Effect of cold food storage techniques on the contents of Microcystins and Cylindrospermopsin in leaves of spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*)

Antonio Casas Rodríguez, Leticia Diez-Quijada, Ana I. Prieto*, Angeles Jos, Ana M. Cameán

Area of Toxicology, Faculty of Pharmacy, University of Sevilla, Profesor García González Nº2, Spain

*Corresponding author:

Ana Isabel Prieto

Area of Toxicology, Faculty of Pharmacy, Universidad de Sevilla, Profesor García González

n°2, 41012 Seville, Spain.

E-mail address: anaprieto@us.es

Tel: +34 954 556762

Fax: +34 954 556422

Abstract

The presence of Cylindrospermopsin (CYN) and Microcystins (MCs) in vegetables is considered as a significant worldwide toxicological risk. Thus, this work aims to assess for the first time the impact of refrigeration (4 ºC) and freezing (-20 ºC) on the levels of CYN, MCs and their mixtures (CYN+MCs) in lettuce and spinach. Samples were spiked with 750 µg cyanotoxins /g dry weight (d.w.). Several storage conditions were studied: refrigeration after 24, 48h and 7 days, and freezing for 7 days, 1 and 3 months. Cyanotoxin concentrations were determined by Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry (UPLC-MS/MS). For CYN, refrigeration at 48h and 7 days was effective to decrease its concentrations up to 26 % and 32 %, respectively, in spinach. For MCs, refrigeration was only effective in lettuce compared to spinach, showing an important decrease of 80.3 % MC-LR and 85.1% MC-YR. In spinach, CYN was stable after 3 months freezing, whereas MC contents were still reduced up to 44%. Overall, cyanotoxins were less stable in the mixture compared to individual toxins for both processes, and the effect of these storage techniques were toxin and food-specific. Further studies of cyanotoxins in foods are required for evaluating the risk for humans.

Keywords: Cyanotoxins; Vegetables; Refrigeration; Freezing; UPLC-MS/MS**.**

1. Introduction

Cyanobacteria are a group of photosynthetic prokaryotes that are present in different geographical areas worldwide living in a broad range of environments, from freshwater and marine to terrestrial ecosystems (Mehdizadeh et al., 2022). Nutrient and climate alterations are supported by the eutrophication and proliferation of harmful algal blooms (HABs) at world level (Glibert et al., 2020), increasing the amount of bioactive secondary metabolites (Prieto et al., 2018). These secondary bioactive metabolites known as cyanotoxins have toxic effects on human and animal health, which could cause acute and chronic diseases (Dulic et al., 2022). Microcystins (MCs) are the most studied and commonly detected worldwide, and they are present in 50-75% of cyanobacterial blooms (Weralupitiya et al., 2022). Cylindrospermopsin (CYN) is gaining importance due to higher incidence and expansion, in line with CYN-producing species (Diez-Quijada et al., 2019a).

With regard to MCs, these cyanotoxins are released by different species of cyanobacteria (Catherine et al., 2017) and they are cyclic heptapeptides with the common structure (D-Ala^{1}-X^{2}-D-MeAsp^{3}-Z^{4}-Adda-Arg^{5}-D-Glu^{6}-Mdha^{7}), in which X and Z are variants of L-amino acid residues at positions 2 (X) and 4 (Z), giving rise to the different structural variations; more than 279 MC-congeners (Bouaïcha et al., 2019; Diez-Quijada et al., 2019b,c). Among them, the three most usual congeners are MC-LR, MC-RR and MC-YR, which differ in the residues (leucine (L), arginine (R) or tyrosine (Y)) in position two and four (Abdallah et al., 2021). MC-LR is the most broadly distributed congener, and the one with the most toxicological studies performed on it (Testai et al., 2016; Pichardo et al., 2005, 2007; Puerto et al., 2009). MCs toxicity is based on the inhibition of serine/threonine phosphatases protein (PP), specifically PP1 and PP2A in animals and plants (Cao et al., 2019 a; Diez-Quijada et al., 2019b,c); moreover, induction of oxidative stress by MCs has been also demonstrated (Prieto et al., 2008).

CYN is produced by different genera of cyanobacteria, being more frequently reported from the genera *Raphidiopsis*, *Aphanizomenon*, *Anabaena* and *Umezakia* (WHO, 2020b). CYN is a stable tricyclic alkaloid formed of a tricyclic guanidine moiety combined with a hydroxymethyluracil group, and is a water-soluble compound (zwitterionic nature) (Pichardo et al., 2017). Inhibition of proteins and glutathione synthesis (Runnegar et al., 1995; Froscio et al., 2003), oxidative stress (Puerto et al., 2011; Guzmán- Guillén et al., 2013) and genotoxicity (Puerto et al., 2018; Diez-Quijada et al., 2019a) have been described

as the main toxicity mechanisms, and recently, its immunomodulatory effects have been reported (Diez-Quijada et al., 2022).

Humans can be exposed to these toxins in several ways, although direct intake of contaminated drinking-water is the main route for the general population, and food (such as edible vegetables) could also be a source of exposure (Abdallah et al., 2021). In that sense, some studies have reported that both, MCs and CYN can be taken up by edible vegetables like spinach (*Spinacia oleracea*) (Llana-Ruiz-Cabello et al., 2019), lettuce (*Lactuca sativa*) (Bittencourt-Oliveira et al., 2016), brown mustard (*Brassica juncea*) (Kittler et al., 2012) or tomato (*Solano lycopersicum*) (Gutierrez-Praena et al., 2014; Corbel et al., 2016). The data available in the literature show that accumulation of MCs in vegetables occurred (1.03-2352.20 µg/kg dry weight (d.w.)) (Prieto et al., 2011; Li et al., 2014; Drobac et al., 2017) as well as of CYN (2.71-49.00 µg/kg fresh weight (f.w.)) (Prieto et al., 2011; Kittler et al., 2012; Cordeiro-Araujo et al., 2017). Several of these studies showed concentrations of these cyanotoxins exceeding the Tolerable Daily Intake (TDI) reported by the World Health Organization (WHO), namely 0.04 µg MCs /kg b.w. (WHO, 2020a) and 0.03 µg/kg b.w. for CYN (WHO, 2020b). Furthermore, it should be noted that cyanobacterial blooms are usually characterized by the presence of multiple toxins (Testai et al., 2016; León and Peñuela, 2019). Therefore, human exposure to these mixtures is highly likely, and its consequences should be further investigated, as suggested by the European Food Safety Authority (EFSA) (Testai et al., 2016).

