Bioaccessibility studies of Fe, Cu and Zn from *Spirulina* dietary supplements with different excipient composition and dosage form

[Estudios de bioaccesibilidad de Fe, Cu y Zn de suplementos dietarios de espirulina con diferente composición de excipiente y forma de dosificación]

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Abstract

Context: The cyanobacteria *Spirulina* is a food supplement according to its high nutritional value. However, the bioaccesibility (Bac) of nutrients like Fe, Cu and Zn from *Spirulina* can be affected by excipients and formulations of supplements.

Aims: To evaluate the Bac of Fe, Cu and Zn from different commercial presentations of *Spirulina*-based dietary supplements according to excipients and formulations.

Methods: Microscopic studies were performed to study *Spirulina* content in dietary supplements. Total Fe, Cu and Zn concentrations were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Bac was calculated as the ratio between total metal concentration and the metal concentration after *in vitro* gastrointestinal digestion.

Results: The *Spirulina* fragments concentration in the analyzed dietary supplements were different, corresponding to $2.21 \times 10^9 - 4.46 \times 10^9$ cell fragments/g (p = 0.021; 95% confidence). Fe, Cu and Zn concentrations in *Spirulina* dietary supplements ranged from $63 \pm 1 - 1066 \pm 7$ µg/g, $1.8 \pm 0.1 - 187.9 \pm 1.9$ µg/g and $3.0 \pm 0.3 - 57.3 \pm 0.6$ µg/g, respectively. Fe, Cu and Zn Bac were higher in the intestinal digestion stage in samples with high *Spirulina* count. Fe Bac was lower in tablets. Cu Bac was higher in the oral phase rather than the intestinal phase when Cu is present in excipients and not in *Spirulina*. Zn Bac decreases when Mg stearate is introduced as an excipient.

Conclusions: The Bac of Fe, Cu, and Zn from *Spirulina* dietary supplements showed to be affected by excipients composition and by the solid dosage form. The different commercial presentations of *Spirulina* affect their nutritional value.

Keywords: bioaccessibility; dietary supplements; excipients; metal accumulation; ICP-DRC-MS; *in vitro* digestion; *Spirulina*.

Resumen

Contexto: La cianobacteria espirulina es un complemento alimenticio según su alto valor nutricional. Sin embargo, la bioaccesibilidad (Bac) de nutrientes como Fe, Cu y Zn de la espirulina puede verse afectada por los excipientes y las formulaciones de suplementos.

Objetivos: Evaluar la Bac de Fe, Cu y Zn de diferentes presentaciones comerciales de suplementos dietéticos basados en espirulina de acuerdo con excipientes y formulaciones.

Métodos: Se realizaron estudios microscópicos para estudiar el contenido de espirulina en suplementos dietéticos. Las concentraciones totales de Fe, Cu y Zn se determinaron por espectrometría de masas de plasma acoplado inductivamente (ICP-MS). Bac se calculó como la relación entre la concentración de metal total y la concentración de metal después de la digestión gastrointestinal *in vitro*.

Resultados: La concentración de fragmentos de espirulina en los suplementos dietéticos analizados fue diferente, correspondiendo a $2.21 \times 10^9 - 4.46 \times 10^9$ fragmentos de células/g (p = 0.021; 95% de confianza). Las concentraciones de Fe, Cu y Zn en los suplementos dietéticos de espirulina variaron entre $63 \pm 1 - 1066 \pm 7$ µg/g, $1.8 \pm 0.1 - 187.9 \pm 1.9$ µg/g y $3.0 \pm 0.3 - 57.3 \pm 0.6$ µg/g, respectivamente. Bac de Fe, Cu y Zn fueron mayores en la etapa de digestión intestinal en muestras con un alto recuento de espirulina. Bac de Fe fue menor en tabletas. Bac de Cu fue mayor en la fase oral que en la fase intestinal cuando Cu está presente en los excipientes y no en la espirulina. Bac de Zn disminuye cuando se introduce estearato de Mg como excipiente.

Conclusiones: La Bac de Fe, Cu y Zn de los suplementos dietéticos de espirulina se vio afectado por la composición de los excipientes y por la forma de dosificación sólida. Las diferentes presentaciones comerciales de espirulina afectan su valor nutricional.

Palabras Clave: acumulación de metales; bioaccesibilidad; digestión *in vitro*; espirulina; excipientes; ICP-DRC-MS; suplementos dietarios.

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Abbreviations: APIs: Actives Pharmaceutical Ingredient; Bac: bioaccessibility; BEC: background equivalent concentration; DRC: dynamic reaction cells; EDS: energy dispersive spectrometer; GI: gastrointestinal; ICP MS: Inductively Coupled Plasma Mass Spectrometry; LOD: limit of detection; LOQ: limit of quantification; MAD: microwave acid digestion; MPAES: microwave induced plasma atomic emission spectrometry; MW: maximum power; P0: phase 0: oral phase; P1: phase 1: gastric phase; P2: phase 2: intestinal phase; RPq: rejection parameter; SEM: scanning electron microscope.

INTRODUCTION

The consumption of dietary supplements has grown exponentially worldwide due to the great interest that consumers have for health and well-being. These are formulated to provide essential nutrients in the diet of healthy people in order to prevent chronic non-transmissible diseases. Among the most consumed are those based on *Spirulina*, this is the name given to the cyanobacteria of the genus *Arthrospira (A. maxima* and *A. platensis*), which have been used since ancient times for their high nutritional value, rich in proteins, vitamins, minerals, fatty acids and pigments (Jubie and Dhanabal 2012, Hosseini et al. 2013, Koyande et al. 2019). Both species can be used as supplementary dietary sources without the risk of metal balance disorder (Kováčik et al., 2008). Fe, Cu and Zn are essential elements for the photosynthetic functions of these cyanobacteria and are also essential for the homeostasis of human physiology (Pfeiffer et al., 2018).

