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**Microalgae amino acid extraction and analysis at nanomolar level using electroporation and capillary electrophoresis with laser-induced fluorescence detection**

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**Running title:** Electroporation and CE–LIF analysis

**Abbreviations:** **a.a.**, amino acid; **DS**, *Dunaliella salina*; **FITC**, fluorescein isothiocyanate;  **$\alpha$ -Ala**,  $\alpha$ -Alanine;  **$\beta$ -Ala**,  $\beta$ -Alanine; **D-Ile**, D-allo-isoleucine; **D-Arg**, D-Arginine; **L-Asn**, L-Asparagine; **L-Cit**, L-Citrulline; **D-Gln**, D-Glutamine; **L-Orn**, L-Ornithine.

**Key words:** amino acid extraction, capillary electrophoresis, electroporation, laser-induced fluorescence detection, microwave-assisted derivatization

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## Abstract

Amino acids play a key role in food analysis, clinical diagnostics and biochemical research. Capillary electrophoresis with laser-induced fluorescence detection was used for the analysis of several amino acids. Amino acid labeling with fluorescein isothiocyanate was conducted using microwave-assisted derivatization at 80°C (680 W) during only 150 s. Good electrophoretic resolution was obtained using a background electrolyte composed of sodium tetraborate buffer (100 mM; pH 9.4) and  $\beta$ -cyclodextrin (10 mM), and the limits of quantification were 3–30 nM.

The developed capillary electrophoresis with laser-induced fluorescence method was used to analyze amino acids in *Dunaliella salina* green algae grown under different conditions. A simple extraction technique based on electroporation of the cell membrane was introduced. A home-made apparatus allowed the application of direct and alternating voltages across the electrochemical compartment containing a suspension of microalgae in distilled water at 2.5 g.L<sup>-1</sup>. A direct voltage of 12 V applied for 4 min gave the optimum extraction yield. Results were comparable to those obtained with accelerated-solvent extraction. The efficiency of electroporation in destroying microalgae membranes was shown by examining the algae surface morphology using scanning electron microscopy. Stress conditions were found to induce the production of amino acids in *Dunaliella salina* cells.

## 1. Introduction

Amino acids are organic compounds that exhibit a major role in many fields (nutrition, health, cosmetics, biochemistry, etc). Several cosmetic products have proved to bring flexibility, softness and elasticity to human skin due to their content in amino acids. Their level in *stratum corneum* which is the outermost layer of the epidermis and the protecting barrier of the skin is a potential marker of this organ health. Amino acid systemic level may be used to diagnose inherited metabolic diseases. Thus, quantitative and qualitative analysis of amino acids is critical [1–3]. Detection of amino acids from different matrices at trace levels is difficult due to their physicochemical properties, such as absence of chromophores, amphotericity, non-volatile nature, high polarity, and complexation with metal ions [4]. Several analytical methods for analyzing amino acids have been reported in the literature based on chromatographic [5–11] and electrophoretic methodologies [12–18]. Different detectors can be used such as UV (UV/VIS) [5, 19, 20], fluorescence [15, 21–26], conductivity [18] and MS detection [11, 16, 27–30]. Recently, CE–MS was used for the identification of amino acids due to its capability to differentiate overlapping peaks with distinct mass-to-charge ratios [29, 30]. CE–LIF is considered as an alternative method and a powerful analytical technique, due to its sensitivity and selectivity [15, 22–26, 31]. According to some reviews that described the advances in amino acid analysis by CE, LIF provides the best LOD among the other detection modes available for CE [14].

Since only few amino acids show strong native fluorescence, a derivatization reaction with suitable fluorogenic reagent is needed to enhance the sensitivity [22, 26]. A wide variety of labeling agents are commonly used such as 6-carboxyfluorescein (6-FAM), 5-(4,6-dichloro-s-triazin-2-ylamino) fluorescein (DTAF) [32] and fluorescein isothiocyanate isomer I (FITC). FITC is widely used as a derivatization reagent for primary amine group. Its derivatives are easily formed and generate strong fluorescence signals with excitation and emission wavelengths (488/520 nm) compatible with an argon ion laser. Derivatization can be obtained

after incubation, in the dark, at room temperature for 16 h [33, 34] or at 50°C for 5 h [35, 36]. Liwei Cao et al. [4] also proposed a microwave (MW)-assisted derivatization approach to accelerate labeling reaction kinetics and hence to reduce derivatization time to 150 s. In inorganic and organic chemistry, microwave technology has been used since the late 1970s and the mid-1980s, respectively. The main advantages of microwave assisted organic synthesis are solvent-free use, shorter reaction times and expanded reaction range which make this technique very interesting [37].

Many studies have been done on the derivatization and separation of standard amino acids, but few papers have been devoted to the separation of non-standard or non-protein amino acids by CE and, to our knowledge, no CE–LIF method have been developed so far to separate and detect these amino acids from microalgae. For this, the analysis of amino acids in *Dunaliella salina* (DS) microalgae was conducted. Indeed, chemical studies on microalgae are complicated by difficulties mainly connected with the influence of nutrients and contaminants in their environment as well as by climatic factors on the biosynthesis of natural products [38]. *DS* microalga is known to be the best commercial source of natural  $\beta$ -carotene. It can accumulate significant amounts of valuable chemicals such as carotenoids, lipids, vitamins and proteins. Additionally, it has a high potential value for biotechnological processes such as treatment of wastewater [39]. In regard to amino acid extraction from microalgae a new method based on low voltage (few V/cm, few mA) was developed. Low voltage (or low current) can induce iontophoresis phenomena implicating the motion of ions across the cell membrane under the influence of the electric field. Electroporation and reverse iontophoresis have been successfully used for extraction of transdermal multi-biomarkers, like urea, prostate-specific antigen (PSA), and osteopontin [40]. Reverse iontophoresis across the skin was also shown to be a useful alternative for non-invasive drug monitoring [41]. Moreover, it was shown that electrically based disruption techniques such as pulsed electric

field (PEF) (20 kV/cm, 1–4 ms) and high-voltage electrical discharge (HVED) (40 kV/cm, 1–4 ms) allow selective extraction of water soluble ionic components and microelements, small molecular weight organic compounds and water soluble proteins in a short time [42, 43].

