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Pressurized liquid extracts from *Spirulina platensis* microalga Determination of their antioxidant activity and preliminary analysis by micellar electrokinetic chromatography☆

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Abstract

In this work, different extracts from the microalga *Spirulina platensis* are obtained using pressurized liquid extraction (PLE) and four different solvents (hexane, light petroleum, ethanol and water). Different extraction temperatures (115 and 170 °C) were tested using extraction times ranging from 9 to 15 min. The antioxidant activity of the different extracts is determined by means of an in vitro assay using a free radical method. Moreover, a new and fast method is developed using micellar electrokinetic chromatography with diode array detection (MEKC–DAD) to provide a preliminary analysis on the composition of the extracts. This combined application (i.e., in vitro assays plus MEKC–DAD) allowed the fast characterization of the extracts based on their antioxidant activity and the UV–vis spectra of the different compounds found in the extracts. To our knowledge, this work shows for the first time the great possibilities of the combined use of PLE–in vitro assay–MEKC–DAD to investigate natural sources of antioxidants.

Keywords: *Spirulina platensis*; Pressurized liquid extraction; Food analysis; Phenols; Carotenoids; Polyphenols; Antioxidants

1. Introduction

The growing interest in natural foods has raised the demand for natural ingredients that in addition to give basic nutritional and energy, are capable to contribute with additional physiological profits as antioxidants, antimicrobial, etc, that is, functional foods or nutraceuticals. In this work, the microalga *Spirulina platensis* is investigated as natural source of antioxidants, an important kind of compounds for the food industry because of their usefulness as a preservation method and their known beneficial effects for health.

One of the research lines of our group is to obtain and characterize natural compounds with antioxidant properties that can be used as ingredients in the food industry [1–5]. Towards this aim, we have developed different purification methodologies based on the use of sub- and supercritical fluid extraction (SFE) to obtain functional compounds from natural sources such as rosemary, olive oil, orange juice, etc It is interesting to mention that traditional extraction methods used to obtain these type of products have several drawbacks, namely, they are time consuming, laborious, have low selectivity and/or low extraction yields. Moreover, these traditional techniques employ large amounts of toxic solvents. At present, new extraction methods able to overcome the above mentioned drawbacks are under study, pressurized liquid extraction (PLE; Dionex trade name ASE for accelerated solvent extraction) being one of the most promising processes [6]. This extraction technique provides higher selec-

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tivity, shorter extraction times and frequently does not require large amounts of toxic organic solvents; furthermore, water can be used as medium polarity solvent when working at high temperatures and moderate pressures, that is, at subcritical conditions. A drawback of PLE is its requirement of special instrumentation in order to get relatively high pressures together with high temperatures. Moreover, there are not data available on solubility of natural compounds in the solvents at the pressures and temperatures employed in PLE.

It has to be mentioned here the large number of known compounds, of both synthetic and natural origin, with antioxidant potential for retarding oxidative damage that have been documented in the literature. Various in vivo and in vitro systems have been devised in order to test these agents for their effectiveness [7]. In vitro systems are by far easier, faster and more cost-effective compared to rodent bioassays, and therefore are the most typically used as initial screening systems (e.g., 2,2-diphenyl-1-picylhydrazyl hydrate) to adequately characterize such fractions. However, the information that they provide is specific for the reactive compound (mostly radicals) selected to perform the assay [8].

On the other hand, the isolated fractions obtained with the mentioned extraction methods are usually composed of active substances plus some other impurities, therefore, a separation-identification step has to be also developed in order to adequately characterize them [9,10]. Among the different analytical procedures employed to analyze these fractions, reversed-phase high-performance liquid chromatography (RP-HPLC) has been widely applied for this purpose [9,10].

Regarding this point, the suitability of this methodology to fully identify the antioxidant fraction of rosemary extracted by SFE [11] or subcritical water (SWE) [12] has also been demonstrated. An important limitation of RP-HPLC is that this technique is not suitable to simultaneously separate highly polar compounds from the less polar ones, since the first ones usually coelute with the dead volume (i.e., unretained) [4,13]. This limitation can reduce considerably the information provided by RP-HPLC regarding the compounds present in the extracts. Besides, it has to be kept in mind that this problem can be even more complex if a relatively polar procedure as subcritical water extraction is employed. Moreover, it has been frequently mentioned in the literature the important antioxidant activity related to polar compounds from e.g., rosemary [14–16], effect that can be logically expected from other different natural sources as microalga.

We have shown in previous works that capillary electrophoresis with diode array detection (CE–DAD) can be used as a good alternative to characterize in a fast way subcritical and supercritical fluid extracts from natural sources. Moreover, CE usually provides shorter analysis times than HPLC, higher efficiencies and, more interestingly, it can provide complementary information about highly polar compounds that cannot be adequately separated by HPLC [4,13]. On the other hand, it is also known that CE is less sensitive than HPLC, while HPLC provides better reproducibility than CE when used with quantitative aims [17].

