

**Capillary electrophoresis-mass spectrometry of *Spirulina platensis* proteins
obtained by pressurized liquid extraction.**

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Running title: PLE-CE-MS of proteins from microalgae

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Abbreviations: Allophycocyanin- α (**APC- α**), allophycocyanin- β (**APC- β**), c-phycocyanin- α (**CPC- α**), c-phycocyanin- β (**CPC- β**), pressurized liquid extraction (**PLE**).

Keywords: Capillary electrophoresis, electrospray, mass spectrometry, ion trap, intact proteins, microalgae, cyanobacteria, food analysis.

Abstract

In this work, the usefulness of capillary electrophoresis-mass spectrometry (CE-MS) to monitor and optimize the pressurized liquid extraction (PLE) of proteins from *Spirulina platensis* microalga is demonstrated. Crude and purified PLE extracts from microalga were analyzed by CE-MS. It was observed that the use of purification protocols of phycobiliproteins (namely, ultrafiltration or precipitation-dialysis-freeze drying) resulted in better CE resolution and MS signals, demonstrating that sample matrix plays an important role on CE-MS of proteins in real samples. Ultrafiltration was found less laborious and much faster than precipitation-dialysis-freeze drying (1 h vs. 48 h). Direct analysis of crude extracts was demonstrated to be also possible by CE-MS, providing less-quality information but enough to characterize PLE extracts in a much faster way. Therefore, the latter protocol was selected to monitor and optimize the extraction process of phycobiliproteins from *Spirulina platensis*. To do that, different extraction conditions were tested, including time, temperature and pressure of extraction, nature of pressurized liquid, distribution of microalga inside the extraction cell, type of packing, etc. It is demonstrated that the combined use of PLE and CE-MS allows the attainment of extracts rich in phycobiliproteins in short extraction times (namely, yields of 20 % can be obtained in less than 2 hours under the optimum PLE process in an automatic way). To our knowledge, this work shows for the first time the usefulness of CE-MS for monitoring and optimizing a PLE process.

1. Introduction

Spirulina platensis is a blue-green alga (or cyanobacteria) whose phycobilisome, i.e., its light-harvesting photosynthetic apparatus, is formed by phycobiliproteins. These proteins are homologous chromoproteins that can have anti-inflammatory [1-3], hepatoprotective [4] and antioxidant properties [1,5-7]. Moreover, phycobiliproteins seem to be also important in photodynamic therapy of various cancerous tumors [8] and in leukemia treatment [9]. Phycobiliproteins also have a variety of uses such as colorants in food (chewing gum, jellies, etc), cosmetics and fluorescent labels for cells and macromolecules in different fluorescence techniques [10,11].

Due to their huge number of applications, extraction and purification of phycobiliproteins from natural sources (mostly from cyanobacteria) have received much attention, developing numerous technological procedures for this purpose [12-23]. However, it is interesting to remark that extraction of phycobiliproteins from cyanobacteria is notoriously difficult because of the resistant multilayered cell wall and the small size of the bacteria [24-26]. This difficulty is the reason why, in spite of the large number of procedures studied so far, no standard technique to quantitatively extract these compounds from microalgae exists [27,28]. Therefore, research on new extraction techniques, as e.g. pressurized liquid extraction (PLE), for purification of phycobiliproteins from cyanobacteria is of high interest. In this sense, PLE can provide fast purification procedures and the possibility to test a large number of extraction conditions because different solvents, temperatures and pressures can be applied.

Logically, the development of any new extraction procedure as the PLE of proteins proposed in this work requires the use of adequate analytical protocols able to characterize the composition of the extracts obtained. Although different analytical procedures have been published to detect phycobiliproteins, these methods frequently used UV spectroscopy [13,16,23,29] and SDS-PAGE of the denatured proteins [13,23,29,30], giving only a rough estimation of the purity, molecular weight and/or nature of proteins. Also, some chromatographic methods have been published to analyze phycobiliproteins [23,31], however, these methods cannot separate the four main biliproteins found in cyanobacteria (i.e., allophycocyanin- α , APC- α ; allophycocyanin- β , APC- β ; c-phycocyanin- α , CPC- α ; and c-phycocyanin- β , CPC- β) [32]. On the other hand, Viskary and Colyer [26,33] could separate three of the four biliproteins in a single run using capillary electrophoresis with UV or laser induced fluorescence detection. Other techniques as electron microscopy and crystallographic studies have also provided important information on structure and structure-function relationship of these phycobiliproteins [32], while ESI-MS has been used to analyze a purified solution containing CPC- α and CPC- β [5]. More recently, a method based on HPLC-MS with the complete resolution of the protein components of phycobilisome from cyanobacterium *Synechocystis 6803* was presented, which required more than 40 min per run [34].

Capillary electrophoresis-mass spectrometry (CE-MS) has shown great possibilities to separate and analyze in a single run proteins from real samples [35-38] including biliproteins [39]. This is because the on-line coupling of capillary electrophoresis (CE) with electrospray ionization mass spectrometry (ESI-MS) yields a powerful method [40,41] in which CE offers high separation efficiency, while ESI-MS allows the

determination of accurate mass for a wide molecular mass range of proteins. Thus, CE-MS can be an attractive analytical procedure to thoroughly monitor the proteinaceous composition of PLE extracts from *Spirulina platensis* due to the high efficiency, specificity and structural information that this analytical method is able to provide [35,36].

The goal of this work is, therefore, to investigate the possibilities of PLE combined with CE-MS to obtain and characterize extracts rich in phycobiliproteins from *Spirulina platensis*.