The analysis of metals in *Spirulina* has been reported, Neher et al. (2018) determined 15 elements in *Spirulina* dietary supplements by microwave-induced plasma atomic emission spectrometry (MPAES). Al-Harbi (2016) determined Pb, As, Cd, Cr, V, Cu and Fe in 25 *Spirulina* commercial products by inductively coupled plasma mass spectrometry (ICP-MS). Recently, dynamic reaction cells (DRC) have been increasingly used in ICP-MS to reduce interferences in a single element, and multi-elemental analysis (Verni et al., 2013). The DRC technology exploits ion–molecule reactions using a variety of reaction gasses.

It is known that excipients present in solid dosage forms can interact with Actives Pharmaceutical Ingredient (APIs) and nutrients. The physical and chemical interactions between nutrients and excipients can affect their chemical nature, stability and bioavailability (Bharate et al., 2016). In *Spirulina* supplements, magnesium stearate is used as a lubricant. Interactions between magnesium stearate and API include potential chemical interactions with impurities (MgO) (Good and Wu, 2017). Dispersing excipients like gelatin can complex essential metals, affecting their bioaccessibility (Bac) (Mikhailov, 2008).

Bac is defined as the fraction of a compound released from its food matrix in the gastrointestinal tract, becoming available for intestinal absorption (Fernández-García et al., 2009). Generally, Bac is established from *in vitro* procedures (Fernández-García et al., 2009). Iron availability from Fe-fortified *Spirulina* culture was studied by *in vitro* digestion/Caco-2 culture model (Puyfoulhoux et al., 2001). Copper and Zinc Bac from *Spirulina* were compared in synthetic supplements (Wojcieszek et al., 2016). The metals' Bac from *Spirulina* has been studied. However, information about excipient effects on this parameter in dietary supplements has not been reported.

Metals (Fe, Cu, Zn) analysis of *Spirulina* supplements was performed by microwave acid digestion (MAD) followed by ICP-DRC-MS determination. Possible interferences present in the samples were minimized by DRC optimization. Biomass determinations in *Spirulina* supplements were performed by optical microscopy. The surface morphology of *Spirulina* and excipients was performed by scanning electron microscopy (SEM). *In vitro* gastrointestinal (GI) digestions of *Spirulina* supplements were performed to determine the Bac of the Fe, Cu and Zn. Finally, the Bac of these metals was related to *Spirulina* biomass, excipients content and different solid dosage forms.

MATERIAL AND METHODS

Instrumentation

An ICP-MS, Perkin-Elmer SCIEX, ELAN DRC-e (Thornhill, Canada) was used. The Argon gas with

a minimum purity of 99.996% was supplied by Air Liquid (Río Cuarto, Córdoba, Argentina), and grade 2.0 / 99% methane was provided by Indura Argentina S.A (Córdoba, Argentina). An HF-resistant and high-performance perfluoracetate (PFA) nebulizer model PFA-ST, coupled to a quartz cyclonic spray chamber with an internal baffle and drain line, cooled with the PC3 system from ESI (Omaha - NE, USA) was used. Tygon black/black 0.76 mm i.d. and 40 cm length peristaltic pump tubing was used. Operating conditions are shown in Table 1.

A microwave digestion system model START-D from Milestone (Sorisole, Italy), and Milestone hermetically sealed 100 mL internal volume, 1 cm wall thickness polytetrafluoroethylene (PTFE) reactors were used for total digestion of the samples. A thermostatized (mesophilic requirements) shaker for the culture of microorganisms from BioDare, was used for the *in vitro* digestion. A 13.000 rpm microcentrifuge Neuation (Gandhinagar, India) was employed for sample processing and supernatant sampling. The *Spirulina* cell count was made with a Leitz Wetzlar DMRB optical microscope and the Neubauer chamber. Scanning electron microscopy images were obtained using an LEO 1450 VP equipped with an EDAX Genesis 2000 energy-dispersive spectrometer (EDS). SPI brand metallizer to coat samples with carbon deposits was used.

Reagents and sample treatment

Distilled and de-ionized water was used, with a resistivity of 18.2 MΩ cm, produced by an Easy pure RF system from Barnstead (Dubuque, IA, USA). Concentrated nitric acid 65% (v/v) from Sigma-Aldrich (Germany) and hydrogen peroxide 30% (v/v) from J. T. Baker (Mexico) were used for samples total digestion. Multi-element calibration standard 3 from Perkin Elmer Pure Plus containing 10 mg/L of Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V and Zn in 5% (v/v) HNO_3 was used for calibration.

For the external calibration using aqueous standards, the standard solutions were prepared in 1.0% (v/v) nitric acid. The analytes concentrations were 0.5, 1, 5, 10, 20, 40, 80, 120 and 180 µg/L. Pepsin from porcine gastric mucosa (powder, ≥250 units /mg solid), pancreatin from porcine pancreas (8× United States Pharmacopeia specifications) from Sigma-Aldrich, double-distilled hydrochloric acid, and sodium bicarbonate from Anedra (Buenos Aires, Argentina) were used in the *in vitro* digestion model.

Samples of *Spirulina* based dietary supplements (capsules, tablets and powders) consisted of different commercial trademarks were purchased in regional pharmaceutical establishments and online platforms of Argentina; they were brought to homogeneous powder using a mortar and pestle of agate to carry out the total digestion and *in vitro* GI digestion. These samples were conserved in the National San Luis University Herbarium with the specimen vouchers # 835, 836, 837, 838, 839, 840 and 841 (UNSL-H). Samples' characteristics are detailed in Table 2.

Table 1. Instrumental operating conditions of the ICP-DRC-MS.

Instrument	Elan DRC-e (Perkin Elmer SCIEX, Thornhill, Canada)
RF power (W)	1050
Plasma gas flow rate (L/min)	15
Auxiliary gas flow rate(L/min)	4
Nebulizer gas flow rate(L/min)	0.81
Scan mode	Peak Hopping
Dwell time (ms)	50
Replicates	3
Isotopes	^{56}Fe, ^{63}Cu, ^{64}Zn

Table 2. *Spirulina* dietary supplements characteristics.