Taking into account both the interest of the food industry in this type of compounds (i.e., natural antioxidants) and the novelty of the combined use of PLE and CE, the goal of this work was, firstly, to demonstrate the usefulness of PLE to provide a high variety of extracts from microalga *Spirulina platensis* employing different polarity solvents together with different extraction temperatures. Secondly, in order to obtain a fast profile of the different pressurized liquid extracts, a new CE–DAD method is developed. Moreover, an in vitro assay is applied to characterize the biological activity of each extract that can be correlated with the CE–DAD profile obtained. To our knowledge, this work shows, for the first time, the great possibilities of the combined use of PLE–in vitro assay–micellar electrokinetic chromatography MEKC–DAD to investigate natural sources of antioxidants.

2. Materials and methods

2.1. Samples and chemicals

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

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% purity) was obtained from Sigma–Aldrich (Madrid, Spain). Sodium dodecyl sulfate (SDS) was purchased from Acros Organics (NJ, USA). Boric acid was obtained from Riedel-De Haën (Hannover, Germany). Methanol and ethanol were obtained from Scharlau Chemie (Barcelona, Spain). Hexane, HPLC grade, provided from Lab Scan (Dublin, Ireland) and light petroleum (b.p. 40–60 °C) was purchased from Panreac Quimica (Barcelona, Spain). The water used was Milli-Q Water (Millipore, Billerica, MA, USA).

2.2. PLE

To perform the extractions with the four different solvents (i.e. hexane, light petroleum, ethanol, and water, see Table 1) an ASE 200 system from Dionex (Sunnyvale, CA, USA) was used, equipped with a solvent controller. Extractions were performed at two different extraction temperatures (115 and 170 °C) and different extraction times (9 and 15 min). Previous to each extraction an extraction cell heat-up was carried out for a given time, which changed according to extraction temperature. Namely, 6 min heat-up was used when extraction temperature was set at 115 °C and 8 min at 170 °C. Likewise, all extractions were performed in 11 ml extraction cells, containing 2.5 g of sample.

Extraction procedure was as follows: (i) sample was loaded into cell, (ii) cell was filled with solvent up to a pressure of 1500 p.s.i (1 p.s.i. = 6894.76 Pa), (iii) heat-up time was applied, (iv) static extraction takes place (i.e. at 9 or 15 min) in which all system valves are closed, (v) cell is rinsed (with

Table 1
Antioxidant activity (given as EC_{50}, μg/ml) and dried extract obtained (mg) depending on the solvent, time and temperature used in the PLE process

Solvent (dielectric constant)	Extraction temperature (°C)	Extraction time (min)	Dried extract obtained (mg)	EC_{50} (μg/ml)
	115	9	43.1	47.5
Hexane (1.9)	115	15	45.1	47.3
	170	9	94.0	86.9
	170	15	95.5	69.4
	115	9	37.6	52.6
Light petroleum (4.3)	115	15	38.6	62.8
	170	9	77.0	91.2
	170	15	66.6	95.6
	115	9	121.7	63.9
Ethanol (24.3)	115	15	166.2	66.8
	170	9	270.1	100.1
	170	15	261.7	98.7
	115	9	160.0	394.5
Water (78.5)	115	15	170.5	373.0
	170	9	285.5	279.3
	170	15	290.0	287.2

Pressure was in all cases 1500 p.s.i.

60 % cell volume using extraction solvent), (vi) solvent is purged from cell with N_2 gas and (vii) depressurization took place. Between extractions a rinse of the complete system was made in order to overcome any carry-over. The extracts obtained were protected from light and stored under refrigeration until dried. For solvent evaporation a Rotavapor R-200 (from Büchi Labortechnik, Flawil, Switzerland) was used when the extracts were obtained with organic solvents and in case of water extracts a Freeze Dryer Unitop 400 SL (from Virtis, Gardiner, NY, USA) was used. Afterwards, different extract solutions were prepared using the same solvent than during extraction, at a known concentration. In the same way, the solutions were stored at 4 °C and protected from light. The water was purged with nitrogen to remove dissolved oxygen prior to the extraction avoiding in this way any possible oxidation [12]. When water was used as extracting solvent, care must also be taken with the clogging of the extractor lines by the extracted material. To avoid clogging, the microalgae was placed inside a filter paper and the extraction procedure was performed as mentioned.