Some foods like vegetables, fish or bivalves are often refrigerated or frozen to extend their storage time. Regarding this, several studies have evaluated the effects that some cold food storage techniques have on the cyanotoxin concentrations (MCs and CYN) in fish (Diez-Quijada et al., 2021). Nevertheless, contradictory results have been reported. Freitas et al., (2014) reported that refrigeration (4 ºC) decreased MC-LR levels in clams but, after freezing (-20 ºC) significant higher concentrations were found. Recently, Diez-Quijada et al., (2021) demonstrated that refrigeration and freezing are effective storage process to reduce the cyanotoxins concentrations accumulated in fish. In this previous work from our laboratory, we reported that longer periods of cold storage time (7 days and 1 month for refrigeration and freezing, respectively) were capable of decreasing cyanotoxin concentrations in tilapia and tench, of which freezing was the most efficient cold food storage procedure. In addition, the toxins showed a different behaviour depending on if they were alone or in a mixture, and the fish species was a variable to take into account. Moreover,

EFSA has pointed out that further efforts are needed to clarify the levels of consumption of these toxins in several scenarios (Testai et al., 2016). This is the first study carried out on vegetables contaminated with cyanotoxins.

Taking all this into account, the objective of this research was to assess for the first time the influence of two storage techniques such as refrigeration (4 ºC) and freezing (-20 ºC) at several intervals of time on the concentrations of MCs (MC-LR, -RR and -YR), CYN and their mixture (MCs + CYN) in edible vegetables spinach (*Spinacia oleracea*) and lettuce (*Lactuca sativa*) leaves contaminated by Ultra performance liquid chromatography-tandem mass spectrometer (UPLC-MS/MS) as determination technique.

2. Materials and methods

2.1. Supplies and chemicals

CYN (95%), MC-LR, MC-RR and MC-YR (99%) standards were supplied by Enzo Life Sciences (Lausen, Switzerland). Methanol (MeOH), acetic acid, dichloromethane (DCM) and formic acid (FA) by Merck (Darmstadt, Germany). Cartridges used for the Solid Phase Extraction (SPE) were Bakerbond® C18 (Dicsa, Andalucía, Spain) and Bond Elut Carbon (Porous Graphitic carbon (PGC)) (Agilent Technologies, Santa Clara, CA, USA). For UHPLC-MS/MS, LC-MS grade reagents employed were water and acetonitrile (ACN) purchased in VWR International (Fontenay-sous-Bois, France), and FA from Fluka (Steinheim, Germany). Standard solutions of toxins were prepared in the following way and utilized to spike the vegetables (lettuce and spinach leaves): MCs (MC-LR, MC-RR and MC-YR) (0.75 µg/mL of each congener, in proportion 1:1:1) in MeOH (100%), CYN (0.75 µg/mL) in Milli-Q water and a multitoxin solution containing all toxins (CYN, MC-LR, MC-RR and MC-YR) (0.75 µg/mL of each toxin, in proportion 1:1:1:1) was prepared in MeOH (20%).

2.2 Vegetables samples and study design

Vegetables (spinach and lettuce) were bought in a supermarket, ready for human consumption. Fresh lettuce and spinach leaves (five samples per experimental group, N=5) were chopped into 1 g fresh weight (f.w.) portions (1.007 ± 0.017 and 1.002 ± 0.003 g f.w. for lettuce and spinach, respectively) and 1 mL stock solution containing 0.75 µg/mL CYN, MCs congeners or a multitoxin mixture was added to each sample. In order to evaluate

exclusively the influence of the storage processes, the vegetables were refrigerated and frozen without prior washing. These concentrations of toxins were chosen according to the levels found in vegetables by Xiang et al., (2019) and in the environment (Yang et al., 2021). The experiments were carried out in quintuplicate for each assay. Furthermore, lettuces and spinach control groups were also spiked with toxins, but these vegetables were not subjected to the storage process.

Two different storage techniques (refrigeration and freezing) were evaluated under the conditions frequently used by consumers of these vegetables (temperature and storage time). Lettuce and spinach leaves were stored in 50 ml falcon tubes and sealed with parafilm at 4 °C for 24, 48h and 7 days. For the freezing process, spinach leaves were saved at -20 °C for 7 days, 1 and 3 months. A negative control group without cyanotoxins was included for each of the procedures assessed. Once each storage process was completed, all samples were frozen and lyophilized before the extraction of the toxins. Preliminary tests were performed to evaluate the influence of the lyophilization process on cyanotoxin contents (Prieto et al., 2018, Toxins, 10, 63) and no effects were reported.

2.3 Toxins extraction from lettuce and spinach samples and SPE

Standard calibration points from control vegetable extracts were assayed to obtain a linear range of 25-750 µg/L for each cyanotoxin. The lyophilized vegetables leaves (0.05g d.w.) were extracted according to several validated methods.

For MCs or CYN+MCs mixture extractions, the method validated by Diez-Quijada et al., (2018) was employed. Briefly; 0.05 g d.w. lettuce or spinach leaves were extracted with MeOH (80%), homogenized, sonicated, and centrifuged. The supernatant was collected and pH adjusted to 11. The samples were purified by a dual SPE system and finally, evaporated and resuspended in MeOH (20%) and analysed by UPLC-MS/MS (Fig.1). For vegetable samples spiked with CYN, the method developed by Prieto et al., (2018) was applied. In summary, 0.05 g d.w. lettuce or spinach were extracted with 10% acetic acid, homogenized, stirred, sonicated and centrifuged. The extracts were purified using PGC cartridges, evaporated, resuspended in Milli-Q water, and analysed.

Some parameters of the previously validated methods for MCs and for the mixture CYN+MCs were: recoveries in the range of 41-93% and RSDIP (%) of 6.92-21.68 (Diez-

Quijada et al., 2018); in the case of CYN, recoveries of 85-104% and RSDIP (%) 12.72-14.70 (Prieto et al., 2018).

