted using a isocratic mobile phase acetonitrile:methanol (85:15 v/v). Lutein standards were used at different concentration ranged from 100 to 500 mg/L.

3. Results and discussions

The extraction of lutein from *Scenedesmus almeriensis* pre-treated biomass were investigated using pressurized fluid extraction at 100 bar and several temperature using different organic solvent. Pressure was tested at 100 bar as it was proven that this pressure is sufficient to achieve maximum extraction yield by using ASE200 (Molino et al., 2018). The organic solvents were chosen on the basis of their polarity properties. Ethanol and acetone, that are recognized as GRAS solvents by the Food and Drug Administration (FDA), were tested for this peculiarity (FDA 2004).
The extraction using the mixture chloroform:methanol (1:1 v/v) was investigated at several temperatures ranging from 20 °C to 80 °C (Figure 2). The quantity of the extracted lutein increased respect to the increase of the temperature but decreased at 80°C. The highest concentration using this mixture was detected at 60°C and was equal to 2.12 mg/g.

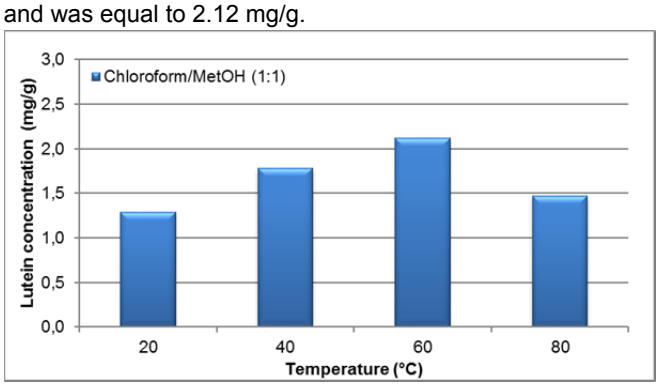

Figure 2: Lutein content at different temperatures, pressure 100 bar, three extraction cycles, using chloroform:methanol 1:1 (v/v)

The mixture of chloroform:methanol demonstrated to be appropriate for the extraction of lutein from *Scenedesmus almeriensis* but the toxic effect of chloroform is not suitable for the extract application as additive and supplement in the food sector.
Notwithstanding hexane was one of the first organic solvents used to study the recovery of lutein from *Scenedesmus almeriensis* (Céron et al., 2008), the lutein recovered in this study using hexane resulted very less. According to Céron et al., 2008 the mechanical pre-treatment trough bead milling with alumina for 5 minutes, followed with alkaline treatment (4 %, KOH, 5 minutes) and solvent extraction using hexane (6-8 L for 1 L of saponified solution) to obtain 95% recovery. Contrary, in this study the recovery of lutein resulted very less at low temperatures as 20 °C and 40 °C while a better extract content was obtained at 60 °C which was equal to 1.24 mg/g (Figure 3).

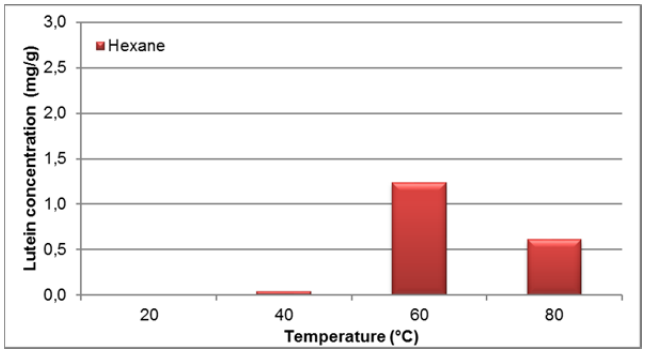

Figure 3: Lutein content at different temperatures, pressure 100 bar, three extraction cycles, using hexane

The best performance was obtained using the GRAS solvent ethanol as shown in figure 4. The lutein content increased with the increase of temperature till to 67 °C when the highest extraction yield was recorded equal to 2.76 mg/g. Gong et al., 2018 studied the extraction of lutein from *Chlorella vulgaris* UTEX 265 wet biomass investigating several parameters (sample size, drying and disruption method, solvent performance). The authors also demonstrated the best performance of extraction using ethanol through soaking for 24 hours.

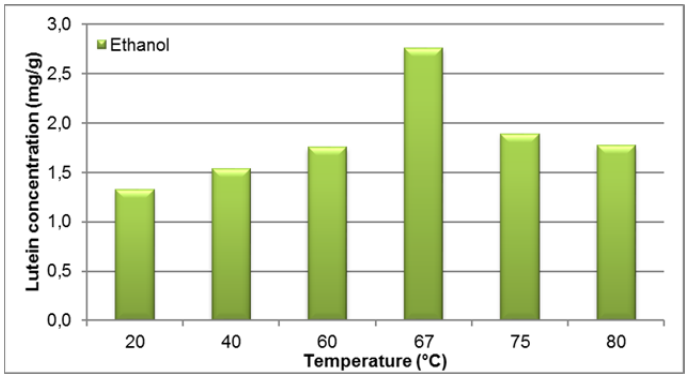

Figure 4: Lutein content at different temperatures, pressure 100 bar, three extraction cycles, using ethanol

Acetone is also a GRAS solvent but less lutein content was recorded as shown in figure 5. Lutein content increased from 20°C to 60 °C when the highest extract content was obtained at a concentration of 1.36 mg/g at 60 °C.