2.3. Antioxidant activity determination (in vitro assay)

Antioxidant activity was measured in all extracts obtained using a method based on a procedure described by Brand-Williams et al. [18]. The method consists of the neutralization of free radicals of DPPH by the extract antioxidants. A solution was prepared dissolving 23.5 mg of DPPH in 100 ml methanol. This solution was stored at 4 °C. To do the measurements, this stock solution was diluted 1:10 on methanol. Different concentrations of the extracts solutions were used. Then 0.1 ml of these solutions were added to 3.9 ml diluted DPPH solution to complete the final reaction medium (4 ml). Due to the colored extracts it was necessary to prepare a control (i.e. blank) that consisted of 0.1 ml of each solution added to 3.9 ml of methanol. The reaction was complete after 4 h at room temperature, and the absorbance was measured at 516 nm in a UV–vis Lambda 2 spectrophotometer from Perkin-Elmer (Wellesley, MA, USA). Methanol was used to adjust the zero. The absorbance value was obtained by subtracting the blank absorbance measurement to the value given by the extracts solution. The method was calibrated using DPPH solutions of different concentration that allowed knowing the DPPH concentration remaining when reaction was finished. This calibration curve ($n = 7$; $r = 0.999$) gave the following equation: [DPPH] = (Abs + 0.0029)/0.0247. For each extract five different concentration solutions were prepared in order to obtain the remaining DPPH concentration when reaction was finished; using these values a curve was generated that allowed the estimation of the extract concentration necessary to achieve a 50% reduction of the initial DPPH concentration. This value is known as EC_{50} (efficient concentration, also called oxidation index) and was utilized to describe the antioxidant activity.

2.4. MEKC–DAD analysis

Buffers were prepared by dissolving into water an appropriate amount of sodium tetraborate with or without SDS. All of them were adjusted to the desired pH by adding aliquots of NaOH 0.1 M and measured by a pH meter (model 692, Metrohm, Herisau, Switzerland), with combined LL pH glass electrode.

Before first use, a new capillary was preconditioned rinsing with 1 M NaOH for 15 min, followed by a 15 min rinse with 0.1 M NaOH and 15 min with deionized water. At the start of an each day, the capillary was conditioned by rising with 0.1 M NaOH for 3 min, followed with the separation electrolyte for 15 min. Between introductions of samples, the capillary was rinsed with water for 2 min followed by a rinse with the separation electrolyte for 2 min. At the end of each day, the capillary was rinsed with deionized water for 5 min.

Table 2
Micellar electrokinetic chromatography

System	P/ACE 5500 with diode array detector
Capillary	Fused silica capillary with 75 μm i.d., 37 cm total length and 30 cm length to the detector
Voltage	15 kV
Temperature	25 °C
Injection	3 s (0.5 p.s.i.)
Detector	Channel A: 200 nm Channel B: 280 nm UV spectra: from 190 to 600 nm
Samples	Concentration: 5 mg/ml for hexane, light petroleum and ethanol extracts Samples obtained with water extraction, concentration: 10 mg/ml
Buffer solution	50 mM sodium tetraborate, 100 mM SDS at pH 8.8

Optimum conditions.

All extracts were analyzed using MEKC. It was performed with a P/ACE System 5500 from Beckman (Palo Alto, CA, USA) equipped with a DAD system. The fused silica capillary used was purchased from Composite Metal Services (Ilkley, UK). The optimum MECK parameters are shown in Table 2.

3. Results and discussion

3.1. Obtaining natural extracts from the microalga *Spirulina platensis* using PLE

In order to obtain a large variety of extracts from the microalga *Spirulina platensis*, PLE and four different solvents (hexane, light petroleum, ethanol and water) were used. The choice of the solvent is based on their different polarity with dielectric constants equal to 1.9 for hexane, 4.3 for light petroleum, 24.3 for ethanol and 78.5 for water. Thus, a large variety of extracts can be obtained by using such different solvents. Different extraction temperatures, 115–170 °C were selected along with different extraction times (9 and 15 min).

Table 1 shows the conditions selected to perform the microalga pressurized liquid extractions with the four solvents along with the antioxidant activities obtained for the different fractions (measured using the DPPH free radical assay EC_{50}) and the extraction yield (given as dry-extract).

From these results it can be deduced that the higher the extraction time, temperature and/or dielectric constant of the solvent the higher the extraction yield. Thus, as can be seen in Table 1, using water and ethanol the yield was clearly much higher than with the non-polar solvents under identical conditions of temperature and time of extraction. This result can be explained through the composition of Spirulina [19]; thus, this microalga is composed of 50–70% of protein and about 15% of carbohydrates [19]. Therefore, it is expected that by using more polar solvents these polar compounds can be extracted in a higher extent, increasing in this way the extraction yield obtained. Also, it can be seen that for the same solvent the extracted amount increases using higher extraction times and/or higher extraction temperatures.

3.2. Development of a new MEKC–DAD method to analyze microalga PLE fractions

As mentioned above, to our knowledge, there is no published work either on the use of PLE to obtain alga extracts or on the use of CE methods to study alga fractions. As a matter of fact, there are very few works dealing with this type of natural source what gives some idea about the novelty of our approach. Therefore, a new CE method had to be developed in order to characterize these new extracts.

In order to carry out this CE optimization, a univariate method was applied using a PLE extract obtained with water at 115 °C. This sample was selected for optimize the separation parameters because in a previous screening it was observed that it provided the most complex pattern (i.e., electropherogram) among the different extracts studied.