The recoveries were different depending on the type of toxin and the extraction method used. Thus, to ensure that the results would be comparable with each other and that they would depend only on the variables studied (refrigeration or freezing), the recovery values were expressed as % toxins found relative to its respective control group (spiked with toxins but not subjected to the storage process).

2.4. Chromatographic conditions

The chromatographic separation was performed using a UPLC Acquity (Waters, Milford, MA, USA) coupled to a Xevo TQS micro (Waters, Milford, MA, USA) consisting of a triple quadrupole mass spectrometer equipped with an electrospray ion source operated in positive mode. UPLC analyses for CYN were performed on 50 x 2.1 mm Acquity BEH C18 1.7 µm column at a flow rate of 0.45 mL min^{-1}. For MCs and the mixture of cyanotoxins the column was a 100 × 2.1 mm XSelect HSS T3 2.5 µm column with a flow rate of 0.45 mL min^{-1}.

Chromatographic separation for CYN, MCs and mixture (CYN+MCs) was carried out using a binary gradient consisting of (A) water and (B) methanol in the case of CYN, and acetonitrile for MCs or the CYN+MCs determination, and both eluents contained 0.1% formic acid (v/v). In the case of CYN, the elution profile was 0% B (0.8 min), linear gradient to 90% B (2.2 min), 90% B (1 min) and lastly 0% B (1 min). For MCs and the mixture, the elution profile was 2% B (0.8 min), linear gradient to 70% B (6.2 min), 100 %B (1 min) and finally 2% B (2 min). The injection volume was 5 µL. Multiple Reaction Monitoring (MRM) was applied in all cases, where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions employed for MC-LR were 996.5/135.0, 996.5/213.1 and 996.5/996.5; for MC-RR 520.2/135.0 and 1039.5/135.0; for MC-YR 1046.5/135.0, 1046.5/213.0 and 1046.5/1046.5; for CYN were 416.2/194.0 and 416.2/176.0, choosing in all cases the first one for quantitation and the others as confirmatory, for each toxin.

For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: capillary voltage: 1.0 kV (for MCs and the mixture) and 3.0 kV (for CYN), source temperature: 500 °C, source desolvation gas flow: 1000 L/h and source cone gas flow: 50 L/h. Standards and samples were dissolved in 20% MeOH for MCs and the mixture and in Milli-Q water for CYN.

2.5 Statistical analysis

A GraphPad InStat software (GraphPad Prism 9 Software Inc., La Jolla, CA, USA) was used for statistical analysis. One-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparison Test was performed. Moreover, Kolmogorov–Smirnov normality tests were used. Results were represented by mean ± standard deviation (SD) of 5 replicates per group. Statistical significance was considered at $p < 0.05$ level.

3. Results and Discussion

The presence of cyanotoxins such as MCs and CYN has been described in different foods for human consumption, such as vegetables irrigated with contaminated water (Testai et al., 2016; Xiang et al., 2019). Vegetables are essential for a balanced diet as they contribute to the supply of vitamins, minerals, antioxidants etc., are a crucial source of nutraceuticals and have a positive effect on human health (Ramya and Priya et al., 2019). For all of this, a significant increase in the consumption of lettuce (up to 183%) and spinach (up to 50%) has been described, being some of the most frequently vegetables consumed in the world (Simko et al., 2014, Brouwer-Brolsma et al., 2020). Furthermore, the presence of CYN and MCs has been described in these vegetables in variable ranges (Prieto et al., 2018; Diez-Quijada et al., 2018; Llana-Ruíz Cabello et al., 2019; Xiang et al., 2019).

On the other hand, vegetables are generally subjected to different conservation processes, such as refrigeration and freezing before consumption. These storage techniques extend the shelf life of foods, which is of significant importance from a health and economic point of view, and for the food industry (Muthukumarappan et al., 2018). However, different studies have shown that the conservation and cooking processes can vary the concentration of MCs and CYN present in foods (Guzmán-Guillén et al., 2011, Guzmán-Guillén et al.,

2017; Prieto et al., 2017; Diez-Quijada et al., 2021). In addition, previous results show that the behaviour of these toxins is not predictable since it can vary depending on several factors such as the biotransformation and conservation process, storage time, type of toxin and food. In relation to the biotransformation of cyanotoxins in plants, Pflugmacher et al. (2001) demonstrated a complete metabolism of MC-LR from the formation of its glutathione conjugate to the degradation of a cysteine conjugate in plants. Moreover, Cao et al. (2019 a) have reported important depuration rates of MC-LR in lettuce (9.5 mg /kg d.w.) and spinach (8.1 mg /kg d.w.). These authors observed a significant increase of GST activity and GR activity, confirming the detoxication mechanism of cyanotoxin by MC-LR-GSH-conjugation in plants (Cao et al., 2019 b). On the other hand, the influence of plant preservation processes on cyanotoxin concentrations has been less studied. In this sense, with the objective to carry out a correct risk assessment, the study of refrigeration and/or freezing process have on MCs and CYN content in vegetables is important. In addition, to ensure that the observed effects on toxin concentration were only a consequence of these preservation processes, the lettuce and spinach samples were spiked and not exposed during their growth, thus ruling out the possible influence of biotransformation processes on the toxins. The recovery percentages of each cyanotoxin subjected to the types and periods of storage (refrigeration or freezing) in lettuce and spinach leaves are shown in Table 1.