In fact, electroporation is an effective process to breach cell membrane barrier by applying a voltage, and has been used for the extraction of intracellular protein and small molecules from bacterial and eukaryotes cells. In an electroporation process, cells are exposed to an external electric field which induces a potential difference across the membrane. The induced transmembrane potential ( $\Delta\psi_E$ ) is given by the following equation [36]:

$$\Delta\psi_E = 1.5 g(\lambda) r E \cos\theta \quad (1)$$

Where  $g(\lambda)$  is a complex function of the membrane and buffer conductivities,  $r$  is the radius of the cell,  $E$  is the electric field intensity and  $\theta$  is the angle between the normal to the membrane surface and the electric field direction.

When  $\Delta\psi_E$  exceeds a threshold, the cell membrane can be irreversibly broken down. As a consequence, cell lysis and intracellular content release occur.

The aim of this study was to develop a CE–LIF method for the sensitive determination of amino acids in microalgae extracts. For this, the fluorescent labeling and the electrophoretic separation of amino acids were optimized, and a new extraction procedure based on electroporation at low voltage was introduced. *Dunaliella salina* (DS) microalgae were used as model algae to evaluate the efficiency of the developed approach.

## 2. Materials and methods

### 2.1. Chemicals and materials

All reagents employed were of analytical grade. D-Glutamine (purity: 98%), D-arginine (purity: 98%), D-allo-isoleucine (purity: 98%), L-asparagine purity: 98%), L-citrulline (purity:

98%), L-ornithine (purity: 99%), L- $\alpha$ -alanine, L- $\beta$ -alanine,  $\alpha$ - and  $\beta$ -cyclodextrin (purity: 90%), FITC isomer I (purity: 98%) and sodium tetraboratedecahydrate ( $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ , purity  $\geq 99.5\%$ ) were purchased from Sigma–Aldrich (Saint-Quentin-Fallavier, France). Ultra-pure water (18 M $\Omega$ .cm) was produced from an Elgasat apparatus (Elga, Villeurbanne, France).

## 2.2. Solutions

All solutions were prepared with ultra-pure water.

–*Separation or BGE*: Unless otherwise stated, borate buffer (10 mM or 100 mM, pH 9.4) was used as a separation BGE by dissolving appropriate amount of sodium tetraborate decahydrate in distilled water. 10 mM of alpha-cyclodextrin ( $\alpha$ -CD) or beta-cyclodextrin ( $\beta$ -CD) were added to the BGE to improve peak resolution.

–*Derivatization buffer (for standard amino acids)*: a borate buffer at 10 mM and pH 9.4 was used for fluorescent labeling of amino acids.

The different buffers were prepared fresh each day. Their pH was checked with a Meterlab PHM201 Portable pH Meter (Radiometer Analytical, Villeurbanne, France).

–*Stock solutions*: Amino acid and FITC stock solutions were prepared respectively at 6 mM and at 10 mM in the derivatization buffer and stored at  $-20^\circ\text{C}$  in the dark. These solutions were diluted appropriately each day in the same buffer.

## 2.3. *Dunaliella salina* microalgae media and cultivation

*Dunaliella salina* strain used in this work was isolated from the Sebkhah of Sidi El Hani (Tunisia).

–*Culture medium*: the culture medium used was the F/2 [44] using artificial seawater ASW. The composition of 1L of F/2 was as following: 996.5 mL ASW; 1 mL  $\text{NaNO}_3$  (75 g.L $^{-1}$ ); 1 mL  $\text{NaH}_2\text{PO}_4$  (5 g.L $^{-1}$ ); 1 mL trace metals (100 mL):  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  0.98 g,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$  2.2 g,  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  1 g,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  18 g,  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$  0.63 g); 0.5 mL vitamin solution (1

L: Biotin 1 mg, 1 mg B<sub>12</sub> and 20 mg Thiamin HCl). Where ASW composition was as following (1 L): NaCl 27 g; MgSO<sub>4</sub> 6.6 g; MgCl<sub>2</sub> 25.6 g; CaCl<sub>2</sub> 1.5 g; KNO<sub>3</sub> 1 g; KH<sub>2</sub>PO<sub>4</sub> 0.07 g; NaHCO<sub>3</sub> 0.04 g; 20 mL Tris buffer (pH 7.6) 1 M.

–*Cultivation conditions*: for normal cultures, nitrogen was at 1.07 g.L<sup>-1</sup> whereas for stressed cultures, the nitrogen was limited at 0.03 g.L<sup>-1</sup>.

Microalgae were harvested using centrifugation under 5000 rpm for 10 min (Jouan BR4I multifunction, Thermo, Illkirch, France) and then the microalgae cells were freeze-dried (lyophilized).