2. Material and methods

2.1. Chemicals

All chemicals were of analytical reagent grade and used as received. Ammonium hydrogen carbonate from Fluka (Buchs, Switzerland), ammonium acetate from Panreac (Barcelona, Spain), ammonium hydroxide from E. Merck (Darmstadt, Germany), acetic acid from Acros Organics (Geel, Belgium), formic acid from Riedel-de Hën (Seelze, Germany), and the organic solvents, methanol, acetonitrile (ACN) and 2-propanol, all from Scharlau (Barcelona, Spain), were used for the CE running buffers and sheath liquids. Buffers were prepared by weighting the ammonium hydrogen carbonate, ammonium acetate or formic acid at the concentrations indicated and adjusting the pH when necessary by adding ammonium hydroxide or acetic acid. The buffers were stored at 4°C and warmed at room temperature before being used. Sodium dodecyl sulfate from Fluka (Buchs, Switzerland) and sodium hydroxide from Panreac (Barcelona, Spain) were used for capillary cleaning procedures before each analysis. Distilled water was deionized by using a Milli-Q system from Millipore (Bedford, MA, USA).

2.2. Samples

Microalgae samples (*Spirulina platensis*) consisted of air-dried microalgae with 6% w/w moisture, from Algamar S.A. (Pontevedra, Spain). Samples were stored under dry and dark conditions.

2.3. PLE extraction procedure

As indicated in the text (see Section 3.3) different extraction conditions were tested including temperature and pressure of extraction, type of solvent, time, volume of extraction cell, type of packing and distribution of the sample within the extraction cell. All PLE experiments were done by triplicate.

The system used to perform the microalga extractions was a Pressurized Liquid Extractor ASE 200 from Dionex Corp. (Sunnyvale, CA, USA). A standard filter paper (73 g/m², Albet, Barcelona, Spain) was used for wrapping the sample inside the extraction cell to prevent the clogging of the system. Furthermore, the distribution of the raw material in one or more packs was studied considering up to 9 packs in which the sample was wrapped inside. Besides, different packings were evaluated, namely, glass beads and Dixon rings. Glass beads were of borosilicate glass and were purchased from Symta S.A. (Madrid, Spain) while Dixon rings were of stainless steel and supplied by Afora S.A. (Spain), all of them with dimensions equal to 3 mm of diameter. The bed voidage (void volume/bed volume) of the different packings was experimentally determined being for Dixon rings equal to 0.83 and for Glass beads, 0.39. Prior to each extraction, sample was sonicated for 1 minute. The amount of sample in all the extractions was 0.850 g of sonicated microalga. Deoxygenated water (by purging with

He for 7 min) was used as extraction solvent (plus ACN or 2-propanol as indicated). The extractions were carried out at 1500 or 3000 psi (1 psi = 6894.76 Pa). The extraction time (10, 15, 30, 45, 60, 75 or 90 minutes), extraction temperature (25, 40, 55, 70, 85 or 100 °C) as well as the volume of the extraction cell (11, 22 or 33 ml) were modified according with the experiment carried out as indicated in each case. The extracts obtained were freeze-dried, and stored protected from light at 4 °C. The extraction procedure was as follows: i) sample was loaded into cell, ii) cell was filled with solvent up to a pressure of 1500 psi, iii) heat-up time was applied (5 minutes in all cases except in extractions at 25 °C in which this step is avoided), iv) static extraction takes place (i.e. extraction time) in which all system valves are closed, v) cell is rinsed (with 60 % cell volume using extraction solvent), vi) solvent is purged from cell with N₂ gas and vii) depressurization took place.

2.4. Protein purification protocols.

All the PLE extracts from *Spirulina platensis* were lyophilized and redissolved in water at the concentrations indicated in each case. They were directly analyzed by CE-MS or submitted to the following purification protocols.

Purification procedure A consisted on the protein precipitation by increasing medium ionic strength. A 10 mL of 20 mg/mL extract solution (in water) was used for this purpose. Solid ammonium sulfate was added to 70 % saturation. Then, it was allowed to stand for 1 hour at room temperature under stirring and protected from light. Subsequently, the extract was centrifuged at 15000 g at 4 °C for 15 minutes. The pellet was resuspended in 5 ml of fresh water and dialyzed (using a 3500 Da membrane cut-

off) for 15 h against distilled water. The dialyzed solution was freeze-dried, a 10 mg/mL solution was prepared in water and analyzed by CE-MS

Purification procedure B consisted on ultrafiltration of 500 μL of 20 mg/mL extract solution (in water) with a Microcon of 10000 Da of cut-off from Millipore (Bedford, MA, USA) by centrifugation at 12500 g for 40 minutes. Both, the filtered solution (with MW < 10000 Da) and the retained fraction were analyzed by CE-MS. The retained fraction was washed with 70 μL of fresh water (centrifugation at 1000 g for 3 minutes) and diluted with five volumes of water (to give a final concentration from 7 to 9 mg/mL, depending on the recovered volume from the filtration process) prior to analysis.

A third purification procedure with organic solvent precipitation of the proteins was also carried out. However, although protein precipitation was done with ethanol and acetonitrile, no redissolution of the precipitated proteins could be achieved.

2.5. Capillary electrophoresis-mass spectrometry.

A capillary electrophoresis apparatus (P/ACE 5010 Beckman Instruments, Fullerton, CA, USA) equipped also with a UV-Vis detector was used. Bare fused-silica capillaries with 50 μm ID were purchased from Composite Metals Service (Worcester, England). The total and detection length of the used capillaries were 89 cm. Injections were made at the anodic end using N_2 at a pressure of 0.5 psi for 36 s. Buffer selected for final separations was 40 mM ammonium hydrogen carbonate at pH 7.8 in water plus acetonitrile:2-propanol (45:50:5, v/v/v). Separation was performed at 25 kV and 25°C.

The CE instrument was controlled by a PC running System GOLD software from Beckman.

