



# Nutritional analysis of Spirulina dietary supplements: Optimization procedure of ultrasound-assisted digestion for multielemental determination



Bárbara D. Neher<sup>a</sup>, Silvana M. Azcarate<sup>b,c</sup>, José M. Camiña<sup>b,c</sup>, Marianela Savio<sup>b,c,\*</sup>

<sup>a</sup> Instituto de Química de San Luis, Av Ejército de los Andes 950, D5700BPB San Luis, Argentina

<sup>b</sup> Facultad Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Av. Uruguay 151, L6300XAI Santa Rosa, La Pampa, Argentina

<sup>c</sup> Instituto de Ciencias de la Tierra y Ambientales de La Pampa (INCITAP), Mendoza 109, L6302EPA Santa Rosa, La Pampa, Argentina

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## ABSTRACT

*Arthrospira platensis* and *Arthrospira maxima* are a type of blue-green microalga used as a dietary supplement (Spirulina). A low time-consuming ultrasound-assisted digestion (UAD) of Spirulina supplements for multielemental determination by microwave induced plasma atomic emission spectrometry (MPAES) was performed. Several parameters such as acid concentration (AC), thermostated water bath (TWB), digestion time (DT) and UAD – probe or bath – affecting the digestion process were evaluated through a full factorial design. Under the optimal conditions – 100 °C for TWB, 5% for AC and 10 min for DT- and selecting the bath as the proper UAD system, the concentrations of 15 analytes (Al, Ba, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, V, Zn) were reported. The values are in accordance with the recommendation established by Food and Drug Administration (FDA) excepting for Cd. The knowledge of Spirulina multielemental composition contributes to an outstanding nutritional and toxicological report for human health.

## 1. Introduction

Spirulina is the common name for diet supplements produced primarily from two species of cyanobacteria, namely *A. platensis* and *A. maxima*. They are grown at large scale in numerous countries for commercial purposes (Cencic and Chingwaru, 2010; FDA, 2017), and are marketed as dietary supplement being considered ‘nutraceuticals’ for their wide use as a rich source of minerals, proteins, antioxidants, polyunsaturated fatty acids, carotenoids, among other (Kepekçi, Polat, Çelik, Bayat, & Saygideger, 2013; Ngo-Matip et al., 2014). Spirulina supplements have been certified as safe and hence can be used as food products (FDA, 2011).

For evaluating Spirulina products quality, it is necessary to analyze constituents such as the content of protein (up to 70%), along with the amount of essential fatty acids, essential amino acids, vitamins, antioxidant pigments (phycobiliproteins, carotenoids), chlorophyll and polysaccharides, it is also significant to assess the nutritional and toxicological mineral content. Trace metals in algal cells are present in extremely small quantities, nonetheless, are an essential component of the phycophysiology. Cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni) and zinc (Zn) are the six most important trace metals required by algae for various metabolic functions (Akbarnezhad,

Mehdi, Abolghasem, & Mehran, 2016).

Currently, microwave induced plasma atomic emission spectrometry (MPAES) is a challenging plasma technique used for numerous trace elements determination (Jankowski & Reszke, 2010). The major advantages are related to the simultaneous multielement detection, high sensitivity, wide range of linear response and low noise level (Jankowski & Reszke, 2010; Zaldarriaga Heredia, Cina, Savio, Gil, & Camiña, 2016). However, developing an appropriate analytical methodology for sample preparation allowing the introduction to MPAES is a critical stage in the analytical process- must regard measurements trueness, facilities and equipment availability, procedure simplicity and determination rapidity.

As an alternative to conventional microwave oven digestion (MW) for sample preparation, ultrasound-assisted digestion (UAD) is currently a growing trend in analytical chemistry (Ilander & Väisänen, 2007; Lima, Da Costa, Ferreira, Richter, & Munoz, 2014; Lima, Lima, Richter, & Munoz, 2016; Priego-Capote & Luque de Castro, 2004a, 2004b; Vudagandla, et al., 2017). Applications of UAD for the extraction of lipids, proteins, flavonoids, carotenoids and aromatic compounds are well reported in literature (Dey & Rathod, 2013; Krug & Piovezani Rocha, 2016). Nevertheless, a study comparing diverse types of ultrasound for mineral digestion on Spirulina supplements has not

\* Corresponding author at: Instituto de Ciencias de la Tierra y Ambientales de La Pampa (INCITAP), Mendoza 109, L6302EPA Santa Rosa, La Pampa, Argentina. Facultad Ciencias Exactas y Naturales, Universidad Nacional de La Pampa, Av. Uruguay 151, L6300XAI Santa Rosa, La Pampa, Argentina.

E-mail address: [msavio@exactas.unlpam.edu.ar](mailto:msavio@exactas.unlpam.edu.ar) (M. Savio).

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been developed yet.

Hence, in this work a rapid and simple UAD for Spirulina sample preparation is proposed. An experimental design was carried out to decide which ultrasonic system will be used and to find the optimal condition for the digestion treatment. In order to assess quality assurance, multielemental determination of Spirulina samples acquired from different pharmaceutical companies was performed by MPAES, reporting the concentration of major, minor and toxic elements.

## 2. Experimental

### 2.1. Reagents

Ultra-pure deionized water (resistivity of 18.2 mΩ) was produced by a Millipore ultra-purifier (Darmstadt, Germany). Concentrated HNO<sub>3</sub> (Merck, Darmstadt, Germany) was obtained by distillation into a Berghof® subboiling distiller system (Eningen, Germany). The calibration step was used in a matrix of 5% HNO<sub>3</sub>, with 100 μg mL<sup>-1</sup> of multielement standards (SPC, Quebec, Canada).

### 2.2. Sampling

Spirulina nutraceutical supplements from different pharmaceutical companies were purchased in Argentina. A total of eleven (11) Spirulina supplements samples were analyzed: five tablets and six capsules. The tablets samples were ground using a mortar and pestle. Meanwhile, the capsules were removed from the wrapping and powders were directly used for analysis.

### 2.3. Analytical procedure for sample preparation

Ultrasound-assisted digestion: Spirulina samples (0.6 g of dry powder) were accurately weighed and transferred to metal-free polypropylene (PP) flasks with screw cap. A strong oxidizing acid, as HNO<sub>3</sub>, is widely used to enhance organic material mineralization, improving sample digestion via formation of element soluble complexes (Krug & Piovezani Rocha, 2016). Thus, the samples were acidified with 5% HNO<sub>3</sub> and placed into a thermostated water bath at 100 °C for 30 min. Then, UAD was performed for 10 min using an ultrasound water bath. The obtained suspensions cooled at room temperature, were diluted to 20 mL with deionized water. Finally, samples were centrifuged for 5 min at 3000 rpm and the supernatant was used for the analytical determinations. Blanks reagents were also run.

