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Microalgae Valorisation via Accelerated Solvent Extraction: Optimization of the Operative Conditions

Antonio Molino[a], Juri Rimauro[a], Patrizia Casella[a], Antonietta Cerbone[a], Vincenzo Larocca[b], Despina Karatza[c], Evangelos Hristoforou[d], Simeone Chianese[c,*], Dino Musmarra[c]

[a]Research Centre of Portici, Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), Piazzale Enrico Fermi 1, 80055 Portici, NA, Italy
[b]Research Centre of Trisaia, Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA), SS Jonica 106 - km 419+500, 75026 Rotondella, MT, Italy
[c]Dipartimento di Ingegneria, Università of Campania "Luigi Vanvitelli", Via Roma 29, 81031, Aversa (CE), Italy
[d]School of Electrical and Computer Engineering, National Technical University of Athens, Zografou Campus, 9, Iroon Polytechniou str, 15780 Athens, Greece
simeone.chianese@unicampania.it

The valorisation of microalgae for the production of valuable biomolecules, for their use in several fields, such as cosmetic, pharmaceutical and animal food, by applying technologies able to avoid any kind of alteration of the biomolecule, is becoming a priority research topic. Several techniques can be used, among them Accelerated Solvent Extraction (ASE) with Generally Recognized As Safe (GRAS) solvents is an interesting technology.
This paper deals with Accelerated Solvent Extraction investigations for extraction of lutein ($C_{40}H_{56}O_2$, carotenoid belonging to the xanthophylls group) from *Haematococcus pluvialis* in red phase with a mechanical pre-treatment method to break the cell wall. Experiments were carried out by comparing performance of several solvents both conventional as Chloroform:Methanol (1:1) and Hexane (class 2 according to FDA classification) and solvents normally accepted in the pharmaceuticals and food industry as Acetone and Ethanol (class 3 of FDA classification) by varying extraction conditions (pressure, temperature and time).

1. Introduction

Lutein ($C_{40}H_{56}O_2$) is a carotenoid mostly used as food additive, supplement in the nutraceutical field and as a conditioning agent in the cosmetics thanks to its abilities as antioxidant and colouring agent. In Europe, it was classified and authorized as a food additive, from EU Regulation (Regulation (EU), 2012). In addition to its use as a food additive, it was also marketed as dietary supplement for its beneficial effects for the protection of the eyes (Noviendri et al., 2011). The use of lutein as food additive, as well as dietary supplement, represents about the 90 % of its worldwide market. In the last few years, this market showed a growing trend, with an estimated value of USD 357.7 Million by 2022. In terms of volume, the market was projected to reach about 2,100 tons by 2022 (Naidoo et al., 2013; Global Market Insight, 2016).
At the actually state, Lutein is extracted from the petals of Tagetes Erecta flower by using hexane as extractive solvent. The produced oleoresins are marketed or refined by a saponification process to convert esters into free forms of Lutein (Cantrill, 2004). However, the production of Lutein from Tagetes Erecta is characterized by several drawbacks, such as seasonality of flowering, land use and a high water consumption, i.e. about 50 - 80 m^3 for each kg of lutein produced (Lin et al., 2015).
Microalgal biomass is an interesting alternative source for production of carotenoids such as Lutein (Sun et al., 2015). Microalgae cultivation usually occurs in large tanks (open ponds) and photobioreactors (Borowitzka, 1999). This last technology allows to overcome the issue related to the competitiveness use of land. In addition, for lutein production, microalgal biomass is more advantageous thanks to its higher lutein content with respect to Tagetes erecta one, achieving productivity till to 5 times higher that Tagetes erecta (Lin et al.,