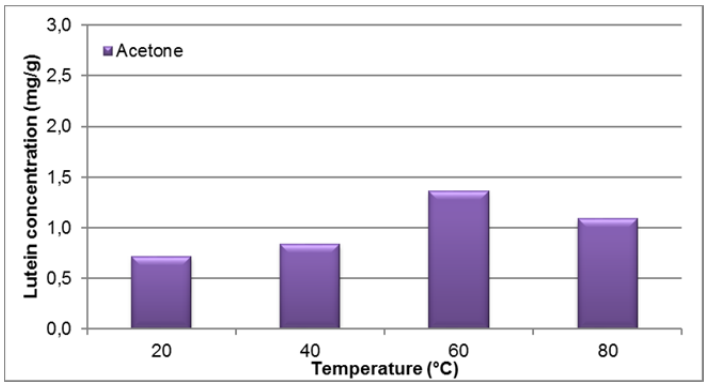

Figure 5: Lutein content at different temperatures, pressure 100 bar, three extraction cycles, using acetone

The results showed that the best performance was detected using ethanol as organic solvent, followed by chloroform:methanol, acetone, and hexane. In table 2, a comparison with other extraction methodologies performed on microalgae for lutein recovery is reported. The comparison evidenced that pretreatment and saponification are fundamental to implement lutein recovery. The quantity of lutein recovered in some studies is similar to that obtained in this study using ethanol as organic solvent. Chan et al., 2016 pretreated *Scenedesmus obliquus* using high pressure cell disruption through French press for 1 minute and seven times. The biomass was saponified using KOH 2.5% (w/v) and the extraction was performed using diethyl ether. The authors obtained in these conditions around 2.51 ± 0.12 mg/g a quantity comparable with that obtained in this study (2.76 mg/g) using ethanol. Cerón García et al., 2018 tested the extraction of lutein from *Scenedesmus almeriensis* using a mixture of ethanol:hexane:water in 77:17:6 (v/v/v) obtaining a lutein recovery equal to 5 mg/g. Ethanol was used as solvent for the pre-treatment of *Chlorella vulgaris* trough ultrasonic bath for the extraction of lutein, and a single step-extraction was performed using 1:3 ether/ethanol (v/v) with 1.25 g KOH/L (Gong et al., 2017). The concentration of lutein was implemented till to 8 mg/g using the optimized condition investigating on the effect of solvent polarity testing different ethanol to ether ratio at three levels: 1:3, 1:1, or 3:1 ether/ethanol (v/v) (Gong et al., 2017). Low et al., 2020 tested a single-step of extraction to recovery lutein from *Scenedesmus sp.* using microwave (250 Watt, rotating speed equal to 250 rpm) and binary phase solvent system with 60% potassium hydroxide solution with acetone in the ratio of 0.1 (ml/ml), for 36 min and 55 °C.

The authors obtained a lutein concentration equal to 11.92 mg/g using a biomass:solvent ratio equal to 0.7 (mg/ml). Lutein extraction was also investigated using tetrahydrofuran as extraction solvent obtaining 5.21 mg/g from *Chlorella sorokiniana* pre-treated mechanically with a French-press (Chen et al., 2016).

Table 2: Comparison between lutein extracted and operative conditions

Microalgae species	Extraction Method Description	Operative conditions	Lutein Recovered	References
Scenedesmus almeriensis	Pretreatment/saponification/ Extraction solvent	Milling with alumina; Extraction solvent mixture ratio of ethanol:hexane:water in 77:17:6 (v/v/v)	5 mg/g	Cerón García et al., 2018
Chlorella vulgaris	Saponification and the extraction steps were conducted simultaneously	2M of ethanolic KOH and dichloromethane sonicator bath, and orbital shaker for extraction	0.69 ± 0.08 mg/g	Este et al., 2017
Chlorella vulgaris	Ultrasonic pre-treatment and extraction combined with saponification	Ultrasound bath with ethanol; ethanolic KOH and solvent extraction with ether/ethanol 1:3 (v/v)	>8 mg/g	Gong et al., 2017
Scenedesmus obliquus	Bead beater cell disruption, water bath extraction method, extraction repetition	diethyl ether for extraction and KOH 2.5% w/v for saponification	2.51 ± 0.12 mg/g	Chan et al., 2012
Chlorella sorokiniana	French press pre-treatment, solvent extraction	tetrahydrofuran as extraction solvent	5.21 mg/g	Chen et al., 2016
Scenedesmus sp.	Microwave assisted binary phase, solvent extraction	using acetone and 60% w/v KOH, single step extraction method	11.92 mg/g	Low et al., 2020
Scenedesmus almeriensis	Bead milling, pressurized fluid extraction, saponification	Ethanol as organic solvent, 67°C, 100 bar	2.76 mg/g	This study

As aforementioned, several extraction solvent methods and organic solvent have been investigated for the recovery of lutein from several microalgae species. The exploitation of pressurized fluid extraction has shown the potentiality to obtain an extract-rich of lutein using GRAS solvent as ethanol and acetone.

4. Conclusions

The presented work highlights the best extraction conditions to obtain lutein from *Scenedesmus almeriensis* microalgae by using pressurized fluid extraction. The highest lutein content, equal to 2.76 mg/g, was recorded using ethanol, a GRAS (Generally recognized solvent), at 60 °C and a pressure equal to 100 bar, and extraction time of 80 minutes. Despite the results obtained, further studies will be needed to optimise the extraction yield of lutein from the microalgae *Scenedesmus almeriensis* by further varying the operating conditions or experimenting with additional pre-treatment technologies.

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