Bioaccessibility and decomposition of Cylindrospermopsin in vegetables matrices after the application of an *in vitro* digestion model

Sara Maisanaba[a], Remedios Guzmán-Guillén[a*], Rocío Valderrama[b], Giuseppe Meca[c] Guillermina Font[c], Ángeles Jos[a], Ana M. Cameán[a].

[a]Área of Toxicology, Faculty of Pharmacy, University of Sevilla, Sevilla, Spain.

[b]Mass Spectrometry Facility, Centro de Investigacion Tecnologica e Investigacion (CITIUS), University of Sevilla, Spain.

[c]Laboratory of Food Chemistry and Toxicology, Faculty of Pharmacy, University of Valencia, Burjassot, Spain.

*Corresponding author: Remedios Guzmán-Guillén

Area of Toxicology, Faculty of Pharmacy, University of Sevilla.C/ Profesor García González, nº 2. C.P. 41012, Seville. Spain.

E-mail address: rguzman1@us.es

Abstract

Research on the human exposure to Cylindrospermopsin (CYN) via consumption of contaminated food is of great interest for risk assessment purposes. The aim of this work is to evaluate for the first time the CYN bioaccessibility in contaminated vegetables (uncooked lettuce and spinach, and boiled spinach) after an *in vitro* digestion model, including the salivar, gastric and duodenal phases and, colonic fermentation under lactic acid bacteria. The results obtained showed that the digestion processes are able to diminish CYN levels, mainly in the colonic phase, especially in combination with the boiling treatment, decreasing CYN levels in a significant way. Moreover, the potential decomposition products in a pure CYN solution and in CYN-contaminated vegetables were evaluated using UHPLC-MS/MS Orbitrap. Under the conditions assayed, only two diastereoisomers of the same fragment with *m/z* 292.09617 have been detected in all the analysed samples, with the exception of digested vegetables. Therefore, in terms of risk assessment, the digestion seems to play an important role in reducing the final bioaccesibility of CYN, and the consumption of cooked vegetables (spinach) would be safer in comparison to raw vegetables.

Keywords: Cylindrospermopsin, bioaccessibility, decomposition products, lettuce, spinach

1. Introduction

Harmful cyanobacterial algal blooms are proliferating world-wide due to anthropogenic nutrient enrichment. They represent a serious threat to the use and sustainability of our freshwater resources due to their ability to synthesize cyanotoxins (Paerl et al., 2011; Merel et al., 2013). Cyanotoxins caused by cyanobacterial blooms have been associated with the death of wildlife and domestic animals, and represent a risk to human health through exposure to contaminated freshwater, ingestion of contaminated drinking water, or by consumption of contaminated food (Rastogi et al., 2014). Cylindrospermopsin (CYN), a zwitterionic and hydrophilic alkaloid toxin consisting of a tricyclic guanidine moiety bridged to a hydroxymethyluracil group (Ohtani et al., 1992), is a naturally occurring cyanotoxin originated by different cyanobacteria such as *Cylindrospermopsis raciborskii, Chrysosporum ovalisporum, Anabaena lapponica* and *Aphanizomenon flos-aquae*, among others (Kokocinski et al., 2017; Buratti et al., 2017). Besides, CYN is the first member of a small group of related alkaloids to be isolated, along with 7-epi-cylindrospermopsin, 7-deoxy-cylindrospermopsin, 7-deoxy-desulfo-cylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin (Wimmer et al., 2014).

Among its mechanisms of toxicity, CYN induces protein synthesis inhibition (Terao et al., 1944), oxidative stress (Gutiérrez-Praena et al., 2011a,b; 2012; Puerto et al., 2011; Guzmán-Guillén et al., 2013), and potentially immunotoxic, genotoxic and carcinogen effects (Buratti et al., 2017; Hercog et al., 2017; Pichardo et al., 2017). In fact, it is currently one of the most studied cyanotoxins on a worldwide scale, with a proposed guideline value of 1 μg L^{-1} in drinking water and a proposed Tolerable Daily Intake (TDI) of 0.03 μg kg^{-1} of body weight based on its potential human health risks (Humpage and Falconer, 2003).

Major routes of human exposure are through ingestion of CYN contaminated drinking water, inhalation while showering, dietary intake via consumption of cyanotoxins in contaminated foods as fish, mollusks, vegetables and algal dietary supplements (Jasim and Saththasivam, 2016; Testai et al., 2016). The accumulation capacity of CYN in different food items has been demonstrated and reviewed several times, mainly in bivalves, crustaceans, gastropods and fish (Gutiérrez-Praena et al., 2013), although the data reported at the moment are still limited according to the European Food Safety Authorithy (EFSA) (Testai et al., 2016). Moreover, few studies are focused on the bioaccumulation and its effects in agricultural crops (Silva and Vasconcelos, 2010; Prieto et al., 2011; Kittler et al., 2012; Corbel et al., 2014). Recently, Cordeiro-Araujo et al. (2017) described the bioaccumulation of CYN in lettuce (*Lactuca sativa L.*) and arugula (*Eruca sativa Mill*.), where its content decreased at the highest concentration assayed. The results indicated that irrigation of both vegetables, lettuce and arugula, with CYN-contaminated water at low concentrations, may constitute a potential human exposure route. This bioaccumulation may be possible due to the absorption of toxins by vegetables, if surface water contaminated with cyanotoxins is used for agricultural purposes, thus representing risks in terms of food safety for human consumers (Prieto et al., 2018).

Lots of vegetables, such as lettuce, spinach, cabbage, and sprouts are minimally processed and they are usually consumed fresh. Moreover, their consumption has been intensified over the last decade due to their high nutritional value, changes in social eating habits, and easy accessibility (Ngnitcho et al., 2017). Although some recommendations and limit values have been already mentioned in order to prevent or manage the possible human health effects induced by CYN exposure under different

scenarios, no suggestions have been proposed in the case of vegetables, even when the risks have been demonstrated (Cordeiro-Araujo et al., 2017).

The evaluation of the risks to human health that a contaminant can suppose, must cover pathways of its direct and indirect exposure, and could be a function of the oral toxicity reference values of the contaminant in question. However, one problem that may arise when calculating risks is over-conservatism, because a perception of risks may not be realistic. This happens, for example, when it is assumed that 100% of the ingested dose of the contaminant is bioaccessible or bioavailable (Meunier et al., 2011; Martínez-Sánchez et al., 2013). Nevertheless, after water or food consumption, metabolic processess are involved in the modification or degradation mechanisms which could cause changes in the ingested contaminant and only a fraction of the initial content would be accessible for absorption (Bordin et al., 2017). This available fraction of a susbtance which is soluble in the gastrointestinal (G.I.) tract is known as oral bioaccessibility and it could be calculated by *in vitro* simulations of the G.I. fluid using different extractants (Rubby et al., 1999; Ng et al., 2010). *In vitro* models allow the screening of various food ingredients or contaminants, before conducting *in vivo* animal or human trials on a limited number of food matrices (Guerra et al., 2012; Ménard et al., 2018). These methods usually include the oral, gastric and duodenal phases, and occasionally colonic fermentation. Among other factors, the presence of digestive enzymes and their concentrations, pH, digestion time, and salt concentrations are considered in each of the mentioned stages. In this sense, the final purpose would be to reproduce physiological conditions *in vivo* (Minnekus et al., 2014).

