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ARTICLE

Stability of Carotenoids in *Scenedesmus almeriensis* Biomass and Extracts under Various Storage Conditions

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Scenedesmus almeriensis biomass is a source of carotenoids, particularly lutein, and is considered to be promising as an alternative source to marigold. One key question concerning alternative sources of lutein is the loss of carotenoids that takes place between harvesting and processing, which in the case of marigold is frequently up to 50%. The work described here involved a study into the stability of the main carotenoids (lutein, violaxanthin, and β-carotene), as well as other components, under different storage conditions. The experiments were carried out with biomass in three forms: frozen, freeze-dried, and spray-dried. The stability of extracts of *Scenedesmus* biomass in acetone and olive oil was also studied. The results show that the most important factor in retaining carotenoids is a low temperature. At −18 °C the loss of carotenoids was negligible after the storage period, regardless of the biomass form used (frozen, freeze-dried, or spray-dried). On the other hand, the carotenoid content and fatty acid profile was increasingly affected with increasing temperature. However, the protein content is unaffected by storage conditions.

KEYWORDS: Stability extraction; lutein production; Scenedesmus almeriensis; carotenoids

INTRODUCTION

Carotenoids are responsible for the beautiful colors of many fruits (pineapple, citrus, tomatoes, mango, paprika) and flowers (marigold, eschscholtzia, narcissus), as well as the color of many birds (flamingo, ibis, canary), insects (ladybird), and marine animals (crustaceans, salmonids). Algae are a rich source of carotenoids and provide these compounds to the aquatic food chain.

The stabilization of carotenoids against oxidation in foods has been of importance for a long time in that their degradation effectively lowers the final product quality in terms of nutritional properties and coloration characteristics. Carotenoid degradation during these processes has usually been observed, and the extent of loss was found to be dependent on the type of treatment, the temperature, and the duration (*1*−*5*).

The stability of carotenoids under different storage conditions has been investigated in different systems, including vegetable juice (*6*), freeze-dried whole egg powder (*7*), spinach and carrots (*8*), spray-dried encapsulated carrot carotenes (*9*), and model systems of simulated aqueous or dehydrated food (*10*, *11*). Although carotenoid degradation was observed in all of these cases, the rate and kinetics of degradation were found to be dependent on both the storage conditions (especially light, temperature, water activity, and oxygen) and the matrix characteristics (liquid or solid state and microenvironment). The effectiveness of spray-drying of β-carotene-rich cells of *Dunaliella* has been studied, and the potential of exogenous antioxidants in preserving the stability of β-carotene has been evaluated (*12*).

Lutein ((3*R*,3′*R*,6′*R*)-β,ε-caroten-3,3′-diol) is an asymmetric dihydroxylated carotenoid that has a wide range of applications in the food industry and medicine. The occurrence of lutein in nature is quite widespread, although the highest levels are found in marigold (*Tagetes erecta* L.) flowers, generally as C14 to C18 even-numbered straight-chain fatty acid esters (*13*). Lutein is a xantophilic compound that is recommended for the prevention of some types of cancer (*14*−*16*), cardiovascular diseases (*17*), and retinal degeneration (*18*, *19*). Lutein is also a food colorant allowed by the EU and reported as E 161 b. Sales of lutein as a feed additive in the US amount to about $150 million per year. Although lutein is present in fruits and vegetables, the estimated daily uptake of 1.5 mg day^{-1} (*20*) is not sufficient to reach the recommended daily uptake of 6 mg day^{-1} (*21*), and thus the consumption of lutein supplements is recommended. The best commercial source of pure lutein is marigold (*Tagetes erecta* L.). However, the lutein content of marigold flowers is very low, 0.03% dry wt, and alternative lutein-rich sources are therefore of interest. In this respect, several microalgae have been proposed as potentially useful to produce lutein, and these include *Muriellopsis* sp. (*22*), *Chlorella zofingensis* (*23*), and *Chlorella protothecoides* (*24*). Recently, a new lutein-rich microalgae strain, *Scenedesmus almeriensis*, has been proposed as a lutein source (*25*). In comparison with higher plants, microalgae have several advantages, because they can be cultivated in bioreactors on a large scale and

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Table 1. Changes in the Recovery of Lutein using a Spray-Drier as a Function of the Processing Temperature[a]

temp (°C)		
inlet	outlet	recovery of lutein (%)
120	68	67.6 ± 3.4
140	80	91.8 ± 6.5
150	90	110.6 ± 10.7
180	105	110.8 ± 9.7
220	132	111.1 ± 7.8

[a] A freeze-dried sample was used as control.

thus could provide a continuous and reliable source of lutein (*26*−*28*).

