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Cloud point extraction of chlorophylls from spinach leaves using aqueous solutions of non-ionic surfactants

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ABSTRACT. Chlorophylls and their derivatives are currently used in a wide range of applications. To replace the volatile organic solvents commonly applied for their extraction from biomass, aqueous solutions of non-ionic surfactants are studied herein in the extraction of chlorophylls from spinach leaves. Aqueous solutions of a wide range of non-ionic surfactants were investigated, allowing us to demonstrate the relevance of their hydrophilic-lipophilic balance (HLB) on the extraction performance and chlorophylls *a*/*b* selectivity, with the best results obtained with surfactants with a HLB ranging between 10 and 13. Furthermore, it was found a relevant impact of the surfactants aqueous solutions towards the biomass disruption, demonstrating that changes in the biomass structure allow a better access of the solvent to the target compounds embedded in the biopolymer matrix. A response surface methodology was then used to optimize operational conditions (surfactant concentration, solid-liquid ratio and temperature), leading to a maximum extraction yield of chlorophylls of 0.94 mg/g. After the extraction step, the chlorophylls-rich extract was concentrated by heating above the surfactant-water cloud point, leading to the separation into two-phases, and to a concentration factor of 9 and a recovery of 97% of chlorophylls in the surfactant-rich phase. The antioxidant activity of the extracts was finally appraised, showing that the antioxidant activity of the aqueous chlorophylls-rich extracts is higher than that obtained with volatile organic solvents. The obtained results show the potential of aqueous solutions of non-ionic surfactants to extract highly hydrophobic compounds from biomass and their potential for a direct use in cosmetic and nutraceutical applications, without requiring an additional recovery or purification step.

KEYWORDS. Solid-liquid extraction; non-ionic surfactants; aqueous solutions; concentration; spinach leaves, chlorophylls; antioxidant activity.

INTRODUCTION

Chlorophylls are the pigments responsible for the green color of fruits and vegetables and play a central role in the primary stage of photosynthesis. Two chemical structures of chlorophyll are present in plants: chlorophyll *a* and chlorophyll *b*, usually in a ratio of 3:1.[1] Chlorophylls are based on a porphyrinic structure, comprising four pyrrole rings, coordinated by a magnesium ion, with a long hydrophobic alkyl chain attached to it – Fig. 1. Chlorophyll *a* contains a methyl group (-CH_3) attached to one pyrrole ring, whereas in chlorophyll *b* the methyl group is replaced by a formyl group (-CHO).[2-3] This difference in their chemical structures is responsible for the blue/green color of chlorophyll *a* against the green/yellow color of chlorophyll *b*.[4] Chlorophylls and their derivatives have been extensively studied due to their unique and valuable properties. They are widely used as natural colorants in the food and cosmetic industries, in energy and medicinal applications,[5] and also attracted the interest of the pharmaceutical industry.[4, 6-7]

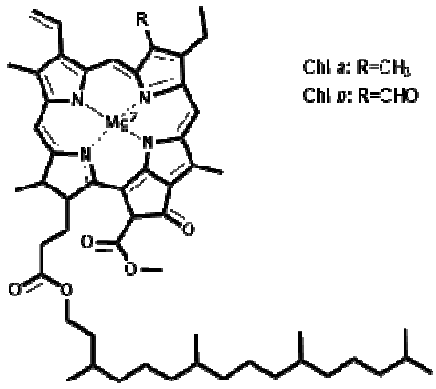

Figure 1. Chemical structure of chlorophylls *a* and *b*.

Several natural sources of chlorophyll can be explored, with spinach, alfalfa meal, and algae as the most studied.[5] For scale extractions, *i.e.* 1–5 g of plant material, acetone, dimethylsulfoxide, dioxane and dimethylformamide are commonly used as preferred solvents. After extraction and filtration, chlorophylls extracts are obtained through drying under vacuum. Medium-scale extractions (up to 1 kg of plant material) are usually performed using fresh spinach, starting by boiling the leaves in water, followed by filtration and extraction with methanol–petroleum ether mixtures. Finally, for large-scale extractions (1–5 kg), pigments are usually extracted with acetone, and further filtered and dried.[5] Some of the solvents currently used are volatile, toxic and flammable, thus leading to industrial risks and to a poor environmental performance, and are of low selectivity resulting in low purity levels and yields.[8] On the other hand, the methods used for extracting chlorophylls typically require high temperatures and are multi-step, leading thus to expensive processes.[9] The most environmentally friendly and biocompatible solvent for extracting chlorophylls from natural sources, while taking into account their potential for applications in food, cosmetic and pharmaceutical areas (and inherent human consumption), is certainly water. However, chlorophylls are highly hydrophobic compounds with low solubility in water.[10] In this context, the use of aqueous solutions of non-ionic surfactants as alternative extraction solvents could be seen as a promising approach. Moreover, their low cloud points could allow an easy concentration and/or purification of the extracts by moderate heating.

Surfactants belong to a class of compounds with amphiphilic nature, formed by a hydrophobic (tail) and a hydrophilic (head) part.[11-14] In aqueous solutions, these molecules are able to spontaneously aggregate, forming micelles above the critical

micellar concentration (CMC).[15] This capacity to form micelles in aqueous media allows the incorporation of hydrophobic molecules in the micelle core, and thus surfactants may improve the extraction/solubilization performance of aqueous solutions.[11, 14, 16] Moreover, aqueous solutions of non-ionic surfactants display low or moderate temperature cloud points, resulting in the formation of two liquid phases upon heating, and allowing these systems to act as extraction and concentration liquid-liquid platforms (Fig. 2). The cloud point is the temperature at which a solution of a surfactant forms a coacervate, separating into two phases: the coacervate, rich in surfactant, and a second phase with a low surfactant concentration.[17] The concentration of target compounds into the coacervate is also possible because this phase is typically of a lower volume than the surfactant-depleted phase.[18-19] Thus, the extracted species solubilized in the micelles can be concentrated simply by changing the system temperature (Fig. 2).[20]