To the extent of our knowledge, only two recent works have evaluated the effects on CYN bioaccessibility after the application of an *in vitro* digestion model in food matrices, specifically in mussels and fish (Freitas et al., 2016; Maisanaba et al., 2017).

However, there are no available studies focused on CYN degradation in vegetables after being submitted to the different digestion phases.

Only few reports are available in the scientific literature concerning the influence of pH, temperature and irradiation on CYN concentrations and its forming products (Adamski et al., 2016a, b), or of cooking treatments (boiling, broiling, steaming and microwaving) in contaminated fish (Guzmán-Guillén et al., 2017; Prieto et al., 2017). Considering the relevant toxic properties of CYN, it would be of interest to investigate the potential degradation products generated in contaminated vegetables after cooking (boiling) and the digestion process, to obtain a more accurate human exposure scenario, following the EFSA recommendations (Testai et al., 2016).

In view of these reports, the aim of the present study was to investigate for the first time the influence of an *in vitro* digestion model to dilucidate CYN bioaccessibility on uncooked lettuce (*Lactuca sattiva*) and spinach (*Spinacia oleracea*), as well as in boiled spinach contaminated with CYN under laboratory conditions. Moreover, the detection of potential CYN by-products in a pure CYN solution and in digested vegetables (uncooked and cooked) was also carried out by Ultra High-Performance Liquid Chromatography-Tandem Mass Spectrometry (UHPLC-MS/MS) Orbitrap.

2. Materials and Methods

2.1. Chemical and reagents

Cylindrospermopsin standard (MW=415.43 g/mol; 95% purity) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in Milli-Q water (1000 µg mL^{-1}) and diluted as required for their use as working solutions (0.005-5 µg mL^{-1}). All chemicals and reagents used in this study were analytical grade materials. For the simulated digestion: formic acid (HCOOH), potassium chloride

(KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts were obtained from Sigma–Aldrich (St. Louis, MO, USA). MRS broth for the bacterial strains growth was supplied by Oxoid (Madrid, Spain).

For the analytical steps: acetonitrile, methanol, trifluoroacetic acid (TFA), acetic and formic acids, dichloromethane of LC–MS grade were purchased from Merck (Darmstadt, Germany). Deionized water (<18MΩ cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA). BOND ELUT® Carbon cartridges (PGC column) (500 mg, 6 mL) and Bakerbond® C18 cartridges (500 mg, 6 mL) were supplied by Agilent Technologies (The Netherlands, Europe) and Dicsa (Andalucía, Spain), respectively.

2.2. *Lactic-acid bacteria (LAB) and growth conditions*

The selected bacterial strains and their growth conditions were described by Maisanaba et al. (2017). The bacterial strains used could be found in the large intestine, being a suitable representation of the real conditions in humans. LAB include a large number of bacterial genera, being *Lactobacillus* and *Bifidobacterium*, two of the best known and most predominant in the large intestine. Eight commercial probiotic strains were used, namely, *Lactobacillus casei* CECT 4180, *Lb. casei rhamnosus* CECT 278T, *Lb. plantarum* CECT 220, Lb. *delbur sub bulgaricus* CECT 4005, *Lb. Salivarus* CECT 4305, *Lb. johnsoni* CECT 289 and *Bifidobacterium breve* CECT 4839T and *B. bifidum* CECT 870T. All of them were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain), in sterile 18% glycerol. For longer survival and higher quantitative retrieval of the cultures, they were stored at -80º C. When needed, recovery

of strains was undertaken by two consecutive subcultures in appropriate growth media prior to use (Laparra and Sanz, 2009; Meca et al., 2012).

2.3. Experimental setup

Commercial fresh lettuce and spinach were bought in a supermarket and transferred to the laboratory. In this work, uncooked lettuce, uncooked spinach and boiled spinach were tested as different matrices. Then, 6 leaves per type of edible vegetable (uncooked lettuce, or uncooked spinach or boiled spinach) (n = 6, average weight per leaf: 5.03 ± 0.08 g fresh weight (f.w.)) were homogenously spiked with 250 µL of a stock solution containing 20 µg CYN mL^{-1} (equivalent to 1 µg CYN g^{-1} f.w.). For spiking the samples, the volume of the CYN stock solution (250 µL) were pipetted over the whole surface of the leaves, as spread as possible, inside of a tube in horizontal position. Then, leaves were left at room temperature until the total absorption and complete absorption of the previously spiked CYN volume. The concentration was selected taking into account the environmental CYN acccumulation data in vegetables (Kittler et al., 2012, Santos et al., 2015) and the sensitivity of the method employed to detect CYN by-products (Guzmán-Guillén et al., 2017, Prieto et al., 2017).

After spiking the samples with CYN (and later the boiling of spinach samples as explained in the following section), 3 samples per matrix were frozen at -80ºC and lyophilized (Cryodos 80 204 model, Telstar, Tarrasa, Spain) before CYN extraction and purification to determine the amount of CYN present in each matrix before digestion, which represent the corresponding controls: uncooked and non-digested lettuce and spinach, and non-digested boiled spinach. The final mean lyophilized weight was 0.91 ± 0.03 g dry weight (d.w.), giving a weight loss of approximately 82% due to lyophilization (and cooking in the case of boiled spinach). The other 3 samples per matrix were submitted to the *in vitro* digestion model to assess CYN bioaccessibility.

Moreover, 3 pure CYN standard solutions (20 µg CYN mL^{-1}) were also tested as different controls (uncooked and non-digested CYN solution; uncooked and digested CYN solution; boiled and digested CYN solution) to assess the behavior of CYN in solution under the digestion process.

2.4. Boiling of spinach leaves

The spiked spinach (n = 6) were boiled for 2 min following the procedure described by Guzmán-Guillén et al. (2017), according to the size of the portions (5 g), maintaining the good appearance of the cooked spinach. Briefly, each spinach leaf was introduced into pots of stainless steel with cool tap water (50 mL) and 2 min were counted once the water reached 100ºC.

2.5. Extraction and purification of CYN from vegetables and boiling waters

Three samples per matrix were extracted and purificated according to Prieto et al. (2018). Briefly, the lyophilized samples were extracted with 6 mL of 10% acetic acid; after ultraturrax homogenization (30 sec), the sample was sonicated and stirred (15 min each process). The resulting mixture was centrifuged (12,000 rpm, 15 min). For purification, BOND ELUT® Carbon cartridges were activated with DCM/MeOH (10/90, acidified with 5% formic acid) (10 mL) and rinsed with Milli-Q water (10 mL), before the sample was passed through the cartridge, washed with Milli-Q water (10 mL), and eluted with DCM/MeOH (10/90, acidified with 5% formic acid) (10 mL). Finally, the eluate was concentrated in a rotary evaporator and resuspended in 1 mL Milli-Q water, prior to its UPLC-MS/MS analysis.

CYN from the waters used for boiling spinach was extracted and quantified by UPLC-MS/MS following the methods described by Guzmán-Guillén et al. (2012, 2017).