An ion-trap mass spectrometer (Esquire 2000, Bruker Daltonik GmbH, Bremen, Germany) equipped with an orthogonal electrospray interface (model G1607A from Agilent Technologies, Palo Alto, CA, USA) was used. Electrical contact at the electrospray needle tip was established via a flow of conductive sheath liquid which consisted of water-2-propanol (75:25, v/v) containing 0.5% (v/v) acetic acid, and delivered at a flow rate of 6 μ L/min by a 74900-00-05 Cole Palmer syringe pump (Vernon Hills, IL, USA). The mass spectrometer was operated in the positive ion mode, and was scanned at 600-2200 m/z range during separation. ESI-MS operating conditions were optimized by adjusting the needle-counter electrode distance. Nebulizer pressure value, temperature, and liquid sheath flow rate and composition, were optimized analyzing the same alga extract by CE-ESI-MS. The optimum nebulizer/drying gas conditions were: 3 psi nitrogen, 10 L/min nitrogen at 100°C. A sheath liquid composed by water-2-propanol (75:25, v/v) containing 0.5% acetic acid was used. The instrument was controlled by a PC running the Esquire NT software from Bruker Daltonics.

2.6. Capillary washing protocol.

All new capillaries were conditioned by flushing with 0.1 M NaOH for 20 min followed by water for 30 min. Initially the capillary washing routine between runs consisted of 3 min with water followed by 3 min with running buffer (all rinses were done using N₂ at a pressure of 20 psi). However, it could be observed that resolution and reproducibility of separation were lost after 5 injections, seemingly due to strong adsorption of solutes

onto the capillary wall [38,42,43]. In order to eliminate any adsorbed compound from algae extracts onto the capillary wall, a 1.5 min washing step using 0.1 M NaOH with 30 mM SDS was used. After this, conditioning was completed by flushing the capillary with water and running buffer for 10 and 4 min, respectively. Using this protocol, the %RSD values for migration times of analytes were lower than 0.8% for five consecutive runs, indicating an adequate capillary reconditioning between runs. Therefore, this latter protocol was used.

3. Results and discussion.

*3.1. CE-MS analysis of **Spirulina platensis** phycobiliproteins.*

The complexity of analysis of entire proteins by CE-MS took us to work in a first step with a sample as pure as possible. For this reason, a PLE extract purified using the procedure A described in Section 2.4 was initially used. In order to obtain adequate CE-MS conditions for the analysis of phycobiliproteins from *Spirulina*, different running buffers were tested. Thus, acetonitrile (0, 10, 20, 30, 40, 50 and 60%), 2-propanol (0, 10, 20 and 30%), methanol (0, 10, 20, 30 and 40%), and several acetonitrile-water-2-propanol mixtures (50:49:1, 50:45:5, 50:40:10), all v/v, were tested with an ammonium bicarbonate buffer at different concentrations (from 30 to 50 mM) and pH values (from 7.2 to 8.2). The BGE consisting of 40 mM ammonium bicarbonate at pH 7.8 (in water) with acetonitrile and 2-propanol (45:50:5, v/v/v) was found to provide optimum CE conditions in terms of resolution and analysis time and it was used to study the ionization parameters at the ESI chamber.

The ionization parameters studied were the sheath liquid composition, sheath liquid flow rate, sheath gas flow-rate, and ESI chamber temperature and dry gas flow-rate. Two different organic solvents (methanol and 2-propanol) plus water were tested as sheath liquid together with acetic acid and formic acid. A strong effect of acid addition to the sheath liquid on the MS signal was observed, since the proteins are separated as anions (pH of BGE is higher than their pI values), but detected as cations (ESI positive). The best MS signals were obtained using water-2-propanol (75:25, v/v) and 0.5% acetic acid. The nebulizer pressure was set to 2, 3, 4, 5, 6, 7 or 8 psi obtaining the best results at 3 psi. Analogously, sheath liquid flow was varied over the range between 2 and 9 $\mu\text{L}/\text{min}$ being the optimum at 6 $\mu\text{L}/\text{min}$. ESI chamber temperature was found to be optimal at 100° C with the best dry gas flow rate at 10 L/min.

Figure 1 shows the CE-MS electropherogram obtained under these optimum CE-ESI-MS conditions. As can be seen in Figure 1, the CE-MS protocol permits the separation of the four phycobiliproteins from *Spirulina platensis* in about 21 min and the attainment of adequate MS spectra for the suitable characterization of these proteins in a single run, namely, the detected proteins are APC- β (peak 1, 17929 Da), CPC- α (peak 2, 18188 Da), CPC- β (peak 3, 19220 Da) and APC- α (peak 4, 17848 Da).

3.2. Effect of different purification protocols of PLE extracts on CE-MS analysis.

Once optimum CE-MS conditions were obtained, the effect of sample preparation on protein analysis was investigated. Since the sample protocol used above for the purification of proteins (precipitation with ammonium sulphate, dialysis and freeze drying of the lyophilized PLE extract) took about 48 hours, a study about the feasibility

of other faster possibilities was carried out. Thus, Figure 2A shows the CE-MS electropherogram corresponding to the direct injection of a crude PLE extract. As can be seen in Figure 2, the CE-MS procedure makes the direct injection of crude PLE extracts possible providing useful information. Moreover, these CE-MS conditions provide adequate MS spectra that make possible the satisfactory assignment of peaks 1, 2 and 4 with enough mass accuracy (i.e., APC- β , CPC- α and APC- α were correctly identified). However, peak 3 could not be assigned as a result of the poor quality of the MS spectra, probably as a result of the large number of interfering compounds. Although the sample concentration used for Figure 1 and Figure 2A was the same, a more purified sample was used in Figure 1 (obtained by precipitation, dialysis and freeze drying of the PLE extract) compared with Figure 2A, in which the crude PLE extract was directly injected. In summary, the conditions shown in Figure 2A provide useful information on the PLE extracts in a much faster way, although the MS signal and quality of the spectra are lower compared with those of Figure 1. This can indicate that some other compounds rather than proteins are extracted by PLE (see MS spectra A3 in Figure 2). Moreover, these other compounds would not only reduce the MS signal but also the CE resolution as can be deduced from the lack of resolution observed between peaks 3 and 4 in Figure 2A. This lack of resolution can be explained through additional dispersion of the electrophoretic band induced by an excess of ions in the crude sample directly injected after PLE extraction. It is likely that further compounds of *Spirulina platensis* (e.g. carbohydrates, oligonucleotides, salts, vitamins [44]) are extracted under PLE conditions, which are not detected by the applied CE-MS protocol. These compounds may affect the EOF, the CE separation and ESI yield. Matrix influence is certainly an important issue to be addressed applying crude extracts to ESI-

MS even by using separation methods like CE or LC, since either the separation or the ESI ionization can be influenced.