Sample	Solid Dosage Form	Tablet weight/ Dosage (mg)	Excipients	Excipients concentration (mg)	*Spirulina* concentration (cells/g)
M_1	Hard gelatin capsules	500	Gelatin	NI	4.04×10^9
M_2	Tablets	500	Povidone	NI	
			Croscarmellose	NI	2.21×10^9
			Silicon-dioxide	NI	
			Dicalcium phosphate	NI	
			Sodium copper chlorophyllin	NI	
			Magnesium stearate	NI	
M_3	Powder	500	NI	NI	4.46×10^9
M_4	Tablets	500	Magnesium stearate	10	3.25×10^9
			Sodium starch glycolate	15	
M_5	Tablets	500	NI	NI	2.96×10^9
M_6	Hard gelatin capsules	300	Magnesium stearate	NI	2.50×10^9
			Gelatin	NI	
M_7	Hard gelatin capsules	500	Magnesium stearate	150	NO

NI: Not informed; NO: Not observed.

Analytical procedure

Spirulina fragments count in dietary supplements

Total *Spirulina* fragments counts were performed manually by an improved Neubauer counting chamber. To this end, 100 mg of each sample was weight and diluted up to 10 mL with ultrapure water. This solution was homogenized by vortex agitation, and 20 µL were introduced in the Neubauer chamber. *Spirulina* fragments were counted in 6 3rd squares of the chamber. Concentration was calculated as the ratio of counted *Spirulina* fragments and the number of counted squares (6). Finally, this ratio was multiplied by 250000, obtaining the *Spirulina* fragments concentration in the solution.

Analysis of dietary supplements of Spirulina by SEM

The samples were reduced to homogeneous powder in an agate mortar, placed on aluminum "stub" and metalized with carbon. The working conditions were acquisition time 120 seconds, an acceleration voltage between 15 and 20 keV, and a working distance of 15 mm.

Mineralization of samples for total metal analysis

Samples (0.2 g dry mass) were digested by MAD with 5 mL of HNO_3 and 3 mL of H_2O_2 (Wojcieszek et al., 2016). The temperature program included two heating steps, i.e., ramp to 200°C (10 min) and hold at 200°C (15 min), with a maximum microwave power of 1000 W. The digest was diluted to a final volume of 50 mL with Milli-Q water. The mineralization of samples was performed in triplicate.

In vitro model for human gastrointestinal digestion

The *in vitro* GI digestion protocol used was that proposed by Davis et al. with some modifications (Davis et al., 1996a;b; Minekus et al., 2014). The procedure was applied for triplicate adding a 0.5 g of sample into 50 mL Erlenmeyer flasks and diluted up to 20 mL with ultrapure water. It should be clear that in this work, the phase corresponding to the mouth was represented by the dilution of the samples in ultrapure water since the different dietary supplements are consumed without chewing. The enzyme with the most significant action in oral phase is α-amylase (salivary lipase), which

hydrolyzes the 1-4 links of glycogen and starch, with an optimum pH of 7.4, this would not have significant effects on *Spirulina* for the limited time to act before it becomes inactive in the stomach by the pH change. After 15 minutes inside the shaker with an agitation of 150 rpm at 36.5 ± 0.5°C (P0), the pH was adjusted at 2 with 150 µL of 6 mol/L hydrochloric acid solutions. Then 2 mL of a freshly prepared gastric solution (0.04 g/mL of pepsin dissolved in 0.1 mol/L hydrochloric acid solution) were added. The gastric digestion was performed in closed reactors inside the shaker at 36.5 ± 0.5°C with a shaking of 150 rpm for 60 minutes. Then they were taken to a freezer at -18°C to stop the enzymatic reaction. Later 1 mL of sample was collected and centrifuged at 5°C and 13000 rpm for 10 minutes. After this treatment, the supernatant is the *in vitro* gastric digest (P1).

The procedure continues by adding 9 mL of freshly prepared intestinal solution (2 mg/mL of pancreatin and 12 mg/mL bile salts dissolved in 0.1 mL sodium bicarbonate) to the rest of phase 1 solution. Intestinal digestion was performed under agitation in a shaker at 36.5 ± 0.5°C and 150 rpm for 120 minutes. Later the enzymatic intestinal digestion was stopped at -18°C. Once again, 1 mL of sample was collected and centrifuged at 5°C and 13000 rpm for 10 minutes. The supernatant resulting from this procedure constitutes the intestinal digestion (P2). Blank reagent samples were also collected at each stage to assess possible contamination.

ICP-DRC-MS measurements

The metal concentration in the samples (total digested and GI digested) was determined by ICP-DRC-MS under the operating conditions shown in Table 1. The mineralized samples were diluted to 1:10 before measurements. Digest from supernatants, gastric and intestinal, were 1:10 diluted before measurements. Calibrations were based on 1% (v/v) nitric acid aqueous standard solutions covering metal concentrations from 0.5 to 180 µg/L for all elements.

Methane was selected as a reaction gas in DRC, based on available information about its action upon interferences for Fe and Cu. The experimental parameters to be evaluated were i) methane flow-rate and ii) the rejection parameter (RPq). The methane flow rate was evaluated from 0.1 to 2 mL/min in 0.1 intervals. The RPq parameter was evaluated from 0.1 to 1 in 0.1 intervals. Background Equivalent Concentration (BEC) was calculated for each experimental condition, corresponding to the analyte's signal compared to the blank's signal. To this aim, two solutions were used: Solution A, blank of the method, gastric plus intestinal solution, and Solution B, blank plus the matrix, spiked with 50 µg/L of Fe and Cu. Optimum cell gas flow rate and rejection factor for each m/z were selected with the 'DRC method development' of the ELAN software according to a maximum limit of 10 counts per second of interferences'signal. The selection of the optimal cell gas flow rates is empirical and based on interference removal and signal-to-noise improvement.

Calculation of bioaccessibility (%)

The Bac of the elements, expressed as a percentage, was calculated using the following [1]:

$$\text{Bac}(\%) = \frac{[A]_{\text{solublefraction}}}{[A]_{\text{total}}} \times 100 \qquad [1]$$

Where Bac(%) is the percentage of metal bioaccessibility, and $[A]_{\text{solublefraction}}$ and $[A]_{\text{total}}$ are the concentrations of the metal after de gastrointestinal digestion and total digestion, respectively.

Statistical analysis

All samples were collected and analyzed in triplicate, and the triplicate tests were statistically similar as paired-samples t-test (p = 0.05). The average results were used to represent the data. Microsoft Excel® was used to test a one-way analysis of variance (ANOVA) at 95% confidence.