2.6. In vitro digestion model

Three samples per matrix (approximately 5 g per sample) were submitted to the following digestion model, according to Maisanaba et al. (2017) with minor modifications (Fig. 1), as well as two of the pure CYN standard solutions described in section 2.3. Briefly, for the saliva/pepsin/HCl digestion, 5 g of uncooked lettuce or spinach, or boiled spinach previously spiked with 20 µg CYN mL^{-1} (1 µg CYN/g f.w.), were mixed with 3 mL of artificial saliva. These mixtures composed by the vegetables and the artificial saliva were introduced in plastic bags, containing 40 g of water and homogenized by Stomacher IUL Instruments (Barcelona, Spain) during 30 sec, simulating the mastication process. Then, a pepsin solution was added and the pH was changed to a value of at least 2, to activate the gastric enzyme. Afterwards, the weight was completed to 50 g by the addition of water, and incubated for 2 h in an orbital shaker (37ºC, 250 rpm). After the gastric incubation period, the pancreatic digestion was simulated by the addition of a mix of pancreatin and bile salts, leaving it for 2 h in an orbital shaker (37ºC, 250 rpm). Finally, to mimic the colonic fermentation, a mixture of the LAB suspensions (previously growth) at a concentration of 10^{12} CFU/mL was incorporated to the duodenal simulate intestinal fluid and incubated for 48 h at 37°C in anaerobic conditions. After each digestion step, aliquots of each one were taken and centrifuged at 4000 rpm, 4ºC, 10 min. The supernatant obtained was filtered by 0.22 µm filters previously to the analytical analysis to quantify and determine CYN bioaccessibility in each digestive fluid.

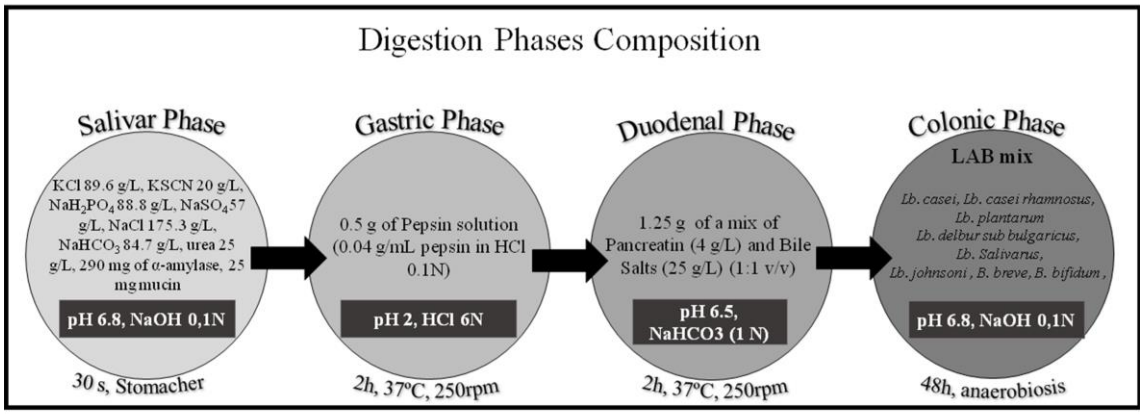

Figure 1. Digestion phases composition and procedure of the applied *in vitro* digestion model.

2.7. Analytical determination

Chromatographic separation was performed using a UPLC Acquity (Waters) coupled to Xevo TQS-micro (Waters) consisting of a triple quadrupole mass spectrometer equipped with an electrospray ion source operated in positive mode, on a 50 x 2.1 mm Acquity BEH C18 1.7 µm column, and at a flow rate of 0.45 mL min^{-1}, according to Prieto et al. (2018). Briefly, the binary gradient consisted of (A) water, and (B) methanol, both with 0.1% formic acid (v/v). Injection volume was 5 µL. Elution profile was: 0 % B (0.8 min), linear gradient to 90% B (2.2 min), 90 %B (1 min) and finally 100 % B (1 min). Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions for CYN were 416.2/194.0 (for quantitation) and 416.2/176.0 (for confirmation). For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: Capillary voltage: 3.0 kV, Source Temperature: 500ºC and source Gas flow: 1000 L h^{-1}.

2.8. Characterization of CYN and its decomposition products before and after digestion by UHPLC-MS/MS Orbitrap

In order to characterize the possible decomposition products of unconjugated CYN produced by the *in vitro* digestion (salivar, gastric, duodenal and colonic phases) for uncooked lettuce and spinach, and boiled spinach, as well as in the CYN standard solutions, these samples were analyzed by UHPLC-MS/MS Orbitrap. All analysis were performed using a Thermo Scientific liquid chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS, connected to a quadrupole-orbitrap Qexactive hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was equipped with a heated-electrospray ionization probe (HESI-II). Xcalibur software was used for instrument control, data acquisition and data analysis. Trace Finder 3.3 software was used for data analysis also. Chromatographic separations were performed using an Acquity UPLC BEH (bridged ethyl hybrid) C18 column (2.1 x 100 mm, 1.7 µm) (Waters), according to Guzmán-Guillén et al. (2017). The column was maintained at 40°C and eluted under the following conditions: 100% of eluent A (2 min) and gradient elution from 100% to 30% of eluent A (10 min) at a flow rate of 0.3 mL min^{-1}. Eluent A was water/formic acid (0.1%, v/v), and eluent B was acetonitrile/formic acid (0.1%, v/v). Injection volume was 5 µL.

MS detection of the CYN decomposition products previously observed by Adamski et al. (2016 a,b), Guzmán-Guillén et al. (2017) and Prieto et al. (2018) was performed with the Q-Exactive Orbitrap mass spectrometer through the accurate *m/z* measurement of each molecule, by scanning from 100 to 1000 *m/z*, in positive and full scan (FS) mode at a resolution of 70,000 (full width half maximum, FWHM at *m/z* 200). Each compound observed was associated with a chemical formula hypothesis. Then, a relative mass error expressed in ppm was calculated, a result lower than 5 ppm being considered as an acceptable value (Makarov et al., 2006). The Q-Exactive Orbitrap was also used to sustain the identity of CYN decomposition products

performing tandem mass spectrometry (MS^2) experiments using the parallel reaction monitoring (PRM) method, which was acquired in positive mode and a resolution of 17500, with an isolation window of 1 *m/z*, and normalized collision energy was set at 35 eV. The selected $[M+H]^+$ were 214.15500, 290.08052, 292.09617, 336.16663, 338.18228, 414.10780, 416.12345 and 434.13401. The identity of the compounds was then sustained by the agreement between theoretical and experimental isotopic patterns, and by the consistency with the resulting product ions formed during MS^2 analysis. HESI source parameters were: spray voltage 3.5 kV, capillary temperature 320ºC, sheath, auxiliary and sweep gas flow rate (N_2) 45, 15 and 2 (arbitrary units) respectively, probe heater temperature 350ºC and S-Lens RF level 50.

Considering the areas of the corresponding chromatographic peaks, a relative comparison among the different vegetable matrices tested and the CYN standard solutions assayed as controls was carried out by selecting the fragments at *m/z* 212.13935 for the molecular ion $[M+H]^+$ 292.09617, as no other ions were detected in the samples.

2.9. Statistical analysis

Results were subjected to one-way analysis of variance (ANOVA) with the statistical package INSTAT, Graph Pad™, and represent mean ± standard deviation (SD) of samples per group. Differences in mean values between groups were assessed by the Tukey's test and were considered statistically significant at $p < 0.001$ level (99.9% confidence interval).