Six different separation buffers were prepared using different concentrations of SDS (i.e., 0, 20, 40, 60, 80 and 100) with 50 mM sodium tetraborate and tested at the same pH (i.e., 9.0). The results of this experiment are given in Fig. 1. As can be seen, the resolution is slightly better using the

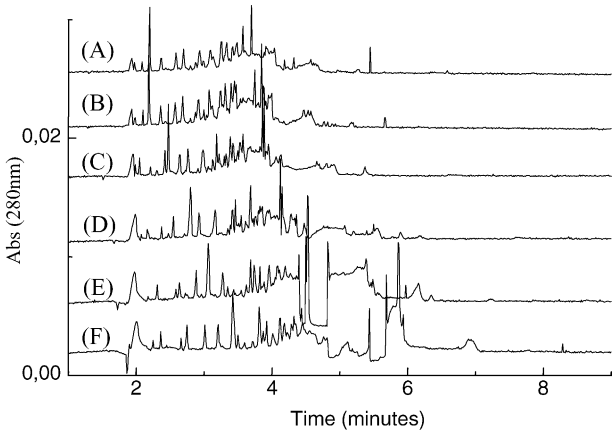

Fig. 1. Effect of concentration of SDS into buffer separation during MEKC analysis of a pressurized liquid extract from *Spirulina platensis* obtained using water at 115 °C for 9 min. Buffer separation: 50 mM sodium tetraborate at pH 9.0 with (A) 0 mM SDS; (B) 20 mM SDS; (C) 40 mM SDS; (D) 60 mM SDS; (E) 80 mM SDS and (F) 100 mM SDS. Other MEKC conditions as in Table 2.

100 mM SDS buffer (Fig. 1F), as can be deduced comparing for instance this electropherogram with that obtained without SDS (Fig. 1A). From these results it can be deduced that there are a large number of compounds carrying a negative charge (the electroosmotic flow is detected around 2 min) and able to absorb at 280 nm. Besides, it can be seen that the inclusion of hydrophobic analyte-SDS interactions during the electrophoretic separation brings about an improvement of their resolution.

Although the use of this buffer composed of 50 mM sodium tetraborate, 100 mM SDS at pH 9 brought about a good separation, still some compounds give rise to broad and overlapped peaks (see e.g. in Fig. 1F the zone between 4 and 6 min). Therefore, a further optimization was required. To do this, the pH of the optimum buffer (50 mM sodium tetraborate, 100 mM SDS) was modified from 8.8 to 9.2 in order to study if these slight variations could improve the separation resolution. As can be seen in Fig. 2, the use of a buffer at pH 8.8 (Fig. 2A) brings about a slight improvement of the separation mostly among the compounds migrating after 4 min. Some other strategies were applied in order to improve this separation. Namely, different organic solvents, different separation temperatures and/or different concentrations of sodium tetraborate were used; however, no noticeable improvement of the separation was observed. Therefore, the optimum buffer solution used to separate these pressurized liquid extracts was composed of 50 mM sodium tetraborate, 100 mM SDS at pH 8.8. Table 2 shows a description of the optimum separation conditions selected to perform the MECK analysis of all the extracts.

Reproducibility of our MEKC method was tested for the same day and three different days using the optimum conditions given in Table 2 and selecting the peaks indicated in Fig. 2. The results obtained are shown in Table 3. As can be seen, the time analysis reproducibility of this new method was adequate as can be deduced from the relative standard deviation values (RSDs) obtained for the same day (lower than 0.19%) and three different days (lower than 0.66%).

Table 3
Method reproducibility intra- and inter-day

Peaks	Same day (n = 5)		Three days (n = 15)	
	Time (min)	RSD (%)	Time (min)	RSD (%)
1	2.66	0.12	2.67	0.40
2	3.57	0.19	3.59	0.57
3	3.94	0.11	3.97	0.65
4	4.12	0.15	4.15	0.66

Peaks are shown in Fig. 2.

3.3. Characterization of microalga extracts using MEKC–DAD and in vitro assays

The developed MEKC method was first used to obtain a rough comparison of the profile from all the pressurized liquid extracts shown in Table 1. It has to be mentioned that the more complex extracts in terms of number of compounds and, therefore, resolution were those obtained at temperatures of 170 °C, and among them the pressurized liquid extracts obtained using water (see above). As an example, Fig. 3 shows a comparison among the electropherograms obtained with the four different solvents at the same extraction conditions (115 °C and 15 min). As can be deduced from Fig. 3, the extracts obtained using hexane (Fig. 3A) and petroleum ether (Fig. 3B) gave a very similar electrophoretic profile as