Many of the commercial algal carotenoid products are oil-based; algal carotenoids are extracted into hot vegetable oil (*29*) and subsequently encapsulated into gelatin capsules. Such an extraction protocol may result in large losses of carotenoids due to incomplete extraction and oxidative destruction during processing. An interesting alternative to carotenoid extraction is the preparation of dried algal powders by spray-drying, thus allowing the use of whole algal cells. This technique is frequently used to dry heat-sensitive products, due to the short drying time. Unlike freeze-drying, which is a time-consuming process that results in a highly hygroscopic powder and is poorly applicable on a large scale, spray-drying results in a dry powder in a relatively short time (*12*). However, studies into the stability of the dried biomass obtained in different ways have not been carried out to date.

The main purpose of the work described here was as to evaluate the preparation of dried algal powders and to study the storage stability of carotenoids derived from microalgae as microalgal dry biomass. This work is focused on studying the stability of the main carotenoid (lutein) from an extract of *Scenedesmus almeriensis* in a matrix such as acetone or olive oil.

MATERIALS AND METHODS

Microorganism Used. The microalga *Scenedesmus almeriensis* was isolated in fresh water from a greenhouse located in Almería, Spain. This strain was identified as new by the "Experimental Phycology and Culture Collection of Algae−SAG" and deposited in the Culture Collection of Algae and Protozoa of the Centre for Hydrology and Ecology, Ambleside, U.K., code CCAP 276/24. Cells were produced in an industrial-sized outdoor tubular photobioreactor (3000 L), in continuous mode at a dilution rate of 0.4 L/day, in March. The cultures were performed at pH 8.0 by on-demand injection of CO_2, and the temperature was maintained at 30 °C by passing thermostated water through a heat exchanger located inside the reactor.

Preparation of Dried Microalgal Biomass. The biomass was harvested daily by centrifugation. The biomass was immediately frozen and stored at −18 °C, and a gram aliquot was then lyophilized and stored at −18 °C. In addition, a continuously agitated algal concentrate was dried using a Buchi Mini spray-drier (Model B-191). This procedure has been optimized here for maximum lutein recovery from *Scenedesmus almeriensis* by using five different inlet temperatures in the range 120−220 °C, which resulted in temperatures in the range 68−132 °C at the outlet, as shown in **Table 1**. The receiving chamber was protected from exposure to light throughout the drying process. A microalgae suspension of 50−60 g L^{-1} was sprayed at a rate of 10 mL min^{-1} using a 35 m^3 h^{-1} air rate. The resulting powder was immediately removed from the receiving vessel following drying and subjected to the stability studies described below.

Stability Experiments using Biomass. Gram aliquots of frozen, freeze-dried, and spray-dried biomass, 42 each, were placed in 25 mL amber bottles (totalling 126 samples) filled with argon gas. Those samples were stored for 6 weeks and analyzed at regular intervals (2−4 days) for their carotenoid content. The results obtained were used to choose the most stable state of the biomass, which turned out to be the freeze-dried form. Once the biomass form was chosen, the effect of storage temperature was studied. For this, gram aliquots of *Scenedesmus almeriensis* freeze-dried biomass were also placed in 126 amber bottles (25 mL) filled with argon gas and divided into 3 42-bottle sets that were respectively stored at 20, 4, and −18 °C for 6 weeks and analyzed to measure their carotenoid content at regular intervals. The evolution of the lipid profile and protein content during storage were also followed.

Stability Experiments of Carotenoid Extracts. Seven vials containing samples of a carotenoid extract obtained as described by Cerón et al. (*30*) were prepared in acetone and in olive oil solution. Those samples were placed in screw-capped dark glass bottles (30 mL) filled with nitrogen gas and stored in the dark at ambient temperature (approximately 20 °C) and at 4 and −18 °C. For the short-term series experiments (a) aliquots were withdrawn an analyzed periodically for 6 weeks, while another series (b) was used in long-term conservation experiments which spanned over 15 months.

Analytical Methods. The carotenoid content of the different samples was determined by HPLC. Prior to HPLC analysis, the solid samples were ground in a mortar with alumina (aluminum oxide, Type A5, Sigma Chemical Co. St. Louis, MO) extracted in acetone and saponified as described in Cerón et al. (*30*). The carotenoids in the extracts thus obtained were analyzed by the chromatographic method described by Mínguez-Mosquera et al. (*31*) (modified by Del Campo et al. (*22*) and then by Cerón et al. (*32*)) using a Shimadzu SPD-M10AV high-performance liquid chromatograph. Separation was performed on a Lichropher 100, RP-18 (5 μm) column (4.6×150 mm). The eluents used were (A) water/methanol (2/8, v/v) and (B) acetone/methanol (1/1, v/v). The pigments were eluted at a rate of 1 mL min^{-1} and detected by measuring absorbance in the range 400−700 nm. Standards of β-carotene and lutein were provided by Sigma Chemical Co., St. Louis, MO. Violaxanthin and zeaxanthin were obtained from DHI LAB (Hørsholm, Denmark), and neoxanthin was provided by ChromaDex LGC Standards, (Barcelona, Spain).