3. Results

3.1. Bioaccessibility of CYN after the in vitro digestion model

First of all, it is important to note that CYN concentration values presented in this work represent the un-bound toxin, as the method employed could only detect free toxin. Results from the digestion of the pure CYN solution (20 µg mL^{-1}) showed a significant decrease from the gastric phase (83% bioaccessibility), being more evident in the duodenal (76%) and colonic (67%) phases. Moreover, the values obtained from each digestion step of lettuce samples were compared to the respective "control group" (1.12 µg g^{-1} f.w.), and the same for digested spinach (1.17 µg g^{-1} f.w.), both of them considered as 100% of CYN bioaccessibility for these matrices when the digestion model was applied ("control vegetable, non-cooked and non-digested") (Fig. 2). However, in the control boiled spinach, CYN concentration was much lower (0.18 µg g^{-1} f.w.), only 14.95% of CYN compared to its non-cooked matrix ("non-digested boiled spinach"). This low concentration was considered as the highest bioaccessibility that could be found in the boiled spinach.

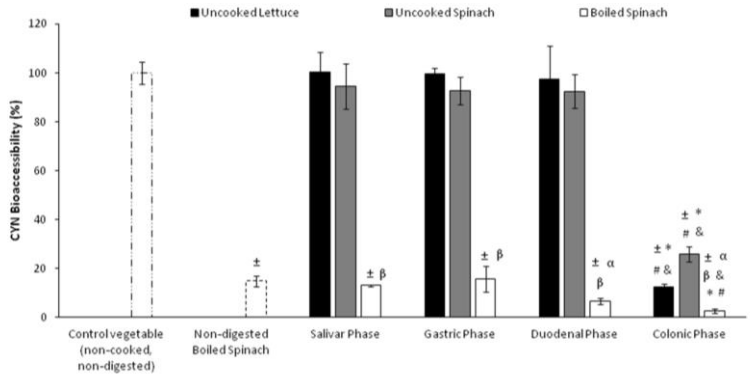

Figure 2. CYN bioaccessibility in control vegetables (non-cooked, non-digested), non-digested boiled spinach, uncooked lettuce (black) and spinach (grey) and in boiled spinach (white). $^{\alpha}$ statistical differences (p<0.001) of boiled spinach compared to non-digested boiled spinach; $^{\pm}$ statistical differences (p<0.001) compared to control vegetable (non-cooked, non-digested); $^{\beta}$ statistical differences

(p<0.001) between boiled and uncooked spinach on its corresponding digestion phase; * statistical differences (p<0.001) in the same matrix compared to salivar phase; # statistical differences (p<0.001) in the same matrix compared to gastric phase; & statistical differences (p<0.001) in the same matrix compared to duodenal phase.

Regarding CYN bioaccessibility after the application of the *in vitro* digestion model, in general, a similar pattern was observed in both uncooked vegetables, obtaining similar values in the salivar, gastric and duodenal phases. In the three first stages, high bioaccessibility percentages (almost 100%) were reached, and no significant differences were found in comparison to the control group (non-cooked, non-digested vegetables). However, a drastic reduction was evidenced after the LAB exposure in the colonic phase. On the other hand, in the boiled spinach, from the initial to the final stage low values were detected, being normal taking into account the concentration detected in its control group (cooked but not digested spinach).

The content (%) of CYN in the salivar fluid resulted variable depending on the sample: from 100±8.1% and 94±9.3% for the uncooked lettuce and spinach, respectively, to 13±0.3% as the worst case for boiled spinach. Significant differences (p<0.001) were obtained between the cooked and the uncooked spinach as well as compared to non-cooked/non-digested control, but no statistical changes were recorded between the fresh vegetables and their control (non-cooked/non-digested).

The gastric digestion showed a similar pattern than the previous analyzed phase. The highest bioaccessibility was obtained for the non-cooked vegetables groups, 99.4±2.7% for lettuce and 92.7±5.8%, for spinach, whereas the bioaccessibility value for CYN in boiled spinach was much lower, 15.7±5.2%. The significant differences obtained were the same that in the before-mentioned salivar phase. Statistical variations

($p<0.001$) were described between the uncooked and cooked spinach as well as with the control group not submitted to any treatment.

Regarding the duodenal digestion, the results were in agreement with those observed in the above-mentioned phases, with the highest decrease of CYN bioaccessibility in the case of boiled spinach (6.5±1.3%), and values above 90% both lettuce and uncooked spinach. Once again, boiled spinach presented significant differences ($p<0.001$) compared to uncooked spinach, non-cooked and non digested control, and in this case as first time, statistical changes ($p<0.001$) were also observed with respect to non-digested /boiled spinach control group.

Finally, the most remarkable changes in the bioaccessibility of CYN were found under the exposure of LAB, being the most drastic stage in all the groups assayed as well as in all the digestive phases. In the colonic fluids, CYN showed the lowest bioaccessibility. Non-cooked vegetables presented reductions of CYN higher than 80% compared to the initial value (final value determined 12.3±1.4%) in the lettuce and more than 70% (final value determined 25.8±3.0%) in non-cooked spinach. The lowest value, 2.8±1.0%, was detected for the cooked spinach. Significant differences ($p<0.001$) in the bioaccessibility of all the CYN-contaminated vegetables when the colonic stage of each experimental group is compared to their respective salivar, gastric and duodenal phases were recorded. Moreover, statistical changes ($p<0.001$) were also obtained between the non-cooked/non-digested control group and the colonic bioaccessibility value of non-cooked vegetables. In the case of the boiled spinach, as in the duodenal phase, significant values ($p<0.001$) were observed between both control groups and the colonic phase of non-cooked spinach.

3.2. Analysis of CYN in water samples

CYN transference to the waters used for boiling spinach was also evaluated, detecting values of 39.8 ± 2.1 μg L^{-1} CYN.

3.3. Study of CYN fragmentation due to the in vitro digestion by UHPLC-MS/MS Orbitrap

As it can be observed in Fig. 3, the mass spectra of the assessed samples revealed the presence of 2 decomposition products (C1-A and C1-B, with approximate retention times of 2.39 and 3.87 min, respectively), which in fact are diastereoisomers of the same fragment with *m/z* 292.09617, in all the analysed samples, with the exception of digested vegetables. The relative abundances of both fragments in comparison with their highest presence in the control CYN solution (100%) are also represented in Fig. 3. In the pure CYN solution, C1-A and C1-B were reduced to 11% and 8% with salivary juices, respectively, and values for both of them in the subsequent phases were almost imperceptible. When this solution was boiled, the reductions in both fragments were still evident from the salivar phase, but less pronounced than in the uncooked solution, leading to 25% and 14% of C1-A and C1-B after salivar digestion. The relative abundances in the following digestion phases were higher than in the case of the uncooked CYN solution, ranging between 10 and 14%. In the uncooked vegetable matrices, medium reductions were detected for these fragments, with relative abundances of 11-19% compared to control CYN solution. In boiled spinach, C1-A and C1-B were not observed in neither the non-digested or in the digestion phase samples, with the exception of a small trace of C1-B in the boiled, non-digested spinach (0.57%). Moreover, no fragments were observed in the different digestion phases from uncooked lettuce and spinach.