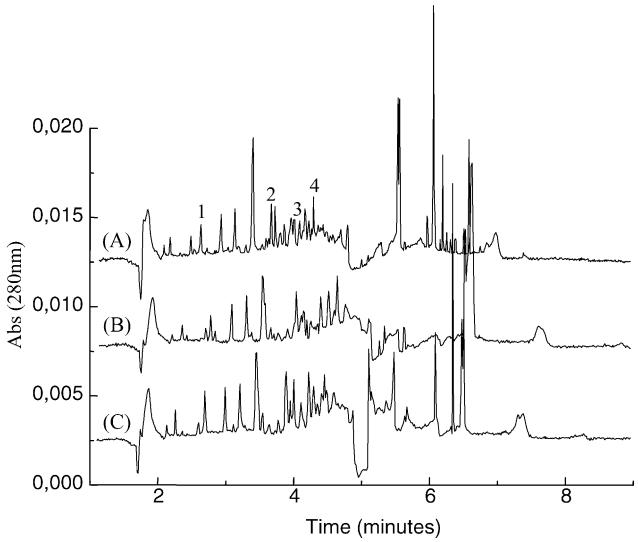

Fig. 2. Effect of buffer separation pH on MECK analysis of a water extract from *Spirulina* microalga. Buffer separation: 50 mM sodium tetraborate, 100 mM SDS at pH: (A) 8.8; (B) 9.0 and (C) 9.2. Other of conditions as in Fig. 1.

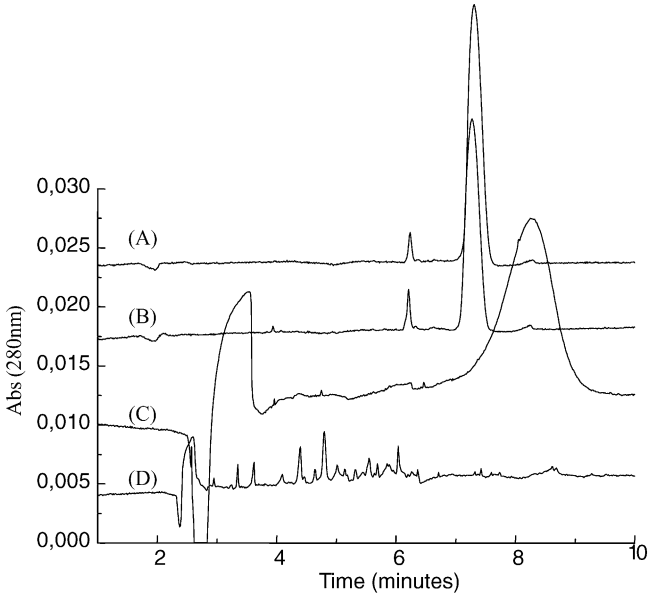

Fig. 3. MEKC comparison of several pressurized liquid extracts obtained using equal temperature (115 °C) and time of extraction (15 min) but different extraction solvent. (A) Hexane; (B) light petroleum; (C) ethanol and (D) water. Separation conditions are indicated in Table 2.