Total N was determined using an elemental analyzer (LECO CHNS-932). The carrier gas was He, and the burning gas was O_2. The results were compared with those obtained on a semimicro Kjeldahl apparatu,s and both methods gave similar results. Total protein was calculated from the evaluated nitrogen by multiplying by 6.25 after deduction of N from nucleic acids and nitrate (*33*).

Fatty acids were determined by gas chromatography as described by Rodríguez-Ruiz et al. (*34*). Total lipids were determined by gravimetry of the extract obtained with chloroform/methanol (2/1 v/v) (*35*).

Statistical Analysis. The statistical significance (*36*, *37*) of the results was investigated using multifactor ANOVA analysis. The independent variables investigated are time, temperature, and solvent used, as indicated. The effects on lutein recovery are considered significant when $p < 0.05$.

RESULTS

Optimization of Conditions for Drying the Biomass in a Spray-Drier. Spray-drying of a suspension of *Scenedesmus almeriensis* cells was carried out under the different conditions shown in **Table 1**, with the lutein content measured in the resulting product (as a percent of the content measured in the reference freeze-dried biomass). Although lutein is considered a thermolabile substance, the best results are obtained at the highest temperatures used, between 150 and 220 °C. Those operating conditions resulted in temperatures at the outlet between 90 and 132 °C, close to or above the boiling point of water, which ensures a complete removal of humidity. On the other hand, the experiments done with the lower temperatures, 120 and 140 °C, give significantly poorer results in lutein recovery.

Therefore, **Table 1** shows that a high temperature between 150 and 220 °C should be used to attain a complete recovery of lutein, although these severe conditions may give rise to certain concerns about the structural integrity of lutein in particular. The chromatograms in **Figure 1a** (freeze-dried biomass compared to spray-drying at the highest temperature) show that no gross molecular changes have taken place in the carotenoids, given the similarity of the peaks obtained, although more subtle changes cannot be

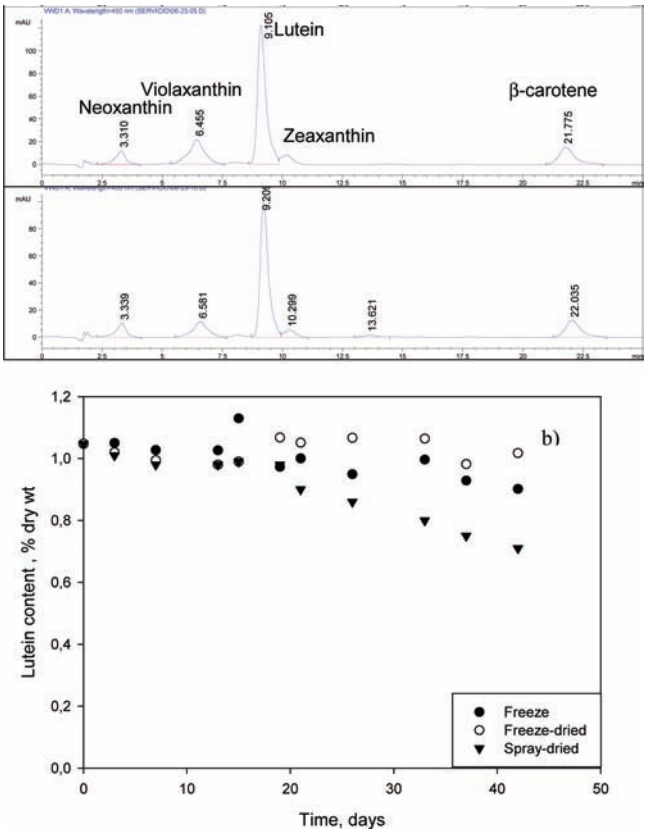

Figure 1. (a) Chromatograms showing the carotenoid profile of freeze-dried (above) and spray-dried (below) biomass of *Scenedesmus almeriensis*. (b) Evolution of lutein content (dry weight) in frozen, freeze-dried, and spray-dried biomass during storage at −18 °C.

denied without further analysis. Under these circumstances it is worth considering spray-drying at 150 °C among the feasible processing alternatives.

Selection of Biomass Stabilization Method. The resistance to degradation of each of the three forms of biomass proposed (frozen, freeze-dried, and spray-dried) is tested by storing them at −18 °C in airtight containers and under an inert atmosphere, as described in Materials and Methods. The evolution of the lutein content, as dry weight, is represented in **Figure 1b**. The results show how the lutein content noticeably decreases in the spray-dried samples after day 20 of storage, with a 30% reduction in the lutein content. The other samples showed a slower degradation rate. The frozen biomass began to degrade from day 32 and deteriorated by only 10%, and in the freeze-dried biomass the lutein remained stable throughout the period of storage. For this reason, we selected the freeze-dried biomass for the subsequent storage experiments.