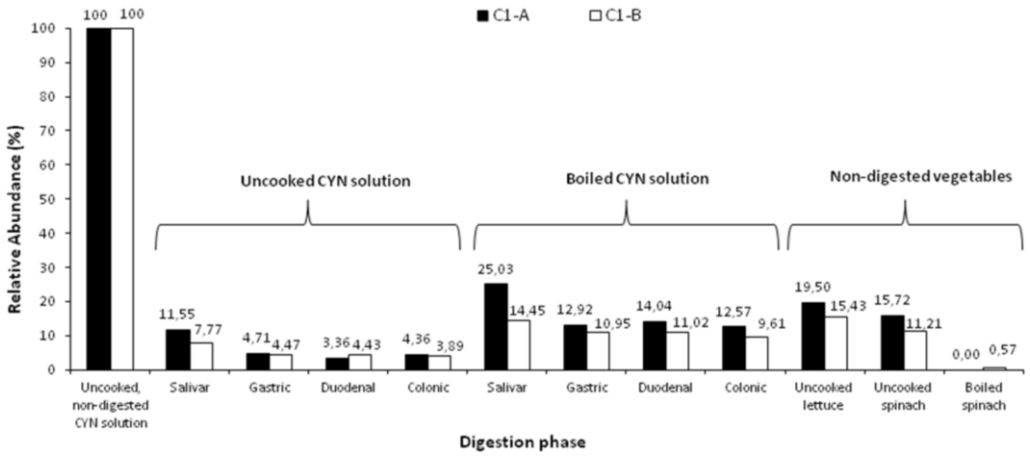

Figure 3. Relative percentage of two diastereoisomers (C1-A and C1-B) of the CYN by-product at *m/z* 292.09617 in control CYN aqueous solutions submitted or not to *in vitro* digestion, and in uncooked and non-digested lettuce and spinach, and non-digested boiled spinach.

4. Discussion

In order to advance in the exposure evaluation of CYN in the frame of the risk assessment, the effects of the digestion phases in CYN-contaminated food and the final bioaccesibility taking into account the different food sources or the food preparations treatments used in the daily life by the consumers should be taken into account. In fact, the ESFA recommends the need to address the effects of food cooking treatments on cyanotoxins, as well as the final bioaccessibility of them after all the processes suffered following the ingestion, by application of the most appropriate analytical methods (Testai et al., 2016). Food digestion is a complex process in which many factors are involved and that has actually aroused the interest of the food industry because there is a growing relationship between food and health and therefore the reduction of the development of certain chronic diseases (Bornhorst et al., 2016). *In vitro* G.I. digestion systems are a valuable tool for understanding the behaviour of food and food

components during human digestion as well as for a better understanding of digestive kinetics (Lucas-González et al., 2018; Ménard et al., 2018). The most drastic mechanical and desintegration changes are produced in the two first main digestion stages, the mouth and the stomach, and, the most notably enzymatic, digestive and absorptive processes are placed in the intestine, considering the small and large intestines (Guerra et al., 2012).

To the extent of our knowledge, this is the first work where the possible alterations and degradation of CYN present in non-cooked lettuce and spinach, as well as in boiled spinach have been evaluated after the application of an *in vitro* digestion model, in order to simulate a real scenario in humans' life, determining in each digestion phase its bioaccessibility. In general, a similar pattern in fresh vegetables was observed, obtaining the most accused changes under LAB exposure and no significant variations were recorded in the salivar, gastric or duodenal phases. By contrast, in the boiled spinach, from the duodenal phase the CYN bioaccesibility significantly decreased, corresponding again the highest loss to the action of LAB.

It is important to note that concentrations of CYN in the non-cooked and cooked vegetables have been evaluated before to the application of the digestion model. In this manner, the real degradation of CYN would be due to the digestive phases and not to the boiling process, in the case of cooked spinach. The loss in the case of boiled spinach could be related with the release to boiling waters or maybe a change in the food structure which could interfere in the caption of the contaminant (Mauvault et al., 2011). In fact, according to the prediction mentioned, a significant concentration of CYN has been detected in boiling waters, being approximately 40 μg L^{-1} CYN, fact that other authors have previously observed in waters from boiled mussels and fish (Freitas et al., 2016; Guzmán-Guilllén et al., 2017). Several authors have demonstrated the capability

of different cooking treatments in the degradation of CYN, describing in most cases a beneficial response by the application of different cooking techniques for reducing the content of CYN in contaminated food depending on the matrix (Freitas et al., 2016; Guzmán-Guillén et al., 2017; Maisanaba et al., 2017; Prieto et al., 2017). Globally, and taking into account all these results, it is suggested that both, cooking and digestion processes, should be considered for a more realistic exposure evaluation of CYN through food consumption, because the final concentration to which humans are exposed to contaminants would be much lower than expected, as it was suggested by Vesanvoort et al. (2005).

In the present study, CYN stability was maintained for the three first digestive phases, performed at 37ºC and a pH range from 2 to 6.8. Stability of CYN has been reported up to 5 weeks in the range 4-50ºC (Chiswell et al., 1999) and under acidic and neutral conditions (pH 3-7) (Adamski et al., 2016a). In this sense, Guzmán-Guillén et al. (2017) recorded higher decreases in CYN concentration in steamed fish compared to boiled fish, and the steamed ones presented the highest pH. It seems that the main representative enzymes involved in the digestion (amylase in the salivar, pepsin in the gastric and pancreatin in the duodenal stages) have played a low role in the case of uncooked vegetables, in agreement with Rocha et al. (2013) who observed that pepsin and pH had little influence on the bioaccessibility of fluoride. By contrast, Freitas et al. (2016) described an absolute degradation of CYN from mouth to duodenal compartments in uncooked mussels of *M. galloprovincialis*. The authors did not discard that the degradation could be related to the binding of CYN to digestive enzymes and its subsequent non detection, as well as, to the very lower concentrations used to intoxicate the mussels (aprox. 10-15 µg/L of CYN per day, 4 d). Therefore, and taking into account differences previously reported related to the specific matrices assayed

(Domingo, 2011), it seems that in fresh food the matrix may have some influence in the digestion process. In comparison to mussels which represents a good source of proteins and polyunsaturated fatty acids (Mohanty et al., 2018), and similarly to most vegetables, lettuce and spinach are not rich source of proteins but provide dietary fiber, minerals, vitamins and bioactive compounds (Kim et al.,2016). Hence, a more detailed study of the influence of these components would be appropriate to dilucidate the potential interactions with CYN.

On the other hand, in the cooked spinach, no significant changes were observed in the salivar and gastric fraction compared to the cooked but not digested control. The significant decrease observed in the duodenal phase indicates that the conditions presented in the digestive phase could also affect CYN degradation joined to the boiling treatment. Thus, the cooking treatment could change the matrix structure and facilitate the action of intestinal enzymes, in contrast to what reported Mauvault et al. (2011) in fish, which have a protein structure compared to the more fibrous one in vegetables. Also in contrast are the results by Freitas et al. (2016), who obtained a slightly, though not significant, higher bioaccessibility of CYN in steamed mussels, compared to the uncooked ones, in all digestion phases. However, according to Colle et al. (2010) thermal treatment of tomato pulp after homogenization did not improve bioaccessibility. In this sense, it is suggested that maybe the entrance of pancreatin and bile salts could be facilitated, causing CYN degradation.