#### 2.4. Instrumentation and operating conditions

All experiments were performed with a CE instrument: AB Sciex PA 800+ equipped with a LIF detector (Brea, CA, USA). The excitation was carried out with an Argon ion laser at a wavelength of 488 nm. The band-pass filter 520 nm was used for emission. Data acquisition and instrument control were carried out using 32 Karat acquisition system software. Separations were carried out using fused-silica capillaries of 60 cm total length (50 cm detection length) purchased from Polymicro Technologies (Phoenix, AZ, USA). The inner diameter was 50 or 75 µm. The capillary was maintained at 25°C and separation was conducted at +25 kV. Hydrodynamic injection mode with 0.5 psi pressure was applied for 5 s.

Each new capillary was conditioned by flushing it with 1 M NaOH (10 min), water (5 min) and BGE (15 min). Between two runs, the capillary was flushed with NaOH 1M (1 min), water (1 min) and BGE (3 min). All rinse cycles were conducted at 20 psi pressure. The buffer vials were renewed every three analyses.

#### 2.5. Derivatization procedure

Pre-capillary derivatization of analytes was applied because labeling reaction is slow and thus inadequate for in-capillary derivatization mode.

Different ratios of FITC and amino acids were tested to evaluate the efficiency of the derivatization. In a 1 mL total volume, the final amino acid concentration was 1  $\mu$ M with concentration ratios of FITC to each amino acid of 2:1, 1:1, 1:5 or 1:20. The mixture vials were capped, homogenized and allowed to stand in the dark at 25°C for 5h or 50°C for 16h. After derivatization, the vials were kept at -20°C. Before CE analysis, the labeling mixture was diluted with the derivatization borate buffer (10 mM, pH 9.5).

The same procedure was conducted for microwave-assisted derivatization using a microwave synthesis labstation (Start Synth, Milestone, Bergamo). Heating was conducted with microwave irradiation under 680 W at constant temperature of 80°C for only 150s.

Blank assays were conducted to evaluate the stability of FITC in the derivatization conditions. For this, FITC was prepared in the derivatization borate buffer and heated at 80°C for 150s by MW or by conventional heating.

## 2.6. Amino acid extraction from *Dunaliella salina* microalgae

-*Electrochemical / electroporation extraction*: a homemade device was used for amino acid extraction studies. It consisted of two parallel platinum electrode wires, each one having a surface area of 0.6 cm<sup>2</sup> (1 cm length x 0.1 cm radius), connected to the positive and negative outlets of a DC (AL841B 30VA – ELC, Annecy, France) or an AC power supply (Evolution F6F12 100VA – Jeulin, Evreux, France). The inter-electrode gap was 1 cm. Two samples of *Dunaliella salina* (DS) cultivated under different conditions (normal and stressed by adding NaCl to medium culture) were analyzed. 50 mg of DS were suspended in 20 mL of distilled water (2.5 g.L<sup>-1</sup>) and placed in glass vessel on a magnetic stirrer. The sample was stirred constantly to prevent cells from settling out of suspension over the time-course of extraction and to maintain steady the diffusion around the electrodes. Extraction was performed by applying voltage (6, 12 or 36 V) to the suspension for certain duration. After each extraction, 1 mL of the suspension (pH ~8.4) was collected, centrifuged and the supernatant was stored

when not in use at  $-20^{\circ}\text{C}$ . Before CE–LIF analysis, FITC was added to the suspension for amino acid derivatization as previously described. Extraction conditions were optimized by quantifying the extracted amino acids by CE–LIF. In optimal extraction conditions, the electrochemical extraction was repeated twice ( $n=2$ ).

The effect of the electric field on the *Dunaliella salina* (DS) cells was evaluated using a scanning electron microscope (Hitachi S-45000, Hitachi High-Technologies Europe GmbH, Japan). Samples were lyophilized and carbon-coated before SEM-FEG (field emission gun) images (obtained at 2 kV voltage).

–*Accelerated solvent extraction (ASE)*: a Dionex accelerated solvent extractor ASE-150 (Thermo Fisher Scientific, Courtaboeuf, France) was used. The lyophilized powder of microalgae (50 mg) was extracted in 5 mL stainless-steel vessel with water (15 mL) as solvent using three static cycles for 10 min each, a flush volume of 70% and a purge with nitrogen gas of 100 s at the end of each extraction. Extractions were carried out at  $130^{\circ}\text{C}$  and under a pressure of 110 bars. Water solvent was evaporated under vacuum using a rotary evaporator (Buchi Labortechnik AG, Switzerland) to obtain a dried crude extract. Extraction was repeated twice ( $n=2$ ). The residue was recovered in 1 mL of derivatization buffer for labeling before CE–LIF analysis. It was stored when not in use at  $-20^{\circ}\text{C}$ .

### 3. Results and discussion

CE–LIF was chosen for the analysis of amino acids extracted by electroporation from *Dunaliella salina* microalgae. The fluorescent labeling of amino acids by fluorescein isothiocyanate as well as electrophoretic separation conditions were optimized to obtain short labeling time and high sensitivity of the CE–LIF method.

#### 3.1. Labeling of amino acids with fluorescein isothiocyanate isomer I

All amino acids (Asn, Gln, Arg, Glu, Orn, Ile, Cit and Ala) selected possess a primary amino group, which may react with the isothiocyanate group of FITC to form the fluorescent derivatives. Labeling with FITC is commonly carried out in borate buffer to minimize the hydrolysis of the labeling agent and consequently the number of undesired fluorescent byproducts [45, 46]. Moreover, the derivatization reaction must be performed under alkaline conditions [47] to favor the nucleophilic addition of the neutral deprotonated amino group to the double bond of the FITC. Therefore, 10 mM sodium tetraborate at pH 9.4 was used as derivatization buffer for developing the amino acid CE–LIF analysis method.