Comparative studies were attained by MW using 0.6 g accurately weighed of dry samples, transferred to a hermetically sealed 100 mL polytetrafluoroethylene tube (PTFE) and added with 5% v/v HNO<sub>3</sub>. Then these tubes were placed in the Anton Paar microwave system and a two-step program (15 min up to 240 °C and then kept by 10 min at 240 °C) was accomplished. Subsequently, all samples were transferred to a 20 mL volumetric tube and diluted with deionized water to mark.

### 2.4. Instrumentation

Multielemental determination was carried out by MPAES Agilent MP 4100 (Santa Clara, USA) which consists of an inert One Neb nebulizer, a double-pass glass cyclonic spray chamber and a SPS3 auto-sampler system. A Czerny-Turner monochromator with a charge-coupled device (CCD) array detector was employed. MPAES 4100 runs “on air”, it uses an on line nitrogen generator (Agilent 4107), which takes the air from the environment and separates it into nitrogen. The plasma gas flow was fixed at 20 L min<sup>-1</sup> and the auxiliary gas flow was fixed at 1.5 L min<sup>-1</sup>. For multielemental determination, common settings were applied: uptake time 13 s, plasma stabilization time with sample aspiration 15 s, read time 3 s (read in triplicates), and wash time 20 s. Automatic background correction was used. The operating parameters such as viewing position and nebulizer pressure were optimized for

each element.

For sample preparation, a Testlab ultrasound water bath (Buenos Aires, Argentina) with a frequency of 40 kHz and a power of 160 W as well as an ultrasound probe with a frequency of 20 kHz and a power of 50 W, were used. Also, MW was carried out using an Anton Paar MW 3000 microwave system (Graz, Austria). The glass and plastic materials used throughout the study were cleaned with 10% nitric acid for 24 h before use and washed with ultra-pure deionized water.

### 2.5. Data analysis

Experimental design, as a collection of statistical and mathematical tools, was useful for developing, improving and optimizing the digestion process using a two-level full factorial design involving four variables influencing the sample digestion. The experimental data were processed by the using statistical software package Unscrambler X 10.3 software (CAMO-ASA, Trondheim, Norway).

All calculations related to calibrations, merit figures, recovery studies, correlations and comparisons, and the corresponding statistical tests were performed using MATLAB (2014b). Environment based on the well-known routines (available in [www.iquironicet.gov.ar/descargas/univar.rar](http://www.iquironicet.gov.ar/descargas/univar.rar)) as well as other homemade routines were used (Olivieri, 2015).

## 3. Results and discussion

### 3.1. Optimization of sample preparation conditions

#### 3.1.1. Screening phase

The effects of ultrasound on digestion yield and kinetics differ depending on the nature of the material to be extracted. *Arthrospira* organisms have a complex organic matrix as consequence of peptidoglycan in the cell wall, moreover in Spirulina nutraceutical supplements is possible to find the presence of excipients used for capsule making (Ciferri, 1983). Thus, when working with complex matrices, the selection of the optimum conditions for the sample pre-treatment is a hard task that requires a considerable amount of time and analysis. For this reason, and in order to find out the best conditions for the Spirulina sample digestion, UAD was selected, and a two-level full factorial design was built taking in account the parameters that simultaneously give information about main contributions and interaction among variables (Dejaegher & Vander Heyden, 2011; Leardi, 2009).

Accordingly, sample digestion could be performed in two general designs of ultrasound-assisted system: water baths or probes. The main factors leading the digestion enhancement with ultrasonic power are the mechanical effects that induce a greater solvent penetration into materials and improve mass transfer, facilitating the sample components release. Ultrasound water baths are frequently used since they are cheaper than probes; even though probes often require shorter digestion time (Krug & Piovezani Rocha, 2016).

The uses of ultrasound have been able to improve, accelerate or automate sample preparation, enhance quality, reduce processing time, chemical and physical hazards as well as being environmentally friendly (Krug & Piovezani Rocha, 2016; Priego-Capote & Luque de Castro, 2004a, 2004b). For this reason, the analyzed factors of UAD systems were: bath (bUAD) and probe (pUAD), joined to acid concentration (AC), thermostated water bath (TWB) and digestion time (DT). These factors were evaluated at two levels each one (Table S1), low (–) and high (+) levels: 1% and 5% for AC, 50 °C and 100 °C for TWB, and 2 min and 10 min for DT, in both, bUAD or pUAD, respectively.

Taking into account that different analytes concentrations are present in Spirulina, a compromise situation to optimize the proposed method was acquired using four of the analytes under study. Thus, the evaluation consisted in the multielemental determination of Ca, K, Mg,