RESULTS AND DISCUSSION

Biomass and excipients analysis of *Spirulina*

Spirulina concentration in dietary supplements can differ in the selected samples according to the different manufacturers. It has been stated that in the process of harvesting, pumping the algal cul-

ture to be filtrate, the filaments of *Spirulina* may become physically damaged, producing short filaments of the cyanobacteria (Habib, 2008). For a better approach of *Spirulina* biomass in dietary supplements, complete cells and cell fragments were counted. Results can be observed in Table 2. *Spirulina* concentration ranged from 2.21×10^9 to 4.46×10^9 cells fragments/g. *Spirulina* concentrations between the different dietary supplements were different (p = 0.021). An elevated concentration of *Spirulina* was quantified in M3 (4.46×10^9 cells fragments/g), compared with sample M2 (2.21×10^9 cells fragments/g). In sample M7 *Spirulina* fragments were not identified or quantified.

Excipients can affect the bioavailability of nutrients from dietary supplements (Bharate et al., 2016). The composition of each sample can be observed in Table 2. Morphological characteristics and texture of the different solid dosage forms are shown in Fig. 1. Trichomes of *Spirulina* are observed in powder (M3) and hard gelatin capsules with powder inside (M1). On the other hand, granules can be observed in tablets (M2, M4 and M5). Granules are formed during the compression process of tablets.

ICP-DRC-MS analysis of metals in *Spirulina* dietary supplements

The ICP operating conditions must be evaluated to favor ionization and to avoid background contribution due to polyatomic ions, oxides and double-charged ions. Double-charged ions as $Ba^{2+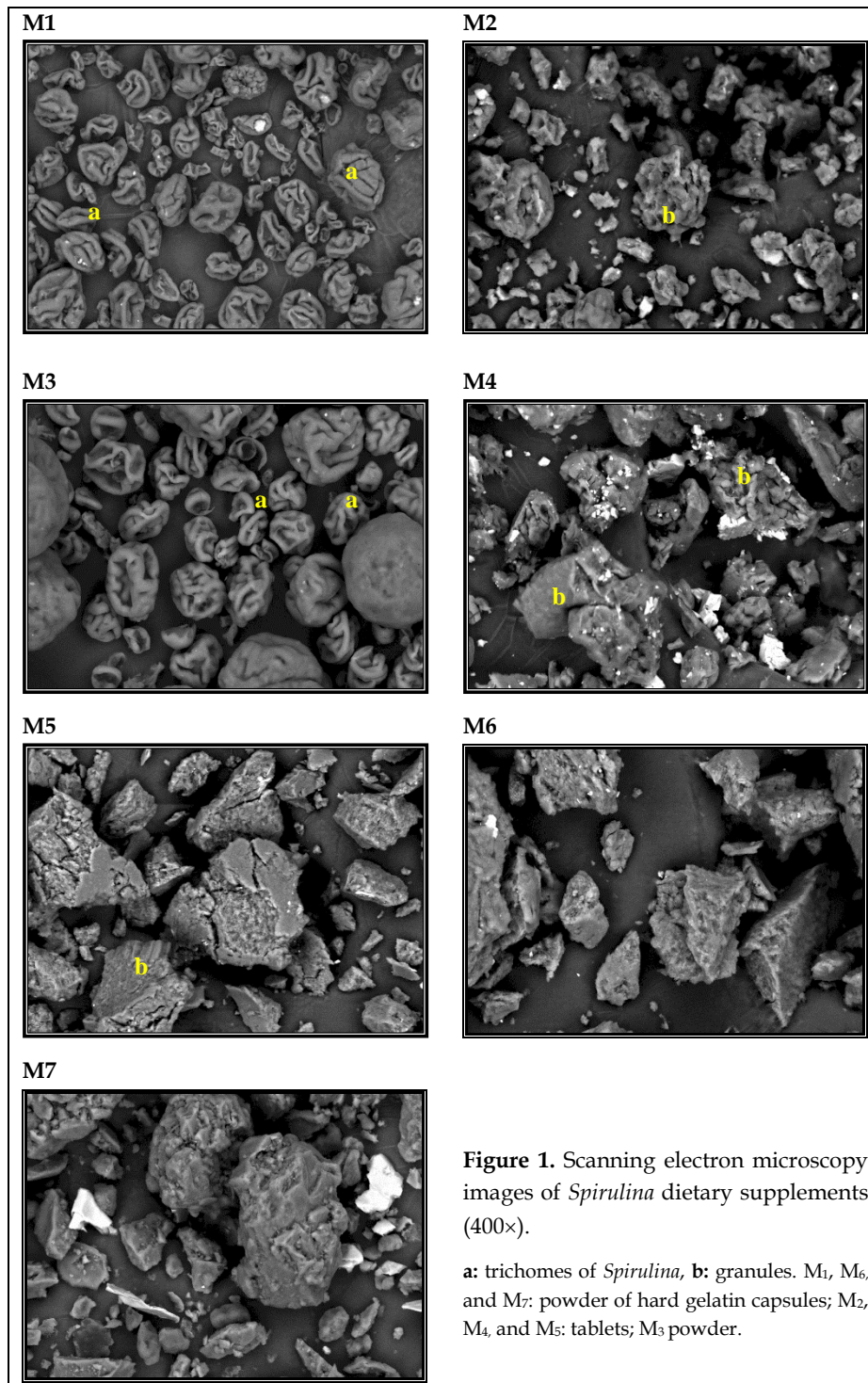}/Ba^+$, oxide ions as CeO^+/Ce, and instrument sensitivity (signal for Mg, In and U), were monitored following the criteria of the instrument manufacturer (Boussiba and Richmond 1980) with a certified standard solution of Mg, In, Ce and U (1 µg/L), and Ba (10 µg/L). The signal variations of each element were monitored according to RF power (750 - 1400 W), and Ar gas flow rate (0.6 to 1.2 L/ min). The final conditions are summarized in Table 1.