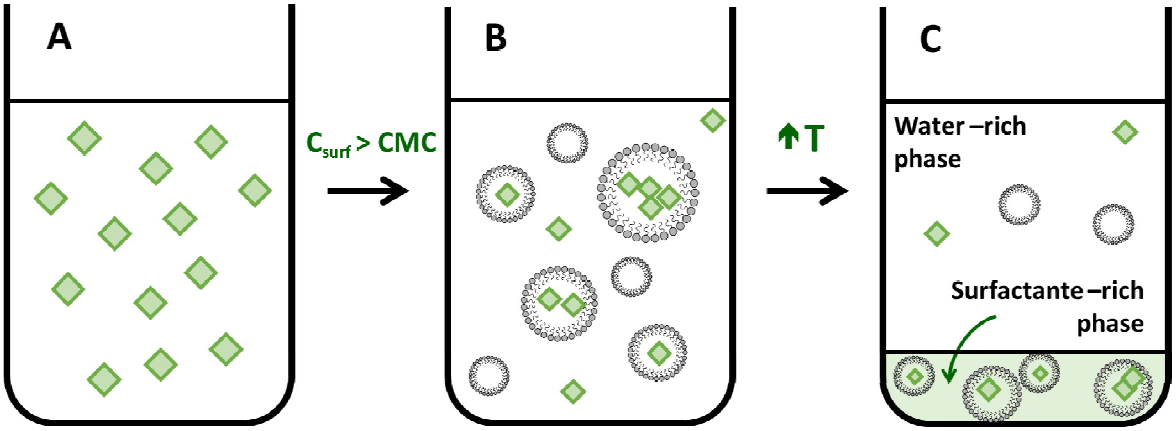

Figure 2. Schematic representation of the CPE. **A)** Initial aqueous solution containing a hydrophobic solute. **B)** Addition of non-ionic surfactants at a concentration higher than the CMC. **C)** Separation into two-phases by temperature changes.

Surfactants have been largely used as household detergents, and in food and personal care industries.[21] Recently they have also found applications in the pharmaceutical industry, as emulsifying and wetting agents in pharmaceutical formulations,[21] and as drug solubilization/delivery systems, e.g. in ophthalmic products.[22] Watanabe et al.[23] were pioneering in reporting the use of non-ionic surfactants for extraction purposes. Since then, surfactants have been successfully used in micelle-based extractions and in the concentration of several compounds, such as metal ions, proteins, and bio-based compounds, from water solutions and biomass.[11, 13, 24-27]

Taking into account the high content of chlorophylls in spinach leaves,[11] and the problems associated to their extraction by conventional methods and solvents, in this work we investigate the use of aqueous solutions of non-ionic surfactants as alternative solvents. Most of the surfactants used in this study are already used in the food, cosmetic and pharmaceutical industries.[28] For instance, sorbitan esters and their ethoxylated derivatives, like Tween 20 and 80, are widely used as food emulsifiers.[21, 29] Tween 20 and 80, as well as ethoxylated alcohols, e.g. Brij 30 and 98, are used in cosmetic lotions and formulations.[28] Other non-ionic surfactants, such as fatty acid esters of sorbitan and their ethoxylated derivatives, Tweens, and Brijs, have also several applications in the pharmaceutical field.[28] In general, non-ionic surfactants have been widely used due to their biocompatible nature, reduced toxicity, and increased stability toward changes in pH and ionic strength, presenting therefore advantages over cationic, anionic or amphoteric surfactants.[22, 28, 30] The main goal of this work is the development of a cost-effective and sustainable process for the extraction and concentration of chlorophylls from biomass using aqueous solutions of non-ionic surfactants instead of the volatile organic solvents

currently used. To this end, an initial screening of various non-ionic surfactants was conducted, and a response surface methodology (RSM) was then applied aiming at optimizing the operational conditions of the extractive process, namely the solid-liquid ratio (*R*, weight of biomass *per* weight of solvent), surfactant concentration (*C*) and temperature (*T*). By heating the extract-surfactant-water solutions at a temperature above their cloud point, two-phase systems are created, allowing us to further concentrate the chlorophylls-rich extract. Finally, the antioxidant activity of the aqueous solutions containing chlorophylls, before and after the concentration step, was determined to evaluate their possible direct use in nutraceutical and cosmetic applications.

EXPERIMENTAL SECTION

Materials. Spinaches were purchased in a local market and immediately washed and frozen for storage. Before extraction, spinach leaves were immersed in liquid nitrogen and ground until a green powder was obtained. Standards of chlorophyll *a* (95% pure) and chlorophyll *b* (99% pure) were purchased from Sigma-Aldrich. Surfactants Brij 98 (Hydrophilic-lipophilic balance (HLB) 15.3), Tween 20 (HLB 16.7), Tween 80 (HLB 15.0) and Triton X-100 (HLB 13.5) were purchased from Sigma-Aldrich. The surfactant Triton X-114 (HLB 12.4) was acquired from Acros Organics. The surfactant Brij 30 (HLB 9.6) was acquired from Fluka. Commercial surfactants C9-C11 6 EO's (HLB 12.4), C12-C15 7EO's (HLB 12.3) and C11-C13 9EO's (HLB 13.2) were kindly supplied by Mistolin, Portugal. The HLB (hydrophilic-lipophilic balance) values of all surfactants used were taken from the literature[27] or from the manufacturers catalogues. The chemical structure of the investigated surfactants, as well as their critical micelle concentration (CMC) values are shown in the Supporting Information. Before use, the water content in

each surfactant was determined by Karl Fisher titration (using Metrohm 831 Karl Fischer). Their water content is shown in the Supporting Information. Mixtures of surfactants with a target HLB value were also investigated, prepared according to the following equation:

$$HLB = HLB_{Brij30}\, w_{Brij30} + HLB_{Brij98} w_{Brij98} \quad (1)$$

Where HLB_{Brij30} and HLB_{Brij98} are the HLB values for the surfactants Brij 30 and 98, and W_{Brij30} and W_{Brij98} are the weight fraction of Brij 30 and Brij 98.

The water employed in all experiments was ultra-pure water, double distilled, passed through a reverse osmosis system and treated with a Milli-Q plus 185 water purification device.

Chlorophylls extraction. Solid-liquid extractions of chlorophyll from spinach leaves were carried out protected from light using a Carousel from Radleys Tech able to both stir and maintain the temperature within ± 0.5 °C. In all experiments the stirring was kept constant at 600 rpm. All aqueous solutions containing known amounts of surfactants and biomass were prepared gravimetrically within ± 10^{-4} g. Several concentrations of surfactant, and different solid-liquid ratio, temperature and times of extraction were investigated. At least three individual samples for each set of conditions were prepared and the amount of extracted chlorophylls quantified.

After the extraction step, the several aqueous solutions and organic solvents were separated from biomass by centrifugation (at 4000 rpm for 30 min, using an Eppendorf

5804 centrifuge). The quantification of chlorophylls in each solution was carried out by UV-Vis spectroscopy, using a microplate reader Synergy HT, BioTek. Calibration curves were prepared using the commercial standards of chlorophylls *a* and *b* in surfactant aqueous solutions. OriginPro 8.0 was used for the spectral deconvolution of the peaks at 649 and 665 nm that correspond to the maximum absorption wavelengths of chlorophylls *b* and *a*, respectively. The absorbance was recorded in duplicate for each sample. The content of chlorophylls in spinach leaves (discussed as extraction yields) was determined according to the total weight of chlorophylls (*a* and *b*) present in the extract divided by the weight of biomass. The selectivity was calculated as the ratio between the content of chlorophyll *a* and the content of chlorophyll *b* in each sample.