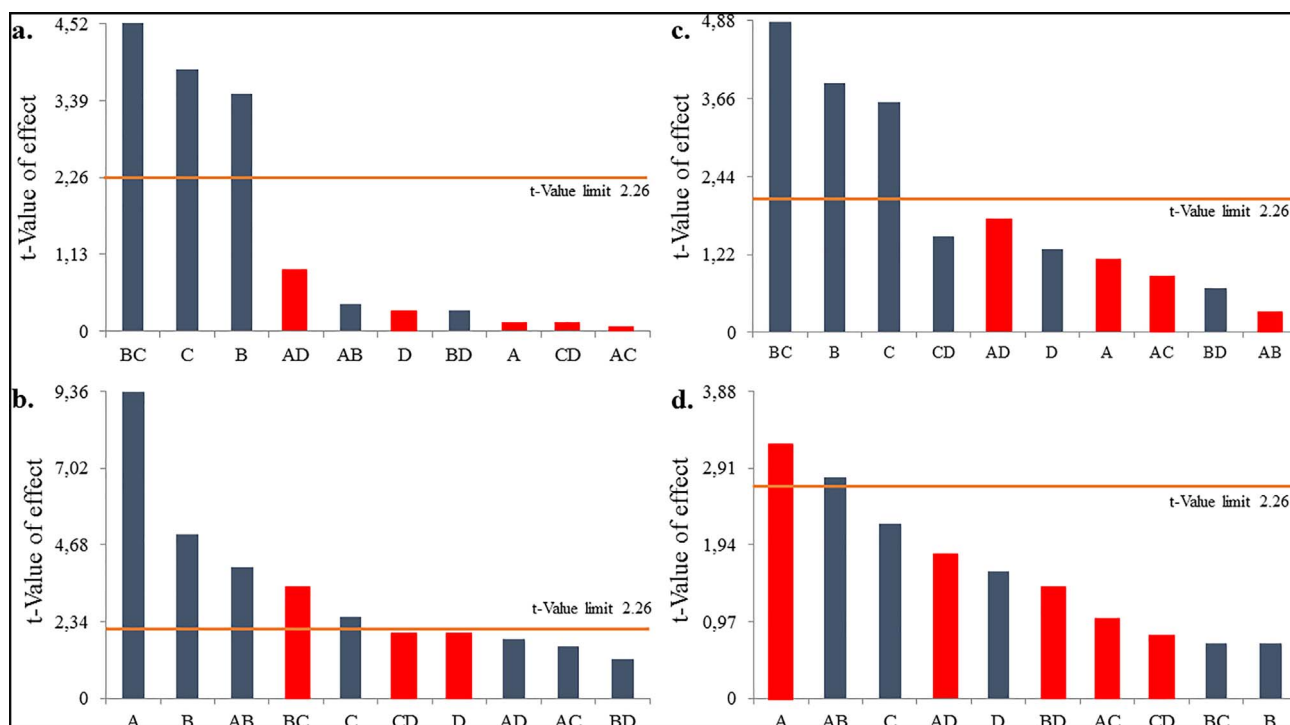


Fig. 1. Pareto chart illustrating main and interaction effects of the factors affecting the digestion system A: thermostated water bath (TWB), B: acid concentration (AC), C: digestion time (DT), and D: ultrasound-assisted digestion systems (UAD), for the (RAS) responses studied of a) Ca, b) Mg, c) K and d) Na.

and Na on *Spirulina* samples, evaluating as design response the relative analytical signal (RAS) of each element. In order to evaluate the model lack of fit, a replicate of the experiments was carried out.

The experiments were performed in randomized order to ensure the results independence and minimize the effects of uncontrolled factors. Then, a screening phase for the determination of the factors affecting the sample digestion was performed on the design results. Analysis of variance (ANOVA) and *p*-value were used to evaluate the significance of the effects, main effects and their mutual interactions on the efficiency of each analyte extraction are depicted in the Pareto charts (Fig. 1). A single variable represent a main effect, while those characterized together indicate an interaction between them (Leardi, 2009). Pareto charts were used to evaluate the significance of the variables used in the experimental design for the chosen response value. Positive values (blue bars) denote a direct proportional relationship of the variable with the response; whereas negative values (red bars) reflect an inverse relationship. Using a confidence value of  $p = 0.05$ , based on a null hypothesis test, values exceeding this limit (horizontal line) are considered as significant to the response values.

Design results demonstrated that the variables TWB, AC, and DT and the interactions between TWB-AC and AC-DT were significant, affecting the response (95% confidence level). The produced interactions between these variables were expected because the longer the digestion process, the longer contact between sample and digestion reagent under specific temperature reached in the system. For this reason, the results showed that minimum DT provides an ineffective digestion for the samples. On the other hand, the use of UAD did not presented significant effects on RAS, thus bUAD or pUAD could be used indistinctively. Consequently, for simplicity and rapidity of procedure besides to avoid possible cross-contamination with the probe, bUAD was chosen for the following optimization phase of *Spirulina* sample digestion conditions.

### 3.1.2. Optimization phase

In order to confirm the optimum conditions, an optimization phase was performed evaluating the relation between independent and

response variables (Long, Zhang & Lei, 2013; Witek-Krowiak, Chojnacka, Podstawczyk, Dawiec, & Pokomeda, 2014). The optimization aim is to find the operating conditions that ensure compliance with the criteria of each response. Thus, TWB, AC and DT factors using bUAD were taken in account to evaluate the most important effects on the system.

The experimental data provided by full factorial design were performed by regression analysis. The relationship between each response and variables was fitted into the following polynomial equations.

$$\text{Ca} = 87.2 - 1.3 \cdot \text{AC} - 0.6 \cdot \text{DT} + 0.4 \cdot \text{AC} \cdot \text{DT}$$

$$\text{Mg} = 70.2 + 0.1 \cdot \text{TWB} - 2.5 \cdot \text{AC} + 0.1 \cdot \text{TWB} \cdot \text{AC}$$

$$\text{K} = 85.3 - 1.5 \cdot \text{AC} - 0.8 \cdot \text{DT} + 0.5 \cdot \text{AC} \cdot \text{DT}$$

$$\text{Na} = 110.5 - 0.3 \cdot \text{TWB} - 4.6 \cdot \text{AC} + 0.5 \cdot \text{DT} + 0.1 \cdot \text{TWB} \cdot \text{AC}$$

being, Ca, Mg, K and Na the predicted responses (RAS), and TWB, AC and DT the linearity and interactive model coefficients.

Analysis of variance was carried out to justify the significance and adequacy of the regression model fit. Statistical significance of the model was established at  $p \leq 0.05$ . The model F-value for RAS of Ca, Mg, K and Na were 15.1, 20.9, 13.2 and 5.4, respectively, indicating that the models were significant. Thus, the determination coefficients ( $R^2$ ) of the models were greater than 0.82 for all responses. Moreover, relatively coefficient of variation reached low values: 3.4, 4.1, 3.7 and 4.4% for Ca, Mg, K and Na models, respectively, showing that the variation was acceptable and satisfactory (Long et al., 2013). Finally, from the evaluation of the residual values, it could be corroborated that the data do not present normality deviation (Fig. S1). In all the cases, response transformation was not required.

In order to visualize the interactive effects of independent variables on responses, three dimensional (3D) response surface plots were developed by the model equations mentioned above. The relationships between the independent variables and response values could be markedly seen in 3D response surfaces, showed in Fig. S2. The variables TWB, AC and DT that affect RAS of each studied analyte were combined in two 3D surface plots while the other variable was kept constant.

Thus, it can be noticed that the RAS for the four study analytes change significantly when the AC is increased from 1% to 5% and when DT augmented from 2 min to 10 min. However, the TWB factor behavior is not equal for all analytes: the higher RAS values for Ca, Mg and K were found at 100 °C while for Na were attained at 50 °C. This shows that the AC had an influence in the extraction, and the use of nitric acid 1% was not enough for a quantitative digestion. On the other hand, the difference between DT is reasonable since increases the time output of bUAD providing more energy and temperature in the suspension sinus (Machado, Dol, Rodríguez-Arce, Cesio, & Pistón, 2016).