In order to decrease the time required for the purification protocol of the PLE extracts, other procedures for protein cleaning were applied prior to CE-MS, as described in Section 2.4. Thus, the precipitates obtained after addition of solid ammonium sulphate to the PLE extract (after re-dissolving the precipitates in water) was injected into the CE-MS. As expected, the high salt content of this sample precludes its CE-MS analysis (data not shown). Higher concentrations of the precipitated extract were used and similar negative results were obtained. Thus, in order to skip the use of salts that clearly interfere with CE (limiting the resolution due to destacking), precipitation of proteins with different organic solvents was explored. However, although protein precipitation was observed with ethanol and acetonitrile (in both cases at 66% of organic solvent), no re-dissolution of the precipitated proteins could be achieved after multiple attempts and, therefore, this procedure was abandoned.

Ultrafiltration of the PLE extract with a 10000 Da membrane was applied as purification procedure following the method described in Section 2.4. Both, the retained and filtered solutions were injected in the CE-MS instrument. No peak was observed in the filtered solution, in agreement with the masses of the phycobiliproteins: which are between 18000-19000 Da and, therefore, do not pass through the membrane. This is corroborated through the analysis of the retained solution whose CE-MS electropherogram is given in Figure 2B. As can be seen, the four biliproteins are detected there and, moreover, the resolution between peaks 3 and 4 has been recovered, probably as a result of the present purification. This cleaning procedure allows

obtaining CE-MS results comparable to those given in Figure 1 (after precipitation-dialysis-freeze drying) with no significant differences in resolution. Moreover, the time required by ultrafiltration (see Section 2.4, protocol B) was only 1 h, which is much faster than the more tedious precipitation-dialysis-freeze drying (protocol A) that required 48 h.

3.3. Study of different PLE conditions to obtain *Spirulina platensis* phycobiliproteins..

In spite of the interesting results shown in Figures 1 and 2, a rough calculation about the extraction yield obtained at these PLE conditions (i.e, using water at 1500 psi and 25 °C for 45 minutes) gave as result a recovery lower than 4% relative to the initial amount of dry microalga (w/w). Therefore, different extraction conditions were tested trying to develop a fast PLE procedure able to provide larger amounts of extracts from *Spirulina platensis* rich in phycobiliproteins.

First, different temperatures of extraction were tested in an extraction cell of 11 ml using water at 1500 psi for 45 min. Figure 3 shows the results obtained in terms of percentage of extract obtained (% yield, Figure 3A) and content of protein in each extract (Figure 3B) calculated by CE-MS as the sum of proteins peak areas. Figure 3C gives some representative CE-MS electropherograms of the PLE extracts obtained at 25 °C, 40 °C and 100 °C. As expected, the higher the temperature of extraction the higher the percentage of extract obtained (see Figure 3A). However, it is also demonstrated that the higher the temperature of extraction the lower the amount of protein obtained as can be deduced from Figures 3B and 3C. Namely, at temperatures higher than 40 °C there is a clear decrease of protein in the extracts detected by CE-MS, probably as a result of the thermal

degradation of these biopolymers. This result is in good agreement with that already observed by Sarada et al [16]; these authors demonstrated by using a rough method based on optical density that biliproteins are highly unstable biopolymers at temperature higher than 45 °C. Therefore, an extraction temperature of 25 °C was selected as adequate for the rest of experiments in this work in order to ensure the integrity of the extracted proteins. However, as can be seen in Figure 3A, the low yield obtained at this temperature (ca. 4 % relative to the initial amount of microalga) needs to be improved.

In order to increase the extraction yield, different organic solvents were used at the selected temperature (25 °C) for the same extraction time (i.e., 45 min) with the aim of investigating the effect of the polarity of the solvent on the extraction yield. The selected pressurized liquids were water, water:isopropanol and water:ACN the last ones tested at two different percentages (namely, 80:20 and 50:50, v:v), covering in this way extraction conditions at high polarity, medium-polarity and low-polarity, respectively. Analysis by CE-MS of these PLE extracts makes possible to achieve interesting conclusions regarding the extraction process. Thus, the PLE yields obtained by using 20 % ACN and 50 % ACN are similar as can be deduced from Figure 4A, however, the quantity of protein extracted is clearly lower using 50 % ACN than using 20 % ACN as can be deduced from Figure 4B, what seems to indicate that at these PLE conditions other compounds different than proteins are being extracted. As can also be deduced from Figure 4, the use of just water provides the best results in terms of yield and amount of proteins; therefore, water was selected as pressurized liquid for the rest of extractions. Moreover, since the yield still remained at the same low value (i.e., ca. 4 %), other PLE conditions were further investigated trying to improve it.

Next, different extraction times were investigated using water as pressurized liquid at 25 °C. Namely, extraction times of 10, 15, 30, 45, 60, 75 and 90 min were studied. Figure 5 shows the yields (Figure 5A) and the amount of protein found in the extracts by CE-MS (Figure 5B) depending on the extraction time. As can be seen in Figure 5A, there is a trend to higher yields at longer extraction times and the same can be observed for the amount of proteins (see Figure 5B). Since no significant difference was observed between 75 and 90 min, the former value was selected in order to speed up the extraction process.

Under these conditions, the effect of the extraction cell volume was also studied using cells of 11, 22 and 33 ml, together with pressurized water at 25 °C for 75 min. No significant difference was observed among the three cells in terms of yield of dry extract and quantity of protein in the extracts obtaining for all cases yields about 5%.

