Food and Chemical Toxicology 158 (2021) 112673

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Influence of refrigeration and freezing in Microcystins and Cylindrospermopsin concentrations on fish muscle of tilapia (*Oreochromis niloticus*) and tench (*Tinca tinca*)

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ARTICLE INFO

Handling Editor: Dr. Jose Luis Domingo

Keywords:
Cylindrospermopsin
Microcystins
Fish
Refrigeration
Freezing
UPLC-MS/MS analyses

ABSTRACT

The consumption of fish contaminated with cyanotoxins is an important public health issue due to their potential adverse effects. The aim of this study was to assess the influence of refrigeration (4 °C) and freezing (−20 °C) on the concentration of Cylindrospermopsin (CYN), Microcystins (MCs) and their combination in tilapia (*Oreochromis niloticus*) and tench (*Tinca tinca*). Fish muscle were spiked with a stock solution of each toxin to reach 750 μg/g dry weight (d.w.). Three different periods of time were investigated for each treatment: 24 h, 48 h and 7 days for refrigeration, and 24 h, 7 days and 1 month for freezing. Samples were extracted and quantified by Ultra Performance Liquid Chromatography - Tandem Mass Spectrometry (UPLC-MS/MS). The results showed that freezing for 1 month produced highest decreases of these toxins in both species in comparison to refrigeration, being CYN the most stable cyanotoxin. Moreover, MCs are more stable to storage processes in the mixtures than alone, and fish species is a factor to take into account in their stability. These findings highlight the need to assess the influence of food storage processes on the presence of cyanotoxins in fish species for a more realistic human health risk assessment.

1. Introduction

As a consequence of global climate change, the increase in nutrient loads, and/or anthropogenic activities, the occurrence of harmful cyanobacterial blooms in waterbodies is increasing worldwide (De la Cruz et al., 2020). They are especially dangerous and require attention because they produce toxic secondary metabolites, called cyanotoxins, that are released to the water (Adamski et al., 2020). Humans can be exposed to cyanotoxins by oral route through consumption of drinking water, foods and dietary supplements contaminated with them (Ríos et al., 2013; Codd et al., 2020). Among cyanotoxins, Microcystins (MCs) and Cylindrospermopsin (CYN) have been the most usually studied (Pichardo et al., 2017; Diez-Quijada Jiménez et al., 2020).

In the environment, MCs concentrations up to 2700 μg/L (Preece et al., 2015) and CYN concentrations up to1050 μg/L (Yang et al., 2021) have been reported. Moreover, these cyanotoxins are able to accumulate in different aquatic organisms such as mollusks and fish inducing several toxic effects (Puerto et al., 2011; Gutiérrez-Praena et al., 2013; Diez-Quijada et al., 2019a,b; Scarlett et al., 2020), and consequently, the consumption of freshwater fish contaminated with cyanotoxins could represent a risk to humans (Drobac et al., 2016; Bormans et al., 2019; Mohamed et al., 2020; Scarlett et al., 2020).

Most studies dealing with the risks associated to the intake of foodstuffs were performed on uncooked/raw products (Domingo, 2011). Nevertheless, edible organisms are usually stored and processed before consumption, which can alter the concentration of cyanotoxins, as it is the case for MCs and CYN (Morais et al., 2008; Zhang et al., 2010; Guzmán-Guillén et al., 2011, 2017; Freitas et al., 2014, 2016; Prieto et al., 2017, 2020). However, the number of studies on the topic remains scarce. Following the recommendations of the European Food Safety Authority (EFSA) for all cyanotoxins, there is the need to quantify them in food, and to evaluate the effect of cooking in their contents, especially in the case of different variants of MCs (not only MC-LR) (Puerto et al., 2009; Testai et al., 2016).