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2015). Some microalgae species, such as *Muriellopsis sp.*, *Haematoccoccus pluvialis*, *Scenedesmus almeriensis*, *Chlorella protothecoides*, *Chlorella zofingiensis*, *Botryococcus braunii*, *Neospongiococcus gelatinosum* and *Chlorococcum citriforme*, were studied because of their aptitude to accumulate Lutein (Ghosh et al., 2015). In particular, *Haematococcus P* could be one of the most suitable species for Lutein production, with an accumulation aptitude in the range of 0.2 - 0.5 % on dry basis, that is 10-15 times higher than Tagetes erecta flower content (Sánchez et al., 2008).
This paper aims to evaluate Accelerated Solvent Extraction (ASE) technology for Lutein extraction from *Haematoccoccus p.* in red phase. The effects of extraction conditions (pressure = 50, 100bar; temperature = 20-100 °C; time = 20-80 minutes) and of several solvents (Chloroform:Methanol (1:1), Hexane, Acetone and Ethanol) on the extraction yield on dry basis of Lutein, identified and quantified by u-HPLC and GC-FID analysis, were investigated.

2. Materials and Methods

Set up of experimental activity for Lutein extraction from *Haematoccoccus p.* in red phase consisted of mechanical pre-treatment of microalgal species, by using a ball mill (Retsch MM400®), in order to break the cell wall, solvent extraction at fixed temperature and pressure, by using Dionex ASE 200 extractor, and separation and quantification, by using Agilent u-HPLC Agilent 1290 Infinity II with Zorbax Eclipse plus C18 column 1.8 µm. All solvents (Chloroform, Methanol, Hexane, Acetone and Ethanol) were of HPLC grade and purchased from Sigma-Aldrich (Saint Louis, Missouri, USA).

Table 1: First experimental series: effect of pressure (extraction time = 20 min)

Pressure [bar]	Temperature [°C]	Solvent
50	50; 100	C:M (1:1)
		Hexane
		Acetone
		Ethanol
100	50; 100	C:M (1:1)
		Hexane
		Acetone
		Ethanol

Table 2: Second experimental series: optimization of extraction conditions (pressure = 100 bar, extraction time = 80 min)

	C.M (1:1)	Hexane	Acetone	Ethanol
Temperature [°C]	50	20	20	50
	58	40	40	58
	67	50	50	67
	75	75	60	75
	88	88	75	88
	100	100	100	100

Chloroform and Methanol were used as mixture (C:M = 1:1), while Hexane, Acetone and Ethanol were used as single compounds. According to Food and Drug Administration (FDA) classification (Food and Drug Administration, 2016), Chloroform and Hexane are solvent of class 2, that should be limited in pharmaceutical products because of their inherent toxicity; Permitted Daily Exposure limits and concentrations are close to 0.1 mg/day and 10 ppm, respectively (Wakelyn and Wan, 2004). Acetone and Ethanol are classified as class 3 solvents (Food and Drug Administration, 2016), which includes no solvent known as human health hazardous at levels normally accepted in pharmaceuticals and food industry. Haematococcus Pluvialis in Red phase was supplied by the Italian Company MICOPERI BLUE GROWTH®. Two series of experiments were performed for the above-said solvents: the former (Table 1) to evaluate the effect of operating pressure (50, 100 bar) at two different extraction temperature (50, 100 °C), keeping constant extraction time (20 minutes), was designed; the latter (Table 2) to optimize the Lutein yield, keeping constant pressure (100 bar), varying extraction temperature in the range of 20-100 °C for Hexane and Acetone and 50-100 °C for C:M (1:1) and Ethanol and performing four consecutive extraction runs of 20 min each for increasing Lutein yield (on dry basis), was designed. Moreover, in order to compare the extraction at a fixed extraction time, the Lutein recovery from the

first extraction run, calculated with respect to the amount of lutein extracted by the method proposed by Li et al. (2012) and measured by using u-HPLC analysis, as it follows was estimated (Eq. 1):

$$\text{Lutein Recovery} = \frac{\text{Lutein Yiedl from first extraction run}}{\text{Total Lutein Yield}} \text{x100} \quad (1)$$

A total amount of Lutein of 7.7 mg/g on dray basis was measured.