Storage under Different Conditions: Evolution of Carotenoid Content. Freeze-dried biomass from *Scenedesmus almeriensis* was kept under different conditions in order to study the degradation of carotenoids with special emphasis in lutein. Two carotenoid extracts from the same biomass in acetone and olive oil (see Materials and Methods) were also included, given their occurrence in lutein recovery processes.

Figure 2 shows the evolution of the content of lutein, violaxanthin, and β-carotene in the freeze-dried biomass at different temperatures. The carotenoids studied degrade over time at different rates and are influenced by temperature in different ways. Lutein and violaxanthin decreased swiftly at ambient temperature, decreasing by approximately 50%. At 4 °C lutein is more stable but violaxanthin also degrades rapidly at this

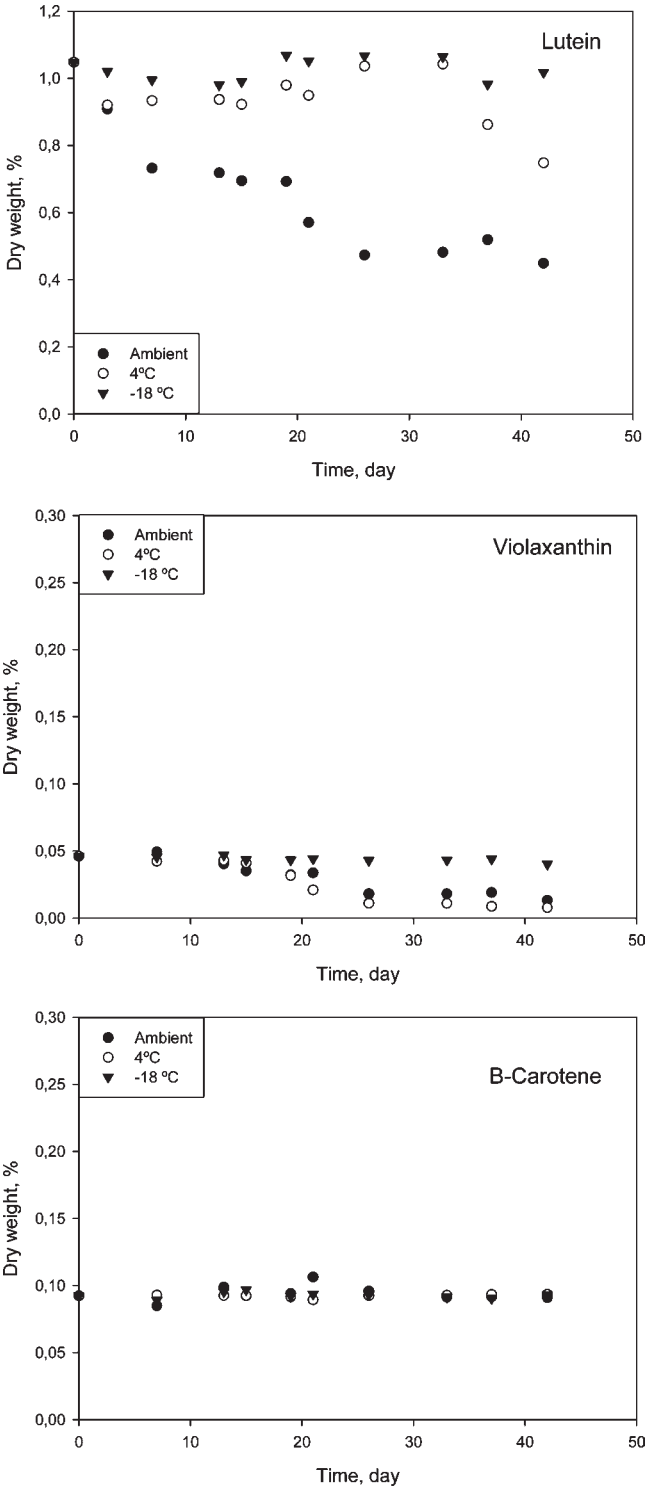

Figure 2. Evolution of the major carotenoids from *Scenedesmus almeriensis* biomass with time for different storage conditions (ambient temperature and 4 and −18 °C) working with freeze-dried biomass.

temperature. A statistical analysis showed that the time-averaged differences in lutein recovery were not significant between the experiments at 4 and −18 °C, which means that either of these temperatures could be used for conservation of the freeze-dried biomass with no significant lutein loss. On the other hand, β-carotene was stable in the range of temperatures tested, showing minimal degradation in the 6 week period of the studies.