The elemental composition of *Spirulina* dietary supplements (Park et al., 2013; Colla et al., 2007) might contribute to the formation of Ca, P, C, and S polyatomic ions overlapping with Fe and Cu ions. Table 3 shows spectral interferences and DRC conditions for the analyzed metals. Formation of N and O polyatomic ions of digestion reagents (water and nitric acid) and Ar polyatomic ions from plasma gas might also be present. The isotope ^{66}Zn did not show a related problem at the time of the analysis. On the contrary, Fe and Cu isotopes suffered from a complicated background contribution due to matrix composition.

The use of methane as reaction gas allows eliminating polyatomic ion interferences through a different mechanism (i.e., neutralization and mass transfer)(de Souza et al., 2017). RPq is a rejection parameter, to adjust the low- and high-mass cutoff regions, thus establishing the dynamic bandpass tunning of DRC. Ions outside these stability boundaries are unstable in the cell and are rejected. The objective was to maximize the analyte signal and suppress the background intensity.

**Table 3.** Spectral interferences and dynamic reaction cell conditions.

Isotope	Spectral interference	CH$_4$ flow rate (mL/min)	RPq
^{56}Fe	$^{16}O^{40}Ar$, $^{40}Ca^{16}O$	1.0	0.40
^{63}Cu	$^{23}Na^{40}Ar$, $^{27}Al^{36}Ar$, $^{15}N^{16}O_3$, $^{14}N^{17}O^{16}O_2$, $^{1}H^{14}N^{16}O^{32}S$	1.0	0.75

Figure 1. Scanning electron microscopy images of *Spirulina* dietary supplements (400×).

a: trichomes of *Spirulina*, **b:** granules. M_1, M_6, and M_7: powder of hard gelatin capsules; M_2, M_4, and M_5: tablets; M_3 powder.

After optimization of ICP-DRC-MS a LOD of 13, 0.1 and 0.6 µg/L were obtained for ^{56}Fe, ^{63}Cu and ^{64}Zn, respectively. LOQ corresponded to 39, 0.4 and 1.9 µg/L were obtained for ^{56}Fe, ^{63}Cu and ^{64}Zn, respectively. The determination coefficient

(R^2) corresponded to 0.999, 0.999 and 0.997 for ^{56}Fe, ^{63}Cu and ^{64}Zn, respectively.

In Fig. 2, it can be observed the background equivalent concentration (BEC) variations of Cu and Fe as a function of RPq and cell gas flow rate

Figure 2. Background equivalent concentration (BEC) variations of Fe and Cu as a function of RPq and cell gas flow rate. Optimized conditions are marked in red dots.
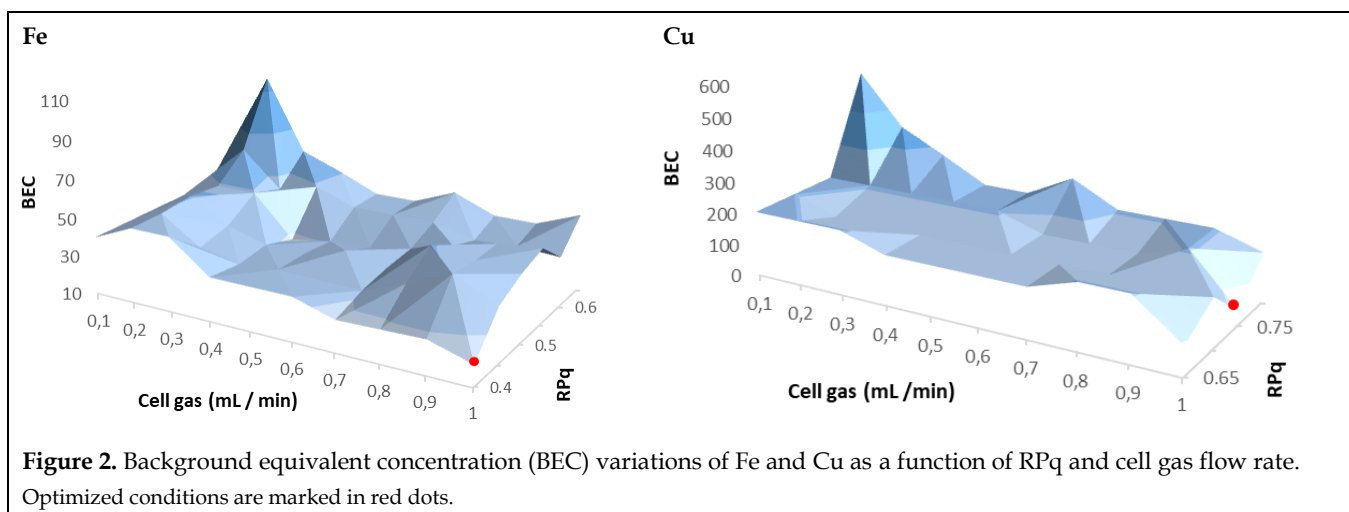
(CH$_4$). BEC decreases as the cell gas flow rate increases. Above 0.7 mL/min, the signal decreased suddenly as a consequence of a poor ion transmission. Table 3 shows elevated RPq parameters obtained for Cu and Fe after optimization. Although at elevated RPq levels, sensibility for these elements decreases, the limit of detection (LODs) were compatible with Cu and Fe concentrations in *Spirulina* dietary supplements.

The optimized method was validated by the analysis of a certified reference material NIST 1570a, spinach leaves, with a certified Cu and Zn concentration of 12.22 ± 0.86 and 82.3 ± 3.9 µg/g, respectively. The determined concentration is in good agreement with the certified values corresponding to 10.91 - 13.66 and 73.2 - 94.0 µg/g (low – high; n = 3) for Cu and Zn, respectively.

Total concentration of Fe, Cu and Zn in dietary supplements based on *Spirulina*

The results of metal concentrations (Fe, Cu and Zn) of 7 samples of dietary supplements based on *Spirulina* are listed in Table 4. The metal concentrations (µg/g) ranged from 63 ± 1 to 1066 ± 7 for Fe; 3 ± 0.3 to 57.3 ± 0.6 for Zn, and 1.8 ± 0.1 to 187.9 ± 1.9 for Cu. Concentrations of Fe, Cu and Zn were significantly different. The analyzed samples show Fe > Zn > Cu, in order of metal concentrations, and results are in a similar range to those reported

by Neher et al. (2018) and other contributions (Belay et al. 2008). The lowest levels of Fe and Cu were found in samples M6 (241 and 1.8 µg/g) and M7 (63.1 µg/g, below LOD), whereas M4 and M6 have the minor concentrations of Zn, 9.5 and 3.0 µg/g, respectively. It is known that the environmental conditions (pH, salinity, temperature, and pollution) can modify the major and trace elements concentrations in *Spirulina* (Park et al., 2013). Likewise, it has also been suggested that certain elements, such as Zn, appear to be incorporated at different rates at different growth stages (Al-Dhabi 2013).