Numerical range optimization of TWB, AC and DT was chosen and RAS for each analyte was maximized. According to these results and considering the best compromise value of the joint response, the optimal digestion conditions were 100 °C for TWB, 5% for AC and 10 min DT to obtain the higher RAS. Thus, the theoretical RAS calculated by polynomial equation and the optimized model factors for Ca, Mg, K and Na were 97.3%, 99.0%, 97.0% and 93.8%, respectively.

Additionally, confirmation experiments in the optimal conditions were carried out. Then, the obtained experimental results were compared with the theoretical results in order to validate the predictive model. The RAS values from the validated experiments of Ca, Mg, K and Na were 93.5%, 95.7%, 92.0% and 89.6%, respectively, which were in close agreement with the predicted results, attaining prediction errors lower than 10%.

### 3.2. Automatic internal instrument optimization

In MPAES, the plasma has axial viewing for optimum sensitivity and best detection limits, even though is vertically-oriented in order to improved matrix handling. The best viewing position in the instrument was assessed for each element wavelength with the aim of attain the best analytical performance. Moreover, to acquire the highest analytical signal for each element, the nebulizer gas pressure is necessary to be optimized in order to transform the sample solution into a fine mist of finely divided droplets. These flows transport the sample into the atomization region. Once optimized the equipment changes automatically for each analyte so the determination is said to be almost simultaneous. The changes of analytical signal as a function of viewing position and gas pressure in nebulizer are resumed in Table 1.

**Table 1**

Figures of merit computed data modeling; and optimized viewing position and nebulizer gas pressure to reach the best analytical signal for each analyte in the MPAES.

Analytes	$\lambda$ (nm)	Viewing Position	Nebulizer Gas Pressure	Sensitivity ( $\times 10^4$ )	LOD [ $\mu\text{g g}^{-1}$ ]	LOQ [ $\mu\text{g g}^{-1}$ ]	RSD [%]
Ag	328.068	-20	220	2.90	0.59	1.74	3.86
Al	396.152	0	220	1.23	113.84	326.10	1.37
Ba	455.403	0	220	5.65	1.08	3.22	4.50
Be	234.861	0	140	34.29	0.65	1.94	2.76
Ca	422.673	-20	240	124.07	5.18	15.42	3.53
Cd	228.802	-10	220	1.33	0.61	1.81	3.32
Co	340.512	-20	220	0.53	0.57	1.71	2.66
Cr	425.433	-20	220	1.67	0.68	2.01	5.80
Cu	324.754	-20	220	6.41	0.62	1.85	3.39
Fe	371.993	-10	220	0.47	287.98	856.74	2.14
K	776.491	-10	220	2.07	831.74	2382.56	1.79
Mg	285.213	-20	240	135.71	1.63	4.85	1.78
Mn	403.076	-10	180	1.63	0.49	1.46	3.52
Na	589.592	-10	240	172.18	1.76	5.24	8.72
Ni	352.454	-10	220	0.99	0.66	1.96	5.00
P	213.618	-10	220	0.02	367.54	1063.04	3.34
Pb	283.305	-20	220	0.19	5.70	16.34	1.86
V	437.923	0	180	0.75	0.69	2.04	4.57
Zn	481.053	-20	140	0.94	0.96	2.87	3.76

$\lambda$ : wavelength; LOD: limit of detection; LOQ: limit of quantification; RSD: relative standard deviation. All parameter were calculated considering 15 calibration points and 3 replicates per sample.

### 3.3. Pretreatment comparison

Analytes extraction depends strongly on their interaction with the reagents and the matrix in which they are found. In addition, the particle size of a solid sample plays a decisive role in the suspensions stability during aspiration, transport and sample introduction, as well as atomization efficiency. It is desirable in MPAES studies that particles are efficiently transported through the sample introducing system, and that the particle decomposition processes as well as the atomization/excitation of the analytes are identical to those obtained with the solutions (Krug & Piovezani Rocha, 2016).

Comparing UAD with MW digestion, MW has a hermetic seal that allows the complete decomposition of the samples at high temperatures and pressures, avoiding analytical volatilization or loss of sample. On the other hand, UAD is an open system, which makes that the temperature and pressure required for complete digestion is not reached efficiently, greatly influencing the particle size of the sample. However, there are studies using UAD as pre-treatment of samples that reach satisfactory outcomes. (Krug & Piovezani Rocha, 2016; Machado, Dol, et al., 2016; Machado, Bergmann & Pistón, 2016; Ruiz Díaz et al., 2015).

In order to evaluate whether the proposed and selected sample preparation (bUAD) is comparable to the conventional MW, a recovery study was assessed. Three samples were randomly selected and spiked with  $5 \mu\text{g g}^{-1}$  and  $100 \mu\text{g g}^{-1}$  for minor elements and major elements, respectively. Digestions were performed in triplicate as indicated in subsection 2.3, and multielement determination was carried out by MPAES. Table 2 expose the analytes recovery percentage, depicting that the recoveries for almost analytes (Ag, Al, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, Pb, V, Zn) were between 85 and 115%. In addition, the bUAD method was statistically compared with MW using a *t* test ( $\alpha = 0.05$ ,  $n = 3$ ), and there were not significant differences between both treatments, demonstrating that bUAD could be proposed as an interesting, quick and non-expensive option for the pretreatment of Spirulina nutraceutical supplements samples.

It is important to highlight that MW system has a 16-position rotor, allowing only few samples at a time, whereas the proposed bUAD enables higher number of samples in the same period of time (50 samples could be digested in the ultrasound bath while performing a digestion in MW). The tubes were placed in the region of higher cavitation intensity, also is according to the literature (Krug & Piovezani Rocha,

**Table 2**

Recovery percentages of the analytes (n = 3) determined after the proposed sample preparation method (bUAD) compared to the conventional (MW).

Analyte	Recovery [%]	
	bUAD	MW
Ag	97.45 ± 2.76	103.21 ± 3.12
Al	93.65 ± 5.08	99.98 ± 1.06
Ba	99.93 ± 0.19	96.14 ± 3.17
Be	105.56 ± 9.45	87.20 ± 9.58
Ca	96.40 ± 2.65	94.24 ± 3.39
Cd	105.12 ± 1.02	96.15 ± 8.03
Cr	96.13 ± 16.57	84.34 ± 10.41
Cu	97.15 ± 2.19	99.43 ± 0.87
Fe	99.96 ± 3.19	98.71 ± 0.54
K	97.10 ± 5.07	98.88 ± 6.36
Mg	99.28 ± 1.86	94.07 ± 4.52
Mn	98.44 ± 0.90	99.47 ± 1.97
Mo	98.70 ± 1.91	99.98 ± 3.22
Na	100.96 ± 9.27	89.83 ± 12.31
Ni	98.97 ± 6.45	89.53 ± 9.37
P	99.88 ± 6.23	98.98 ± 5.20
Pb	99.02 ± 2.74	92.57 ± 6.21
V	97.99 ± 2.89	89.08 ± 5.09
Zn	100.09 ± 7.02	98.47 ± 9.54

Values expressed as mean ± Confidence Interval (CI).