In relation to CYN concentration changes, in the case of lettuce, the present work showed that the refrigeration does not modify the concentration of CYN in any of the experimental times assessed (24h, 48h and 7 days) (Fig. 2a). Similarly, Diez-Quijada et al., (2021) reported not changes in CYN concentrations in fish species (tilapia and tench) refrigerated, under the same laboratory conditions. By contrast, for spinach a significant decrease was reported by refrigeration at 48h and 7 days (Fig. 3a). Consequently, the refrigeration technique could affect CYN concentration depending on the vegetable species

considered. According to the data provided by the US Department of Agriculture (USDA, 2022), there are some differences in the main composition of these vegetables. Lettuce has 97.7 % water and 0.26 % total lipids compared to 92.4 % water and 0.6 % total lipids in spinach. This could explain, in part, the higher stability of CYN in lettuce versus spinach because CYN is a more hydrophilic molecule than MCs. After freezing, no significant changes in CYN content (92-99% recoveries) were observed for the periods evaluated (7 days, 1 month and 3 months) (Fig. 4a). These results are opposite to those obtained for the same toxin in the case of fish subjected to the same preservation process, with significant CYN decreases after 48h, 7 days and 1 month (remaining up to 70% in tilapia and up to 20% in tench) (Diez-Quijada et al., 2021). Freitas et al., (2016) did not observe differences in the content of CYN present in mussels refrigerated for 24 and 48 h. These results agree to those obtained in lettuce in this study for the same refrigeration periods. By contrast, Freitas et al. (2016) observed an increase in CYN levels after freezing periods of 48h (up to 52.5%) and 7 days (up to 57.7%). However, the freezing process in spinach did not result in significant differences in CYN content after the same period (7 days), and longer ones (1 and 3 months). These discrepancies could be partially explained by the CYN exposure conditions employed. Thus, Freitas et al. (2016) exposed mussels by immersion with cyanobacterial extracts (10-15 µg/L) for 4 days, and these authors justified the significant increases of CYN observed due to the potential cell disruption and protein denaturation caused by freezing, which entailed a more efficient CYN extraction. Moreover, according to our previous results, after application of several cooking techniques to fish, significant reductions of the toxin contents in tilapia spiked with 50 ng/g d.w. of CYN were observed (Guzmán-Guillén et al., 2017; Prieto et al., 2017).

Regarding to MCs mixture, the refrigeration was effective in decreasing MCs concentrations significantly in the case of lettuce compared to the control group for all the

time periods assessed (24h, 48h and 7 days) (Fig. 2b). These results are in agreement with those reported by Diez-Quijada et al., (2021) in refrigerated MC-spicked tench that showed decreases in the levels of these MCs for all the times studied compared to the control group. In the present work similar decreases were observed for MC-LR and MC-YR, and a less prominent reduction for MC-RR content when comparing to the other MCs. By contrast, Diez-Quijada et al., (2021) reported a higher reduction for MC-RR compared with other MCs-congeners, in tench.

For spinach (Fig. 3b) the refrigeration didn´t produce any change in the concentrations of these toxins in relation to the control group, contrary to the results obtained in lettuce. Similar to the previous explanation with CYN, the MCs (less hydrophilic molecules than CYN) are more stable in vegetables with a lower water content and a higher % of total lipids. The differences observed in the decrease of MCs content in vegetable matrices (lettuce and spinach) did not show in the case of fish species (tilapia versus tench) where the authors described a significant decrease of toxins under similar laboratory conditions (Diez-Quijada et al., 2021). Data reported in lettuce in the present work are in accordance with Freitas et al., (2014), who reported a significant decrease in the concentration of MC-LR after similar periods of time (48h and 72h) at 4 ºC in clams. Similarly, in mussels exposed by feeding to a MCs producer *Microcystis aeruginosa* strain for 4 days, significant decreases were observed in the toxins levels after 24, 48 and 72h at 4 ºC (Morais et al., 2008). In the case of freezing, significant changes were observed only after 3 months of treatment (Fig.4b), with recoveries of 56 % (MC-LR), 57% (MC-RR) and 65% (MC-YR). This procedure of freezing was more effective in terms of time for fish and mussels, with more early changes on the concentration of MCs (decreased) after 48h in tilapia and mussels, and after one month in tench (Morais et al., 2008, Diez-Quijada et al., 2021). By contrast, in clams after periods of 1 week and 1 month there was an increase in

the concentration of MC-LR (Freitas et al., 2014). These differences in the content of MCs between foods have also been observed when they are subjected to diverse types of cooking (boiling and microwaves). Thus, in tilapias, boiling proved to be the most effective process to reduce these toxins, while in mussels it was the use of microwave technique. However, results obtained by Zhang et al. (2010) in bighead carp after boiling, showed higher concentrations of MCs in boiled muscle than unboiled controls.

Since the simultaneous presence of CYN and MCs is becoming more frequent in waters (León et al., 2019; Li et al., 2022) and taking into account their transference in vegetables (Codd et.,al, 1999; Crush et al., 2008; Llana-Ruiz-Cabello et al., 2019) it is important to study the effects of CYN+MCs mixture in them. In relation to refrigeration in lettuce spiked with CYN+MCs mixture, a similar behaviour in relation to the individual toxins was observed, showing only a decrease in the levels of MCs, with the highest reduction for MC-LR (Fig. 2c). For CYN no modifications were reported with respect to the control group. Similar results were reported in tilapia and tench for CYN; however, this technique was less effective for the mixture of MCs with CYN in comparison to the MCs (Diez-Quijada et al., 2021). For the refrigerated spinach, this method was effective in decreasing cyanotoxins levels, after 48h or 7 days of treatment, showing significant decreases for all toxins (Fig. 3c) with recovery values between 46 to 48% for CYN, 46 to 40% for MC-LR, 69 to 62% for MC-RR and 55 to 48% for MC-YR for 48h and 7 days, respectively. In general, in vegetables assayed in the present work the conservation at 4 ºC is more effective to decrease these toxins when they are in mixture, contrary to the results previously observed in fish (Diez-Quijada et al., 2021). Freezing proved to be highly effective in reducing the content of all toxins in the mixture at all times studied (7 days, 1 month and 3 months) in relation to the control group in spinach (Fig. 4c). Considering all the results obtained (single toxins versus toxins mixture) freezing is only effective in

decreasing CYN in presence of MCs. However, in relation to CYN it is important to take into account that these differences could be due to the application of different extraction methods (Prieto et al., 2018 vs. Diez-Quijada et al., 2018), the CYN recoverie values in the mixture being lower (range 45-69%, Diez-Quijada et al., 2018) than that obtained for CYN individually (range 85-104%, Prieto et al., 2018).

In tilapia, similar results were reported when toxins were evaluated in an individual manner as well as in the mixture. According to Chiswell et al., (1999) the decomposition of CYN was more rapid in presence of bloom extracts components in comparison to Milli-Q water, which could be due to enzyme or pigment-promoted degradation. However, in tench, freezing showed to be more effective for CYN alone than in the mixture, in contrast to the MCs (Diez-Quijada et al., 2021). Consequently, it is important to know the type of toxins that may be present in food, the kind of food, etc., because they can modify the effectiveness of preservation processes.