3. Results

Results of the first extraction investigation series for each solvent, in terms of Lutein yield on dry basis as function of pressure, are reported in Figure 1. As shown, the amount of lutein extracted depends on operating pressure, as, keeping constant temperature, the higher the pressure the higher the Lutein yield. For C:M, Hexane and Acetone the effect of pressure at 50 °C was mainly marked; while for Ethanol, effects of pressure at 50 °C and 100 °C was comparable. For C:M, Lutein yield of about 2 mg/g_d and 2.2 mg/g_d were achieved at 50 bar and 100 bar, respectively. For Hexane, Lutein yield on dray basis of about 3.5 mg/g_d and 3.8 mg/g_d were achieved at 50 bar and 100 bar, respectively. For Acetone, Lutein yield on dray basis of about 3.9 mg/g_d and 4.3 mg/g_d were achieved at 50 bar and 100 bar, respectively. For Acetone, Lutein yield on dray basis of about 3.9 mg/g_d and 4.3 mg/g_d were achieved at 50 bar and 100 bar, respectively. For Ethanol, Lutein yield on dry basis of about 2.3 mg/g_d and 2.6 mg/g_d were achieved at 50 bar and 100 bar, respectively. As reported, the highest extraction for Acetone was observed.
Optimization of extraction condition (second experimental series) at 100 bar pressure was carried out.

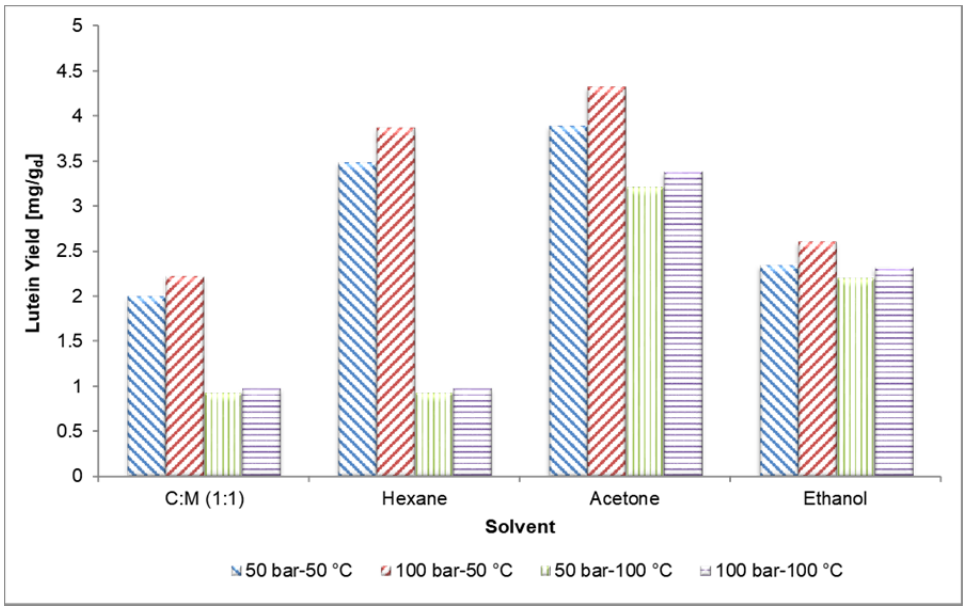

Figure 1: Results of the first experimental series – effect of pressure (extraction time = 20 min)

Results of the second extraction investigation series in terms of both total Lutein yield (on dry basis) and Lutein recovery from first extraction run (Eq. 1) as function of temperature, keeping constant pressure, for C:M (1:1), Hexane, Acetone and Ethanol are reported in Figures 2, 3, 4 and 5, respectively. For all solvents, total Lutein yield increased with temperature till a maximum value, beyond which yield reduced; the minimum total Lutein yield at the highest temperature was observed. This phenomenon may be explained by considering the thermal instability of carotenoids, as reported by several authors (Chafer et al., 2004; Krichnavaruk et al., 2007; Ruen-ngam et al., 2010). For C:M (Figure 2), the highest total Lutein yield, about 5.9 mg/g_d, was found at 67 °C with a total extraction time of 80 min; in terms of Lutein recovery (Eq.1), from an extraction time of 20 minutes, the highest value, of about 58 % (≅ 4.4 mg/g_d), at 75°C was recovered; minimum total Lutein yield of about 0.97 mg/g_d was found. For Hexane (Figure 3), the highest total Lutein yield, close to 3.9 mg/g_d, was found at 50 °C with a total extraction time of 80 min; in terms of Lutein recovery (Eq.1), from an extraction time of 20 minutes, the highest value, of about 50 % (≅ 3.3 mg/g_d) at 50 °C, was recovered; minimum total Lutein