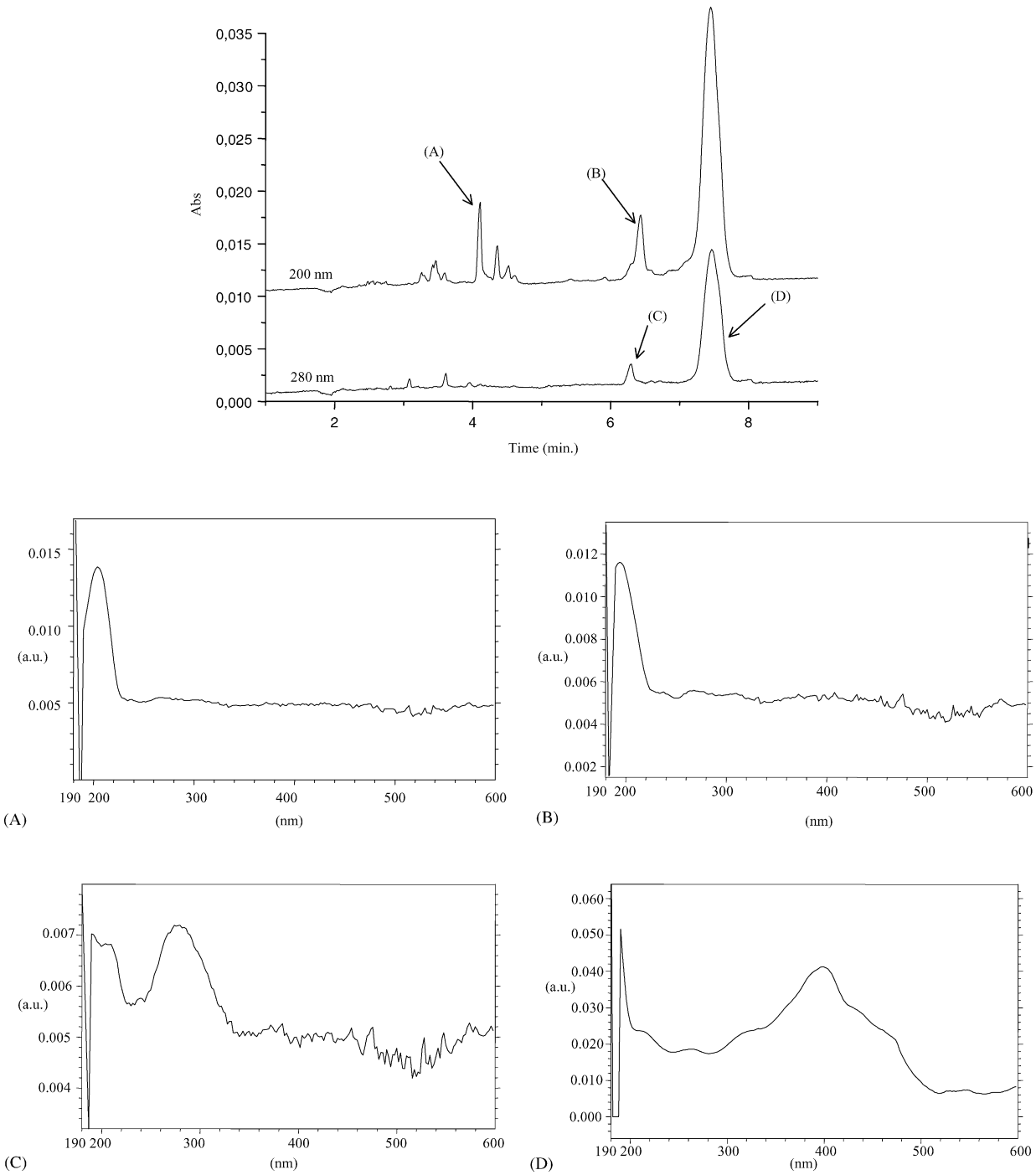

Fig. 4. Comparison of MEKC electropherograms obtained from the same hexane extract (obtained at 170 °C for 15 min) recorded at 200 and 280 nm. UV spectra of four selected peaks (from A to D) are shown. All separation conditions as in Fig. 3.

could be expected from their very similar dielectric constants (1.9 and 4.3, respectively). Interestingly, the ethanol extract (Fig. 3C) shows a MEKC electropherogram clearly different from that obtained with hexane (Fig. 3A), light petroleum (Fig. 3B) and water (Fig. 3D), being the last one the most complex in terms of number of compounds extracted, what can probably be related to the high dielectric constant of water compared with the other solvents.

A further comparison can be established by using the EC_{50} values obtained from the DPPH in vitro experiments of these extracts. Thus, although similar antioxidant capacity seems to be obtained for the microalga extracts obtained using hexane and light petroleum (compare EC_{50} values in Table 1), it can be deduced that in general a slightly better antioxidant activity (i.e., lower EC_{50} values) were obtained for hexane microalga extracts than for light petroleum microalga extracts. This dif-