Response surface methodology. A RSM was applied to simultaneously analyze various operational conditions and to identify the most significant parameters on the chlorophylls extraction yield. In a 2^k RSM there are k factors that contribute to a different response and the data are treated using a second order polynomial equation:

$$y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i<j} \beta_{ij} X_i X_j \qquad (2)$$

where y is the response variable and β_0, β_i, β_{ii} and β_j are the adjusted coefficients for the intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are independent variables. This model allows drawing surface response curves, and through their analysis, the optimal conditions can be determined. Based on the results obtained in the initial screening with several non-ionic surfactants, the commercial ethoxylated alcohol C11-C13 9EO's was selected to perform a 2^3 factorial planning with the aim of

optimizing the extractive process of chlorophylls from spinach leaves. The 2^3 factorial planning used is described in the Supporting Information. The obtained results were statistically analyzed with a confidence level of 95%. Student's t-test was used to check the statistical significance of the adjusted data. The adequacy of the model was determined by evaluating the lack of fit, the regression coefficient (R^2), and the F-value obtained from the analysis of variance (ANOVA). The Statsoft Statistica 10.0© software was used for all statistical analyses and for representing the response surfaces and contour plots.

Scanning electron microscopy (SEM). The SEM pictures, used to evaluate the morphology of the spinach leaves before and after extraction, were acquired using a FEG-SEM Hitachi S4100 microscope (after carbon evaporation) with a 25 kV acceleration voltage.

Cloud point concentration of chlorophylls. The extracted chlorophylls present in the surfactant aqueous solutions were further concentrated by heating them above their cloud point (65 ± 1 ºC), leading to the formation of two liquid phases. To this end, aqueous solutions were kept in an air oven at the desired temperature for *ca*. 1 h. The phases were carefully separated and the recovery of chlorophylls determined according to the weight of total chlorophylls present in the concentrated solution to that in the aqueous solution before the concentration step.

Antioxidant activity assays. The antioxidant activity of the different chlorophylls-rich extracts was determined using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) scavenging assay.[31] The antioxidant activity is expressed in IC_{50} values, defined as the

inhibitory concentration of the chlorophylls-rich extract required to decrease the initial DPPH radical concentration by 50%.[32] Taking into account the IC_{50} definition, a lower IC_{50} value reflects a better DPPH radical scavenging activity, i.e. a better antioxidant activity of the extract. Further details are given in the Supporting Information.

RESULTS AND DISCUSSION

Effect of the surfactant type on the extraction of chlorophylls. A screening of aqueous solutions of several surfactants at concentrations above their CMC was carried out in order to evaluate the most promising surfactants for the extraction of chlorophylls. The studied surfactants are listed in the Experimental Section, and their chemical structures and CMC values are provided in the Supporting Information. The same operational conditions were kept in all experiments, namely a surfactant concentration of 3.3 mM, a spinach-solvent weight fraction ratio of 1:50 (R=0.02) and an extraction time of 30 min at 25°C. The impact of different surfactants on the extraction yield of chlorophylls is presented in Fig. 3A, and compared with the extraction yield obtained using pure water at the same conditions. The respective extraction yields are provided in Table 1. The results obtained show that the amount of extracted chlorophylls using aqueous solutions of non-ionic surface-active compounds (at low concentrations) is significantly higher than that achieved with water, demonstrating the importance of surface-active compounds to increase the extraction yield of highly hydrophobic compounds from biomass, such as chlorophylls. However, the amount of extracted chlorophylls largely depends on the surfactant type. Among the studied surfactants, tritons and ethoxylated alcohols perform better for the extraction of chlorophylls from spinach leaves (up to 0.66 ± 0.03 mg/g). On

the other hand, aqueous solutions of surfactants from the Brij and Tween families lead to a lower extraction yield, and only slightly better than that obtained with water.