The most severe changes in CYN bioaccesibility were observed under the LAB action, decreasing the CYN concentration detected from the duodenal to the colonic phase in a 70% in non-cooked vegetables and 65% in the cooked spinach. Several functions have been described for LAB, highlighting the fermentation of plants, fish, meats and milk and turn them into tasty food products with increased shelf life, or, more

important in our case, other LAB help digesting food and create a healthy environment in the intestine (Teusink and Molenaar, 2017). In fact, LAB count with the 'Generally Regarded as Safe (GRAS)' status according to the US Food and Drug Administration (Gálvez et al., 2007). Several LAB have been reported for its successful applications to bind and remove toxic contaminants from food and water (Halttunen et al., 2007). This remarkable action produced by LAB in CYN reduction in vegetables is in agreement with that previously reported in cooked fish, and even non detected values were shown in the boiled fish (Maisanaba et al., 2017). Manubolu et al. (2014) also described the potential of rumen microorganisms to reduce microscystins and nodularin, suggesting that the microbial flora may preserve or protect against cyanotoxin intoxication.

Freitas et al. (2016) suggested that the binding of CYN to digestive enzymes and its subsequent non-detection could be a possibility, because they obtained the same decreasing trend in CYN availability in pure solution and in intoxicated mussels. In vegetables, either cooked or not, the own vegetables components, such as fiber, could be hampering the action of the digestive enzymes. In addition, boiling spinach might have changed the matrix, as hypothesized before, and facilitated the action of enzymes in some way.

In relation to the characterization of CYN-decomposition products, and taking into account the fragmentation spectra by PRM, the fragments C1-A and C1-B observed in the present work are the same that were previously reported in fish cooked by boiling, steaming, microwaving and broiling (named C-2A and C-2B in those works) (Guzmán-Guillén et al., 2017; Prieto et al., 2018). These fragments also coincided with that detected in cultures of *C. raciborskii* influenced by high temperatures and UV-B irradiation in alkaline conditions (Adamski et al., 2016a, b), although in this case the authors only identified one fragment at 3.85 or 3.87 min, which would correspond to

C1-B in the present work. According to Adamski et al. (2016b), the fragmentation pathway of the molecule with *m/z* 292.09617 could result from the excision of the uracil ring from the tricyclic guanidine moiety with the subsequent insertion of a hydroxyl group and racemization. In the present work, 88% and 92% reductions were observed in the relative abundances of C1-A and C1-B after salivar digestion, followed by 95%, 97% and 96% reductions for the gastric, duodenal and colonic phases, respectively for C1-A, and 96% from the gastric phase for C1-B.

To our knowledge, in two previous studies, more fragments were detected in cooked fish: seven fragments in boiled and steamed fish (Guzmán-Guillén et al., 2017), and similarly seven fragments in broiled fish and eleven after microwaving (Prieto et al., 2017). The fact that more CYN by-products were detected in these previous works in fish compared to the vegetables in the present work, could be explained by differences in toxin concentration employed in the toxin spiking step. While in those works, high CYN concentrations (40 μg g^{-1} d.w.) were tested to assure the visualization of fragments, in the present study, a lower and more environmentally realistic concentration was chosen (1 μg g^{-1} f.w., equivalent to 20 μg g^{-1} d.w.). Another fact that could explain the hampered visualization of decomposition products could be the own matrix, due to the presence of fiber in vegetables, in comparison to fish products, rich in proteins.

5. Conclusions

In conclusion, the present work reports for the first time the effects on CYN degradation and its bioaccessibility occurred in CYN-contaminated fresh and cooked vegetables subjected to an *in vitro* digestion model, as well as, the CYN decomposition products obtained after the simulated digestion. The effects of the main four digestive steps have been taken into account (salivar, gastric, duodenal and colonic phases) in all

the experimental groups, being the LAB action the most efficient in reducing CYN levels in all of them, and also the small intestine only in the cooked spinach.. Moreover, 2 decomposition products which are diastereoisomers of the same fragment with *m/z* 292.09617 have been detected in all the analysed samples, with the exception of digested vegetables.

According to the data obtained, this study shows the importance of the digestion in the degradation of CYN and also the crucial role of the matrix employed, taking one more step in CYN risk assessment. More studies should be carried out in other matrices, with higher CYN concentrations, and with other dietary components (fiber, proteins, etc.) in order to explore in more detail the behavior and fate of CYN after the digestion processes.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to acknowledge the Ministerio de Economía y Competitividad of Spain (AGL2015-64558-R, MINECO/FEDER, UE) for its financial support.

References

Adamski, M., Zmudzki, P., Chrapusta, E., Bober, B., Kaminski, A., Zabaglo, K., Latkowska, E., Bialczyk, J., 2016a. Effect of pH and temperature on the stability of cylindrospermopsin. Characterization of decomposition products. Algal Res. 15, 129–134.

Adamski, M., Zmudzki, P., Chrapusta, E., Kaminski, A., Bober, B., Zabaglo, K., Bialczyk, J., 2016b. Characterization of cylindrospermopsin decomposition products formed under irradiation conditions. Algal Res. 18, 1–6.

Bordin, K., Saladino, F., Fernández-Blanco, C., Ruiz, M.J., Mañes, J., Fernández-Franzón, M., Meca, G., Luciano, F.B., 2017. Reaction of zearalenone and a-zearalenol with allyl isothiocyanate, characterization of reaction products, their bioaccessibility and bioavailability *in vitro*. Food Chem. 217, 648–654.

Bornhorst, G. M., Gouseti, O., Wickham, M. S. J., Bakalis, S., 2016. Engineering digestion: Multiscale processes of food digestion. J. Food Sci. 81, 534–543.

Buratti, F.M., Manganelli, M., Vichi, S., Stefanelli, M., Scardala, S., Testai, E., Funari, E., 2017. Cyanotoxins: producing organisms, occurrence, toxicity, mechanism of action and human health toxicological risk evaluation. Arch. Toxicol. 91, 1049-1130.

Chiswell, R.K., Shaw, G.R., Eaglesham, G., Smith, M.J., Norris, R.L., Seawright, A.A., Moore, M.R., 1999. Stability of cylindrospermopsin, the toxin from the

cyanobacterium *Cylindrospermopsis raciborskii*: effect of pH, temperature, and sunlight on decomposition. Environ. Toxicol. 14, 155-161.

Colle, I., Van Buggenhout, S., Van Loey, A., and Hendrickx, M., 2010. High pressure homogenization followed by thermal processing of tomato pulp influence on microstructure and lycopene *in vitro* bioaccessibility. Food Res. Int. 43, 2193-2200.

Corbel, S., Mougin, C., Bouaïcha, N., 2014. Cyanobacterial toxins: modes of actions, fate in aquatic and soil ecosystems, phytotoxicity and bioaccumulation in agricultural crops. Chemosphere 96, 1–15.

Cordeiro-Araujo, M.K., Chia, M.A., Bittencourt-Oliveira, M.C., 2017. Potential human health risk assessment of cylindrospermopsin accumulation and depuration in lettuce and arugula. Harmful Algae 68, 217-223.