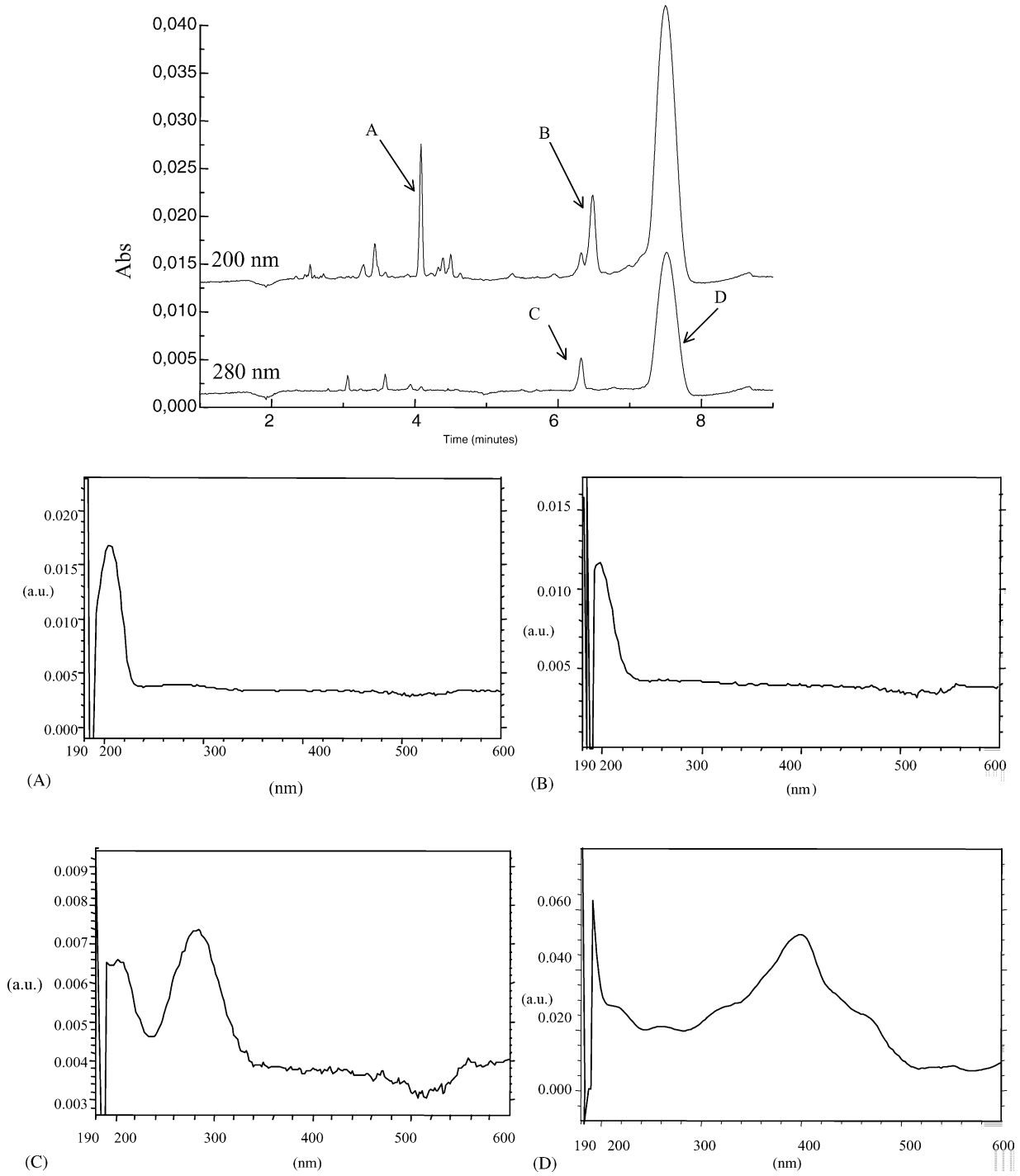

Fig. 5. Comparison of MEKC electropherograms from the same light petroleum extract (obtained at 170 °C for 15 min) recorded at 200 and 280 nm. UV spectra of four selected peaks (from A to D) are shown. All separation conditions as in Fig. 3.

ference can be correlated to the higher amount of the main compound (with a migration time of ca. 7.5 min in Fig. 3) that can be extracted using hexane. In order to corroborate this point, a deep study of these two extracts was carried out by MEKC–DAD. Thus, the electropherograms obtained at 200 and 280 nm together with the UV–vis spectra of the majority compounds, marked as A, B, C and D, are provided for hexane (Fig. 4) and for light petroleum (Fig. 5). These two figures corroborate the high similarity suggested above between these two Spirulina extracts. Moreover, using the peak purity capability provided by the diode array detector and software it could be deduced that compound D is not a pure peak (data not shown). However, from the different UV–vis spectra obtained (see UV–vis spectra taken at the