Recently, Dinh et al. (2021) evaluated cyanotoxin stability in different storage periods (up to 365 days) in ultrapure water, chlorine-treated drinking water, and surface water at various temperatures (20 °C, 4 °C and –20 °C). Cyanotoxin recoveries oscillated between 81-113% for CYN, 88-113% for MC-LR, 77-105% for MC-RR and 82-114% for MC-YR. In general, regardless of matrix type, storage process (4 ºC or -20 ºC) and periods (28 days or 365 days), the cyanotoxins recoveries were similar. These differences could be explained because the greater complexity of the food (spinach versus water), which could favour the degradation of these toxins under storage conditions and highlights the importance to study the effects of these conservation techniques in different contaminated food with cyanotoxins.

4. Conclusions

The present work evaluated for the first time the effects of refrigeration and freezing on the levels of CYN, MCs and their combination in edible vegetables (lettuce and spinach). Results showed that the toxin decrease depends on several parameters as the type of cyanotoxin, CYN was more stable than MCs in refrigerated lettuce obtaining recoveries of 74-90 % and 14-75 %, respectively; the vegetable (i.e. higher CYN decrease in refrigerated spinach than in lettuce showing a decrease of 32 % versus 26 %, respectively), the preservation technique (i.e. CYN decrease in refrigerated spinach with 32% after 7 days and no changes after freezing), the presence of different toxins in the food matrix (i.e. CYN decreased 60% after 3 months in frozen spinach is only observed in the mixture), and time evaluated (mainly the decrease is observed at longer times). Thus, it is of interest to further investigate how human exposure to cyanotoxins can be modified by food treatments to perform a more realistic risk evaluation.

Credit authorship contribution statement

Antonio Casas Rodríguez: Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft. **Leticia Diez-Quijada:** Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft. **Ana I. Prieto**: Data curation; Formal analysis; Investigation; Methodology; Software; Writing - original draft; Writing - review & editing. **Ana M. Cameán**: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision Writing – original draft, Writing – review & editing. **Angeles Jos**: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Fig. 1. Chromatograms obtained by UPLC-MS/MS of the different cyanotoxins (CYN, MC-LR, MC-RR, and MC-YR) in A) lettuce (refrigerated at 4ºC for 1 week) and B) spinach samples (freezing at -20ºC for 3 months).

Fig. 2. Effects of refrigeration on contaminated lettuce (Lactuca sativa) spiked with 1 mL of a standard solution containing 0.75 µg/mL of each toxin (CYN, MCs and CYN + MCs) and refrigerated at 4 ºC for 24 h, 48 h and 1 week. CYN (a), MCs (b), and CYN + MCs (c). Values are expressed as the mean ± SD (N=5). The significant levels observed are ***p < 0.001 compared to the control group, &p < 0.05 and &&&p < 0.001 compared to 24h, #p < 0.05, ##p < 0.01 and ###p < 0.001 when comparing to MC-LR within the same period, +p < 0.05, ++p < 0.01 and +++p < 0.001 when comparing to MC-RR within the same period.

Fig. 3. Effects of refrigeration on contaminated spinach (Spinacia oleracea) spiked with 1 mL of a standard solution containing 0.75 µg/mL of each toxin (CYN, MCs and CYN + MCs) and refrigerated at 4 ºC for 24h, 48h and 1 week. CYN (a), MCs (b) and CYN + MCs (c). Values are expressed as the mean ± SD (N=5). The significant levels observed are ***p < 0.001 compared to the control group, aaap < 0.001 compared to 24h, #p < 0.05 and ##p < 0.01 when comparing to MC-LR within the same period, ++p < 0.01 when comparing to MC-RR within the same period.

Fig. 4. Effects of freezing on contaminated spinach (Spinacia oleracea) spiked with 1 mL of a standard solution containing 0.75 µg/mL of each toxin (CYN, MCs and CYN + MCs) and freezing at -20 ºC for 1 week, 1 month and 3 months. CYN (a), MCs (b) and CYN + MCs (c). Values are expressed as the mean ± SD (N=5). The significant levels observed are **p < 0.01 and ***p < 0.001 compared to the control group, &p < 0.05 and &&&p < 0.001 compared to 1 week, $p < 0.05 and $$$p < 0.001 compared to 1 month, ##p < 0.01 when comparing to MC-LR within the same period.

Table 1. Cyanotoxins recoveries % (CYN; MC-LR, -RR and -YR and CYN + MCs mixture) after refrigeration (4 ºC) or freezing (-20 ºC) at three different periods of time in leaves of edible vegetables spinach (Spinacia oleracea, N=5) and lettuce (Lactuca sativa, N=5) contaminated under laboratory conditions. The significant levels observed are **p < 0.01 and ***p < 0.001 compared to the control group, &p < 0.05 and &&&p < 0.001 compared to 24h, $p < 0.05 and $$$p < 0.001 compared to 1 month, #p < 0.05, ##p < 0.01 and ###p < 0.001 when comparing to MC-LR within the same period, +p < 0.05, ++p < 0.01 and +++p < 0.001 when comparing to MC-RR within the same period.

Fig. 1.