yield of about 1 mg/g_d was found. For Acetone (Figure 4), the highest total Lutein yield, close to 4.6 mg/g_d, was found at 40 °C with a total extraction time of 80 min; in terms of Lutein recovery (Eq.1), the highest value, of about 58 % (≅ 4.7 mg/g_d) at 40 °C, was recovered. For Ethanol (Figure 5), the highest total Lutein yield, close to 5.8 mg/g_d, was found at 67 °C with a total extraction time of 80 min; in terms of Lutein recovery (Eq.1), the highest value, of about 72 % (≅ 5.5 mg/g_d) at 67 °C, was recovered.

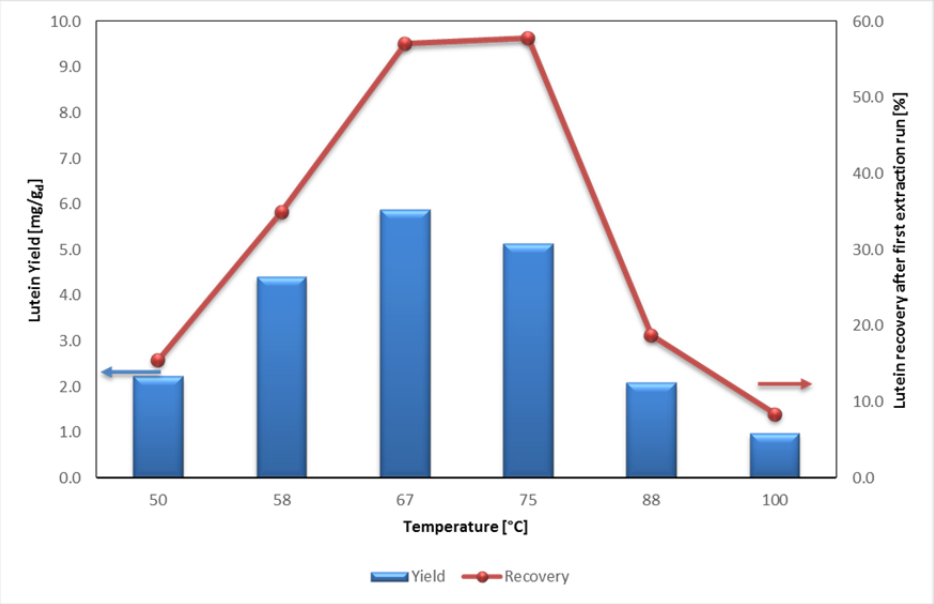

Figure 2: Optimization of Lutein extraction – effect of temperature (Solvent = C:M (1:1); pressure = 100 bar)

