Accelerated Solvent Extracts from *Spirulina Platensis* Microalga: Determination of their Antioxidant Activity and Analysis by Micellar Electrokinetic Chromatography.

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ABSTRACT

In this work, different extracts from the microalga *Spirulina platensis* are obtained using Accelerated Solvent Extraction (ASE) and four different solvents (hexane, petroleum ether, ethanol and water). Different extraction temperatures (115°C and 170°C) were tested using extraction times ranging from 9 to 15 minutes. 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 analyze 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 ASE-*in vitro* assay-MEKC-DAD to investigate natural sources of antioxidants.

KEYWORDS: ASE, capillary electrophoresis, phenols, carotenoids, polyphenols, natural compounds, alga, subcritical water.

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 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 [6]. *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., DPPH) to adequately characterize such fractions.

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. 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 [7,8]

Regarding this point, the suitability of this methodology to fully identify the antioxidant fraction of rosemary extracted by SFE [9] or subcritical water (SWE) [10] 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) [11,12]. 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 [13-15], 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 can not be adequately separated by HPLC [16,17].

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 ASE and CE, the goal of this work was, firstly, to demonstrate the usefulness of ASE 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 ASE-extracts, a new CE-DAD method is developed. Moreover, an in-vitro assay is applied to characterize the biological activity of each extract which 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 ASE-*in vitro* assay-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 weight, from Algamar S.A. (Pontevedra, Spain), stored under dry and dark conditions.

2,2-Diphenyl-1-picrylhydrazyl hydrate (DPPH, 95% putity) was obtained from Sigma-Aldrich (Madrid, Spain). Sodium dodecyl sulfate (SDS) was purchased from Acros Organics (New Jersey, USA). Boric acid was obtained from Riedel-De Haën AG (Hannover, Germany). Methanol and ethanol were obtained from Scharlau Chemie S.A. (Barcelona, Spain). Hexane, HPLC grade, provided from Lab Scan (Dublin, Ireland) and petroleum ether was purchased from Panreac Quimica S.A. (Barcelona, Spain). The water used was Milli-Q Water (Millipore Corporation, Billerica, MA, USA).

2.2 Accelerated Solvent Extraction (ASE).

To perform the extractions with the four different solvents (i.e. hexane, petroleum ether, ethanol, and water, see Table 1) an Accelerated Solvent Extraction system ASE 200 from Dionex Corporation (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 minutes). Previous to each extraction an extraction cell heat-up was carried out for a

given time, which changed according to extraction temperature. Namely, 6 minutes heat-up was used when extraction temperature was set at 115°C and 8 minutes at 170°C. Likewise, all extraction 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 psi, iii) heat-up time was applied, iv) static extraction takes place (i.e. at 9 or 15 minutes) 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. 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 AG, 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. When water was used as extracting solvent, care must 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. The procedure followed was the next: 23.5 mg of DPPH were weighted and dissolved on 100 ml methanol. This solution was stored at 4°C. To do the

measurements, this stock solution was diluted 1:10 on methanol. Different concentration of the extracts solutions were used. 0.1 ml of this solutions were added to 3.9 ml diluted DPPH solution to complete the final reaction medium (4 ml). Due to the coloured extracts it was necessary to prepare a control (i.e. blank) which consisted of 0.1 ml of each solution added to 3.9 ml of methanol. The reaction was complete after 4 hours at room temperature, and the absorbance was measured at 516 nm in a UV/VIS Lambda 2 spectrophotometer from Perkin Elmer Inc. (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 which allowed to know the DPPH concentration remaining when reaction was finished. This calibration curve ($n=7$; $r=0.9999$) 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 EC50 (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 by rising with 1M NaOH for 15 min, followed by a 15 min rinse with NaOH 0.1 M and 15 min with deionized water. At the start of a 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 minutes followed by a rinse with the separation electrolite for 2 min. At the end of each day, the capillary was rinsed with deionized water for 5 min.

All extracts were analysed using Micellar Electrokinetic Chromatography. It was performed with a P/ACE System 5500 from Beckman Inc. (Palo Alto, CA, USA) equipped with Diode Array Detector. The fused silica capillary used was purchased from Composite Metal Services Ltd. (Ilkley, UK). The optimum MECK parameters are shown in Table 2.

3. RESULTS AND DISCUSSION

*3.1 Obtention of natural extracts from the microalga *Spirulina platensis* using ASE.*

In order to obtain a large variety of extracts from the microalga *Spirulina platensis*, accelerated solvent extraction (ASE) and four different solvents (hexane, petroleum ether, 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 petroleum ether, 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 °C to 170 °C were selected along with different extraction times (9 and 15 minutes).

Table 1 shows the conditions selected to perform the microalga ASE extractions with the four solvents along with the antioxidants activities obtained for the different fractions (measured using the DPPH free radical assay EC50) 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 to analyze microalga ASE-fractions.