The PLE procedure used so far has consisted in: i) introducing a known amount of microalga *Spirulina platensis* within the extraction cell in a single pack; ii) filling with water the extraction cell; iii) pressurizing at 1500 psi; iv) heating at the desired temperature; and v) allowing the contact (microalga-water) for a given extraction time. Considering the strong influence of the procedure to pack the extraction cell in the static PLE efficiency, an increase of the surface of contact between microalga and water was intended by splitting the same amount of microalga into different packs within the extraction cell keeping all the other conditions as above. Thus, Figure 6 shows the results obtained using different number of packs in terms of yield (Figure 6A) and amount of protein determined by CE-MS (Figure 6B). As it can be seen in Figure 6, the higher the number of packs the higher the yield obtained during the extraction and the higher the amount of proteins detected in the extracts. This result can easily be explained considering

that the surface of contact microalga-water increases with the number of packs what improves the extraction yield of the PLE procedure, getting values higher than 8% as demonstrated in Figure 6A when 9 packs were used.

Interestingly, since the yield attained under the optimized conditions was not very high (i.e., 8 %), consecutive static extractions were intended with the same sample and using fresh solvent able to accelerate the mass transfer improving the extraction efficiency. However, under these PLE conditions the microalga packs collapse and block the extraction cell preventing any subsequent extraction. Based on these results, the use of different packings within the extraction cell was tested in order to improve the resistance of the sample packs to collapse, reducing the probability of blocking the cell and increasing consequently the number of consecutive static extractions that can be done with the same sample. Two packings (glass beads and Dixon rings) were tested together with the 9 packs of microalga. A comparison between both packing materials demonstrated that the amount of protein that they provide was very similar (see Figure 7A), while the extraction yield was slightly higher using glass beads than using Dixon rings. Considering the different properties of the packings in terms of porosity (bed voidage) and packing density (1508 kg/m^3 for glass beads compared to 456 kg/m^3 for Dixon rings) and the similar results obtained, it seems clear that the packing material acts only as solid support to keep the integrity of the sample inside the extraction cell, and no other effect on diffusion or mass transfer can be associated to their use. Since yields obtained using glass-beads-packing were slightly better than using Dixon rings, the first packing was used for the next experiment.

As expected, the use of glass beads within the extraction cell also allowed repeating the extraction process for the same sample several times before the PLE cells gets blocked by the microalga. This procedure to pack the sample inside the extraction cell greatly influences the extraction efficiency considering that better results can be expected when the matrix is subjected to several consecutive static extractions rather than to a single longer static extraction. For instance, Figure 7A also shows the amount of protein detected by CE-MS in the first and sixth extraction carried out using glass beads as packing material. As can be seen in Figure 7A, under these conditions a higher amount of protein was obtained in the 6th extraction compared to the 1st one; this can be related to the rupture of a higher number of *Spirulina* cells induced by the previous pressurized extractions, what could give rise to a higher amount of protein molecules released to the surrounding water during the subsequent extractions. Interestingly, it was observed that the maximum number of extractions that could be done with the same sample depended on the static extraction time used for each cycle. Thus, it was possible to repeat 7 times the PLE process using cycles of 15 min for each extraction before the cell gets blocked. It was possible to carry out 6 extractions using cycles of 30 min, while only two repetitions were allowed using cycles of 75 min. Figure 7B shows the yields obtained under these conditions depending on the static extraction time used for each cycle. Also in Figure 7B, the total extraction time is given on the top of the bars for each case. As can be seen, an optimum yield of more than 20 % was obtained by using cycles of 15 min requiring a total extraction time of 105 min. Since the best results were obtained at the fastest studied cycle (i.e., 15 min) a still faster cycle (i.e., 10 min) was tested obtaining slightly worse results, therefore, the extraction cycle of 15 min was considered as optimum.

In order to test the influence of the extraction pressure on the extraction yield, an additional experiment was carried out under the last described conditions using higher extraction pressure (i.e., 3000 psi). Namely, microalgae was distributed into 9 packs, using glass beads within a 22 ml extraction cell. Seven extraction cycles of 15 min were performed at room temperature applying the mentioned pressure of 3000 psi. A total extraction yield of 20 % was achieved under these conditions. Therefore, since no yield improvement was observed compared to the previous pressure used (1500 psi), the lower pressure value was selected as optimum.

The optimum PLE conditions achieved in this work (i.e., pressurized water at 1500 psi and 25 °C, microalga distributed in 9 packs, using glass beads as packing material and 15 min of extraction time, repeating the process 7 times) apart of providing higher yields also give extracts richer in phycobiliproteins. This enrichment is clearly demonstrated comparing the first and the last PLE extracts analyzed under identical CE-MS conditions, namely, compare electropherograms of Figure 2B and Figure 7C (see y-axis). Thus, the electropherogram given in Figure 2B (corresponding to one of the first extracts) shows protein peaks intensities about three times lower than those shown in Figure 7C (corresponding to one of the last extracts), what demonstrates the protein enrichment that the optimum PLE extraction conditions provide.

Concluding remarks

In this work, it has been demonstrated for the first time that the combined use of PLE and CE-MS is a valuable procedure to obtain and characterize proteins from natural sources of major interest in medical and food applications. It has been shown that the

combined method PLE-CE-MS facilitates the extraction of phycobiliproteins from *Spirulina platensis* microalga through and adequate characterization of the extracts, increasing in that way the amount and purity of proteins obtained. To our knowledge, this work shows for the first time the great possibilities of CE-MS to monitor and optimize pressurized liquid extraction procedures.

Acknowledgements

Financial support from AGL2002-04621-C02-02 project (CICYT, Ministerio de Ciencia y Tecnología) is acknowledged. M.H. thanks to MCYT for a FPI grant. C.S. thanks to Consejería de Educación y Cultura (Comunidad de Madrid) for a fellowship.