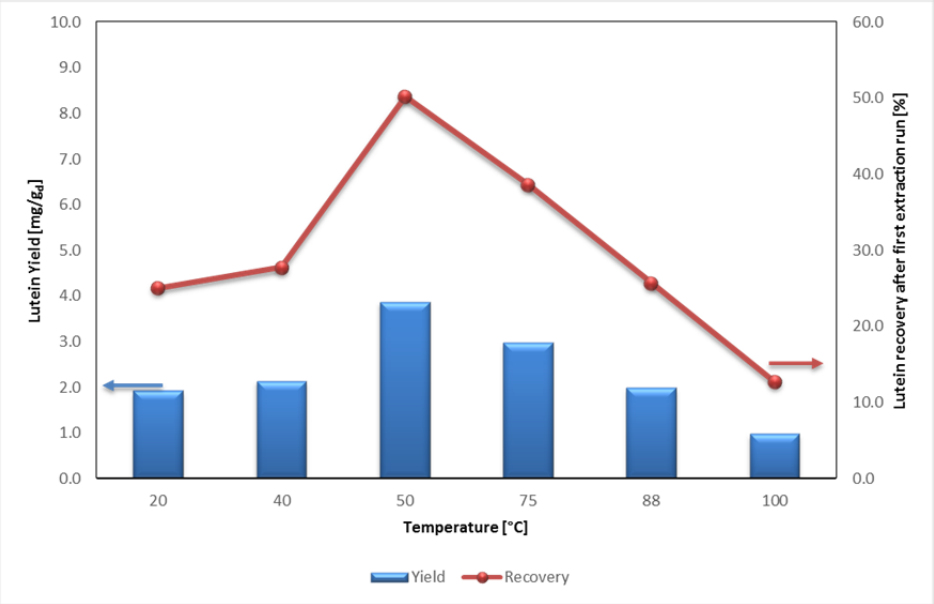

Figure 3: Optimization of Lutein extraction – effect of temperature (Solvent = Hexane; pressure = 100 bar)

Comparison between solvents in terms of Lutein yield (dry basis), at extraction times of 20 min and 80 min, in Table 3 is reported. The highest value of about 5.9 mg/g_d for C:M (1:1) (temperature = 67 °C; pressure = 100 bar; time = 20 min) was found; while at 20 minutes Lutein yield close to 4.4 mg/gd was found. However, it is worth highlighting that, based on FDA classification, Chloroform is a solvent to be limited.
Hexane showed the lowest extraction performance both at 20 minutes (≅ 3.3 mg/g_d) and 80 minutes (≅ 3.9 mg/g_d) at 50 °C.

For Acetone, at 40 °C, from an extraction time of 20 minutes, Lutein yield of ≅ 4.5 mg/g_d was achieved; increasing extraction time to 80 minutes, Lutein yield of ≅ 4.6 mg/g_d was found. For Ethanol, at 67 °C, from an extraction time of 20 minutes, Lutein yield of ≅ 5.5 mg/g_d was achieved; increasing extraction time to 80 minutes, Lutein yield of ≅ 5.8 mg/g_d was found. As pointed out, for Acetone and Ethanol, an extraction time of 20 minutes was effective to achieve Lutein recovery higher than 95 %.

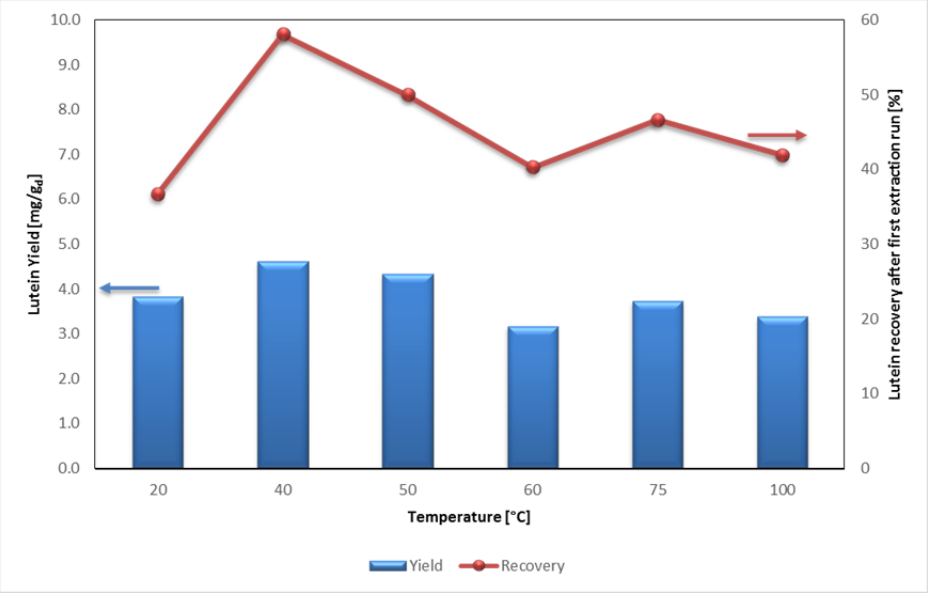

Figure 4: Optimization of Lutein extraction – effect of temperature (Solvent = Acetone; pressure = 100 bar)