With respect to the effects of cooking on cyanotoxins concentrations, contradictory results have been found so far. Thus, microwave cooking reduced MCs contents in mussels, while boiling caused no significant alteration (Morais et al., 2008). In contrast, boiled or microwaved clams showed higher concentrations of free MC-LR (Freitas et al., 2014). Similarly, contradictory results were also reported in fish: MCs

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https://doi.org/10.1016/j.fct.2021.112673
Received 14 September 2021; Received in revised form 8 November 2021; Accepted 11 November 2021
Available online 18 November 2021

concentrations were significantly higher in boiled muscle of bighead carp (Zhang et al., 2010), whereas boiling caused a reduction in MCs (MC-LR, –RR and -YR) concentrations in tilapia fish muscle (*Oreochromis niloticus)* (Guzmán-Guillén et al., 2011). Concerning CYN, cooking processes (boiling, steaming and microwaving) did not cause a significant change in CYN concentration in mussels. However, this cyanotoxin was found in the cooking water (Freitas et al., 2016). In fish, Guzmán-Guillén et al. (2017) observed that CYN concentrations decreased in tilapia fish muscle after steaming and boiling, being CYN also detected in the cooking water. In accordance with these results, decreased CYN levels were also reported in fish muscle after microwaving, broiling and boiling compared to the control group (Prieto et al., 2017, 2020).

Regarding the effects of food storage on cyanotoxins concentrations, only few studies have been performed, and again contrary results have been reported. A reduction of detectable MCs in mussels after storage at room temperature (20 °C), refrigeration (4 °C) and freezing (−20 °C) has been reported (Morais et al., 2008). Freitas et al. (2014) observed a decrease in free MC-LR concentration in refrigerated (4 °C) clams but a significant higher concentration after freezing (−20 °C). Similarly, in mussels, CYN concentration did not significantly differ from the control group after refrigeration, whereas it was significantly higher after freezing (Freitas et al., 2016). As far as we know, no studies have been carried out in the case of contaminated fish. Thus, to evaluate the influence of common storage practices on the concentration of single cyanotoxins and their mixtures in fish is worth of research.

Concerning MCs or CYN concentrations in fish and mollusks, their determination was performed by Liquid Chromatography-Mass Spectrometry (LC-MS) (Zhang et al., 2010; Guzmán-Guillén et al., 2011), as LC-MS has been widely proved suitable for their analysis in a variety of matrices (blooms, cyanobacterial cultures, biological samples (Cameán et al., 2004, Ruíz et al., 2005). However, the technique of choice for the analysis of different cyanotoxins (MCs, CYN, etc.) in complex matrices is the Ultra Performance Liquid Chromatography - Tandem Mass Spectrometry (UPLC-MS/MS), because of its specificity and sensitivity (Adamski et al., 2016a,b; Pekar et al., 2016; Guzmán Guillén et al., 2017; Prieto et al., 2017; Diez-Quijada Jiménez et al., 2020).

In view of this, the aim of this work was to investigate for the first time the effect of common storage practices of fish such as refrigeration (4 °C) and freezing (−20 °C) at three different periods of time, on the concentration of free CYN, MCs (MC-LR, –RR and -YR) and their combination in muscle of two different fish species (Tilapia, *Oreochromis niloticus*, and Tench, *Tinca tinca*) contaminated under laboratory conditions by UPLC-MS/MS.