The stability of the carotenoid extracts prepared in acetone and olive oil is shown in **Figure 3**. The acetone extracts were measured

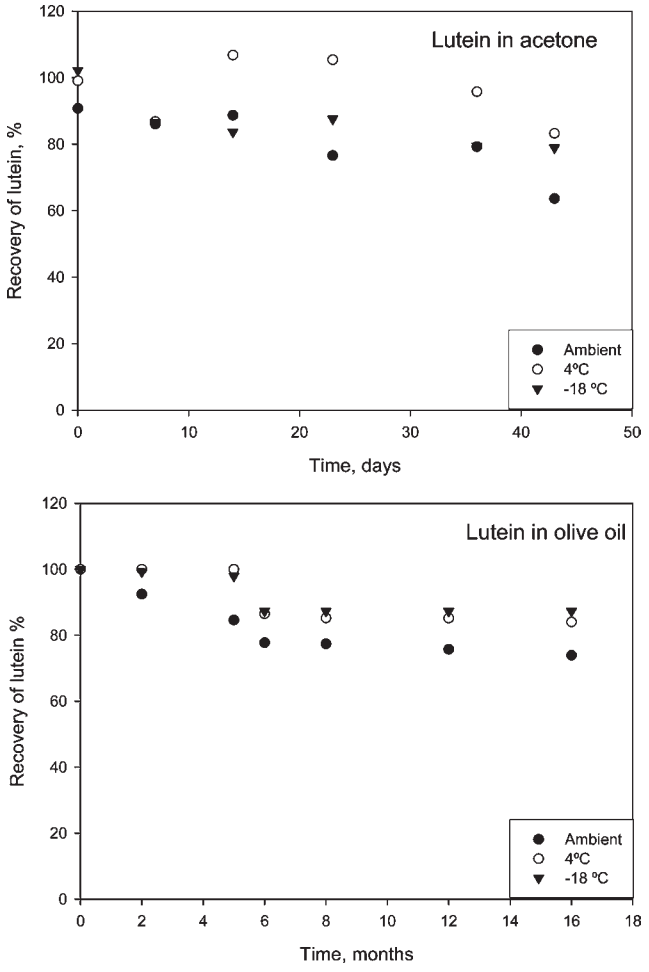

Figure 3. Evolution of the recovery of lutein from extracts of *Scenedesmus almeriensis* in different solvents (acetone and olive oil) at different temperatures (ambient and −18 °C).

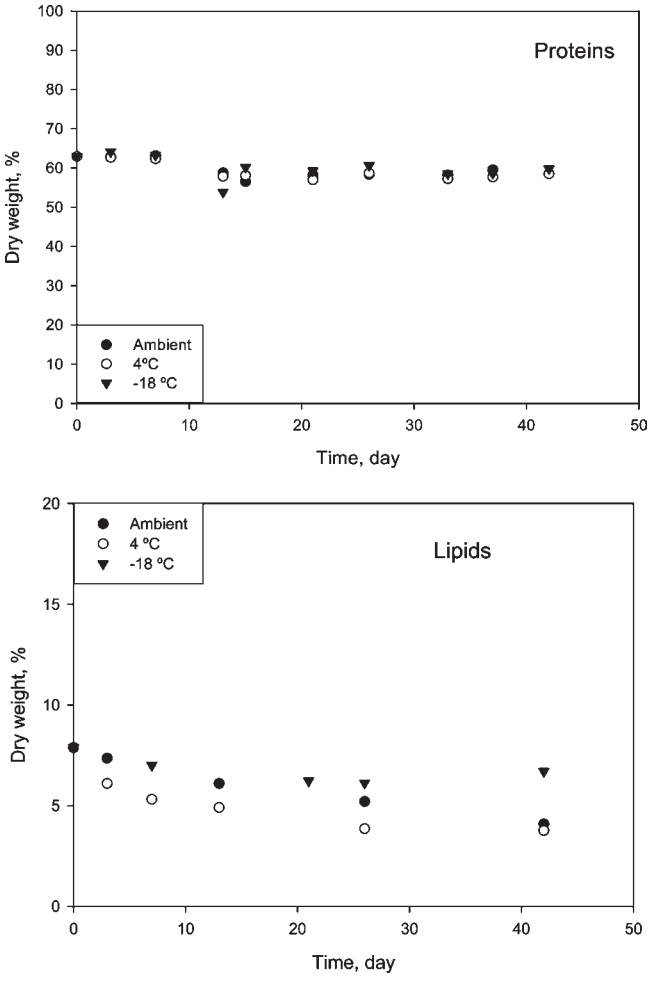

Figure 4. Evolution of lipid and protein content with time for *Scenedesmus almeriensis* freeze-dried biomass under different storage conditions (ambient temperature and 4 and −18 °C).

over the same 6 week period as the solid samples but the time span of the study of the olive oil extracts was prolonged to 16 months, because preliminary results indicated that a high stability in olive oil should be expected. The lutein in olive oil extracts was stable for 5 months at temperatures of 4 and −18 °C, showing no significant change, while a 20 °C a steady decrease was observed. After 6 months, the extracts in olive oil kept at 4 °C and −18 °C lost around 15% and remained unchanged until the end of the 16 month period. At ambient temperature the loss of lutein content after 16 months was 25%. With regard to the extracts prepared in acetone, at ambient temperature we observed a steady decrease in lutein content, ending in a 40% loss after the 6 week period. At −18 °C a similar result was observed but with a lower rate of lutein degradation, ending in a final loss of 20%. An ANOVA analysis was done to investigate the effect of time, temperature, and solvent on the preservation of lutein. The results showed that the temperature and type of solvent did not have a significant effect, which means that the variable that accounts for the changes in lutein content is storage time.