Different conditions were tested to optimize the yield of the labeling reaction, which was evaluated by CE using corrected peak-areas of each derivative. The parameters studied were the ratio of FITC and amino acids, and the reaction time and temperature. The concentration of FITC was optimized while keeping amino acid concentration constant (1  $\mu$ M). The tested ratios of FITC to amino acids were 2:1, 1:1, 1:5 and 1:20. The obtained mixture was homogenized and allowed to react in the dark at room temperature (25°C) for 5h or at 50°C for 16 h. The use of low concentration of FITC resulted in poor sensitivity. The increase in the concentration of FITC induced an increase in signal magnitude of amino acid and also of additional interfering signals. Blank assays (section 2.5) confirmed that these additional peaks are mainly due to impurities found in the FITC standard used (purity 98%). The electrophoretic profiles obtained for the blank assay before and after heating were almost identical. Conventional heating had thus little influence on FITC stability. Therefore, the FITC/analyte ratio of 1:20 was chosen all along the study as a compromise between detection sensitivity and interference from FITC.

The efficiency of the derivatization reaction depends on both time and temperature. For this, the labeling reaction was successively conducted at 25, 37 and 50°C (Figure 1) using convective heating. The highest derivative concentrations were achieved in 5 h at 50°C

whereas 16 h were needed to obtain similar results at 37°C. Further experiments were conducted to reduce the derivatization time. Performing microwave-assisted derivatization allowed the acceleration of the labeling reaction that was completed within only 150 s at 680 W and 80°C (Figure 1). Comparable results in terms of sensitivity were obtained by conventional convective heating but with longer reaction times (120 times). These results are consistent with literature, demonstrating that microwaves can be very efficient for remarkably accelerating fluorescent tagging [37, 48]. As for conventional convective heating, blank assays conducted (section 2.5) confirmed that MW heating had low effect on FITC stability. For the remaining of this study, microwave-assisted derivatization of amino acids with FITC in alkaline borate buffer was used for CE–LIF analysis.

### 3.2. Analysis of labeled amino acids by CE with LIF detection

Borate buffer was used as the BGE because it provides a stable EOF as reported by several authors [4]. As shown in Figure 2.a, a good separation was obtained for a standard mixture containing 1  $\mu$ M of D-Gln, D-Arg, L-Orn, L-Cit,  $\beta$ -Ala and L-Asn using 10 mM borate buffer (pH 9.4) as BGE. Peaks for all analytes were baseline resolved in less than 10 min. L- $\alpha$ -Alanine and L- $\beta$ -alanine could not be separated in these conditions. Several attempts were made to achieve full separation by varying pH value (8.0, 8.6, 9.0, 9.4, 10.0, 10.5 and 11.0) but no significant improvement was obtained. Native  $\alpha$ - and  $\beta$ -cyclodextrins (CD) were added to the BGE. The  $\alpha$ -CD did not improve resolution whereas  $\beta$ -CD improved Ala isomers resolution. For this,  $\beta$ -CD concentration (2.5, 5, 7.5, 10 and 15 mM) as well as the borate buffer concentration (10, 20, 30, 50, 75 and 100 mM) were optimized. Best results were obtained using the following BGE: 100 mM sodium tetraborate and 10 mM  $\beta$ -cyclodextrin at pH 9.4. Excess current of 240  $\mu$ A was observed with 75  $\mu$ m internal diameter capillary engendering high Joule heating whereas acceptable current of 140  $\mu$ A was obtained with a 50  $\mu$ m capillary. Figure 2.b shows an electropherogram obtained in these conditions

for the analysis of D-Ile, D-Gln, L-Asn,  $\beta$ -Ala and  $\alpha$ -Ala. Additional peaks observed on electropherograms are due to FITC impurities and not to the heating technique that had trivial influence on compounds stability. The developed CE-LIF method was validated in terms of linearity. Calibration linear curves were obtained for the different studied amino acids with  $r^2$  higher than 0.9975. Excellent sensitivity was found with a LOQ of a few nM for the different amino acids (3–9 nM). LOQs were obtained by injecting and analyzing each labeled amino acid at the estimated value for  $S/N=10$ . This so-called instrumental LOQ is different from the limit of derivatization which expresses the minimum amount of the analyte that can react with the fluorescent reagent. Repeatability was also satisfactory with RSD on migration times and on peak areas inferior to 1.2 and 1.9% ( $n=6$ ), respectively.

### 3.3. Analysis of amino acids in microalgae after electroporation extraction

The previously developed CE-LIF method was applied to the analysis of amino acids in *Dunaliella salina* microalgae. These microalgae are well-known source of carotenoids ( $\beta$ -carotene), lipids, vitamins and proteins. Electrical treatment of algae has been recently shown by Dagherir et al. [49] to be very efficient in damaging cell membranes helping lipid extraction from microalgae. Extraction efficiency is a function of the applied voltage intensity and the time when the voltage is applied. Different direct voltages were applied (6, 12 and 36 V) for different durations (Table 1). At low voltage 6 V DC, no extraction was obtained due certainly to the incapacity of this electric field to damage the microalgae membrane. For higher direct voltages (12 and 36 V), extraction was successful and three amino acids (D-Arg, L- $\alpha$ -Ala and L- $\beta$ -Ala) were detected and identified by CE-LIF. To further understand this process, different extraction times were studied. The Figure 3 summarizes extraction results obtained by electroporation of amino acids from *Dunaliella salina* green algae. As it can be seen, the best extraction results were obtained at 12 V DC for 4 min. When this voltage was applied for a longer time (10 min), the amount of extracted

amino acids decreases (−20%) due certainly to their degradation by electrochemical reactions in contact with the electrode surface. Figure 4 shows the electropherograms for the analysis of *Dunaliella salina* extracts obtained by applying 12 V DC voltage for different times. Several amino acids have been identified such as D-Arg, L- $\alpha$ -Ala and L- $\beta$ -Ala. Identification was done by migration time matching and by spiking the samples with labeled amino acids. On the other hand, good extraction was also obtained when applying pulses of higher voltage *i.e.* 36 V DC during only 20s.