2016; Mason & Lorimer, 2002; Munoz, Oliveira, & Angnes, 2006; Nascentes, Korn, Sousa & Arruda, 2001). Therefore, in spite of the fact that microwave oven is secure for users, allows the complete decomposition of the samples at high temperatures and pressures; the UAD has the advantages of improve productivity, procedure simplicity and determination rapidity as tested in this work.

### 3.4. Analytical performance

#### 3.4.1. Trueness

For this validation protocol the trueness should be evaluated using a certified reference material. However, as it was not available in our laboratory, trueness was evaluated through addition and recovery tests with acceptance criteria between 85% and 115% (Camera et al., 2017). In this study, almost all the analytes reached recovery percentage within this ranges, as it is shown in Table 2.

#### 3.4.2. Precision

Precision describes the closeness of a set of independent measurements obtained from an experimental procedure and it is related only to dispersion and not to deviation from a correct or reference value. In this work precision for validation of the method was established as the relative standard deviation (RSD) of the measurements. The RSD obtained using the three calibration curves was between 1.37% for Al to 8.72% for Na, attaining good precision and meeting the established acceptance criterion (Table 1).

#### 3.4.3. Linearity

Table 3 presents the results corresponding to the linearity study. After of corroborate normality of the data, Levene test was performed in order to evaluate the homogeneity of variances, indicating as result the homoscedasticity of the data. For the linearity assessment, the goodness of fit was tested by comparing the variance of the lack of fit against the pure error variance: the fit of each model was estimated by application of an *F*-test.  $R^2$  coefficient major than 0.99 were obtained from each element calibration curve.

A three-sample validation set of three concentration levels was built considering concentrations different than those used for calibration set (Table S2). The validation samples were prepared as previously described for the calibration samples, and the root mean square error of prediction (RMSEP) values were below 2% in all cases.

**Table 3**

Linearity analysis.

Analyte	Parameter				
		Linearity range ( $\mu\text{g g}^{-1}$ )	Slope ± SD <sup>a</sup>	Fexp <sup>b</sup>	R <sup>2</sup>
Ag	1.74–120.00	29022 ± 368	1.596	0.998	0.188
Al	326.10–2000.00	12291 ± 191	2.381	0.997	0.068
Ba	3.22–120.00	56532 ± 563	1.170	0.999	0.389
Be	1.94–40.00	342868 ± 5671	0.890	0.999	0.587
Ca	15.42–300.00	1240664 ± 18193	1.146	0.998	0.413
Cd	1.81–120.00	13302 ± 389	1.564	0.999	0.199
Co	1.71–120.00	5285 ± 49	1.392	0.998	0.267
Cr	2.01–120.00	16652 ± 223	1.291	0.999	0.317
Cu	1.85–120.00	64145 ± 816	2.097	0.999	0.082
Fe	856.74–12000.00	4726 ± 64	0.786	0.999	0.694
K	2382.56–20000.00	20741 ± 235	2.243	0.998	0.082
Mg	4.85–300.00	1357110 ± 13170	1.145	0.999	0.405
Mn	1.46–120.00	16308 ± 210	1.382	0.998	0.272
Na	5.24–600.00	1721787 ± 30124	0.897	0.999	0.596
Ni	1.96–120.00	9945 ± 113	1.598	0.998	0.188
P	1063.04–12000.00	219 ± 2	0.781	0.999	0.698
Pb	16.34–60.00	1892 ± 53	0.915	0.997	0.575
V	2.04–120.00	7551 ± 93	1.384	0.998	0.271
Zn	2.87–120.00	9364 ± 98	1.546	0.998	0.205

<sup>a</sup> SD = standard deviation values.

<sup>b</sup> *F*-test for linearity determination.  $F_{\text{tab}} = 2.892$ .

<sup>c</sup> Since the p-value of the lack of adjustment is greater than or equal to 0.10, the model is adequate for the observed data.

In order to appraise whether the recoveries were statistically different than 100%, a hypothesis test was applied. The recoveries are considered statistically different than 100% when experimental  $t$  ( $t_{\text{exp}}$ ) value exceeds the critical  $t_{(\alpha, \nu)}$  value at level  $\alpha$  and  $\nu = n - 1$  grades of freedom ( $n =$  number of samples) (Olivieri, 2015). Considering 95% confidence level,  $t_{\text{exp}}$  values for all analytes in the validation samples were lower than the critical value  $t_{(0.025, 2)} = 4.303$ , indicating the recoveries were not statistically different than 100%.

#### 3.4.4. Limit of detection and quantification

According to the latest IUPAC recommendations, the estimation of the limit of detection (LOD) should comply with two conditions: (1) it should be based on the theory of hypothesis testing, taking into account the probabilities of false-positive and false-negative decision, and (2) it should include all the different sources of error, both in calibration and prediction steps which could affect the final result (Olivieri & Allegrini, 2014). Limit of detection and limit of quantification values for the 15 analytes are presented in Table 1. Moreover, the table displays the figures of merit for trace element determination by MPAES after treating samples by bUAD. The recommended procedure reaches LOD between  $0.49 \mu\text{g g}^{-1}$  and  $831.74 \mu\text{g g}^{-1}$ ; and LOQ between  $1.46 \mu\text{g g}^{-1}$  and  $2382.56 \mu\text{g g}^{-1}$  for Mn and K, respectively.

### 3.5. Analytical application

Spirulina nutraceutical supplement products are not presented as a conventional food (Cencic & Chingwaru, 2010). Under the Dietary Supplement Health and Education Act (DSHEA, 1994), the manufacturer is responsible for ensuring that its products are safe before they are marketed; while the FDA regulates both, finished dietary supplement products and dietary ingredient (FDA, 2017).

Therefore, trace elements are significant for their nutritional and/or toxicological properties, contributing to quality characterization and diet adequacy (Bilandžić, Sedak, Đokić, Božić, & Vrbic, 2015). Trace elements are prone to contaminate Spirulina products and some could produce the end of algae growth (Arunakumara, Zhang & Song, 2008; Siva Kiran, Madhu, Satyanarayana, Kalpana, & Subba Rangaiah, 2017). To cite some cases, Ni, Cu, and Zn are common fertilizer components or

**Table 4**  
Mean analyte concentrations (n = 3) and standard deviations found in Spirulina supplements [ $\mu\text{g g}^{-1}$ ].