Domingo, J.L., 2011. Influence of cooking processes on the concentrations of toxic metals and various organic environmental pollutants in food: a review of the published literature. Crit. Rev. Food Sci. Nutr. 51, 29–37.

Freitas, M., Azevedo, J., Carvalho, A.P., Mendes, V.M., Manadas, B., Campos, A., Vasconcelos, V., 2016. Bioaccessibility and changes on cylindrospermopsin concentration in edible mussels with storage and processing time. Food Control 59, 567-574.

Gálvez, A., Abriouel, H., López, R.L., Omar, N.B., 2007. Bacteriocin-based strategies for food biopreservation. Int. J. Food Microbiol. 120, 51-70.

Guerra, A., Eienne-Mesmin. L, Livrelli, V, Denis, S., Blanquet-Diot, S., Alric, Mo. 2012. Relevance and challenges in modeling human gastric and small intestinal digestion. Trends Biotechnol., 30, 591-600.

Gutiérrez-Praena, D., Pichardo, S., Jos, A., Cameán, A.M., 2011a. Toxicity and glutathione implication in the effects observed by exposure of the liver fish cell line PLHC-1 to pure cylindrospermopsin. Ecotox. Environ. Saf. 74, 1567–1572.

Gutiérrez-Praena, D., Jos, A., Pichardo, S., Cameán, A.M., 2011b. Oxidative stress responses in tilapia (*Oreochromis niloticus*) exposed to a single dose of pure cylindrospermopsin under laboratory conditions: influence of exposure route and time of sacrifice. Aquat. Toxicol. 105, 100–106.

Gutiérrez-Praena, D., Pichardo, D., Jos, A., Moreno, F.J., Cameán, A.M., 2012. Biochemical and pathological toxic effects induced by the cyanotoxin Cylindrospermopsin on the human cell line Caco-2. Water Res. 46, 1566–1575.

Gutiérrez-Praena, D.; Jos, A.; Pichardo, S.; Moreno, I.M.; Cameán, A.M. 2013. Presence and bioaccumulation of microcystins and cylindrospermopsin in food and the effectiveness of some cooking techniques at decreasing their concentrations: A review. Food Chem. Toxicol. 53, 139-152.

Guzmán-Guillén, R., Prieto, A.I., González, A.G., Soria-Díaz, M.E., Cameán, A.M., 2012. Cylindrospermopsin determination in water by LC-MS/MS: Optimization and validation the method and application to real samples. Environ. Toxicol. Chem. 12, 1-6.

Guzmán-Guilllén, R., Prieto, A.I., Vasconcelos, V.M., Cameán A.M., 2013. Cyanobacterium producing cylindrospermopsin cause oxidative stress at environmentally relevant concentrations in sub-chronically exposed tilapia (*Oreochromis niloticus*). Chemosphere 90, 1184-1194.

Guzmán-Guillén, R., Maisanaba, S., Prieto Ortega, A.I., Valderrama-Fernández, R., Jos, A., Cameán, A.M., 2017. Changes on cylindrospermopsin concentration and characterization of decomposition products in fish muscle (*Oreochromis niloticus*) by boiling and steaming. Food Control 77, 210–220.

Halttunen, T., Salminen, S., Tahvonen, R., 2007. Rapid removal of lead and cadmium from water by specific lactic acid bacteria. Inter. J. Food Microbiol. 114, 30–35.

Hercog, K., Maisanaba, S., Filipic, M., Jos, A., Cameán, A.M., Zegura, B., 2017. Genotoxic potential of the binary mixture of cyanotoxins microcystin-LR and cylindrospermopsin. Chemosphere 189, 319-329.

Humpage, A.R., Falconer, I.R., 2003. Oral toxicity of the cyanobacterial toxin Cylindrospermopsin in male Swiss albino mice: determination of no observed adverse effect level for deriving a drinking water guideline value. Environ. Toxicol. 18, 94-103.

Jasim, S. Y., Saththasivam, J., 2017. Advanced oxidation processes to remove cyanotoxins in water. Desalination 406, 83-87.

Kim, M.J., Moon, Y., Tou, J.C., Mou, B., Waterland, N.L. 2016. Nutritional value, bioactive compounds and health benefits of lettuce (*Lactuca sativa* L.). J. Food Compos. Anal. 49, 19-34.

Kittler, K., Schreiner, M., Krumbein A., Manzei, S., Koch, M., Rohn, S., Maui, R. 2012. Uptake of the cyanobacterial toxin cylindrospermopsin in *Brassica* vegetables. Food Chem. 133, 875-879.

Kokociński, M., Cameán, A.M., Carmeli, S., Guzmán-Guillén, R., Jos, A., Mankiewicz-Boczek, J., Metcalf, J.S., Moreno, I., Prieto, A.I., Sukenik, A., 2017. Cylindrospermopsin and Congeners, in: Meriluoto, J., Spoof, L., and Codd, G.A. (Eds.), Handbook on Cyanobacterial Monitoring and Cyanotoxin Analysis. John Wiley & Sons, Ltd, United Kingdom, pp. 127-137.

Laparra, J.M., Sanz, Y., 2009. Comparison of *in vitro* models to study bacterial adhesión to the intestinal epithelium. Lett. Appl. Microbiol. 49, 695–701.

Lucas-González, R., Viuda-Martos, M., Pérez-Álvarez, J.A., Fernández-López, J., 2018. *In vitro* digestion models suitable for foods: Opportunities for new fields of application and challenges. Food Res. Inter. 107, 423-436.

Maisanaba, S., Saladino, F., Font, G., Jos, A., Cameán, A.M., Meca, G., 2017. Bioaccesibility of Cylindrospermopsin from cooked fish muscle after the application of an *in vitro* digestion model and its bioavailability. Food Chem. Toxicol. 110, 360-370.

Manubolu, M., Madawala, S.R.P., Dutta, P.C., Malmlof, K., 2014. *In vitro* biodegradation of cyanotoxins in the rumen fluid of cattle. BMC Veter. Res. 10, 110–117.

Maulvault, A.N., Machado, R., Afonso, C., Lourenço, H.M., Nunes, M.L., Coelho, I., Langerholc, T., Marques, A., 2011. Bioaccesibility of Hg, Cd and as in cooked black scabbard fish and edible crab. Food Chem. Toxicol. 49, 2808–2815.

Makarov, A., Denisov, E., Lange, O., Horning, S., 2006. Dynamic range of mass accuracy in LTQ orbitrap hybrid mass spectrometer. J. Am. Soc. Mass. Spectr., 17, 977-982.

Martínez-Sánchez, M.J., Martínez-López, S., Martínez-Martínez, L.B., Pérez-Sirvent, C., 2013. Importance of the oral arsenic bioaccesibility factor for the characterising the risk associated with the soil ingestion in a mining-influences zone. J. Environ. Manage. 16, 10-17.

Meca, G., Manes, J., Font, G., Ruiz, M.J., 2012. Study of the potential toxicity of enniatins A, A1, B, B1 by evaluation of duodenal and colonic bioavailability applying an *in vitro* method by Caco-2 cells. Toxicon 59, 1–11.