As a general trend, the higher metal concentration was observed in samples with a higher concentration of *Spirulina* cells (samples M1, M3 and M4; Table 2). Whereas sample M7, where *Spirulina* cells were not found, showed the lowest metal concentration. Higher Cu contents in sample M2 (Table 4) reveals the additional Cu of excipients (chlorophyllin of Na and Cu) indicated in the commercial formulation (Table 2).

***In vitro* bioaccessibility of Fe, Cu, and Zn**

Bac of trace elements in dietary supplements based on *Spirulina* is critical from the nutritional, toxicological, and pharmacological perspective. Numerous investigations (Fukada et al., 2011, Fernández-García et al., 2009, Angelino et al., 2017,

Table 4. Global analyte concentration determined in *Spirulina* sold as a dietary supplement in Argentine.

Sample	Fe μg/g	Cu μg/g	Zn μg/g
M1	1066 ± 7[a]	6.7.0 ± 0.1[a]	26.9 ± 0.5[a]
M2	407 ± 4[a]	187.9 ± 1.9[a]	15.2 ± 0.3[a]
M3	737 ± 6[a]	4.4 ± 0.1[a]	17.6 ± 0.3[a]
M4	963 ± 7[a]	2.5 ± 0.1[a]	9.5 ± 0.4[a]
M5	480 ± 4[a]	2.8 ± 0.1[a]	57.3 ± 0.6[a]
M6	241 ± 3[a]	1.8 ± 0.1[a]	3.0 ± 0.3[a]
M7	63 ± 1[a]	<DL	11.6 ± 0.4[a]
p	4.12×10^{-26}[b]	6.00×10^{-24}[b]	7.34×10^{-23}[b]

[a]Data represent the mean ± $t_{(0.05;2)}$S/√n with n=3 [b]Significant difference ($p<0.05$).

M_1, M_6 and M_7: powder of hard gelatin capsules; M_2, M_4 and M_5: tablets; M_3 powder.
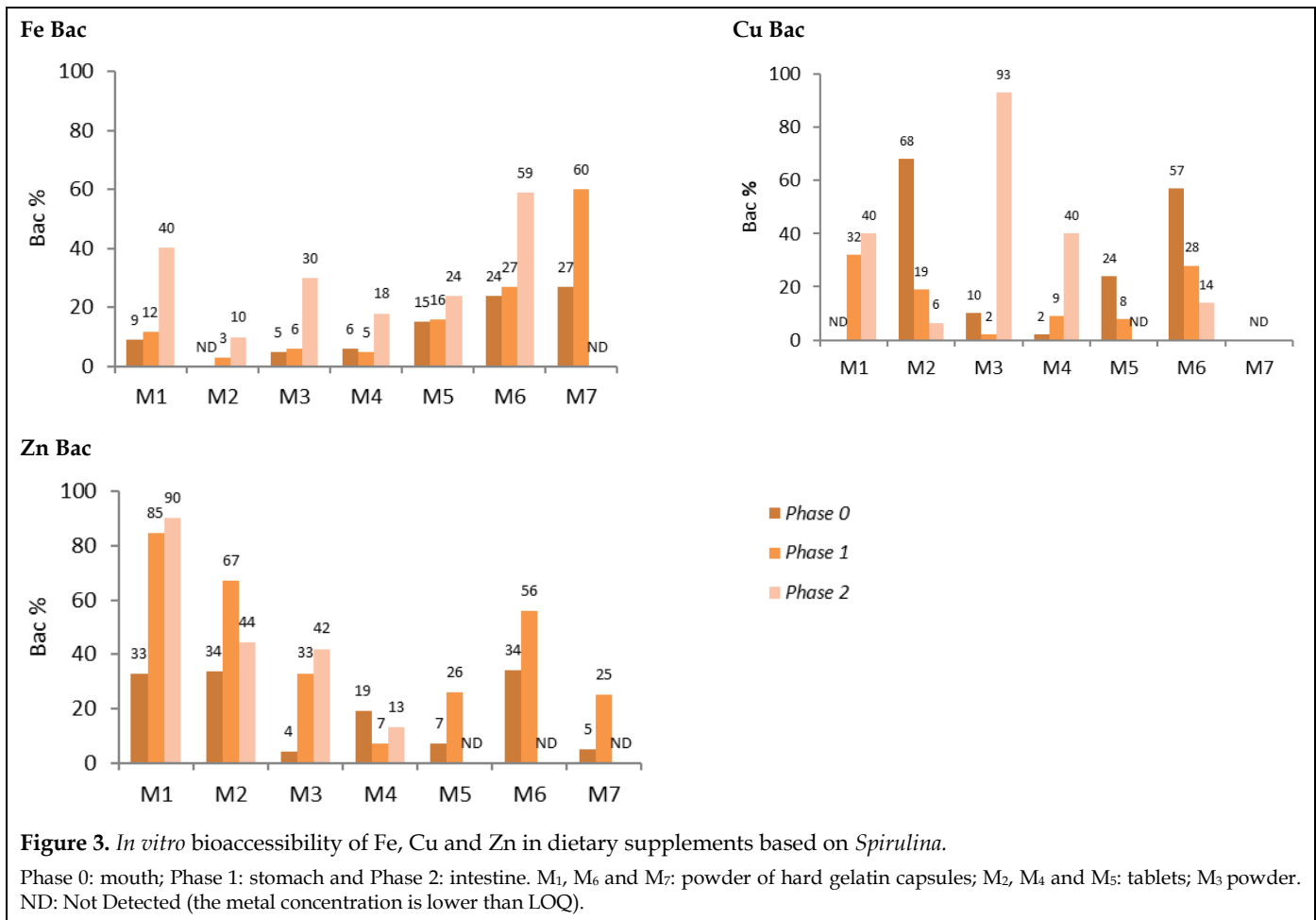
Kafaoglu et al., 2016) have focused on the *in vitro* Bac of minerals (Hg, Pb, As, Cd, Ca) in food, but only a few works reported this parameter of Fe, Cu and Zn in dietary supplements (Wojcieszek et al., 2016; Kafaoglu et al., 2016, Cases et al. 2001). The obtained values of Bac of metals, that represent oral, gastric, and intestinal conditions are quoted in Fig. 3.