As mentioned above, to our knowledge, there is no published work either on the use of ASE to obtain alga extracts nor 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, an univariate method was applied using a ASE 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 Figure 1. As can be seen, the resolution is slightly better using the 100 mM SDS buffer (Figure 1F), as can be deduced comparing for instance this electropherogram with that obtained without SDS (Figure 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 minute 2) and able to absorb at 280 nm. Besides, it can be seen that the inclusion of hydrophobic interactions analyte-SDS 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 Figure 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 Figure 2, the use of a buffer at pH 8.8 (Figure 2A) brings about a slight improvement of the separation mostly among the compounds migrating after minute 4. 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 ASE 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 MEKC analysis of all the extracts.

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 ASE 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 ASE extracts obtained using water (*vide supra*). As an example, Figure 3 shows a comparison among the electropherograms obtained with the four different solvents at the same extraction conditions (115°C and 15 minutes). As can be deduced from Figure 3, the extracts obtained using hexane (Figure 3A) and petroleum ether (Figure 3B) gave a very similar electrophoretic profile as could be expected from their very similar dielectric constants (1.9 and 4.3, respectively). Interestingly, the ethanol extract (Figure 3C) shows a MEKC electropherogram clearly different from that obtained with hexane (Figure 3A), petroleum ether (Figure 3B) and water (Figure 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 EC₅₀ 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 petroleum ether (compare EC₅₀ values in Table 1), it can be deduced that in general a slightly better antioxidant activity (i.e., lower EC₅₀ values) were obtained for hexane-microalga extracts than for petroleum ether-microalga extracts. This difference can be correlated to the higher amount of the main compound (with a migration time of ca. 7.5 min in Figure 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 (Figure 4) and for petroleum ether (Figure 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 apex of peak D and given in Figures 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) can not be ruled out.

From Table 1 it can be deduced that the lowest activity was obtained when using water as extracting solvent in the ASE procedure. The MEKC-DAD profile obtained for this type of extracts (see Figure 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 Figure 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 petroleum ether. 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 EC₅₀ values given in Table 1, it can be also deduced that ethanol extracts posses a good antioxidant activity comparable in some cases to that obtained with petroleum ether and slightly worse that that obtained with hexane. This property can be used as an additional advantage taking into account that ethanol, unlike hexane or petroleum ether, 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 petroleum ether. Therefore, by using mixtures ethanol:water as ASE solvent it is expected to obtain a high yield along with a higher antioxidant power. This work is now being carried out at our lab and it will be the subject of a forthcoming paper.

4. CONCLUSIONS

In this work, the high potential of the combined use of ASE-*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 ASE extracts from the microalga have been obtained using as solvents hexane, petroleum ether, 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 ASE process. Besides, the new MEKC-DAD method developed is reproducible (Table 3), and allows to obtain a fast profile of the main compounds obtained under different extraction conditions which could be favourably used to optimize the ASE extraction conditions in a feed-back-like procedure.

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FIGURE LEGENDS

Figure 1. Effect of the concentration of SDS into the buffer separation during the MEKC analysis of an ASE 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) 80 mM SDS and E) 100 mM SDS. The rest of MEKC conditions as in Table 2.

Figure 2. Effect of the 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. The rest of conditions as in Figure 1.

Figure 3. MEKC comparison of the ASE extracts obtained using equal temperature (115 °C) and time of extraction (15 min) but different extraction solvent. A) hexane; B) petroleum ether; C) ethanol and D) water. Separation conditions are indicated in Table 2.

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

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

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

Table 1. Antioxidant activity (given as EC50, µg/ml) and dried extract obtained (mg) depending on the solvent, time and temperature used in the ASE process. Pressure was in all cases 1.500 psi.

SOLVENT (dielectric constant)	Extraction Temp. (°C)	Extraction time (minutes)	Dried extract obtained (mg)	EC50 (µg/ml)
HEXANE (1.9)	115	9	43,1	47,5
	115	15	45,1	47,26
	170	9	94,0	86,9
	170	15	95,5	69,41
PETROLEUM ETHER (4.3)	115	9	37,6	52,56
	115	15	38,6	62,84
	170	9	77,0	91,2
	170	15	66,6	95,55
ETHANOL (24.3)	115	9	121,7	63,85
	115	15	166,2	66,75
	170	9	270,1	100,08
	170	15	261,7	98,68
WATER (78.5)	115	9	160,0	394,48
	115	15	170,5	372,95
	170	9	285,5	279,32
	170	15	290,0	287,16

Table 2: Micellar Electrokinetic Chromatography. Optimum conditions.

System:	P/ACE 5500 with Diode Array Detector
Capillary:	Fused silica capillary with 75 μm i.d., 37 cm l_t and 30 cm l_d
Voltage:	15 KV
Temperature:	25°C
Injection:	3 seconds (0.5 psi)
Detector:	Channel A: 200 nm Channel B: 280 nm UV Spectra: from 190 to 600 nm
Samples:	Concentration: 5 mg/ml for hexane, petroleum ether 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

Table 3. Method reproducibility intra- and inter-day. Peaks are shown on *Figure 2*.

Peaks	Same day (n=5)		Three days (n=15)	
	Time (min.)	% RSD	Time (min.)	% RSD
Peak 1	2.659	0.123	2.669	0.404
Peak 2	3.566	0.193	3.585	0.575
Peak 3	3.936	0.106	3.965	0.651
Peak 4	4.119	0.154	4.147	0.661

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Figure 5

Figure 6.