2. Materials and methods

2.1. Chemical and reagents

MC-LR, MC-RR and MC-YR (99% purity) and CYN (95% purity) commercial standards were purchased from Enzo Life Sciences (Lausen, Switzerland). All chemicals and reagents used in this work were analytical grade materials. Deionized water (18.2 MΩ cm^{-1} resistivity) was acquired from an ultrapure water purification system (NANOpure Diamond™, Bamstead, USA). HPLC-graded methanol (MeOH), dichloromethane (DCM), formic acid (FA), acetonitrile (ACN), trifluoroacetic acid (TFA) and acetic acid were supplied by Merck (Darmstadt, Germany). Bakerbond® C18 cartridges (500 mg, 6 mL, Dicsa, Andalucía, España), Bond Elut Carbon cartridges (Porous Graphitic carbon (PGC)) (500 mg, 6 mL, Agilent Technologies, Santa Clara, CA, United States) and OASIS HLB cartridges (500 mg, 6 mL, Waters, Milford, MA, USA) were used for Solid Phase Extraction (SPE). Nanosep® Centrifugal Devices with Bio-Inert® Membrane (modified nylon) 0.45 μm were supplied by PALL Corporation (New York, USA) and 25 mm Syringe Filters with 0.2 μm cellulose acetate membrane were purchased from VWR (International Radnor, Pennsylvania, USA). LC–MS grade reagents were used for UHPLC–MS/MS analyses: water and ACN, obtained from VWR International (Fontenay-sous-Bois, France), and FA from Fluka (Steinheim, Germany). Standard stock solutions of each toxin prepared as follows and used to spike the fish muscles: CYN (1.5 μg/mL) in Milli-Q water, MCs (1.5 μg/mL of MC-LR, MC-RR and MC-YR) in 100% MeOH, and a standard multitoxin solution (1.5 μg/mL) containing the four cyanotoxins (CYN, MC-LR, MC-RR and MC-YR) in 20% MeOH.

2.2. Fish samples and experimental design

Fish (Tilapia, *Oreochromis niloticus* and Tench, *Tinca tinca*) were obtained from a local supermarket, ready for human consumption. Each fish muscle sample was cut into 4 g fresh weight (f.w.) portions (4.16 ± 0.04 and 4.13 ± 0.01 g f.w., for Tilapia and Tench, respectively) and each portion was spiked with a stock solution (500 μL of 1.5 μg/mL, equivalent to 750 μg/g d.w) containing CYN, MCs (MC-LR, MC-RR and MC-YR) or a mixture of the four studied cyanotoxins (CYN, MC-LR, MC-RR and MC-YR). To ensure a concentration of 750 μg/g d.w. in each fish sample, these toxins were directly spiked by injecting them into the muscle and individually homogenised. These toxin concentrations were selected according to the highest levels found in the environment (Preece et al., 2015; Yang et al., 2021). The assays were always performed by quintuplicate ($n = 5$) for each experimental condition. Moreover, fish control groups spiked with CYN, MCs or cyanotoxins mixture (500 μL of a solution containing 1.5 μg/mL of each toxin) not submitted to any storage procedure were included.

Two storage procedures were investigated: refrigeration and freezing. To test the refrigeration process, samples of fish muscle were refrigerated at 4 °C for 24, 48 h and 7 days. In the case of freezing, samples were stored at −20 °C for 48 h, 7 days and 1 month. For each of these tested procedures, a control group without toxins was included. All samples were frozen (−80 °C) and lyophilized (Cryodos −80 model, Telstar, Terrassa, Spain) before cyanotoxins were extracted.

2.3. Cyanotoxins extraction and clean up procedures (SPE)

First, the UPLC-MS/MS method (Guzmán-Guillén et al., 2017; Diez-Quijada et al., 2018) was examined for the analysis of CYN, MC-LR, MC-RR and MC-YR, obtaining mass spectra. Standard calibration points (50–1500 μg/L) were prepared from control fish extracts, to reach linear ranges of 25–750 μg/L, equivalent to 25–750 μg/g d.w., in the different regression equations obtained for each toxin. Fish muscles samples previously lyophilized were extracted following different validated analytical methods according to the type of cyanotoxin. In the case of CYN, they were extracted according to the method of Guzmán-Guillén et al. (2015b) with minor modifications. For this, 1 g dry weight (d.w.) was extracted with 20 mL Milli-Q water/acetonitrile (30:70 v/v) containing 0.5% TFA (v/v). After homogenization with ultraturrax and sonication, the sample was centrifuged (3700 rpm, 15 min); the whole process was repeated once again and the extracts were pooled. Once the extracts had been obtained, the purification step performed out using a combined SPE system consisting of C18 and PGC columns, as described in the cited work. At the end, the extracts were evaporated to dryness and resuspended in 1 mL Milli-Q water. Then, they were filtered through Nanosep® Centrifugal Devices (0.45 μm, 13200 rpm, 10 min), and through a syringe filter (0.22 μm) and analysed by UPLC-MS/MS.