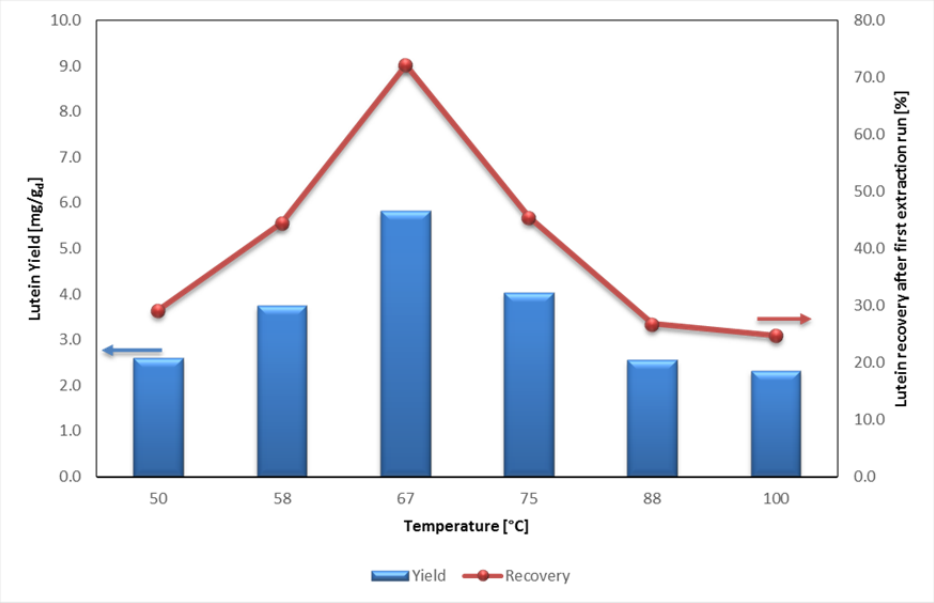

Figure 5: Optimization of Lutein extraction – effect of temperature (Solvent = Ethanol; pressure = 100 bar)

Among considered solvents, for an extraction time of 20 minutes, Ethanol (67 °C) resulted the solvent with the best extraction performance, allowing to increase Lutein yield till to ≅ 40 %. At an extraction time of 80 minutes, C:M (67 °C) and Ethanol (67 °C) showed comparable extraction aptitude, demonstrating that solvents as Ethanol that is safer (class 3 of FDA) than Chloroform (class 2 of FDA) can be a valuable substitute of conventional solvents, such as Chloroform or its mixture.

Table 3: Extraction performance comparison: optimized conditions (pressure = 100 bar)

Solvent	Temperature [°C]	Extraction time [min]	Lutein yield [mg/g_d]	Extraction time [min]	Lutein yield [mg/g_d]
C:M (1:1)	67	20	≅ 4.4	80	≅ 5.9
Hexane	50	20	≅ 3.3	80	≅ 3.9
Acetone	40	20	≅ 4.5	80	≅ 4.6
Ethanol	67	20	≅ 5.5	80	≅ 5.8

4. Conclusions

Extraction of Lutein form *Haematococcus p.* in red phase, by ASE technology, comparing several solvents, was investigated. The effects of pressure, temperature and time, on Lutein yield (dry basis) for each solvent, were explored. Optimized extraction conditions, allowing to achieve Lutein recovery higher than 95 %, for all considered solvents were defined. C:M (1:1), required temperature, pressure and time of 67 °C, 100 bar and 80 minutes, respectively, to extract about 5.9 mg/g_d of Lutein. Hexane (75 °C, 100 bar, 80 minutes) showed the lowest extraction performance (≅ 3.9 mg/g_d). With respect to solvents of class 3 as Acetone and Ethanol, the highest extraction performance by Ethanol was found: for Acetone, Lutein yield of about 4.5 mg/g_d at 100 bar, 40 °C and 20 minutes, was achieved, while for Ethanol, Lutein yield of about 5.5 mg/g_d at 100 bar, 67 °C and 20 minutes, was reached. At an extraction time of 20 minutes, Ethanol (67 °C) resulted the solvent with the highest extraction performance, allowing to increase Lutein yield till to ≅ 40 %.

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