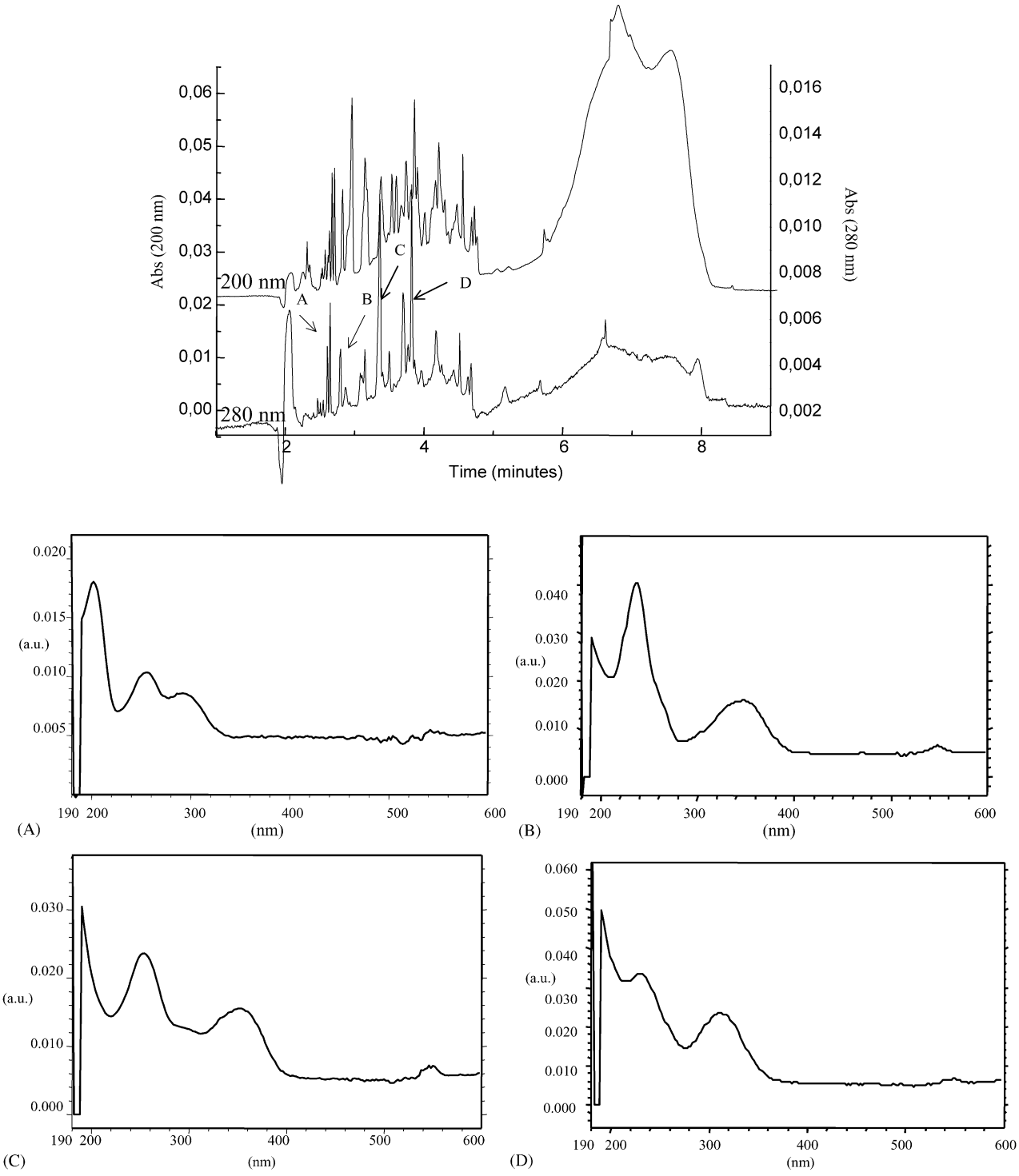

Fig. 6. Comparison of MEKC electropherograms from the same water extract (obtained at 170 °C for 15 min) recorded at 200 and 280 nm. UV spectra of four selected peaks (from A to D) are shown. All separation conditions as in Fig. 3.

apex of peak D and given in Figs. 4 and 5) it could be deduced that the major D compound belongs to the carotenoid family, probably β-carotene, which has been demonstrated to exist in *Spirulina platensis* [19] in a large amount and to possess antioxidant activity. This result seems to confirm that the antioxidant activity of these extracts is mostly linked to this compound; however, some additional effect coming e.g. from compound C (showing a typical polyphenolic UV–vis spectrum) cannot be ruled out.

From Table 1 it can be deduced that the lowest activity was obtained when using water as extracting solvent in the PLE procedure. The MEKC–DAD profile obtained for this

type of extracts (see Fig. 6) shows a very complex pattern indicating that these compounds are mostly polar substances carrying some negative charge. This point is further corroborated by the UV–vis of some majority compounds that show a typical polyphenolic profile. However, by comparing the peak areas of these compounds with those from the main compounds of Figs. 4 and 5 a clear quantitative difference is observed, moreover, considering that the concentration of the water samples injected in MEKC–DAD is two-fold the concentration of the other extracts obtained with hexane or light petroleum. This clear quantitative difference in the composition of these extracts can explain their different antioxidant activity. Therefore, in order to improve the antioxidant activity of the microalga extracts obtained with water some modification of its extraction capabilities is required.

Interestingly, from the EC_{50} values given in Table 1, it can be also deduced that ethanol extracts possess a good antioxidant activity comparable in some cases to that obtained with light petroleum and slightly worse than that obtained with hexane. This property can be used as an additional advantage taking into account that ethanol, unlike hexane or light petroleum, is generally considered as GRAS (i.e., generally recognized as safe) and therefore, can be used as safe solvent for the food industry. Moreover, the yields obtained with ethanol (i.e., dried extract obtained in Table 1) are comparable to those obtained with water and much higher than those obtained with hexane and light petroleum. Therefore, by using mixtures ethanol–water as PLE solvent it is expected to obtain a high yield along with a higher antioxidant power. This work is now being carried out at our laboratory and it will be the subject of a forthcoming paper.

4. Conclusions

In this work, the high potential of the combined use of PLE–in vitro assay–MEKC–DAD has been demonstrated, to our knowledge for the first time, to characterize natural extracts. To do this, the microalga *Spirulina platensis* has been chosen as raw material and pressurized liquid extracts from the microalga have been obtained using hexane, light petroleum, ethanol and water as extracting solvents. The procedure permits to characterize the antioxidant activity of the different Spirulina extracts in a fast way obtaining some interesting information about the composition of the different extracts. It has also been shown that such extracts composition can be tailored by modifying the solvent (i.e., the dielectric constant) during the PLE process. Besides, the new MEKC–DAD method developed is reproducible, and allows to obtain a fast profile of the main compounds obtained under different extraction conditions which could be favorably used to optimize the PLE conditions in a feed-back-like procedure.

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