Evolution of Lipids, Protein, and Fatty Acid Profile. Figure 4 shows the content of total lipids and protein for the three storage conditions. The lipid content clearly decreases during the storage, whereas the protein content does not. Close to 50% of total lipid content is lost at 4 °C and ambient temperature, while the loss is only 15% at −18 °C. Since no metabolism can be active in the freeze-dried biomass, this has to be attributed to chemical or biochemical (enzymatic) modifications that turn lipids insoluble

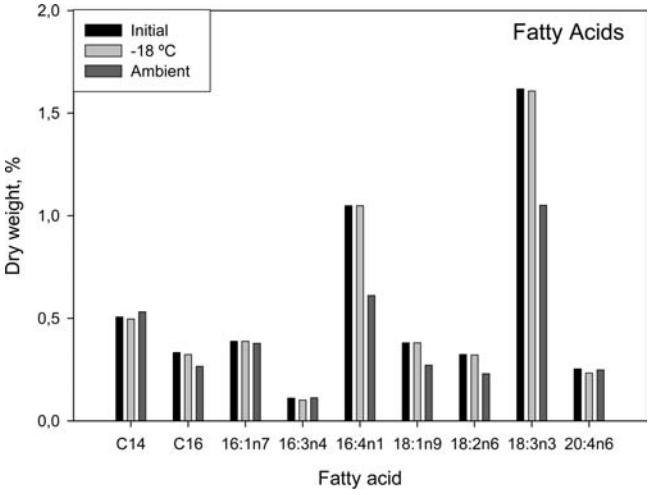

Figure 5. Fatty acid profile from *Scenedesmus almeriensis* frozen biomass: comparison between the most significant assays (ambient temperature and −18 °C at day 42) and the initial conditions.

in the extracting mixture used in the lipid analysis method (described in the corresponding section).

The fatty acids profile of *Scenedesmus almeriensis* (**Figure 5**) is typical of green algae, mainly showing 14:0, 16:0, 16:4n1, and 18:3n3 along with minor levels of monounsaturated (16:1n7,

18:1n9) and polyunsaturated fatty acids (16: 3, 18:2 and 18:3n3). A similar profile was found by Wiltshire et al. for another *Scenedesmus* (*38*). A comparison of the fatty acid profile between the first day and the last day of storage is given in **Figure 5**. The fatty acids that form the majority of the sample are the most markedly affected by temperature (16:4n1, 18:3n3, 18:1n9, and 18:2n6). Storage at ambient temperature leads to the degradation of components 18:3n3 and 16:4n1 by 30% and almost 50%, respectively. The contents of the other fatty acids remain the same.

DISCUSSION

Stability is a major concern in the design of extractive processes, particularly when dealing with a labile substance obtained from biomass. Carotenoids in general and lutein in particular can be easily altered by factors such as heat, light, and oxygen, given its delicate structure, its antioxidant properties, and its capacity to act as a blue light filter. Such substances must be handled with care through every step of the extraction process in order to avoid unbearable losses or the appearance of undesired or harmful derivatives. Thus, immediate stabilization is usually a must.

Freezing, freeze-drying, and spray-drying are among the most suitable stabilization techniques for the purpose described here. Freezing is the least expensive, because it requests the simplest equipment and does not need the necessary drying step. Freeze-drying is expensive in investment and operation but is a well-tested alternative, likely to preserve labile substances because it takes place under low temperature and high vacuum. Finally, spray-drying is a cost-effective operation with a high processing potential, but it requires the use of temperatures well above ambient and the presence of air and thus oxygen and its effects on *Scenedesmus almeriensis*, or any other lutein-containing microalgal biomass, have to be tested.