To conclude, best extraction was obtained at lower and long-lasting voltage (12 V DC x 4 min). More precisely, extraction is probably due to iontophoresis phenomenon implicating the motion of ions across the cell membrane under the influence of an electric field. For analytes such as amino acids, both electromigration as well as electroosmosis contribute to the iontophoretic extraction across the damaged membrane, resulting in increased analyte extraction. In other terms, extraction is mainly due to electroporation (increasing cell permeability) as well as to amino acid migration from the cell into the surrounding solvent.

Finally, an alternative voltage (12 V AC, 50 Hz) has been tested applied for 4 or 10 min to perform the extraction by electroporation but did not engender any extraction as confirmed by CE-LIF. Indeed, when using alternative voltage, the iontophoretic extraction was reduced. Only reversible pores which reclosed rapidly may be obtained in these conditions.

In the optimum conditions (DC 12 V x 4 min), the reproducibility of the extraction method was excellent with RSD inferior to 3.9% on peak areas ( $n=3$ ). SEM-FEG images of the *Dunaliella salina* microalgae cells before and after electrical treatment show the change in cell morphology and illuminate the morphological impacts on disrupted cells. After application of the electrical field, the shapes of the cells became less defined confirming electroporation.

To confirm the obtained results, the conventional extraction method ASE was conducted using the same conditions as for the electroporation extraction (50 mg of *Dunaliella salina* extracted with 20 mL of water). D-Arg and L-Ala were extracted using both methods, but approximately eight times lesser using ASE than electroporation; 0.1 versus 0.8  $\mu\text{M}$  for D-Arg and 1.0 versus 9.2  $\mu\text{M}$  for L-Ala. These results prove the efficiency of the developed extraction method based on electroporation. Moreover, it is worthy to note that the sensitivity of the CE–LIF method was essential to directly detect amino acids in the extracts at low range of nanomolar with no need of any pre-concentration step.

The herein developed electroporation–CE–LIF method was then used to study the effect of the cultivation conditions on the amino acid quantity in microalgae (Figure 5). Indeed, the environmental constrains increase the productivity of biologically active molecules by microalgae [38]. The same amino acids were found in the two cultivations. However, our results showed that the imposed stress conditions induced the production of a higher quantity of amino acids in *Dunaliella salina* than in normal conditions. For both D-Arg and L-Ala, the concentration is ten times higher in the stressed algal cultivation. Indeed, it has been shown that alanine accumulation in plants and animals in response to exposure to a variety of stress conditions is a general phenomenon. Alanine is a universal first stress signal expressed by cells[50, 51].

#### **4. Concluding remarks**

A new CE–LIF method was developed for the determination and the identification of non-standard and standard amino acids. Microwave-assisted derivatization (680 W, 80°C) was used to minimize the fluorescent labeling time to 150 s. This analytical process has been successfully applied for the determination of amino acids in microalgae extracts. A novel extraction technique for amino acid based on electroporation of cell membrane has been

introduced. Best extraction is obtained either after a long exposure of the vegetal cells to a low voltage (12 V DC, 4 min) or a short exposure to a higher voltage (36 V DC, 20 s), both of which gives good extraction yields for amino acids. The developed electroporation-CE/LIF method was used to study the influence of the cultivation conditions on amino acid expression in microalgae. Our results showed that stress conditions induced the production of amino acids in *Dunaliella salina* confirming that alanine is a universal first stress signal expressed by cells.

In this study, we present a proof-of-concept demonstration of a green approach to extract and analyze amino acids in microalgae. Further optimization will be necessary to identify more than two amino acids in microalgae and to expand this approach for studying varied molecular families in other algae. Several algae products have attracted great interest due to their potential practical application as pharmaceutical agents, cosmetic ingredients, energy sources, valuable food constituents, and future materials for nanotechnology.

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*The authors declare that there are no conflicts of interest.*

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List of figures

Figure 1. Effect of heating procedure and time upon derivatization yield of amino acids by FITC.

The derivatization yield 100% corresponds to the maximum peak area obtained using microwave-assisted derivatization.