Supplement	Al	Ba	Ca	Cd			Cr
A	< LOD	< LOQ	60.01 $\pm$ 1.09	< LOQ			< LOD
B	1270.02 $\pm$ 13.79	< LOD	41.35 $\pm$ 1.4	2.01	$\pm$	0.05	< LOD
C	< LOQ	36.22 $\pm$ 0.95	277.35 $\pm$ 5.33	< LOQ			3.32 $\pm$ 0.07
D	< LOQ	< LOD	34.94 $\pm$ 1.6	2.14	$\pm$	0.08	< LOD
E	< LOD	3.79 $\pm$ 0.16	39.94 $\pm$ 1.19	1.82	$\pm$	0.01	< LOD
F	< LOD	5.09 $\pm$ 0.06	60.24 $\pm$ 0.27	1.85	$\pm$	0.01	< LOD
G	< LOQ	28.84 $\pm$ 0.36	454.05 $\pm$ 9.52	2.02	$\pm$	0.05	3.74 $\pm$ 0.06
H	< LOD	23.94 $\pm$ 0.2	195.79 $\pm$ 3.32	1.86	$\pm$	0.08	< LOQ
I	< LOD	4.61 $\pm$ 0.13	82.59 $\pm$ 6.49	1.74	$\pm$	0.03	< LOD
J	< LOQ	16.94 $\pm$ 0.78	103.28 $\pm$ 8.79	1.88	$\pm$	0.03	2.98 $\pm$ 0.06
K	< LOD	17.71 $\pm$ 0.26	285.03 $\pm$ 6.64	2.00	$\pm$	0.03	0.95 $\pm$ 0.04
Supplement	Cu	Fe	K	Mg			Mn
A	832.26 $\pm$ 115.6	< LOD	6414 $\pm$ 130	880	$\pm$	31.3	6.34 $\pm$ 0.56
B	< LOD	< LOD	< LOD	1062	$\pm$	32.9	< LOD
C	2.72 $\pm$ 0.1	889.7 $\pm$ 15.4	11670 $\pm$ 141	2271	$\pm$	30.6	75.8 $\pm$ 2.51
D	< LOD	< LOD	< LOD	11	$\pm$	0.22	< LOD
E	< LOQ	< LOD	9420.5 $\pm$ 54.8	1501	$\pm$	105	20.9 $\pm$ 0.95
F	2.45 $\pm$ 0.19	< LOQ	10644 $\pm$ 64.2	1745	$\pm$	20	35.1 $\pm$ 0.86
G	7.94 $\pm$ 0.06	905.8 $\pm$ 14.3	14058 $\pm$ 119	2086	$\pm$	32.7	91.2 $\pm$ 1.3
H	11.98 $\pm$ 0.44	< LOQ	16610 $\pm$ 256	2118	$\pm$	41.3	119 $\pm$ 2.97
I	< LOQ	< LOQ	18756 $\pm$ 376	1947	$\pm$	43	47.6 $\pm$ 1.4
J	3.8 $\pm$ 0.22	< LOQ	18314 $\pm$ 413	2311	$\pm$	115	109 $\pm$ 2.63
K	7.8 $\pm$ 0.16	< LOD	12950 $\pm$ 73.6	1264	$\pm$	47.9	46 $\pm$ 1.05
Supplement	Na	Ni	P	V			Zn
A	154.9 $\pm$ 7.99	5 $\pm$ 0.04	2740.4 $\pm$ 38.5	< LOQ			8928 $\pm$ 399
B	31.73 $\pm$ 2.15	7.72 $\pm$ 0.28	< LOD	< LOQ			< LOQ
C	187.07 $\pm$ 4.05	3.47 $\pm$ 0.07	7391.7 $\pm$ 106	< LOQ			13.8 $\pm$ 0.77
D	5.25 $\pm$ 0.07	8.91 $\pm$ 0.15	< LOQ	< LOD			< LOQ
E	418.69 $\pm$ 31.07	2.03 $\pm$ 0.03	4279.7 $\pm$ 56.3	< LOQ			4.9 $\pm$ 0.58
F	335.11 $\pm$ 5.49	3.71 $\pm$ 0.03	5153.1 $\pm$ 102	< LOQ			6.82 $\pm$ 1.68
G	96.46 $\pm$ 1.66	2.75 $\pm$ 0.02	9887.1 $\pm$ 163	< LOQ			20.7 $\pm$ 0.24
H	553.49 $\pm$ 26.09	5.13 $\pm$ 0.32	7379.2 $\pm$ 138	2.14	$\pm$	0.02	20.1 $\pm$ 0.23
I	798.06 $\pm$ 66.48	< LOQ	8422.8 $\pm$ 205	< LOQ			5.66 $\pm$ 0.33
J	590.48 $\pm$ 20.25	3.1 $\pm$ 0.06	11539 $\pm$ 337	< LOQ			11.7 $\pm$ 0.18
K	137.64 $\pm$ 2.53	3.91 $\pm$ 0.04	2590.8 $\pm$ 130	< LOQ			17 $\pm$ 0.15

< LOD values found below of detection limit.

< LOQ values found below of quantification limit.

Values expressed as mean  $\pm$  CI.

Ag, Be, Co and Pb were lower LOD.

contaminants, but they are considerably less toxic and have a narrow range of optimum concentrations for algae and cyanobacteria, at least in the case of *Cladophora* and *Spirulina (Arthrospira)* (Doshi, Seth, Ray, & Kothari, 2008). Sn, Cr and Al are not a common hazard, but local conditions must be evaluated before they can be completely eliminated as a possible risk.