For MCs, fish samples previously lyophilized were extracted following the method of Guzmán-Guillén et al. (2011) according to Dai et al. (2008) with minor modifications. The lyophilized muscle (1 g d.w.) was extracted with 10 mL of water with EDTA-Na_2 (0.01 M)- 5% acetic acid, sonicated (3 min, 0 °C) and centrifuged (3700 rpm, 15 min, 25 °C). This procedure was repeated twice using 5 mL of extractant. Once the extracts had been obtained, they were purified using Oasis HLB cartridges, as explained in the cited work. Later, extracts were evaporated to dryness and redissolved in 1 mL of 100% MeOH, filtered through

Nanosep® Centrifugal Devices (0.45 μm, 13200 rpm, 10 min), and analysed by UPLC-MS/MS.

Fish samples spiked with the mixture of the four cyanotoxins were extracted according to the multitoxin method developed by Diez-Quijada Jiménez et al. (2020) with minor modifications. Briefly, 1 g d. w. of fish was extracted with 20 mL 90% MeOH, sonicated and stirred for 15 min each step, and centrifuged (3700 rpm, 15 min, 25 °C), and the process was repeated once again. Later, the supernatants were pooled and concentrated in a rotary evaporator to reach 15% MeOH, and then the extracts were cleaned using a dual SPE system with C18 and PGC cartridges. Finally, the extracts were evaporated to dryness and resuspended in 1 mL 20% MeOH and analysed by UPLC-MS/MS.

The parameters of the validated methods applied in this work were: for MCs, recoveries of 91–103% and %RSD of 4.82–18.8 (Dai et al., 2008); for CYN, recoveries of 94–104% and %RSD of 6.7–11 (Guzmán-Guillén et al., 2015b), and for the mixture, recoveries of 70.37–114.03% and %RSD of 2.61–13.73 (Diez-Quijada Jiménez et al., 2020).

2.4. Chromatographic conditions

Chromatographic separation was carried out using a UPLC Acquity (Waters) coupled to a Xevo TQS micro (Waters) being made up of a triple quadrupole mass spectrometer equipped with an electrospray ion source, operating in positive mode. UPLC analyses for CYN were conducted on a 50 × 2.1 mm Acquity BEH C18 1.7 μm column. The column used for MCs and for the mixture of cyanotoxins was a 100 × 2.1 mm XSelect HSS T3 2.5 μm column. The flow rate was 0.45 mL min^{-1}. Chromatographic separation for CYN, MCs and mixture were performed using a binary gradient consisting of (A) water and (B) methanol in the case of CYN or acetonitrile in the case of MCs and the mixture, 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). In all cases, the injection volume was 5 μL, and Multiple Reaction Monitoring (MRM) was applied, where the parent ions and fragments ions were monitored at Q1 and Q3, respectively. The transitions employed were 416.2/194.0 and 416.2/176.0 for CYN; 996.5/135.0, 996.5/213.1 and 996.5/996.5 for MC-LR; 520.2/135.0 and 1039.5/135.0 for MC-RR; and 1046.5/135.0, 1046.5/213.0 and 1046.5/1046.5 for MC-YR, selecting in all cases the first one for quantitation and the others as confirmatory.

For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: capillary voltage: 3.0 kV (for CYN) and 1.0 kV (for MCs and the mixture), 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 Milli-Q water for CYN, in 100% MeOH for MCs and in 20% MeOH for the mixture.

2.5. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparison Test was performed verified with the normality test (Kolmogorov–Smirnov) and homogeneity of variances test (Bartlett) using GraphPad InStat software (GraphPad Prism 9 Software Inc., La Jolla, CA, USA), representing mean ± standard deviation (SD) of 5 samples per group. Statistical significance was considered at $p < 0.05$ level.