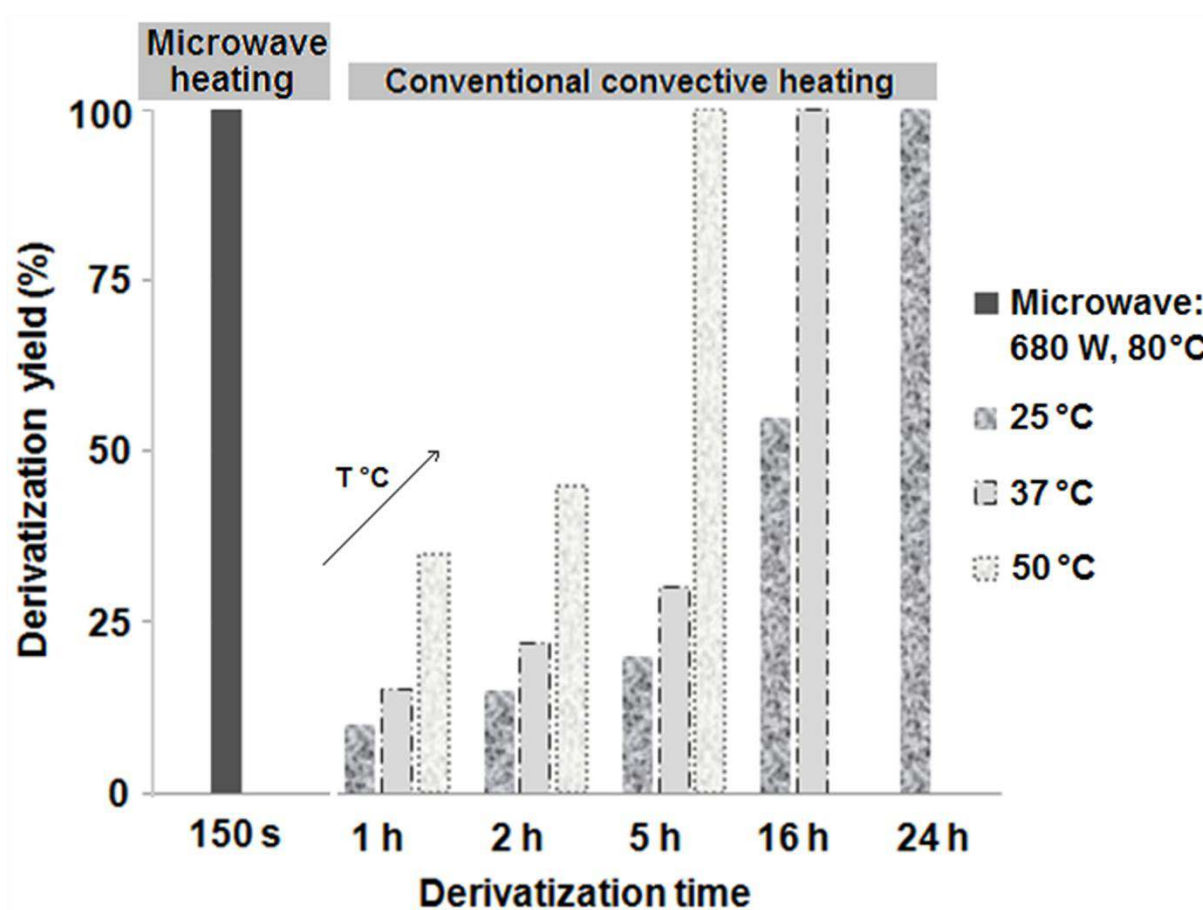


Figure 2. Separation of standard mixtures of amino acids labeled with FITC by CE-LIF.

Separation voltage: + 25 kV; hydrodynamic injection: 0.5 psi for 5 s; excitation and emission wavelengths: 488/520 nm; amino acid concentration: 100 nM. [FITC]/[a.a.] = 1:20

(a) BGE: 10 mM sodium tetraborate buffer (pH 9.4); capillary: 60 cm x 50  $\mu$ m x 75  $\mu$ m I.D.; derivatization: 50°C x 5 h.

(b) BGE: 100 mM sodium tetraborate buffer + 10 mM  $\beta$ -CD (pH 9.4); capillary: 60 cm x 50  $\mu$ m x 50  $\mu$ m I.D.; microwave assisted derivatization: 150 s x 680 W.