Jayawardene et al. (2010) indicated variations of Pb, As, Cd and Hg Bac in five traditional Indian medicine samples in both gastric and intestinal phases. The differences were related to sampling compositions and pH (gastric pH 2 and intestinal pH 7) of the two phases. In this sense, marked variations in Fe, Cu and Zn Bac for the analyzed samples of *Spirulina* dietary supplements, in the different phases, could be explained by differences in the composition and pH, differences in the biological matrix of the samples, and presences of excipients or impurities. *In vitro* conditions, such as the pH, solution compositions, and precipitation reactions in the medium influence the extractability of metal.

The Bac of Fe (Fig. 3) ranged from 2.8 to 27% in oral conditions, 3 to 60% in the gastric conditions, and 3 to 40% in the intestinal conditions. In Fig. 3, it can be observed an increase in the Fe release under intestinal conditions for samples M1 to M6. An elevated Fe Bac in the intestinal phase encompasses Fe absorption in the upper parts of the gut,

notably in the duodenum and the proximal jejunum (Collins and Anderson, 2012). Fe Bac decreases when *Spirulina* cells concentration is low (sample M2 and M7) and in tablets (samples M2, M4 and M5) because the compression applied affects the dissolution kinetics (Díaz et al., 2012). Sample M7 with no *Spirulina* fragments did not show Fe Bac at the intestinal phase despite showing Bac in oral and gastric phases, probably of Fe from excipients or impurities. Fe from *Spirulina* cells has a higher intestinal Bac than Fe from excipients.

The Bac of Cu showed differences between the analyzed samples (Fig. 3), ranged from nondetectable levels up to 68% in oral conditions, up to 32% in the gastric conditions, and up to 93% in the intestinal conditions. In samples with a higher concentration of *Spirulina* cells (M1, M3 and M4) it is observed a higher Bac in the intestinal phase. M7, where no *Spirulina* fragments were observed did not show any Bac at different GI stages. Wojcieszek et al. (2016) reported the copper ions binding to amino acids, such as aspartic acid, phenylalanine, proline and tyrosine, forms different hydrophobic, soluble and bioavailable complexes. This fact could explain the variability in the behavior of Cu between the different samples. The higher Bac of Cu in the oral phase, from M2 sample (Fig. 3), results from the additional excipient chlorophyllin of Na and Cu.

Figure 3. *In vitro* bioaccessibility of Fe, Cu and Zn in dietary supplements based on *Spirulina*.

Phase 0: mouth; Phase 1: stomach and Phase 2: intestine. M_1, M_6 and M_7: powder of hard gelatin capsules; M_2, M_4 and M_5: tablets; M_3 powder. ND: Not Detected (the metal concentration is lower than LOQ).

Zinc Bac increases in the intestinal phase in samples with a higher *Spirulina* cell count (Sample M1 and M3) and with no magnesium stearate. Samples M4, M5, M6, and M7 with low *Spirulina* cells concentration showed a decreased Zn Bac in all digestion stages. Particularly in M4, M6 and M7, the low Zn Bac can be explained by the presence of Mg stearate. Wojcieszek et al. (2016) suggest that stearic acid deteriorates Zn Bac by the formation of insoluble Zn stearate.

CONCLUSIONS

In this work, the Bac of Fe, Cu and Zn at oral, gastric, and intestinal phases after *in vitro* digestion of dietary supplements based on *Spirulina* were successfully determined by ICP-DRC-MS. DRC technology was used to warrantee accurate results by decreasing interferences effects from a complex matrix such as *Spirulina* dietary supple-

ments. The analyzed samples showed Fe, Cu and Zn concentration variations consistent with the *Spirulina* cells count and the presence of excipients and impurities. The scanning electron microscopy study showed the textures and morphologies of the different solid dosage forms. In tablets, granules were observed; and in powders, the *Spirulina* trichomes.

Bac studies of Fe, Cu and Zn showed that samples with high *Spirulina* cell count increase digestion in the intestinal phase. In tablets of *Spirulina*, Fe Bac decreases according to compression, affecting the dissolution kinetics. Cu Bac showed differences between the digestion stages according to Cu from excipients or *Spirulina*. Zn Bac in the intestinal phase is affected by the presence of magnesium stearate in the formulations.

According to these observations, it is advisable to consider excipients and solid dosage form of

Spirulina dietary supplements since Fe, Cu and Zn Bac are affected, decreasing the nutritional value of this superfood.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Al-Dhabi NA (2013) Heavy metal analysis in commercial *Spirulina* products for human consumption. Saudi J Biol Sci 20: 383–388.

Al-Harbi NA (2016) Heavy metals concentration in commercially available *Spirulina* products. Biosci Biotechnol Res 9: 43–51.

Angelino DM, Cossu AM, Zanoletti M, Chiavaroli L, Brighenti F, Del Rio D, Martini D (2017) Bioaccessibility and bioavailability of phenolic compounds in bread: a review. Food Funct 8: 2368–2393.

Belay A, Gershwin ME, Belay A (2008) *Spirulina* in human nutrition and health. CRC Press Boca Raton.

Bharate SS, Bharate SB, Bajaj AN (2016) Interactions and incompatibilities of pharmaceutical excipients with active pharmaceutical ingredients: a comprehensive review. J Excip Food Chem 1 (3): 1131.

Boussiba S, Richmond AE (1980) C-phycocyanin as a storage protein in the blue-green alga *Spirulina platensis*. Arch Microbiol 125(1-2): 143–147.