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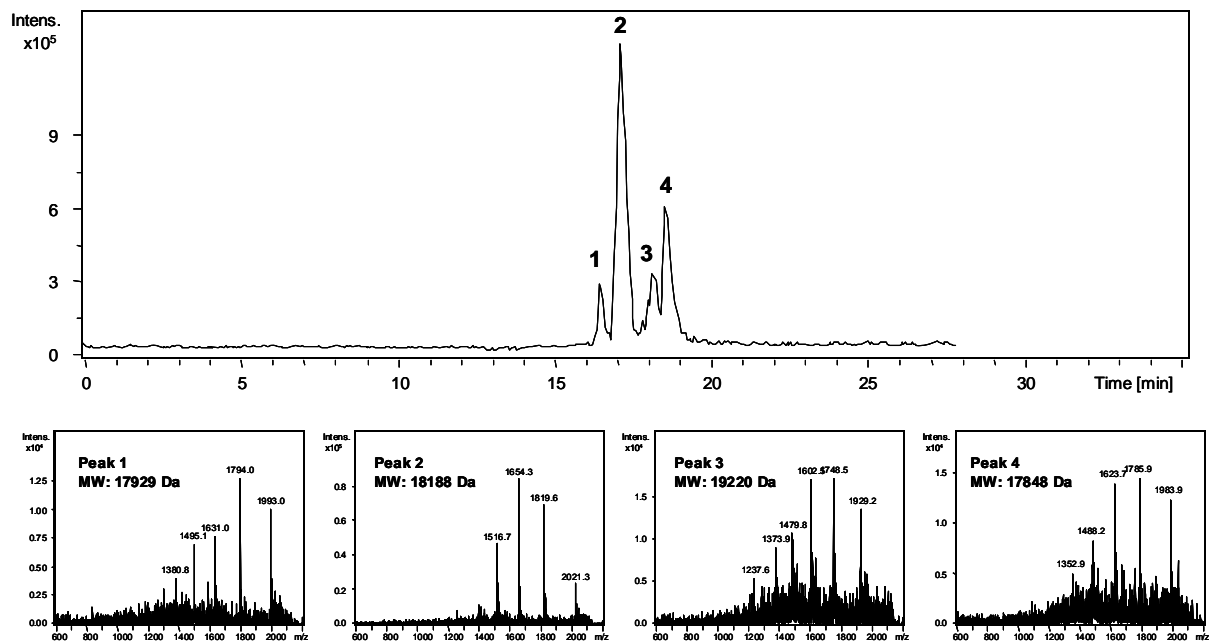


Figure 1

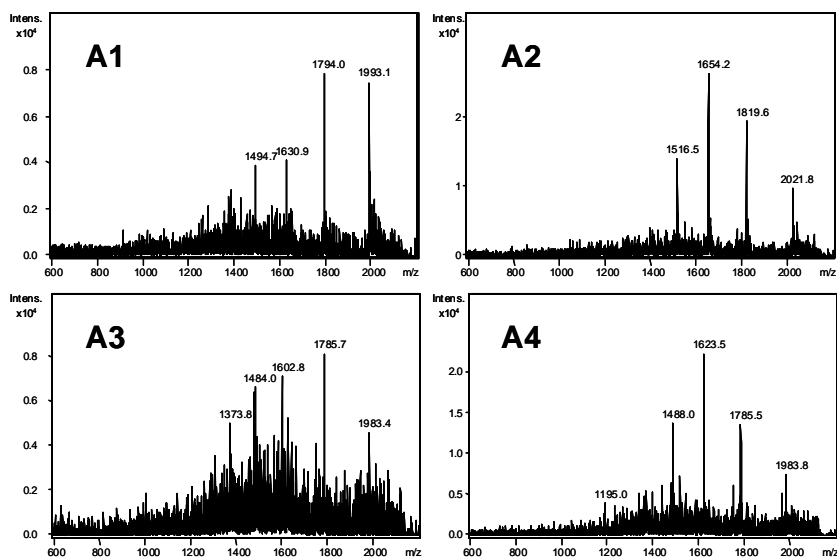
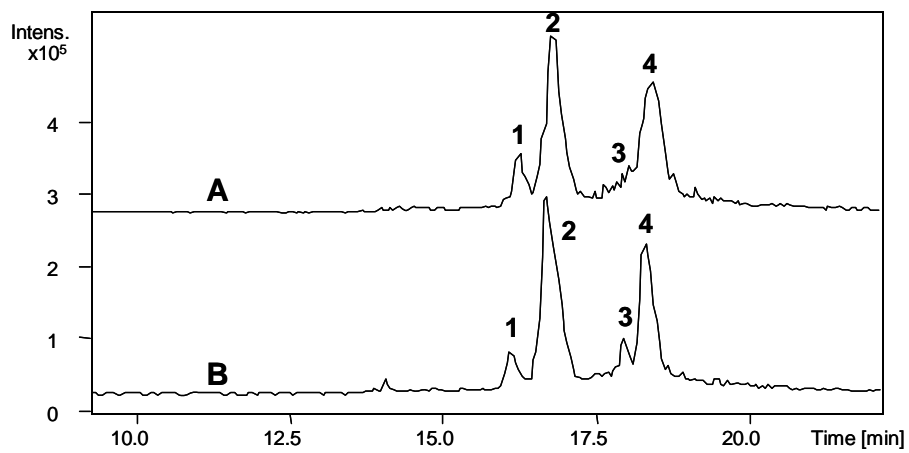