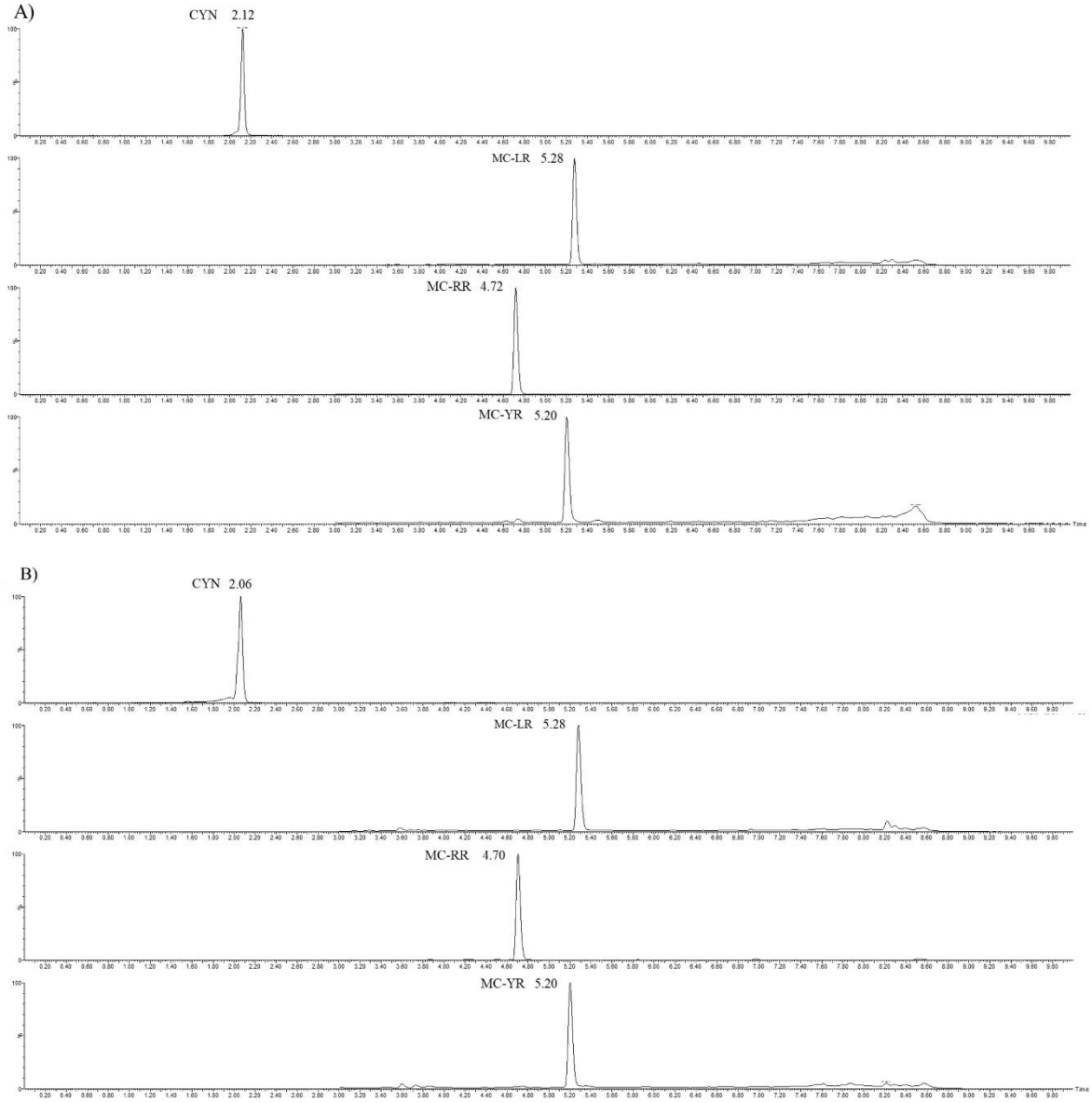

A)
CYN 2.12
MC-LR 5.28
MC-RR 4.72
MC-YR 5.20
B)
CYN 2.06
MC-LR 5.28
MC-RR 4.70
MC-YR 5.20

Fig. 2.