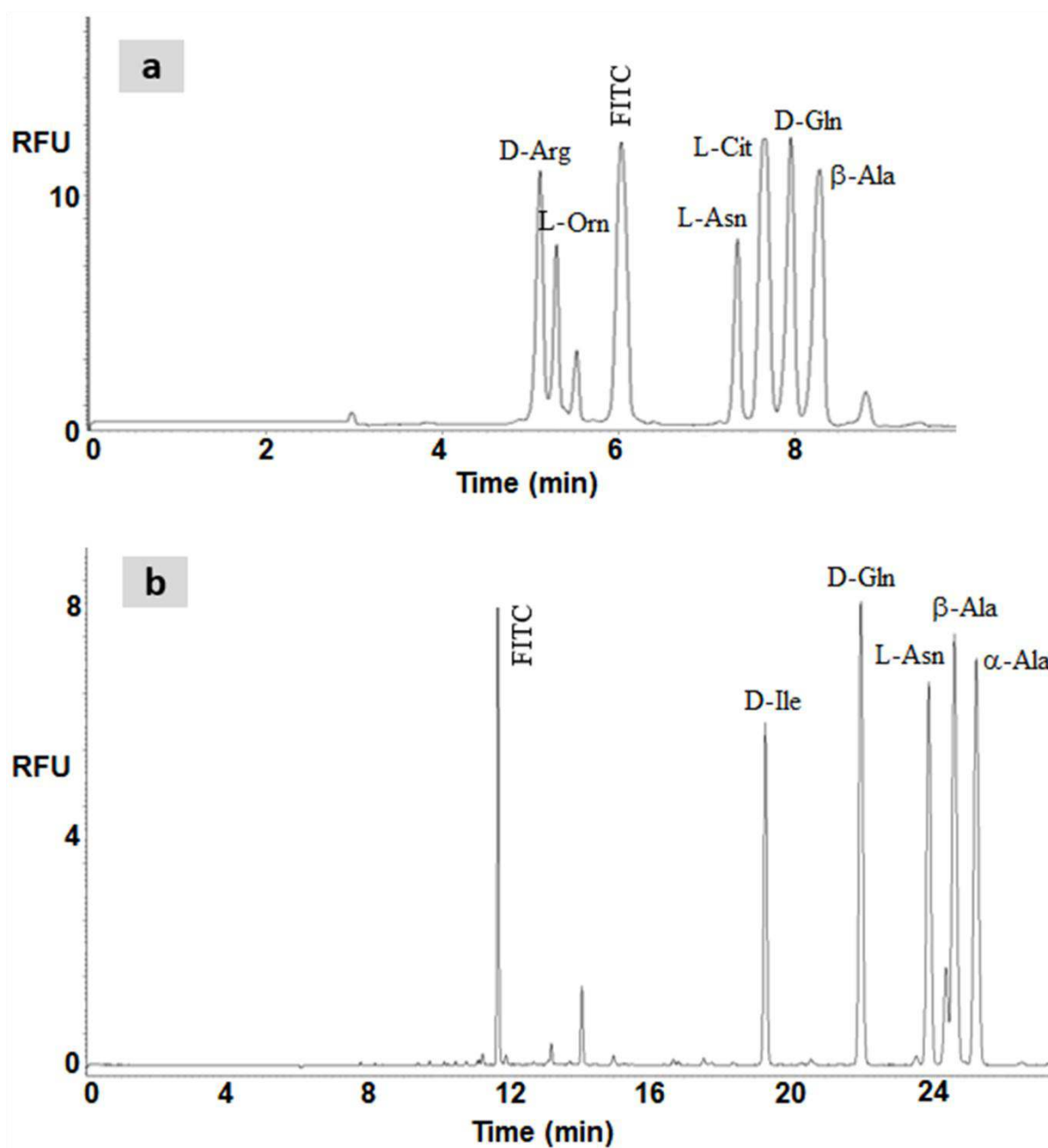


Figure 3. Effect of voltage and extraction time on the concentration of amino acids extracted from *Dunaliella salina* microalgae by electroporation.

Extraction conditions: 50 mg microalgae suspended in 20 mL water. Other conditions: see Figure 2.b.

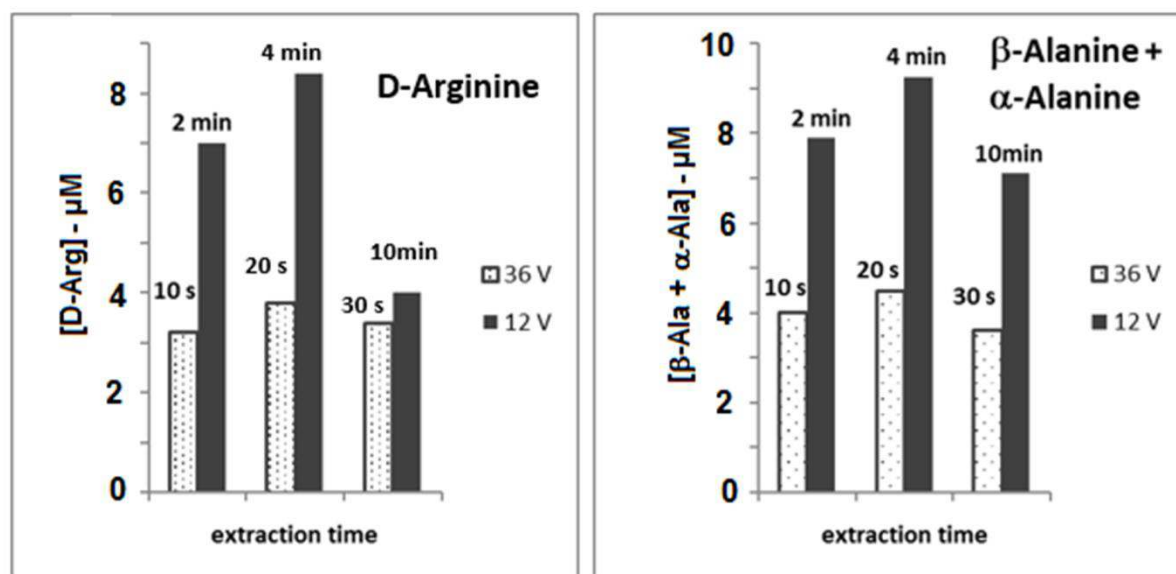


Figure 4. Amino acids identified in *Dunaliella salina* microalgae sample extracted by electroporation.

Other conditions: see Figure 3.