Figure 2

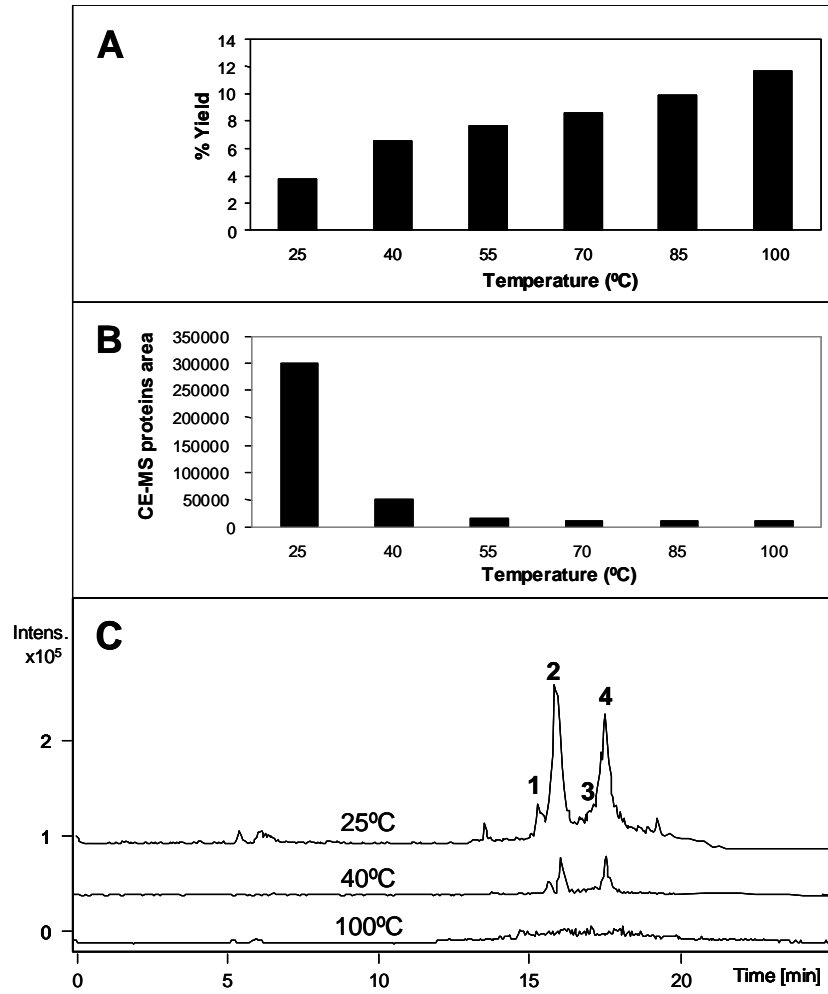


Figure 3

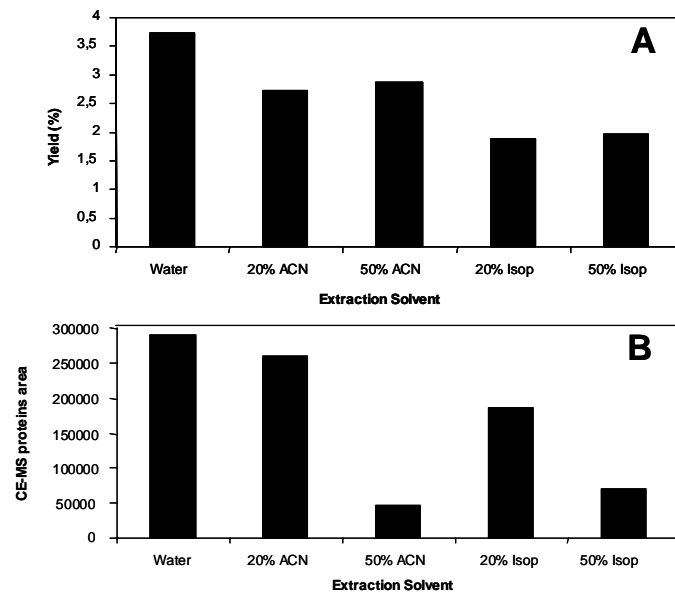


Figure 4

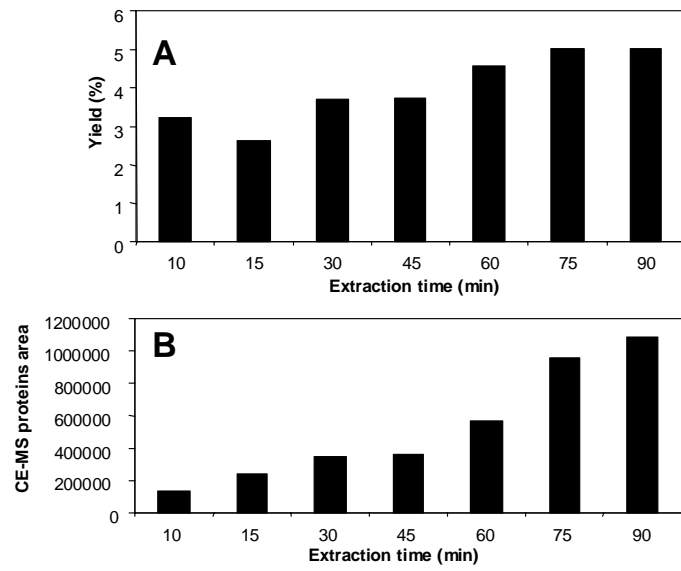


Figure 5

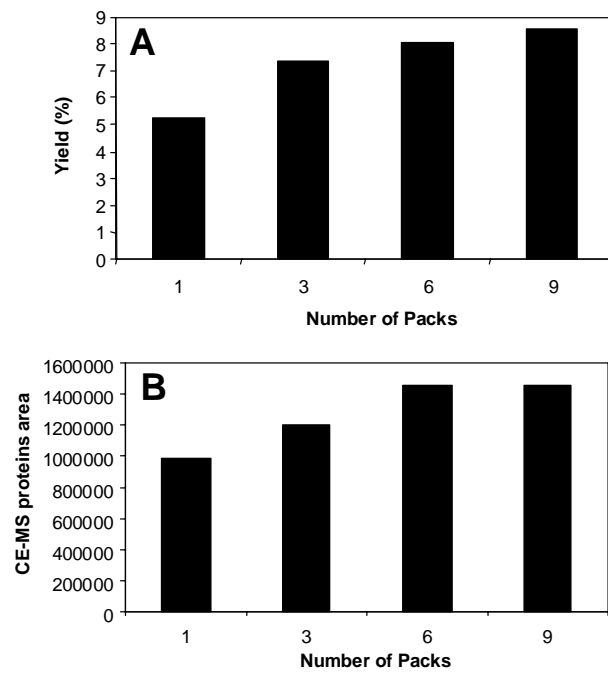


Figure 6

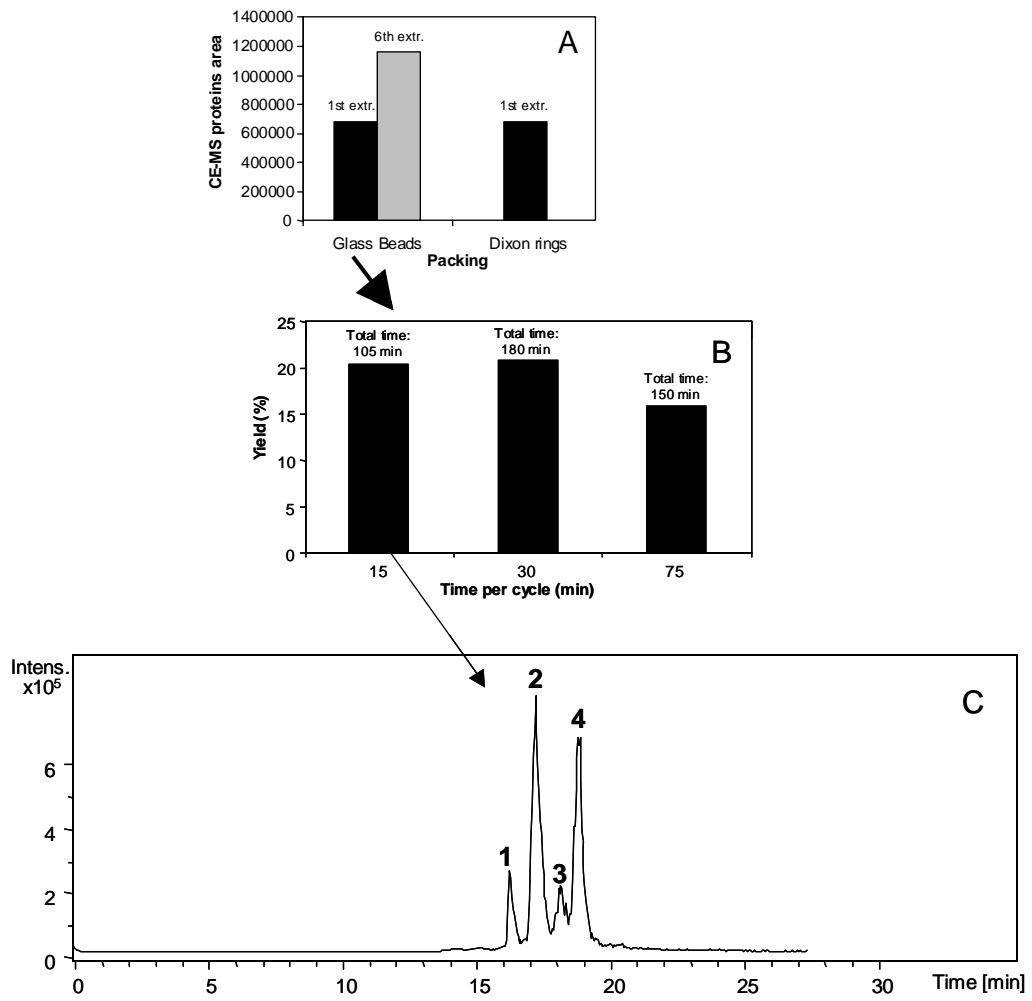


Figure 7

Figure Legends

Figure 1. Base peak electropherogram and MS spectra of a PLE purified extract (10 mg/mL) from *Spirulina platensis* at optimum CE-ESI-MS conditions. Bare silica capillary (90 cm l_t, 50 μm I.D.), running buffer: 40 mM ammonium hydrogen carbonate in acetonitrile-water-2-propanol (50:45:5%, v/v/v), running voltage: +25 kV, injection: 36 s at 0.5 psi. MS positive ion mode (-4000 V), sheath liquid: water-2-propanol (75:25%, v/v) with 0.5% acetic acid at 6 μL/min, nebulizer pressure: 3 psi, 10 L/min dry gas at 100 °C (see text for further details).

Figure 2. Comparison of CE-MS base peak electropherograms of the same PLE extract after different treatments. (A) Direct injection of the crude PLE extract (10 mg/mL) and (B) after 10000 Da ultrafiltration (8 mg/mL). All CE-MS conditions as in Figure 1.

Figure 3. Effect of PLE extraction temperature on: A) yield and B) quantity of protein extracted from *Spirulina platensis* using water at 1500 psi for 45 min within an extraction cell of 11 ml. C) CE-MS base peak electropherograms of three different PLE extracts obtained at the indicated temperatures. All CE-MS conditions as in Figure 1.