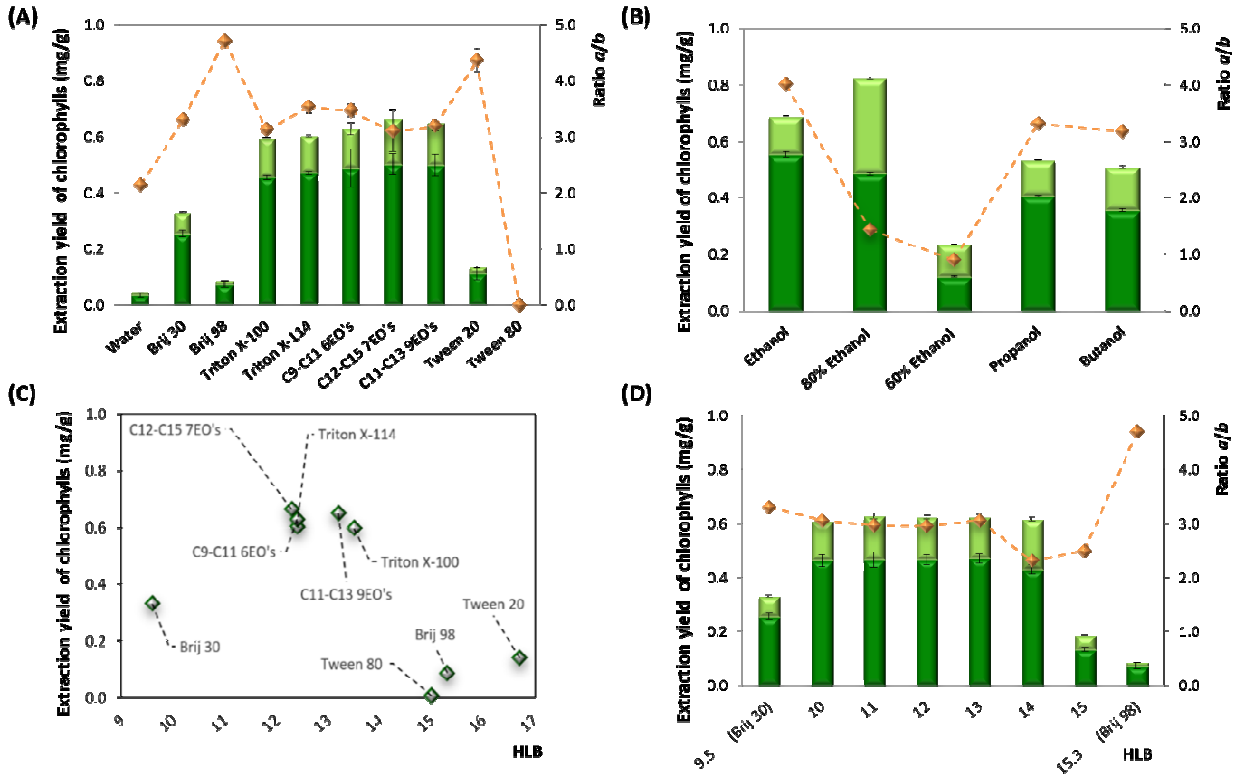

Figure 3. Extraction yield of chlorophyll *a* (■) and chlorophyll *b* (■) from spinach leaves using **(A)** several surfactants aqueous solutions (surfactant concentration=3.3 mM; *R*=0.02; *t*=30 min; *T*=25 °C) and water; and **(B)** organic solvents (*R*=0.02; *t*=30 min; *T*=25 °C) and ratio of chlorophyll *a*/*b* (◆). **(C)** Relationship between the HLB values of surfactants and the total extraction yield of chlorophylls from spinach leaves. **(D)** Extraction yield of chlorophyll *a* (■) and chlorophyll *b* (■) from spinach leaves using mixtures of Briji 30 and Briji 98 with different HLB values (*R*=0.02; *t*=30 min; *T*=25 °C) and ratio of chlorophyll *a*/*b* (◆).

Table 1. Extraction yield of chlorophyll *a* and chlorophyll *b* and ratio of chlorophyll *a*/*b* from spinach leaves (surfactant concentration=3.3 mM; *R*=0.02; *t*=30 min; *T*=25 °C), and HLB values of the studied surfactants.

Solvent	HLB	Amount of chlorophylls (mg/g)			Ratio *a*/*b*
		Chl *a*	Chl *b*	Chl Total	
Pure water	---	0.03 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	2.15 ± 0.01
Brij 30	9.60	0.25 ± 0.01	0.08 ± 0.01	0.33 ± 0.01	3.31 ± 0.05
Brij 98	15.30	0.07 ± 0.01	0.01 ± 0.01	0.09 ± 0.01	4.71 ± 0.01
Triton X-100	13.50	0.45 ± 0.01	0.14 ± 0.01	0.60 ± 0.01	3.14 ± 0.05
Triton X-114	12.40	0.47 ± 0.01	0.13 ± 0.01	0.60 ± 0.01	3.55 ± 0.12
C9-C11 6EO's	12.40	0.49 ± 0.07	0.14 ± 0.02	0.63 ± 0.09	3.47 ± 0.10
C12-C15 7EO's	12.30	0.50 ± 0.04	0.16 ± 0.03	0.66 ± 0.07	3.10 ± 0.37
C11-C13 9EO's	13.20	0.50 ± 0.04	0.15 ± 0.01	0.65 ± 0.05	3.19 ± 0.09
Tween 20	16.70	0.11 ± 0.02	0.03 ± 0.01	0.14 ± 0.03	4.37 ± 0.21
Tween 80	15.00	0.01 ± 0.01	0.00 ± 0.00	0.01 ± 0.01	0.00 ± 0.00

The extraction of chlorophylls from spinach leaves also was performed using volatile organic solvents, namely ethanol, propanol and butanol, under the same operational conditions for comparison purposes. Mixtures of ethanol/water were additionally studied aiming at tailoring the polarity of the solvent. As shown in Fig. 3B, alcohols with shorter aliphatic moieties perform better in the extraction of chlorophylls from spinach leaves (0.69 ± 0.06 mg/g with pure ethanol). Detailed data are provided in the Supporting Information. Nevertheless, water-ethanol mixtures in adequate compositions (80% of ethanol) lead to higher extraction yields of both chlorophylls (up to 0.82 ± 0.05 mg/g), while a higher water content (60% of ethanol) leads to a decrease on the recovery of the target biocompounds. It should be however highlighted that the first mixture is more appropriate to extract chlorophyll *b*.

The results obtained show that the extraction of chlorophylls using aqueous solutions of non-ionic surfactants, namely ethoxylated alcohols, is more successful with low concentrations of C11-C13 9EO's (3.3 mM), allowing to achieve extraction yields of (0.66 ± 0.03 mg/g). These results open new perspectives on the development of more sustainable and cost-effective solvents and processes for the extraction of chlorophylls from bioresources.

To better understand the role of the various aqueous solutions of surfactants, the relationship between the extraction yield and the HLB value of the surfactants was evaluated, with the results obtained depicted in Fig. 3C. Surfactants with HLB values ranging between 12 and 14 are the most effective in the extraction of chlorophylls, being observed a significant decrease in the amount of extracted chlorophylls when using surfactants with HLB values outside this range.

To further confirm if the extraction yields obtained are due to any particular chemical structural feature of the surfactant that would lead to specific chlorophyll-surfactant interactions or just the result of a micelle-mediated phenomenon, where the HLB would play the leading role, the extraction of chlorophylls was performed using aqueous solutions of mixtures of Brij 30 and Brij 98, with HLB values of 9.5 and 15.3, respectively, allowing to obtain surfactant mixtures with tailored HLB values (from 10 to 15). The mixtures of surfactants with the desired HLB were prepared according to Eq. (1). The same operational conditions were kept in all experiments, namely a spinach-solvent ratio of 1:50 (R=0.02), and an extraction time of 30 min at 25 °C. The results obtained, shown in Fig. 3D and Table 2, confirm that the maximum extraction yields are obtained with surfactants with a HLB value between 10 and 14. For the HLB value of 15 there is a

decrease on the amount of chlorophylls extracted. These results confirm that no specific chlorophyll-surfactant interactions are present since mixtures of surfactants perform as well as pure surfactants of different chemical structure, as long as the HLB values are kept between 10 and 14.

Table 2. Extraction yield of chlorophyll *a* and chlorophyll *b* and ratio of chlorophyll *a*/*b* from spinach leaves using mixtures of Briji 30 and Briji 98 with different HLB values (surfactant concentration=3.3 mM; R=0.02; t=30 min; T=25 °C).