3. Results and discussion

Worldwide demand for fish products is continuously increasing (Carrassón et al., 2021). In fact, from 1990 to 2018, there was a global rise in aquaculture production of 527% (FAO, 2020). Accumulation of CYN and MCs in different fish species such as tilapia or tench, as well as the possibility of exceeding the provisional TDI established by World Health Organization (2020a,b) for humans (0.03 and 0.04 μg/kg b.w. for CYN and MC-LR, respectively) have been well reviewed by different authors (Gutiérrez-Praena et al., 2013; Chen et al., 2021). Furthermore, different studies highlighted the importance of the occurrence of these cyanotoxins in fish farms and recommended their monitoring to reduce the exposure of fish to CYN and MCs (Gutiérrez-Praena et al., 2013; Drobac et al., 2016; Mohamed et al., 2020).

For risk assessment purposes, a realistic estimation of human exposure to these toxins is essential. In this sense, EFSA indicated that additional efforts were necessary to elucidate the levels of human exposure to cyanotoxins under different scenarios (Testai et al., 2016). On the other hand, different processes to which food are subjected before consumption have shown to produce important variations in the concentration of contaminants (Domingo, 2011). In addition to cooking, fish is a perishable product, and it is normally subjected to different conditions of refrigeration and freezing during storage in order to extend its shelf life and quality (Freitas et al., 2014).

This work investigates, for the first time, the effects of refrigeration and freezing processes on the concentration of CYN, MCs and their mixture in two fish species. To ensure that the differences observed in toxin concentrations were due exclusively to the refrigeration or freezing processes, the samples were spiked with the toxins. Thus, possible conjugation reactions of these toxins (that would occur *in vivo*) can be excluded in our investigation.

The recovery values were different according to the type of toxin and the evaluated experimental group, reaching recoveries up to 80.4% for CYN, and 46.4% for MCs. For this reason, and to ensure that the results can be comparable among them, they were expressed as % toxins found relative to its respective control group.

In general, the results showed that the most effective food storage process for the reduction of cyanotoxins in fish muscle is freezing. Thus, the refrigeration process did not modify the concentration of CYN compared to the respective control groups in both fish species, tilapia (Fig. 1a) and tench (Fig. 2a). On the contrary, significant CYN decreases have been found after 48 h, 7 days and 1 month of freezing in tilapia (remaining 73.5%, 69.3% and 82.2%) (Fig. 3a) and tench (remaining 59.3%, 32.5% and 20.3%) (Fig. 4a), respectively.

Regarding CYN, no differences were observed in its stability between the tested fish species, tilapia or tench, after both conservation processes. In fact, the high stability of CYN to light and over a large range of temperature and pH (acidic and neutral conditions) has been reported, which might have significant consequences for aquatic environments (Campos et al., 2013; Guzmán-Guillén et al., 2014; 2015a). According to our results, Freitas et al. (2016) reported no significant differences in CYN concentrations after 24 and 48 h of refrigeration at 4 °C in mussels intoxicated with cyanobacterial crude extracts for 4 days under laboratory conditions. By contrast, the same authors showed a significant increase in CYN concentration in the mussels after 48 h, 7 days and 1 month of freezing at −20 °C. Contrary to their work, in the present study, CYN was in its unconjugated form, and it was possible to detect this toxin in all experimental groups, including the contaminated control group. Actually, Freitas et al. (2016) explained that the increase in CYN concentrations detected in the freezing group could be due to cell disruption and protein denaturalization produced by this process.