Table 1 presents data on the immediate effect of spray drying on the lutein content of *Scenedesmus almeriensis*, showing that the best results are obtained at temperatures well above the feasible minimum. Although it could be expected that the operation at lower temperatures should favor the preservation of thermolabile substances such as lutein, it is clear that the operations at higher temperature were better for preserving the lutein content. It is difficult to explain the reasons for this from the experiments presented, but it must be borne in mind that the temperature of the droplets never gets as high as that of the air stream due to the evaporation of water. Actually, the temperature of the biomass droplets is limited by the boiling point of the solvent and in practice can be kept well below the boiling point of water, due to the air−water interaction (psychometrics). It is also important to highlight that the residence time of the microalgal material is very short, between 1.0 and 1.5 s, as stated in the handbook of the equipment. Under the experimental conditions described, a constant gas stream of 35 $m^3 h^{-1}$ (measured at approximately 20 °C and 75% relative humidity, as taken from the surroundings), the residence time changes because air dilates with temperature and the volumetric air rate is thus higher at higher temperatures. Nevertheless, the variations are small. It can be calculated from the properties of moist air that the mean volumetric flow rate in the spray drier varies from 46 $m^3 h^{-1}$ in the experiment at the lowest temperature (taking the air temperature as the mean between the inlet, 120 °C, and the outlet, 68 °C) to a maximum of 53 $m^3 h^{-1}$ at the highest temperature (220 °C at the inlet and 132 °C at the outlet). This means there is a maximum difference of 22% in volumetric flow and thus in residence time for the experiments presented, which can hardly account for the differences found. On the other hand, the temperature of operation also determines the speed of the drying process and the final humidity of the product. A complete drying of the product is ensured, because the experiments in this work were designed for a maximum evaporation rate of 540 g h^{-1} of water, well under the maximum capacity of the equipment used (1000 g h^{-1}), but the higher the temperature, the faster the drying is completed and the lower the final humidity content. It is very difficult to obtain even an estimate of the drying speed in the experiments presented, because the size of the droplets for the microalgal suspensions is unknown and the drying process at such high temperatures and under turbulence conditions is difficult to describe, but it is clear that when the air temperature is above the boiling point, the process must be considerably faster. The results in **Table 1** show that the best results are obtained in the experiments in which this happens. Even in the experiments with the inlet temperature at 150 °C and the outlet at 90 °C the process takes place above the water boiling point most of the time. Thus, it can be suggested that with regard to lutein preservation in a spray-drying process a short drying time is more important than the temperature of the operation and that a minimum air temperature near 100 °C at the outlet should be recommended for *Scenedesmus almeriensis*. This differs from the results presented by Mendes Pinto et al. (*39*), who found that the spray-drying of *Haematococcus pluvialis* using an temperature of 180 °C at the inlet and 115 °C at the outlet degraded the total carotenoids to a large extent. In contrast, Orset et al. showed that spray drying of *Dunaniella salina* was a stable process in terms of preserving the algal carotenoid content and composition (*12*).

The long-term effects of the stabilization process have been studied by keeping the samples at −18 °C under the conditions of packaging and inert atmosphere described in Materials and Methods. As **Figure 1** shows, it can be concluded that any of the stabilization processes used (freezing, freeze-drying, and spray-drying) give satisfactory results after 20 days, but then the spray-dried biomass suffers a continued loss of lutein that can be estimated from **Figure 1** as 7−8% every 10 days of storage. The frozen biomass experiments a moderate 10% total loss after the 6 week period, which averages 2−3% every 19 days, while the freeze-dried samples can be stored for a longer time, showing no significant loss of lutein. Therefore, freeze-drying is the most effective conservation technique, under the described conditions, but when shorter storage periods are needed, spray drying should also be considered because of its lower cost. It is also clear that freeze-dried powder is more stable than spray-dried, which may be explained due to the more porous structure of the spray-dried material (*40*, *41*), which allows an easier penetration of oxygen and probably causes some air to remain embedded in the dry biomass: hence, the necessity of lower temperatures in this case (*42*) if a prolonged storage period is needed. The slight increase in the recovery of pigments shown in **Figure 1** could be explained by an increase in the extractability of the carotenoid pigments reported by Mantoura et al. (*43*).

The stability of different carotenoids in freeze-dried biomass has been studied next under different storage conditions. As shown in **Figure 2**, the stability of the three carotenoids considered, β-carotene, lutein, and violaxanthin, differ significantly, which highlights that the results obtained from lutein cannot be used to extrapolate for others, but in every case, the lower the storage temperature, the better the preservation obtained. However, to be able to design an extraction process, it is also important to measure the stability of carotenoids in intermediate steps, such as dissolutions in organic solvents, and in a final product, such as a suspension in vegetable oil. Focusing on lutein, **Figure 3** shows the stability of extracts of this carotenoid in acetone and in virgin olive oil at different temperatures. The results have been qualitatively noted in the Results, but for comparison purposes it is

Table 2. Rate Constant for the First-Order Degradation of Lutein (day^{-1}) in Processed Biomass and in Acetone and Olive Oil Extracts[a]

	k				
temp (°C)	frozen biomass	freeze-dried biomass	spray-dried biomass	acetone extract	olive oil extract
ambient		7.5×10^{-3}		6.9×10^{-3}	6.1×10^{-4} (1.6×10^{-4})
4		3.0×10^{-3}		4.6×10^{-3}	4.0×10^{-4} (6.5×10^{-5})
−18	2.9×10^{-3}	6.7×10^{-4}	8.1×10^{-3}	2.1×10^{-3}	3.2×10^{-4} (6.7×10^{-7})

[a] Values shown in parentheses correspond to long-term experiments (time >3 months).