Cases J, Vacchina V, Napolitano A, Caporiccio B, Besancon P, Lobinski R, Rouanet JM (2001) Selenium from selenium-rich *Spirulina* is less bioavailable than selenium from sodium selenite and selenomethionine in selenium-deficient rats. J Nutr 131: 2343–2350.

Colla L, Oliveira Reinehr MC, Reichert C, Costa JAV (2007) Production of biomass and nutraceutical compounds by *Spirulina platensis* under different temperature and nitrogen regimes. Bioresour Technol 98: 1489–1493.

Collins JF, Anderson GJ (2012) Molecular Mechanisms of Intestinal Iron Transport. Johnson LR, Ghishan FK, Kaunitz JD, Merchant JL, Said HM and Wood JD (eds). Physiology of the Gastrointestinal Tract, 5th ed. Boston. Academic Press (71): 1921–1947.

Davis A, Ruby MV, Bloom M, Schoof R, Freeman G, Bergstrom PD (1996a) Mineralogic constraints on the bioavailability of arsenic in smelter-impacted soils. Environ Sci Technol 30: 392–399.

Davis A, Ruby MV, Goad P, Eberle S, Chryssoulis S (1996b) Mass balance on surface-bound, mineralogic, and total lead concentrations as related to industrial aggregate bioaccessibility. Environ Sci Technol 31: 37–44.

de Souza JR, da Silva L, da Rocha MS, Saint'Pierre TD (2017) Dynamic reaction cell-ICP-MS as a powerful tool for quality control of a Se-enriched dietary supplement. Food Anal Meth 10: 3088–3097.

Díaz DC, Estevan MdCL, Díaz MC (2012) Manual de tecnología farmacéutica. España: Elsevier.

Fernández-García E, Carvajal-Lérida I, Pérez-Gálvez A (2009) *In vitro* bioaccessibility assessment as a prediction tool of nutritional efficiency. Nutr Res 29: 751–760.

Fukada T, Yamasaki S, Nishida K, Murakami M, Hirano T (2011) Zinc homeostasis and signaling in health and diseases. J Biol Inorg Chem 16: 1123–1134.

Good D, Wu Y (2017) Chapter 1: Excipient characterization. Properties, functionality, and applications in research and industry. In Pharmaceutical Excipient. John Wiley & Sons, Inc, pp. 20–51.

Habib MAB (2008) Review on culture, production and use of *Spirulina* as food for humans and feeds for domestic animals and fish. FAO Fisheries and Aquaculture Circular No. 1034. Rome, Italy: FAO Publications.

Hosseini SM, Shahbazizadeh S, Khosravi-Darani K, Mozafari MR (2013) *Spirulina paltens*is: Food and function. Curr Nutr Food Sci 9: 189–193.

Jayawardene I, Saper R, Lupoli N, Sehgal A, Wright RO, Amarasiriwardena C (2010) Determination of *in vitro* bioaccessibility of Pb, As, Cd and Hg in selected traditional Indian medicines. J Anal Atom Spectrom 25: 1275–1282.

Jubie S, Dhanabal SP (2012) Gas chromatography-mass spectrometry analysis and antibacterial activity of fatty acid mixture of *Spirulina platensis*. J Pharma Sci Res 4: 1836–1838.

Kafaoglu B, Fisher A, Hill S, Kara D (2016) Determination and evaluation of element bioaccessibility in some nuts and seeds by *in-vitro* gastrointestinal method. J Food Compos Anal 45: 58–65.

Kováčik J, Bačkor M, Kadukova J (2008) Physiological responses of *Matricaria chamomilla* to cadmium and copper excess. Environ Toxicol 23: 123–130.

Koyande AK, Chew KW, Rambabu K, Tao Y, Chu DT, Show PL (2019) Microalgae: A potential alternative to health supplementation for humans. Food Sci Hum Wellness 8: 16–24.

Mikhailov OV (2008) Gelatin-immobilized metal complexes: synthesis and applications. J Coord Chem 61: 1333–1384.

Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, Carriere F, Boutrou R, Corredig M, Dupont D (2014) A standardised static *in vitro* digestion method suitable for food–an international consensus. Food Funct 5: 1113–1124.

Neher BD, Azcarate SM, Camiña JM, Savio M (2018) Nutritional analysis of *Spirulina* dietary supplements: Optimization procedure of ultrasound-assisted digestion for multielemental determination. Food Chem 257: 295–301.

Park YI, Labrecque M, Lavoie JM (2013) Influence of elevated CO_2 and municipal wastewater feed on the productivity, morphology, and chemical composition of *Arthrospira* (*Spirulina*) *platensis*. ACS Sustain Chemi Eng 1: 1348–1356.

Pfeiffer T, Camagajevac I, Maronic D, Maksimovic I (2018) Regulation of photosynthesis in algae under metal stress. Environ Photosynth Future Prospect (13): 261–286.

Puyfoulhoux G, Rouanet JM, Besançon P, Baroux B, Baccou JC, Caporiccio B (2001) Iron availability from iron-fortified *Spirulina* by an *in vitro* digestion/Caco-2 cell culture model. J Agr Food Chem 49: 1625–1629.

Verni ER, Moyano F, Martinez LD, Lapierre AV, Gil RA (2013) Handling spectral interferences and matrix effects in DRC-ICPMS to assess the elemental profile in human serum samples after dissolution with formic acid. J Anal Atom Spectrom 28**:** 1655–1659.

Wojcieszek J, Witkos K, Ruzik L, Pawlak K (2016) Comparison of copper and zinc *in vitro* bioaccessibility from cyanobacteria rich in proteins and a synthetic supplement containing gluconate complexes: LC-MS mapping of bioaccessible copper complexes. Anal Bioanal Chem 408: 785–795.

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Contribution	Principe MV	Permigiani IS	Della Vedova MC	Petenatti E	Pacheco P	Gil RA
Concepts or ideas				x		x
Design				x		x
Definition of intellectual content				x		x
Literature search	x					
Experimental studies	x	x	x			
Data acquisition	x	x	x			
Data analysis	x	x	x		x	
Statistical analysis	x	x	x			
Manuscript preparation	x				x	
Manuscript editing	x				x	
Manuscript review	x	x	x	x	x	x