Important differences in MCs concentrations were observed between fish species contaminated with these toxins depending on the food storage process. In the case of tilapia (Fig. 1b), from 24 h of refrigeration only MC-RR showed a significant decrease in its concentration (remaining at 24 h: 60.3%; 48 h: 44.4%; 7 days: 7.4%), being the less stable toxin after the refrigeration process. Longer refrigeration periods were needed to observe a decrease in the case of MC-YR (48 h: 69.9% and 7 days: 19.4%) and MC-LR (7 days: 34.2%). The refrigeration process was more effective in the case of tench (Fig. 2b) in comparison to tilapia, observing a decrease in the concentration of the three MCs for all periods of time (for 24 h: MC-LR: 44.4%; MC-RR: 26.3%; MC-YR: 55.8%; for 48 h: MC-LR: 62.2%; MC-RR: 44.1%; MC-YR: 64.9%; for 7 days: MC-

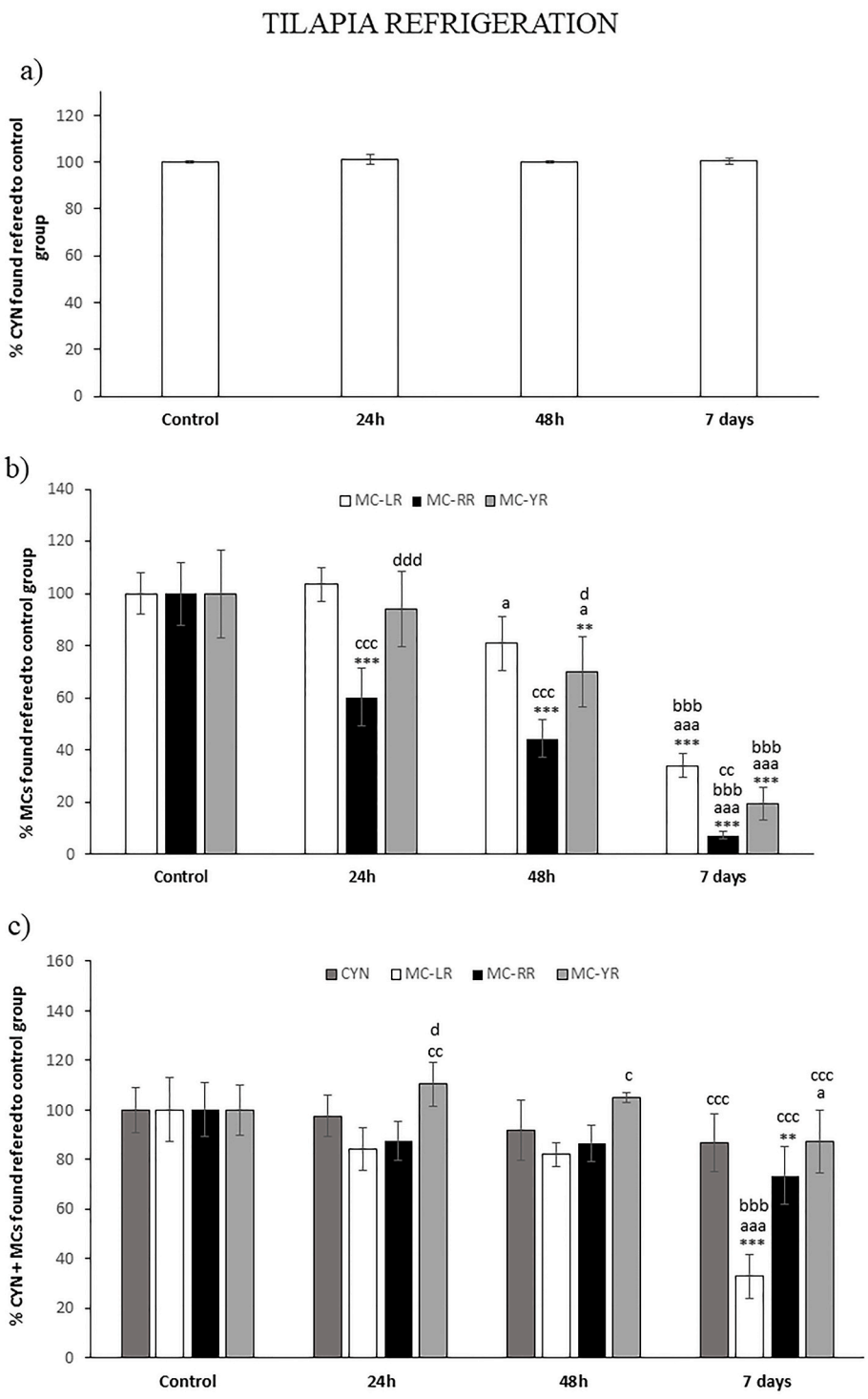

Fig. 1. Effects of refrigeration on contaminated tilapia fish muscle (*Oreochromis niloticus*) spiked with 500 μL of a standard solution containing 1.5 μg/mL of each toxin (CYN, MCs and CYN + MCs) and refrigerated at 4 °C for 24 h, 48 h and 1 week. CYN (a), MCs (b) and CYN + MCs (c). Values are expressed as the mean ± SD (N = 5). The significant levels observed are $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group, ^{a} $p < 0.05$ and $^{aaa}p<0.001$ compared to 24 h, ^{bbb} $p < 0.001$ compared to 48 h, $^{c} < 0.05$; $^{cc}p<0.01$ and $^{ccc}p<0.001$ when comparing to MC-LR within the same period, $^{d}p < 0.05$ and $^{ddd}p<0.001$ when comparing to MC-RR within the same period.