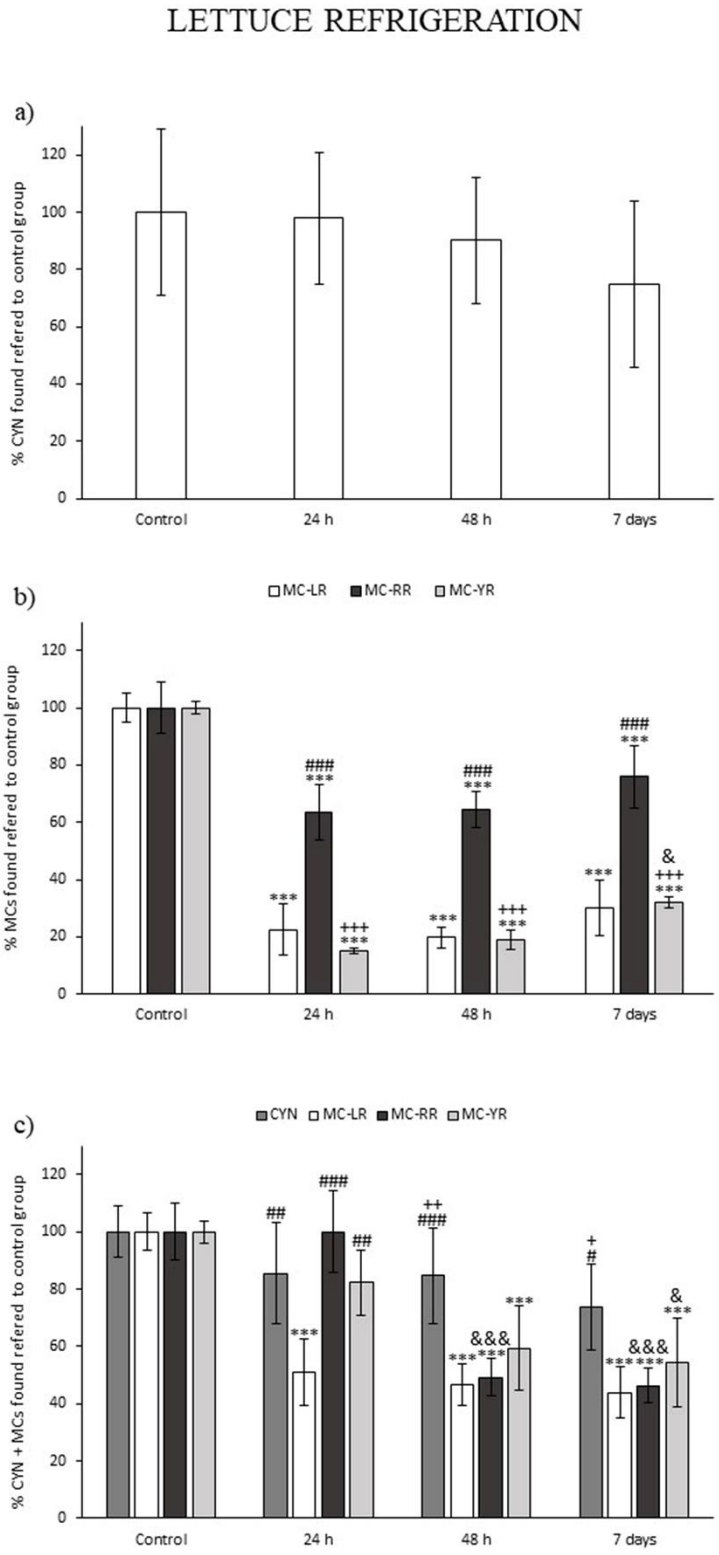
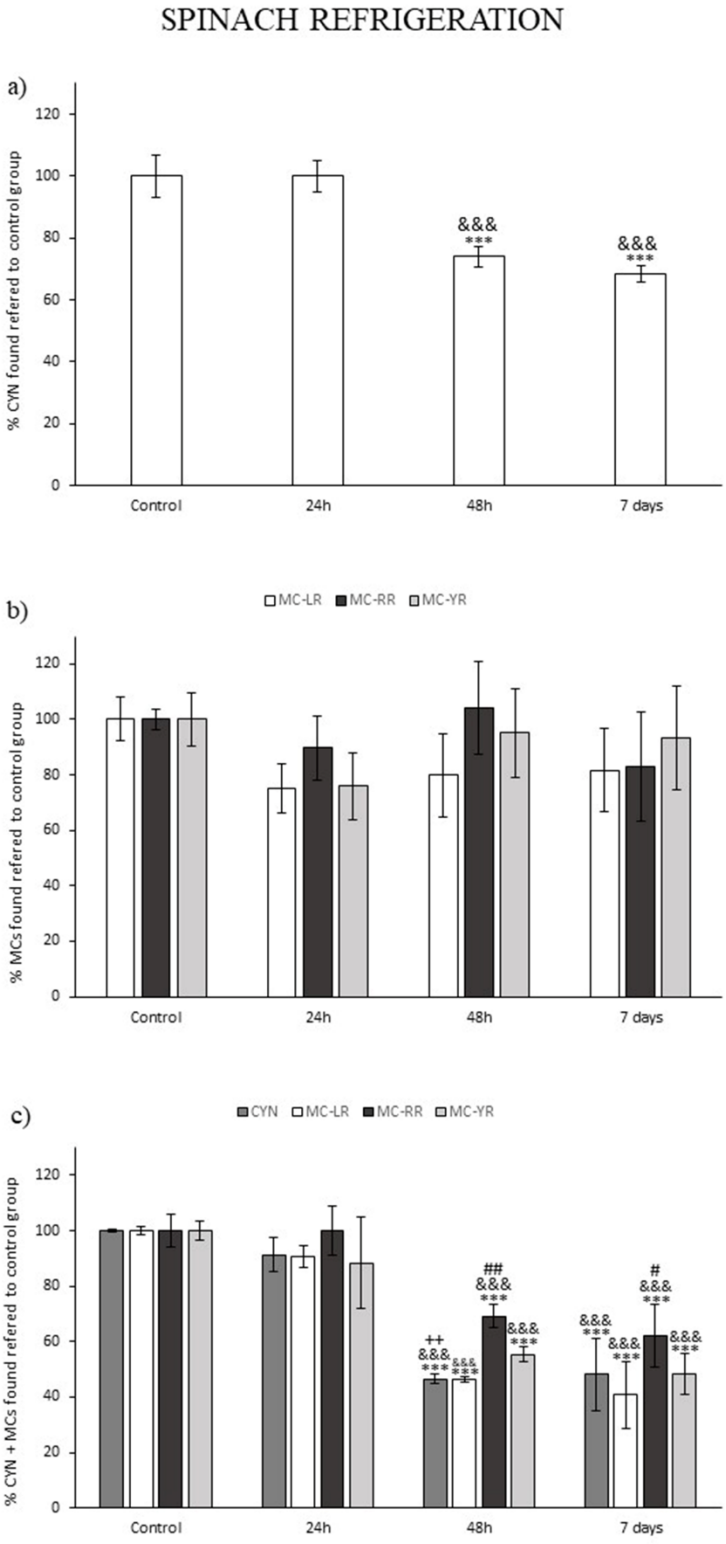

LETTUCE REFRIGERATION
a)
% CYN found refered to control group
Control
24 h
48 h
7 days
b)
MC-LR
MC-RR
MC-YR
% MCs found refered to control group
c)
CYN
MC-LR
MC-RR
MC-YR
% CYN + MCs found refered to control group

Fig. 3.


SPINACH REFRIGERATION
a)
% CYN found refered to control group
120
100
80
60
40
20
0
&&&
***
&&&
***
Control
24h
48h
7 days
b)
MC-LR
MC-RR
MC-YR
% MCs found refered to control group
120
100
80
60
40
20
0
Control
24h
48h
7 days
c)
CYN
MC-LR
MC-RR
MC-YR
% CYN + MCs found refered to control group
120
100
80
60
40
20
0
++
&&&
***
&&&
***
##
&&&
***
&&&
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Control
24h
48h
7 days

Fig. 4.