HLB/Solvent	Extraction yield of chlorophylls (mg/g)		Ratio *a*/*b*
	Chl *a*	Chl *b*	
9.5 (Brij 30)	0.25 ± 0.01	0.08 ± 0.01	3.31 ± 0.01
10	0.46 ± 0.02	0.15 ± 0.01	3.06 ± 0.01
11	0.47 ± 0.03	0.16 ± 0.01	2.97 ± 0.01
12	0.47 ± 0.02	0.16 ± 0.01	2.96 ± 0.01
13	0.47 ± 0.02	0.15 ± 0.01	3.06 ± 0.06
14	0.43 ± 0.02	0.19 ± 0.01	2.31 ± 0.01
15	0.13 ± 0.01	0.05 ± 0.01	2.50 ± 0.01
15.3 (Brij 98)	0.07 ± 0.01	0.01 ± 0.01	4.71 ± 0.01

The ratio between the extracted chlorophyll *a* and chlorophyll *b* with all the investigated solvents is shown in Fig. 3, and is given in detail in Tables 1 and 2. Taking into account that the ratio of chlorophylls *a* and *b* in plants is around 3:1,[1] the extractive process is selective if their ratio is higher than 3. All surfactants, with the exception of Tween 80, are able to isolate higher amounts of chlorophyll *a*, especially Brij 98 and Tween 20, where a ratio of 4.71 and 4.37, respectively, was achieved, despite the low amount of chlorophylls extracted. Compared to pure water, where this ratio is 2.15, it is

possible to conclude that the use of specific surfactants can be advantageous in terms of selectivity. Regarding the tested alcohols as pure solvents, ethanol is the solvent which provides the highest selectivity (ratio of 4.01). A similar effect was reported by Hojnik et al.[7] for the extraction of chlorophylls from stinging nettle (*Urtica dioica L.*). Nevertheless, and although some water-ethanol mixtures can result in higher extraction yields, the selectivity decreases when these mixtures are employed. In summary, the selectivity achieved with aqueous solutions of surfactants is higher when using aqueous solutions of non-ionic surfactants, with the chlorophylls *a*/*b* ratio higher than 3 for most of the surfactants investigated, and reaching values up to 5 with an aqueous solution of Brij 98 (HLB 15.3). Based on the optimum HLB values to enhance both the extraction yield and selectivity, which are between 10 and 13, C11-C13 9EO's was selected for the further optimization of the operational conditions of the extraction process, as discussed below.

Optimization of the operational conditions by RSM. The univariate methods carried out before for the optimization of the operational conditions do not consider the interaction between different factors and may not correspond to the overall optimized process. With the aim of optimizing the extractive process of chlorophylls from spinach leaves and to identify the most significant conditions (surfactant concentration, spinach-solvent weight ratio and temperature), a RSM applying a 2^3 (3 factors and 2 levels) factorial planning was performed. This type of strategy allows the exploitation of the relationship between the response (amount of chlorophylls extracted) and the independent variables that may improve the extraction efficiency. The factorial planning was performed using aqueous solutions of C11-C13 9EO's, with a constant extraction time of

30 min. The effect of the extraction time was object of a preliminary study, using a 3.3 mM C11-C13 9EO's aqueous solution, with extractions carried out between 10 and 60 min – *cf.* the Supporting Information. The results obtained show that the extraction time (in the time range studied) has no major influence on the chlorophylls extraction yield and selectivity, where the extraction yield reaches a maximum at 20 min followed by a plateau up to 60 min.

The results obtained according to the RSM applied with the combined effects of solid-liquid ratio and surfactant concentration, solid-liquid ratio and temperature, and surfactant concentration and temperature, are depicted in Fig. 4. Variance analysis (ANOVA) was used to estimate the statistical significance of the variables and the interaction between them. The experimental conditions, the model equation, the experimental extraction yields of chlorophylls and respective calculated values, as well as the complete statistical analysis, are provided in the Supporting Information. No significant differences were observed between the experimental and calculated responses, supporting a good description of the experimental results by the statistical models. According to the statistical analysis shown in the Supporting Information and the data depicted in Fig. 4, it is shown that the three studied operational conditions are significant variables for the chlorophylls extraction yield. An increase in the C11-C13 9EO's concentration, in the extraction temperature, or in the solvent volume, all contribute to increase the amount of extracted chlorophylls.

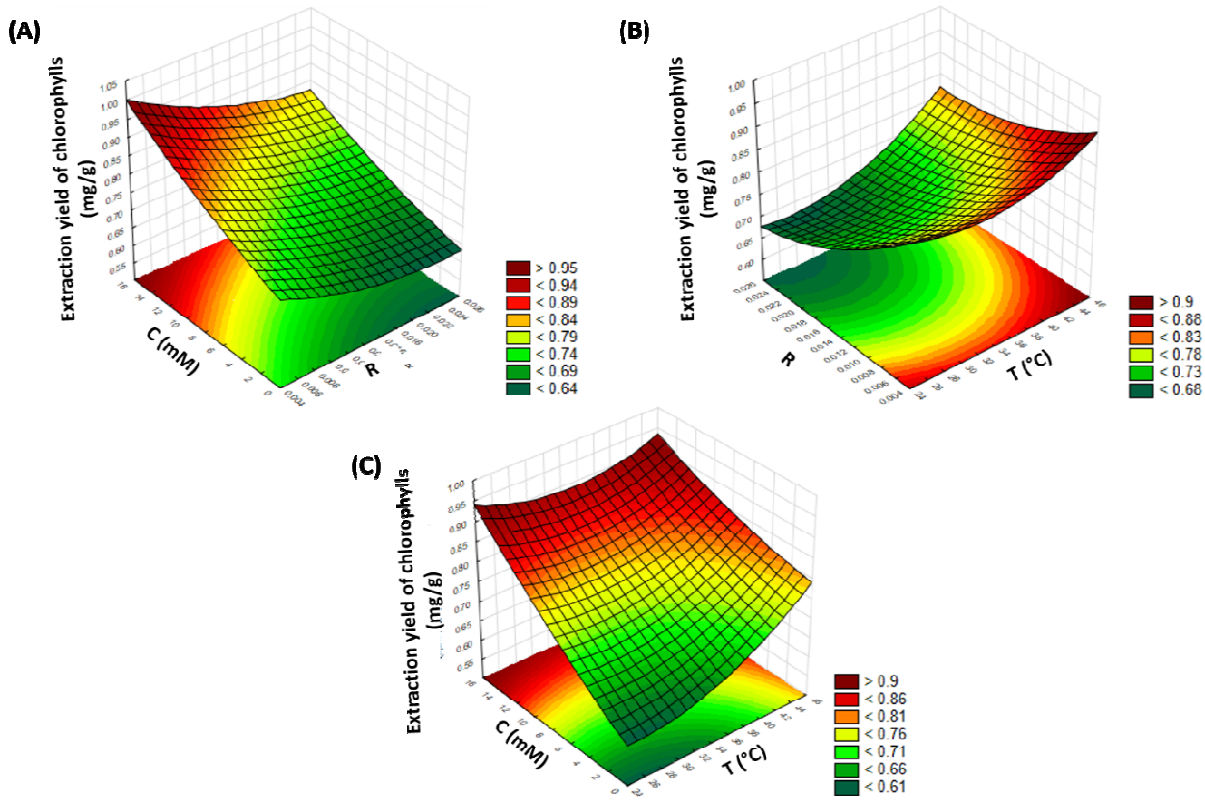

Figure 4. Response surfaces corresponding to the chlorophylls extraction yields with the following combined parameters: **(A)** solid-liquid ratio and surfactant concentration; **(B)** solid-liquid ratio and temperature; and **(C)** surfactant concentration and temperature.