interesting to obtain the rate of degradation using a kinetic model, which also allows evaluating the statistical significance of the changes. For this, the data of lutein concentration vs time can be used to obtain the parameter models by a regression analysis. The zero-order ($c = c_0 - kt$) and first-order kinetics ($c = c_0 \exp(-kt)$) have been tested for their ability to fit the degradation of lutein shown in **Figures 2** and **3**. Both models are capable of explaining the results with similar precision, the first-order model being slightly better. This similarity is not surprising, because in all the experiments the degradation progress is in its first stages (less than 30% lutein is lost in most experiments and some lose as little as 5%), and in that case the decreasing exponential of the first-order model approaches a linear decrease which becomes indistinguishable from the zero-order kinetics even in the presence of low noise (random measurement error). To test properly the difference of the two models proposed, the experiments should be prolonged for a much longer time, which is of no interest for the purposes of this study. **Table 2** shows the k constant for the first-order degradation of lutein for the different products and temperatures tested.

It is necessary to take into account that the determination coefficients obtained for some of the regressions shown in **Table 2** are low, particularly for the experiments at −18 °C ($0.15 < r^2 < 0.3$) and at 4 °C ($0.3 < r^2 < 0.6$), while the coefficients are somewhat higher for the experiments at ambient temperature ($0.85 < r^2 < 0.95$), but this does not necessarily mean that the model used does not represent well the degradation kinetics. The determination coefficient represents the amount of variability that is accounted for by the model and gives a measure of how good the model is for making predictions. This means that the variations in lutein concentration at −18 °C can be attributed to time in a small proportion, between 15% and 30%, but an ANOVA analysis carried out on these data show that among all the variances tested (temperature, time, and process) only time had a significant influence on lutein content and therefore the rest of the variability should be attributed to unexplained factors such as errors in sampling and storage and the limited accuracy of the analytical methods. This happens because the variations observed with time are small in comparison to the variability carried about by the experimental process, which is actually satisfactory but is unable to give measures of enough precision and accuracy to obtain a good regression at low temperature in the time interval studied. On the other hand, at ambient temperature the determination coefficients are much higher because the variations of lutein content with time are greater than random errors. Taking into account that the ANOVA analysis shows that time is the only variable causing significant changes and that the error is uniformly distributed across the interval (from a residue analysis not shown), it can be concluded that the first-order model and the data presented in **Table 2** constitute a good method to evaluate average changes in lutein content and to compare with other methods and conditions.

Gouveia et al. (*44*) tested the stability of carotenoids present in microalgal biomasses such *Chlorella vulgaris* and *Haematococcus pluvialis* and also assessed their acetone extracts under different storage conditions. They obtained data similar to ours for the loss of carotenoids from the biomass at −18 °C, with values around 7% for freeze-dried biomass after 1 month. This means $k = 2.2 \times 10^{-3}$ day^{-1}, which compares well with the results presented here for *Scenedesmus almeriensis*. On the other hand, the results given by this author for the stability of carotenoids of *Haematococcus pluvialis* extracted in acetone are considerably worse.

The stability of the extracts of lutein in olive oil is remarkable, with an expected loss of under 1% in 1 month and less than 15% in a year and a half, as observed from the experimental data. The degradation data of the extracts in olive oil has been split into two sets, according to what is shown in **Figure 3**. In those data the variation of lutein concentration experiences a rather steep change after month 6, which is out of the global tendency and would be better attributed to some accidental circumstance interrupting the programmed storage method, such an uncontrolled elevation of temperature or exposure to light. Thus, in **Table 2** the degradation constant k after month 6 is also calculated and its value is shown in parentheses. In either case, the shelf life of lutein in olive oil predicted by the model is extremely prolonged even at ambient temperature: 90% of lutein is conserved after 6 months and 75% after 15 months. If the parameters calculated for the long-term data are used, 90% of the lutein is conserved after 18 months of storage and more than 75% after 6 years.

Scenedesmus almeriensis biomass could be an interesting raw material for aquaculture feedstocks with regard to the lutein content, particularly in the form of freeze-dried biomass for stability reasons. Data shown in **Figure 4** indicate also that the protein content is also very well conserved during the storage period shown and that it is appropriate for use in formulations for aquaculture (*45*). The variation in lipids, shown in **Figure 4**, is noticeable, and it remains to be determined what kind of lipids are lost and what chemical modifications are undergone. An examination of the variation of the fatty acid profile displayed in **Figure 5** shows that the substances mainly degraded are the fatty acids 18:3n3 and 16:4n1 by 30% and almost 50%, respectively. Those are polyunsaturated fatty acids, quite susceptible to oxidation, which suggests that the main changes taking place in the stored biomass are oxidative and in this case the preservation of the biomass quality in general, and of lutein in particular, could be enhanced with the use of antioxidants, although it remains to be determined how these additives could be incorporated in the microalgal biomass. This possibility could be particularly important for the spray-dried biomass, which presents a poor stability that could be attributed to its porosity and thus to its higher exposure to oxygen. In this case the addition of a suitable antioxidant to the microalgae suspension prior to spray drying is a possibility worth exploring.

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