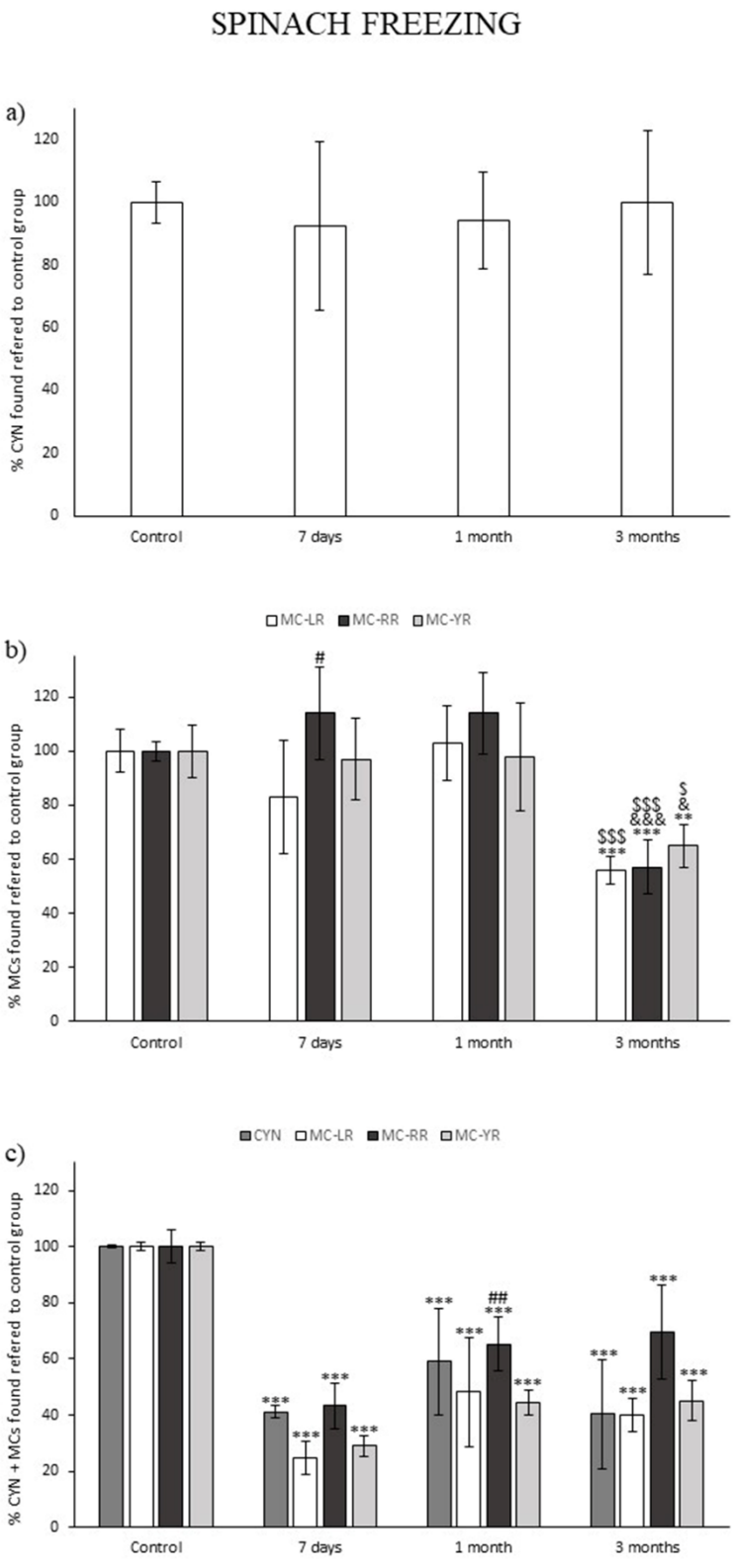
SPINACH FREEZING
a)
% CYN found refered to control group
Control
7 days
1 month
3 months
MC-LR
MC-RR
MC-YR
b)
% MCs found refered to control group
c)
CYN
% CYN + MCs found refered to control group

Table 1.

Cyanotoxins recoveries (%)	**Lettuce samples (4 ºC)**			**Spinach samples (4 ºC)**			**Spinach samples (-20 ºC)**		
Time of storage	**24h**	**48h**	**7 days**	**24h**	**48h**	**7 days**	**7 days**	**1 month**	**3 months**
CYN alone	98.00 ± 22.95	90.20 ± 22.05	74.82 ± 29.04	100.00 ± 5.21	74.03 ± 3.20$^{***\&\&\&}$	68.61 ± 2.66$^{***\&\&\&}$	92.37 ± 27.02	94.28 ± 15.51	99.95 ± 22.98
MCs mixture									
MC-LR	22.44 ± 8.88***	19.74 ± 3.74***	30.13 ± 9.67***	75.00 ± 8.83	79.99 ± 15.03	81.74 ± 15.13	83.00 ± 21.03	103.00 ± 14.01	56.00 ± 4.97$^{***\$\$\$}$
MC-RR	63.51 ± 9.49$^{***\#\#\#}$	64.51 ± 6.21$^{***\#\#\#}$	75.90 ± 10.85$^{***\#\#\#}$	89.83 ± 11.56	104.00 ± 16.79	82.97 ± 19.62	114.00 ± 17.04$^{\#}$	114.00 ± 15.02	57.00 ± 9.96$^{***\&\&\&\$\$\$}$
MC-YR	14.88 ± 0.99^{***+++}	18.89 ± 3.44^{***+++}	32.01 ± 1.83$^{***+++\&}$	75.87 ± 11.92	95.07 ± 15.94	93.44 ± 18.70	97.00 ± 15.00	98.00 ± 19.95	65.00 ± 8.05$^{**\&\$}$
CYN+MCs mixture									
CYN	85.48 ± 17.69$^{\#\#}$	84.66 ± 16.79$^{\#\#\#++}$	73.71 ± 14.99$^{\#+}$	91.31 ± 6.21	46.53 ± 1.86$^{***\&\&\&++}$	48.17 ± 13.08$^{***\&\&\&}$	41.07 ± 2.19***	59.08 ± 18.97***	40.34 ± 19.29***
MC-LR	51.02 ± 11.76***	46.70 ± 7.11***	43.96 ± 9.14***	90.49 ± 3.91	46.21 ± 1.02***	40.78 ± 12.13$^{***\&\&\&}$	24.65 ± 5.87***	48.27 ± 19.47***	40.00 ± 6.02***
MC-RR	100.00 ± 14.38$^{\#\#\#}$	49.27 ± 6.47$^{\#\#\#\&\&\&}$	46.25 ± 6.14$^{\#\#\#\&\&\&}$	100.00 ± 9.10	69.23 ± 4.39$^{***\&\&\&\#\#}$	62.10 ± 11.35$^{***\&\&\&\#}$	43.32 ± 8.07***	65.36 ± 9.64$^{***\#\#}$	69.52 ± 16.64***
MC-YR	82.23 ± 11.52$^{\#\#}$	59.43 ± 14.64***	54.58 ± 15.50$^{***\&}$	88.30 ± 16.54	55.32 ± 2.77$^{***\&\&\&}$	48.31 ± 7.46$^{***\&\&\&}$	29.09 ± 3.60***	44.55 ± 4.40***	45.00 ± 7.11***