Table 4 exposes the concentrations of the 15 analytes found in the 11 Spirulina nutraceutical supplement products analyzed. Ni concentrations were below the level agreed by the FDA in all samples (Egan, Bolger & Carrington, 2007). In the case of Cd, concentrations were above the levels allowed by the FDA, this could be explained by the algae ability of bioaccumulating this element (Al-Homaidan, Alabdullatif, Al-Hazzani, Al-Ghanayem, & Alabbad, 2015). Regarding to Cu and Zn concentration, they were within the allowed FDA range, only a supplement showed high level due to addition of these analytes as fortification by the company. However, taking into account the amount of supplement pills ingested per day the values, they are within the recommended established by Codex Alimentarius Commission (CODEX, 2008) and Latin American countries (García-Rico, Leyva-Perez, & Jara-Marini, 2007). In the case of supplement B, it showed high level of Al, while the remaining samples showed concentrations lower than the quantification limits. Again, the level of Al for supplement B, not exceed the recommended daily intake level. The same case for V concentrations, they were lower than quantification limit for the majority of the samples, except for H supplement. Six of the 11

supplements (A, B, D, E, F, I) exhibited Cr level below detection limits. Meanwhile, C, G, J and H supplements confirm lower concentrations according to FDA. In the case of Ag, Be, Co and Pb, the analyzed Spirulina samples were below the detection limit. The levels detected in the present paper fit within the concentration ranges observed in other studies (Al-Dhabi, 2013; Ortega-Calvo, Mazuelos, Hermosin, & Saiz-Jimenez, 1993). There were, however, higher values for Mg than those previously reported (Al-Dhabi, 2013). Nevertheless, the values not exceed the recommended daily intake level (CODEX, 2008; Planes et al., 2002). Regarding to Ba, Ca, Fe, K, Mn, Na and P, concentrations of all samples were found below the recommended by CODEX.

It is important to mention that the supplements B and D are marketed as Spirulina, nevertheless, we observe the presentation as a whitish powder, very different from the bluish green of the rest of the other brands tested. Therefore we could not conclude that the quantified analytes represent the composition of the microalga for these samples.

The outcome of this study reveals that minerals elements in Spirulina samples are lower or complied the recommended daily intake level (RDI-mg/daily) established by Codex Alimentarius Commission (CODEX). All analytes are in accordance with the recommendation established by FDA, with exception of Cd that has a higher concentration than the suggested.

#### 4. Conclusions

Microwave induced plasma atomic emission spectrometry (MPAES) was used for first time for multielemental determination of Spirulina nutraceutical supplements in order to assess its quality assurance. Spirulina supplement samples were suitably mineralized by UAD, enabling their introduction to the MPAES. Experimental design was developed for improving and optimizing the digestion process. UAD using bath system is fast, simple, accurate and less expensive than MW. The concentrations of Al, Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, P, V, Zn in Spirulina dietary supplement be found in accordance with the recommendation established by FDA, excepting for Cd. Regarding to food safety monitoring, the proposed method is an attractive alternative that could be easily implemented in laboratories for routine analysis of nutraceutical supplements, considering mainly the multielement analysis capabilities of MPAES, with the advantage of being straightforward and in good agreement with green chemistry.

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#### Conflicts of interest

The authors declare that they have no competing interests.

#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2018.03.011>.