The temperature of extraction and the concentration of surfactant have a positive effect on the response, while the solid-liquid ratio has a negative effect (data shown in the Supporting Information). The use of low concentrations of surfactant is important to improve the economic viability of the process, as well as to improve the biocompatible nature of the aqueous solution. The obtained data suggest that the surfactant concentration is only relevant up to a given value. The increase of the surfactant concentration up to 12.4 mM has a positive effect on the extraction yield of chlorophylls, being followed by a plateau for higher surfactant concentrations. Similar patterns were observed by Hosseinzadeh et al.[11] in the extraction of phenolic compounds from fruit extracts, where

surfactant concentrations higher than 7 mM do not lead to changes in the extraction efficiency. The extraction temperature seems to have the same effect on the amount of extracted chlorophylls; above 41°C, there is no increase in the response result. On the other hand, lower solid-liquid ratios lead to higher amounts of extracted chlorophylls, with no plateau observed with this variable.

The maximum extraction yield of chlorophylls obtained was of 0.94 ± 0.03 mg/g, for an extraction time of 30 min, an extraction temperature of 41 ºC, a surfactant concentration of 12.4 mM and a solid-liquid ratio of 0.07. We also applied these conditions to extract chlorophylls with pure ethanol, obtaining 0.98 ± 0.01 mg/g, a value similar to that obtained with aqueous solutions of surfactants. Values of 1.04 mg/g of chlorophylls extracted from dried spinach leaves were reported with ethanol-water mixtures (ethanol at 93%), at a temperature of 43 °C, and with and extraction time of 258 min.[12] In summary, our data demonstrate that aqueous solutions of non-ionic surfactants, at concentrations ca. 12.4 mM allow high extraction yields of chlorophylls, with potential economic and energy-saving advantages.

Scanning electron microscopy (SEM) was used to investigate the morphology of spinach leaves, before and after the extraction procedure. Details on the experimental procedure are given in the Supporting Information. The SEM images of spinach leaves before and after the extraction carried out with water, an aqueous solution of C11-C13 9EO's, and ethanol, are shown in Fig. 5. The sample that was in contact with pure water seems to be less affected than the ones treated with ethanol and aqueous solutions of surfactant. In addition to the improved solubility of chlorophylls in organic solvents and in aqueous solutions of surface-active compounds, this change in the biomass structure,

which allows a better access of the solvent to the target compounds embedded in the biopolymer matrix, seem also to be responsible for the improved extraction of chlorophylls achieved with aqueous solutions of C11-C13 9EO's.

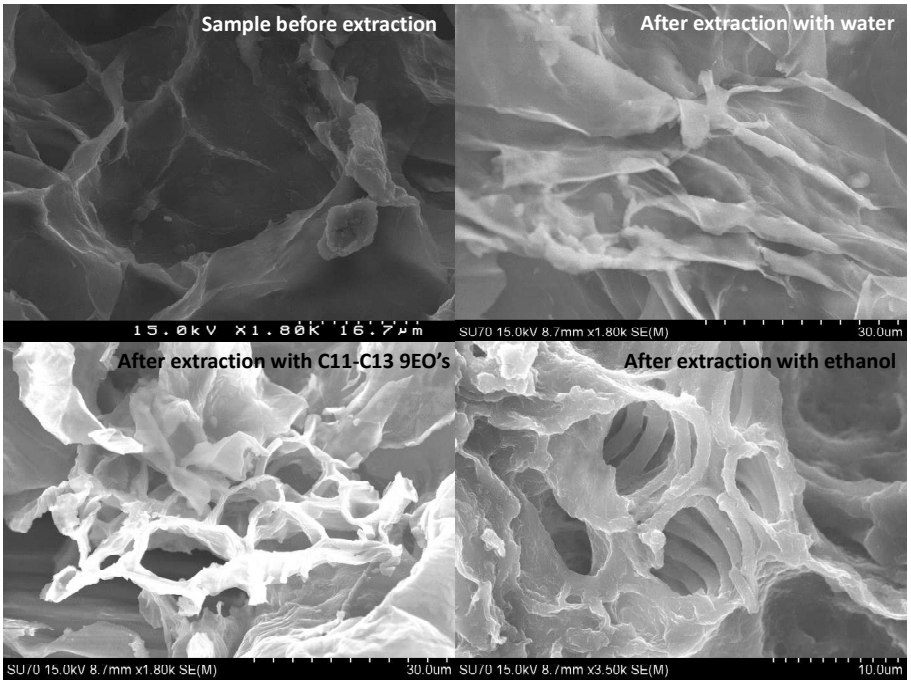

Figure 5. SEM images of the original spinach leaves, and of spinach leaves after the extraction with water, with an aqueous solution of C11-C13 9EO's at 12.4 mM and with ethanol.

Cloud point concentration of chlorophylls. After demonstrating that aqueous solutions of non-ionic surfactants are promising solvents to extract chlorophylls from biomass, we further investigated their concentration while envisaging their application in nutraceutical and cosmetic products. This step is relevant to decrease the water content. Aqueous solutions of the studied non-ionic surfactants display lower critical solution temperature (LCST) type phase diagrams, associated to the coacervation of the surfactant micelles which results in the formation of two phases upon an increase in temperature, thus

allowing the concentration of the chlorophylls-rich extract. This can be seen as an additional advantage when compared to extractions carried out with volatile organic solvents, that require the evaporation of the organic solvent. The aqueous solution which led to a maximum extraction yield of chlorophylls (0.94 ± 0.03 mg/g, obtained with an extraction time of 30 min, an extraction temperature of 41 ºC, a surfactant (C11-C13 9EO's) concentration of 12.4 mM, and a solid-liquid ratio of 0.07) was placed for 1h at 65 ºC, leading to the formation of a small volume surfactant-rich phase enriched in chlorophylls and a large volume water-rich phase (depleted in surfactant and chlorophylls) - Fig. 6. With this approach we were able to concentrate chlorophylls by a factor of 9 and to achieve a recovery of 97 %.

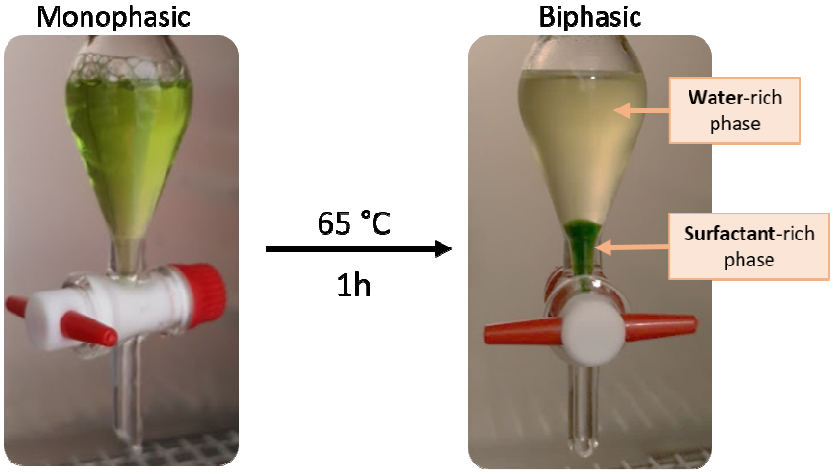

Figure 6. Scheme of the process used to concentrate chlorophylls.