LR: 41.0%; MC-RR: 10.3%; MC-YR: 29.5%). Although a mild increase in toxins concentration was observed after 48 h compared to 24 h, it was only significant for MC-RR. This could be explained because the period considered between 24 and 48 h is small, and the variability derived from the experimental process itself. Similar results were reported in *Mytillus galloprovincialis* exposed to cyanobacterial cells of *Microcystis aeruginosa* for 4 days, and later stored at 4 °C for 24, 48 and 72 h, detecting MCs by ELISA (Morais et al., 2008). However, under the same conditions, LC-MS analysis performed by Freitas et al. (2014) only showed this effect in *Corbicula fluminea* exposed to MC-LR producing cells of *Microcystis aeruginosa* after 48 and 72 h, but not in the case of 24 h, coinciding with our results in tilapia after 24 h and in tench after 48 h. In our study, a higher period was also assessed (7 days) which showed a significant decrease in MCs concentrations in comparison to 24 and 48 h in both fish species. Moreover, this is the first study involving three MCs congeners and evaluating the effect that the refrigeration process has in each of them, and among MCs variants within the same period. In this sense, MC-RR was the most sensitive to refrigeration in both fish species. These differences observed between the MCs variants studied could be due to their chemical structure and to the composition of the matrix in which they are included. Previously, MC-RR has demonstrated to be more sensitive than MC-LR and MC-YR when these cyanotoxins were