#### References

- Akbarnezhad, M., Mehdi, S. M., Abolghasem, K., & Mehran, J. B. (2016). Bioaccumulation of Fe<sup>2+</sup> and its effects on growth and pigment content of spirulina (*Arthrospira platensis*). *AAEL Bioflux*, *9*, 227–238.
- Al-Dhabi, N. A. (2013). Heavy metal analysis in commercial Spirulina products for human consumption. *Saudi Journal of Biological Sciences*, *20*, 383–388.
- Al-Homaidan, A. A., Alabdullatif, J. A., Al-Hazzani, A. A., Al-Ghanayem, A. A., & Alabbad, A. F. (2015). Adsorptive removal of cadmium ions by Spirulina platensis dry biomass. *Saudi Journal of Biological Sciences*, *22*, 795–800.
- Arunakumara, K. K. I. U., Zhang, X., & Song, X. (2008). Bioaccumulation of Pb<sup>2+</sup> and its effects on growth, morphology and pigment contents of Spirulina (*Arthrospira*) platensis. *Journal of Ocean University of China*, *7*, 397–403.
- Bilandžić, N., Sedak, M., Đokić, M., Božić, Đ., & Vrbić, A. (2015). Content of macro- and microelements and evaluation of the intake of different dairy products consumed in Croatia. *Journal of Food Composition and Analysis*, *40*, 143–147.
- Camera, A. S., Passos Arcênio, P., Pacheco Filho, W. O., Maranhão, T. A., Santos de Oliveira, F. J., & Azzolin Frescura, V. L. (2017). Method development and validation for sulfur determination via CS molecule in petroleum green coke by high resolution continuum source molecular absorption spectrometry. *Microchemical Journal*, *134*, 301–308.
- Cencic, A., & Chingwaru, W. (2010). The role of functional foods, nutraceuticals, and food supplements in intestinal health. *Nutrients*, *2*, 611–625.
- Ciferri, O. (1983). Spirulina, the edible microorganism. *Microbiological Reviews*, *47*, 551–578.
- Código Alimentario Argentino – CODEX 2008 < <https://www.biol.unlp.edu.ar/alimentosysalud/CAPITULO-XVIIICA.pdf> > , < [http://www.anmat.gov.ar/alimentos/codigoa/CAPITULO\\_XVIII.pdf](http://www.anmat.gov.ar/alimentos/codigoa/CAPITULO_XVIII.pdf) > .
- Dejaegher, B., & Vander Heyden, Y. (2011). Experimental designs and their recent advances in set-up, data interpretation, and analytical applications. *Journal of Pharmaceutical and Biomedical Analysis*, *56*, 141–158.
- Dey, S., & Rathod, V. K. (2013). Ultrasound-assisted extraction of β-carotene from Spirulina platensis. *Ultrasonics Sonochemistry*, *20*, 271–276.
- Dietary supplement health and education act of 1994 public law 103–417, < [https://ods.od.nih.gov/About/DSHEA\\_Wording.aspx](https://ods.od.nih.gov/About/DSHEA_Wording.aspx) > .
- Dietary Supplements- U.S. Food and Drug Administration, < <https://www.fda.gov/Food/DietarySupplements/default.htm> > 2017 < <http://www.fda.gov/downloads/food/ingredientspackaginglabeling/gras/noticeinventory/ucm269772.pdf> > 2011
- Doshi, H., Seth, C., Ray, A., & Kothari, I. L. (2008). Bioaccumulation of heavy metals by green algae. *Current Microbiology*, *56*, 246–255.
- Egan, S. K., Bolger, P. M., & Carrington, C. D. (2007). Update of US FDA's Total Diet Study food list and diets. *Journal of Exposure Science & Environmental Epidemiology*, *17*, 573–582.
- García-Rico, L., Leyva-Perez, J., & Jara-Marini, M. E. (2007). Content and daily intake of copper, zinc, lead, cadmium, and mercury from dietary supplements in Mexico. *Food and Chemical Toxicology*, *45*, 1599–1605.
- Ilander, A., & Väisänen, A. (2007). An ultrasound-assisted digestion method for the determination of toxic element concentrations in ash samples by inductively coupled plasma optical emission spectrometry. *Analytica Chimica Acta*, *602*, 195–201.
- Jankowski, K. J., & Reszke, E. (2010). *Microwave induced plasma analytical spectrometry*. Royal Society of Chemistry.
- Kepekçi, R. A., Polat, S., Çelik, A., Bayat, N., & Saygideger, S. D. (2013). Protective effect of Spirulina platensis enriched in phenolic compounds against hepatotoxicity induced by CCl<sub>4</sub>. *Food Chemistry*, *141*, 1972–1979.
- Krug, F. J., & Piovezani Rocha, F. R. (2016). *Métodos de preparo de amostras para análise elemental*. Brasil: Sociedade Brasileira de Química.
- Leardi, R. (2009). Experimental design in chemistry: A tutorial. *Analytica Chimica Acta*, *652*, 161–172.
- Lima, A. F., Da Costa, M. C., Ferreira, D. C., Richter, E. M., & Munoz, R. A. A. (2014). Fast ultrasound-assisted treatment of inorganic fertilizers for mercury determination by atomic absorption spectrometry and microwave-induced plasma spectrometry with the aid of the cold-vapor technique. *Microchemical Journal*, *118*, 40–44.
- Lima, A. F., Lima, F. F., Richter, E. M., & Munoz, R. A. A. (2016). Combination of sonication and heating for metal extraction from inorganic fertilizers prior to microwave-induced plasma spectrometry determinations. *Applied Acoustics*, *103*, 124–128.
- Long, A., Zhang, H., & Lei, Y. (2013). Surfactant flushing remediation of toluene contaminated soil: Optimization with response surface methodology and surfactant recovery by selective oxidation with sulfate radicals. *Separation and Purification Technology*, *118*, 612–619.
- Machado, I., Bergmann, G., & Pistón, M. (2016). A simple and fast ultrasound-assisted extraction procedure for Fe and Zn determination in milk-based infant formulas using flame atomic absorption spectrometry (FAAS). *Food Chemistry*, *194*, 373–376.
- Machado, I., Dol, I., Rodríguez-Arce, E., Cesio, M. V., & Pistón, M. (2016). Comparison of different sample treatments for the determination of As, Cd, Cu, Ni, Pb and Zn in globe artichoke (*Cynara cardunculus* L. subsp. *Cardunculus*). *Microchemical Journal*, *128*, 128–133.
- Mason, T. J., & Lorimer, J. P. (2002). *Applied sonochemistry: The uses of power ultrasound in chemistry and processing*. Wiley-VCH 314p.
- Munoz, R. A. A., Oliveira, P. V., & Angnes, L. (2006). Combination of ultrasonic extraction and stripping analysis: An effective and reliable way for the determination of Cu and Pb in lubricating oils. *Talanta*, *68*(850).
- Nascentes, C. C., Korn, M., Sousa, C. S., & Arruda, M. A. Z. (2001). Use of ultrasonic baths for analytical applications: A new approach for optimisation conditions. *Journal of the Brazilian Chemical Society*, *12*, 57–63.
- Ngo-Matip, M.-E., Pieme, C. A., Azabji-Kenfack, M., Biapa, P. C. N., Germaine, N., Heike, E., ... Ngogang, J. Y. (2014). Effects of Spirulina platensis supplementation on lipid profile in HIV-infected antiretroviral naive patients in Yaounde - Cameroon: A randomized trial study. *Lipids in Health and Disease*, *13*, 191.
- Olivieri, A. C. (2015). Practical guidelines for reporting results in single- and multi-component analytical calibration: A tutorial. *Analytica Chimica Acta*, *868*, 10–22.
- Olivieri, A. C., & Allegrini, F. (2014). IUPAC-consistent approach to the limit of detection in partial least-squares calibration. *Analytical Chemistry*, *86*(15), 7858–7866.
- Ortega-Calvo, J. J., Mazuelos, C., Hermosin, B., & Saiz-Jimenez, C. (1993). Chemical composition of Spirulina and eukaryotic algae food products marketed in Spain. *Journal of Applied Phycology*, *5*, 425–435.
- Planes, P., Rouanet, J. M., Laurent, C., Baccou, J. C., Besançon, P., & Caporiccio, B. (2002). Magnesium bioavailability from magnesium-fortified spirulina in cultured human intestinal Caco-2 cells. *Food Chemistry*, *77*, 213–218.
- Priego-Capote, F., & Luque de Castro, M. D. (2004a). Analytical uses of ultrasound I. Sample preparation. *TrAC Trends in Analytical Chemistry*, *23*, 644–653.
- Priego-Capote, F., & Luque de Castro, M. D. (2004b). Analytical uses of ultrasound: II. Detectors and detection techniques. *TrAC Trends in Analytical Chemistry*, *23*, 829–838.
- Ruiz Díaz, J. D., Savio, M., Moyano, M. F., Verni, E. E., Martínez, L. D., & Gil, R. A. (2015). *Ultrasonic-assisted dissolution of vegetable oils with tetrabutylammonium hydroxide for multielemental analysis by inductively coupled plasma mass spectrometry*. *Atomic Spectroscopy*. PerkinElmer Inc.
- Siva Kiran, R. R., Madhu, G. M., Satyanarayana, S. V., Kalpana, P., & Subba Rangaiah, G. (2017). Applications of Box-Behnken experimental design coupled with artificial neural networks for biosorption of low concentrations of cadmium using Spirulina (*Arthrospira*) spp. *Resource-Efficient Technologies*, *3*, 113–123.
- Vudagandla, S., Kumar, N. S., Dharmendra, V., Asif, M., Balaran, V., Zhengxu, H., & Zhen, Z. (2017). Determination of Boron, Phosphorus, and Molybdenum content in biosludge samples by Microwave Plasma Atomic Emission Spectrometry (MP-AES). *Applied Sciences*, *7*, 264–274.
- Witek-Krowiak, A., Chojnacka, K., Podstawczyk, D., Dawiec, A., & Pokonka, K. (2014). Application of response surface methodology and artificial neural network methods in modelling and optimization of biosorption process. *Bioresource Technology*, *160*, 150–160.
- Zaldarriaga Heredia, J., Cina, M., Savio, M., Gil, R. A., & Camiña, J. M. (2016). Ultrasound-assisted pretreatment for multielement determination in maize seed samples by microwave plasma atomic emission spectrometry (MPAES). *Microchemical Journal*, *129*, 78–82.