The effect of the chlorophylls concentration on the cloud point temperature of the surfactant-water system was also studied. The surfactant solution at 12.4 mM has a cloud point temperature of about 60 ºC. However, with the presence of other compounds, in this work spinach extracts, the cloud point temperature is affected. For the aqueous solution with a chlorophylls concentration of 6.61 mg/L (after 1 cycle of extraction), the cloud

point temperature is 65 ºC – the temperature used to perform the concentration step. With the increase of the chlorophylls concentration, i.e. after more than one cycle of extraction with the same solvent and fresh biomass, the cloud point temperature increases. After 9 cycles of extraction (61.1 mg/L of chlorophylls), the surfactant aqueous solutions were no longer able to phase separate at the highest temperature tested of 85 ºC. Detailed data are given in the Supporting Information.

Antioxidant activity of the surfactant-chlorophylls extracts. The surfactants studied in this work are currently used in food supplements and cosmetic formulations.[21, 29] Therefore, we finally evaluated the antioxidant activity of the surfactant-chlorophylls extracts to appraise the possibility of directly using these extracts without any additional isolation/recovery step. The antioxidant activity of the initial C11-C13 9EO's aqueous solutions containing chlorophylls, of the chlorophylls extracts obtained after the concentration step, and of the extracts obtained with organic solvents, was determined using the DPPH radical scavenging assay, with ascorbic acid as reference. The antioxidant activity of the aqueous surfactant solution was also determined as a control. The results obtained are depicted in Fig. 7. Detailed data are given in the Supporting Information. The extracts obtained with ethanol and acetone show similar IC_{50} values (1.56 ± 0.04 and 1.68 ± 0.08 μg/mL at 1.5 h, respectively), while the extract with the aqueous surfactant solutions displays lower IC_{50} values (1.20 ± 0.11 μg/mL at 1.5 h) and are not influenced by the presence of the surfactant (as confirmed by the null IC_{50} value obtained with the aqueous solution of surfactant used as control). After the concentration of the extract, the IC_{50} value increases slightly (1.33 ± 0.10 μg/mL at 1.5 h); yet, it

remains lower than those obtained with organic solvents, emphasizing the better antioxidant properties of the surfactant-based extracts.

In general, all chlorophyll-rich extracts display a higher antioxidant capacity than ascorbic acid (Fig. 7). Hunter et al.[33] showed that pure chlorophylls present a high antioxidant activity (chlorophyll *a* and chlorophyll *b* as 2.11 and 1.69 ascorbic acid equivalents). The obtained extracts are highly concentrated in chlorophylls (with a purity level around 80%, as determined by HPLC-DAD; data and experimental procedure given in the Supporting Information), thus supporting their high antioxidant activity. However, it should be kept in mind that other compounds commonly found in spinach leaves can be simultaneously extracted, contributing to the high antioxidant activity observed. According to Ligor et al.[34] a high content of polyphenol acids and flavonoids in spinach leaves may be responsible for the high antioxidant activity of the respective extracts. In summary, these results support the possibility of using directly the surfactant-rich phase containing chlorophylls in nutraceutical and cosmetic applications, instead of carrying additional recovery or purification steps.

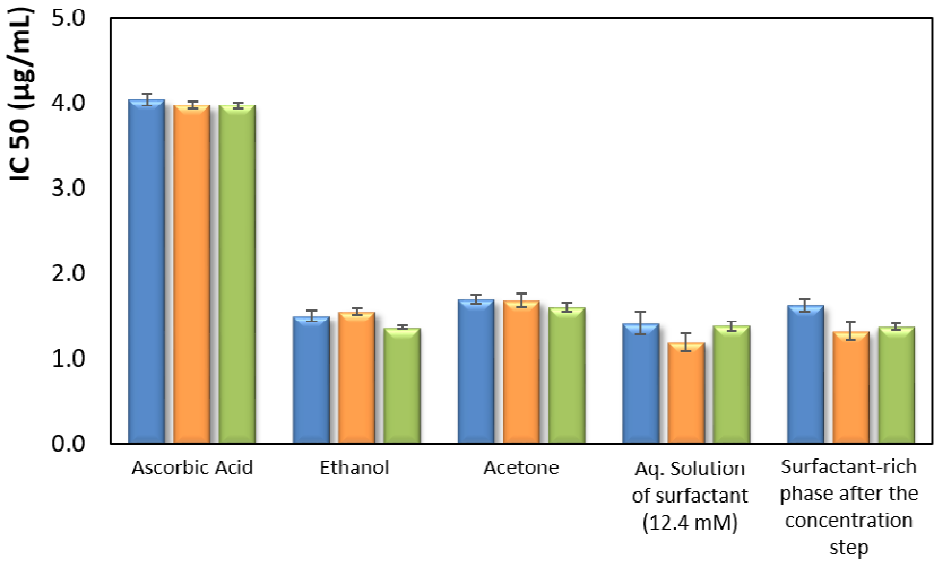

Figure 7. IC_{50} values (µg/mL) after 0.5 (■), 1.5 (■) and 2h (■) of exposure to DPPH.

Fig. 8 summarizes the developed extraction-concentration process of chlorophylls using aqueous solutions of non-ionic surfactants, while envisaging the application of this process to recover natural chlorophylls that can be used (at a lower cost) in food, nutraceutical, cosmetic or pharmaceutical applications. It should be highlighted that after the extraction of chlorophylls, which has shown to be successful by non-ionic surfactant aqueous solutions, the remaining biomass can be further used in other applications within an integrated biorefinery approach.