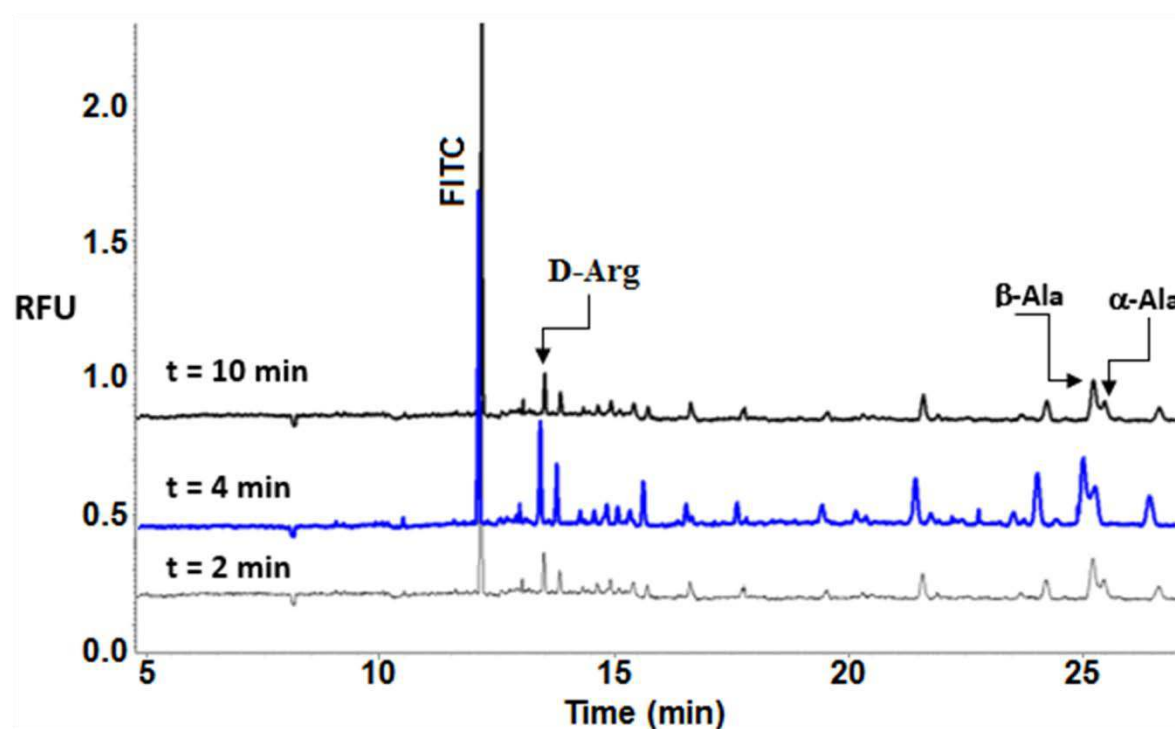


Figure 5. Effect of microalgae cultivation conditions (normal and stressed) on the amount of total extracted amino acids.

Extraction conditions : 12 V DC x 4 min. Other conditions: see figures 2 and 3 and section 2.3.

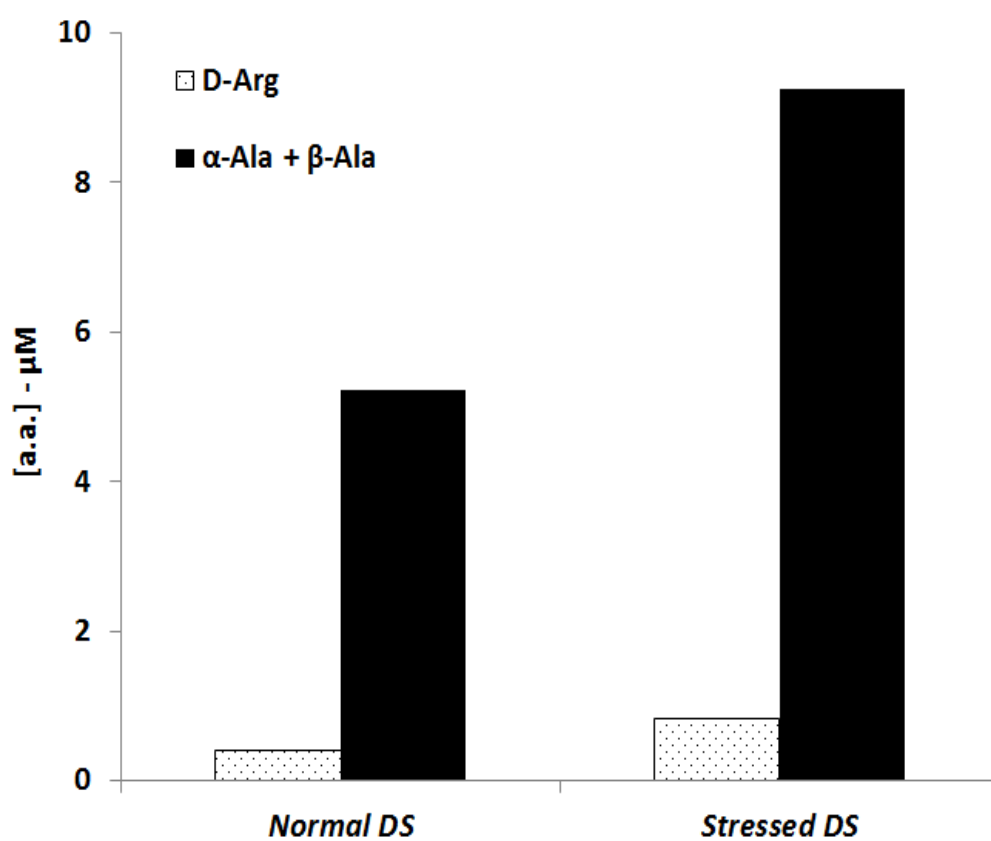


Table 21. Electrochemical treatment (voltage and extraction time) tested for the extraction of amino acids from *Dunaliella Salina* microalgae.

Voltage (V)	Extraction time	Amino acids identified
6	2, 4, 6, 10 min	No extraction
12	2, 4, 6, 10 min	D-Arg, $\alpha$ -Ala, $\beta$ -Ala
36	10, 20, 30, 40, 50 s	D-Arg, $\alpha$ -Ala, $\beta$ -Ala
12 (alternative voltage)	2, 4, 6, 10 min	No extraction