Figure 4. Effect of PLE extraction solvent on: A) yield and B) quantity of protein extracted from *Spirulina platensis*. PLE conditions: water was used together with the indicated solvents (v/v) at 1500 psi for 45 min within an extraction cell of 11 ml. All CE-MS conditions as in Figure 1.

Figure 5. Effect of PLE extraction time on: A) yield and B) quantity of protein extracted from *Spirulina platensis* using water at 1500 psi and 25 °C within and extraction cell of 11 ml. All CE-MS conditions as in Figure 1.

Figure 6. Effect of the number of packs in which the microalga is distributed within the PLE extraction cell on: A) yield and B) quantity of protein extracted. PLE conditions: water at 1500 psi and 25 °C, single extraction for 75 min within a cell of 22 ml. All CE-MS conditions as in Figure 1.

Figure 7. A) Effect of packing material on the quantity of protein extracted by PLE from *Spirulina platensis*. B) Effect of the extraction time (extraction cycle and total time) on the yield obtained. Other PLE conditions: *Spirulina* distributed in 9 packs, water at 1500 psi and 25 °C for the indicated extraction times (for each cycle and total extraction time) within an extraction cell of 22 ml. C) CE-MS base peak electropherograms of the *Spirulina* extract obtained at optimum PLE conditions after 10000 Da ultrafiltration. All CE-MS conditions as in Figure 2B.