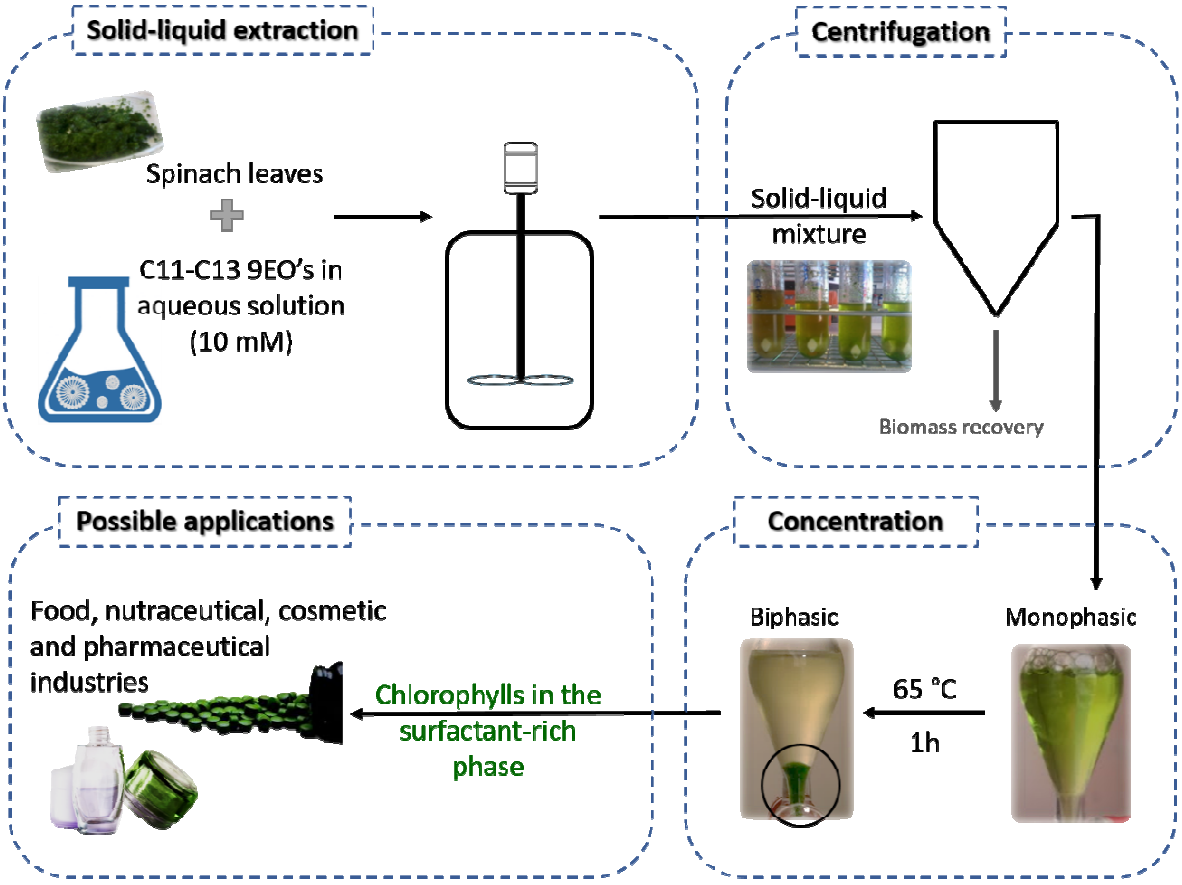

Figure 8. Scheme of the developed extraction-concentration process for chlorophylls from spinach leaves using aqueous solutions of non-ionic surfactants.

CONCLUSIONS

Chlorophylls and their derivatives have been extensively investigated for food, nutraceutical, cosmetic and pharmaceutical/medicinal applications. However, and in spite of their natural abundance, typical methods for chlorophylls extraction require the use of volatile organic solvents. Aiming at developing a cost-effective and more sustainable approach for the extraction of chlorophylls from biomass, in this work we investigated aqueous solutions of non-ionic surfactants as alternative solvents. After a preliminary screening where several surfactants were studied, it was found that ethoxylated alcohols,

namely C11-C13 9EO's, or mixtures of surfactants with HLB values between 10 and 14, lead to the higher extraction yields. A RSM was then applied, revealing that the surfactant concentration, the solid-liquid ratio and the extraction temperature play a significant role on the extraction yield, with a maximum value of extracted chlorophylls of 0.94 mg/g. This value was obtained using an aqueous solution of C11-C13 9EO's at 12.4 mM, a solid-liquid ratio of 0.007, a temperature of 41°C, and 30 min of extraction time. The concentration of the chlorophylls extract in a surfactant-rich phase was then achieved by an increase in temperature leading to a concentration factor of 9 and a recovery of 97 %. Finally, it was found that the chlorophylls-rich extracts in the aqueous surfactant solutions display a higher antioxidant activity than those obtained with volatile organic solvents. The gathered results support the idea that aqueous solutions of surfactants containing chlorophylls may have the potential to be safely and directly used in cosmetic or nutraceutical applications.

ASSOCIATED CONTENT

Supporting Information. Chemical structure of the studied non-ionic surfactants, additional experimental procedures, experimental points used in the factorial planning, model equations, yields of chlorophyll obtained experimentally and respective calculated values, and statistical analysis connected to the response surface methodology.

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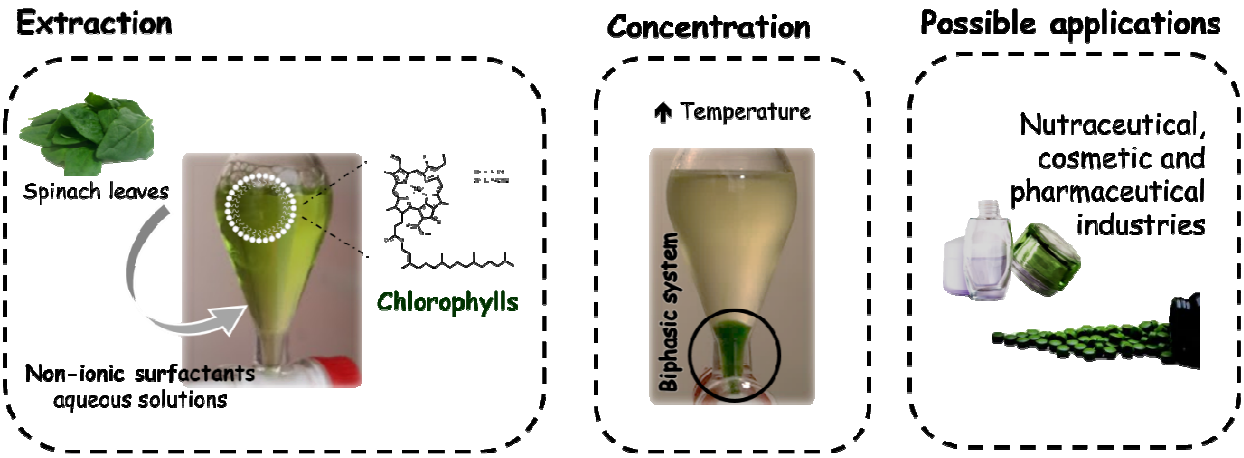
SYNOPSIS



Aqueous solutions of non-ionic surfactants rich in natural chlorophylls may be directly used in cosmetic and nutraceutical applications.