Menárd, O., Bourlieu, C., De Oliveira, S.C., Dellarosa, N., Laghi, L., Carrière, F., Capozzi, F., Dupont, D., Deglaire, A., 2018. A first step towards a consensus statuc *in vitro* model for simulating full-term infant digestion. Food Chem. 240, 338-345.

Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., Thomas, O., 2013. State ofknowledge and concerns on cyanobacterial blooms and cyanotoxins. Environ. Int. 59, 303–327.

Meunier, L., Koch, I., Reimer, K.J., 2011. Effect of particle size on arsenic bioaccessibility in gold mine tailings of Nova Scotia. Sci. Total Environ. 409, 2233-2243.

Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carriere, F., Boutrou, R., Corredig, M., et al.,Dupont, D., Dufour, C., Eggeg, L., Golding, M., Korakaya, S., Kirklus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., Ménard, O., Recio, I., Santos, C.N., Singh, R.P., Vegarud, G.E., Wickman, M.S., Weitchies, W., Brodkorb, A., 2014. A standardised static *in vitro* digestion method suitable for food – an international consensus. Food Funct. 5, 1113–1124.

Mohanty, B.P., Mahanty, A., Ganguly, S., Mitra, T., Karunakaran, D., Anandan, R. Nutritional composition of food fishes and their importance in providing food and nutritional security. Food Chem. *In press.*

Ng, J.C., Juhasz, A.L., Smith, E., Naidu, R., 2010. Contaminant Bioavailability and Bioaccessibility- Part 1: A Scientific and Technical Review, CRC CARE Technical Report no. 14, CRC for Contamination Assessment and Remediation of the Environment, Adelaide, Australia.

Ngnitcho, P.-F.K., Khan, I., Tango, C.N., Hussain, M.S., Oh, D.H., 2017. Inactivation of bacterial pathogens on lettuce, sprouts, and spinach using hurdle technology. Innov. Food Sci. Emerg. Technol. 43, 68–76.

Ohtani, I., Moore, R.E., Runnegar, M.T.C., 1992. Cylindrospermopsin: a potent hepatotoxin from the blue-green alga *Cylindrospermopsis raciborskii*. J. Am. Chem. Soc. 114, 7941–7942.

Paerl, H. W., Hall, N. S., Calandrino, E.S., 2011. Controlling harmful cyanobacterial blooms in a world experiencing anthropogenic and climatic-induced change. Sci Total Environ. 409, 1739–1745.

Pichardo, S., Cameán, A.M., Jos A., 2017. *In vitro* Toxicological Assessment of Cylindrospermopsin: A review. Toxins 9, 402.

Prieto, A., Campos, A., Cameán, A., Vasconcelos, V., 2011. Effects on growth and oxidative stress status of rice plants (*Oryza sativa*) exposed to two extracts of toxin-producing cyanobacteria (*Aphanizomenon ovalisporum* and *Microcystis aeruginosa*). Ecotox. Environ. Safe. 74, 1973–1980.

Prieto, A.I., Guzmán-Guillén, R., Valderrama-Fernández, R., Jos, A., Cameán, A.M., 2017. Influence of cooking (microwaving and broiling) on cylindrospermopsin concentration in muscle of Nile Tilapia (*Oreochromis niloticus*) and characterization of decomposition products. Toxins 9, 177.

Prieto, A.I., Guzmán-Guillén, R., Díez-Quijada, L., Campos, A., Vasconcelos, V., Jos, A., Cameán, A.M., 2018. Validation of a method for CYN determination in vegetables: Application to real samples such as lettuce (*Lactuca sativa* L.). Toxins 10, 63.

Puerto, M., Jos, A., Pichardo, S., Gutiérrez-Praena, D., Cameán, A.M., 2011. Acute effects of pure cylindrospermopsin on the activity and transcription of antioxidant enzymes in tilapia (*Oreochromis niloticus*) exposed by gavage. Ecotoxicology 20, 1852-1860.

Rastogi, R.P., Sinha, R.P., Incharoensakdi, A., 2014. The cyanotoxin-microcystins: current overview. Rev. Environ. Sci. Biotechnol. 13, 215–249.

Rubby, M.V., Schoof, R., Brattin, W., Goldade, M., Post, G., Harnois, M., Mosby, D.E., Casteel, S.W., Berti, W., Carpenter, M., Edwards, D., Cragin, D., Chappell, W., 1999. Advances in evaluating the oral bioavailability of inorganics in soil for use in human health risk assessment. Environ. Sci. Technol. 33, 3697-3705.

Santos, C., Azevedo, J., Campos, A., Vasconcelos, V., Pereira, A.L. 2015. Biochemical and growth performance of the aquatic macrophyte *Azolla filiculoides* to sub-chronic exposure to cylindrospermopsin. Ecotoxicology 24, 1848-1857.

Silva, P., Vasconcelos, V., 2010. Allelopathic effect of *Cylindropermopsis raciborskii* extracts on the germination and growth of several plant species. Chem. Ecol. 26, 263-271.

Testai, E.; Buratti, F.M.; Funari, E.; Manganelli, M.; Vichi, S.; Arnich, N.; Biré, R.; Fessard, V.; Sialehaamoa, A., 2016. Review and analysis of occurrence, exposure and toxicity of cyanobacteria toxins in food. EFSA Supporting Publications. 13, 1–309. https://doi.org/10.2903/sp.efsa.2016.EN-998.

Teusink, B., Molenaar, D. 2017. Systems biology of lactic acid bacteria: For food and thought. Curr. Opin. Syst. Biol. 6, 7-13.

Versantvoort, C.H.M., Oomen, A.G., de Kamp, E.V., Rompelbeerg, C.J.M., Sips, A.J.A.M., 2005. Applicability of an *in vitro* digestion model in assessing the bioaccessibility of mycotoxins from food. Food Chem. Toxicol. 43, 31–40.

Wimmer, K.M., Strangman, W.K., Wright, J.L.C., 2014. 7-Deoxydesulfocylindrospermopsin and 7-deoxy-desulfo-12-acetylcylindrospermopsin: Two new cylindrospermopsin analogs isolated from a Thai strain of *Cylindrospermopsis raciborskii*. Harmful Algae 37, 203–206.

Figure captions

Figure 1. Digestion phases composition and procedure of the applied *in vitro* digestion model.

Figure 2. CYN bioaccessibility in control vegetables (non-cooked, non-digested), non-digested boiled spinach, uncooked lettuce (black) and spinach (grey) and in boiled spinach (white). $^{\alpha}$ statistical differences (p<0.001) of boiled spinach compared to non-digested boiled spinach; $^{\pm}$ statistical differences (p<0.001) compared to control vegetable (non-cooked, non-digested); $^{\beta}$ statistical differences (p<0.001) between boiled and uncooked spinach on its corresponding digestion phase; * statistical differences (p<0.001) in the same matrix compared to salivar phase; $^{\#}$ statistical differences (p<0.001) in the same matrix compared to gastric phase; $^{\&}$ statistical differences (p<0.001) in the same matrix compared to duodenal phase.

Figure 3. Relative percentage of two diastereoisomers (C1-A and C1-B) of the CYN by-product at *m/z* 292.09617 in control CYN aqueous solutions submitted or not to *in vitro* digestion, and in uncooked and non-digested lettuce and spinach, and non-digested boiled spinach.