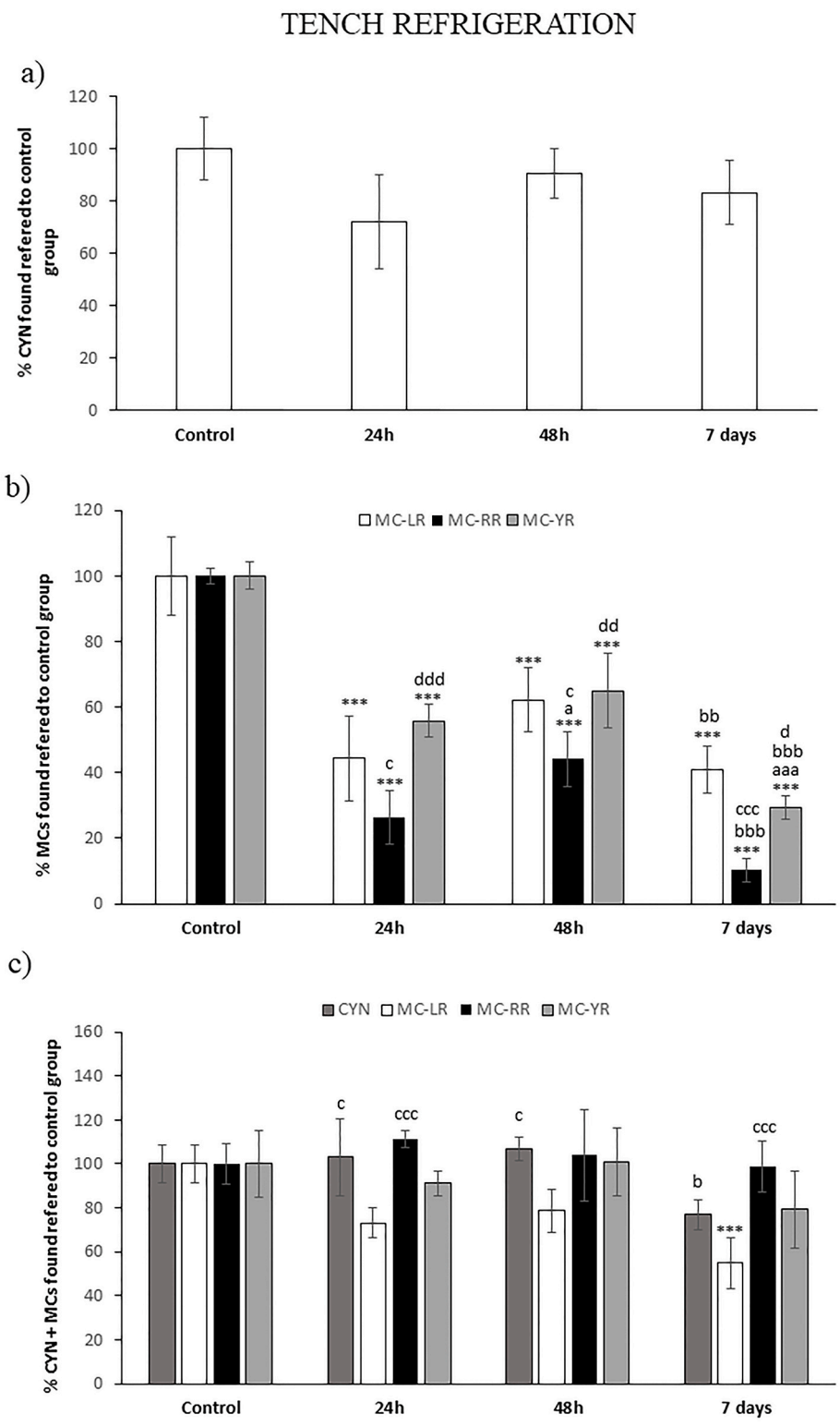

Fig. 2. Effects of refrigeration on contaminated tench fish muscle (*Tinca tinca*) spiked with 500 μL of a standard solution containing 1.5 μg/mL of each toxin (CYN, MCs and CYN + MCs) and refrigerated at 4 °C for 24 h, 48 h and 1 week. CYN (a), MCs (b) and CYN + MCs (c). Values are expressed as the mean ± SD (N = 5). The significant levels observed are ***$p < 0.001$ compared to the control group, $^{a}p < 0.05$ and $^{aaa}p<0.001$ compared to 24 h, $^{b}p < 0.05$; $^{bb}p<0.01$ and $^{bbb}p < 0.001$ compared to 48 h, $^{c} < 0.05$ and $^{ccc}p<0.001$ when comparing to MC-LR within the same period, $^{d}p < 0.05$, $^{dd}p<0.01$ and $^{ddd}p<0.001$ when comparing to MC-RR within the same period.

submitted to *in vitro* digestion processes (Moreno et al., 2004).

However, the freezing storage process was more effective in reducing MCs concentrations in tilapia in comparison to tench.

Thus, the results in tilapia showed a significant reduction for the three MCs for all the periods assessed (MC-LR: 13.4%, 12.7% and 9.3%; MC-RR: 28.7%; 18.9% and 11.0%; MC-YR: 23.7%, 14.6% and 13.9%, for 48 h, 7 days and 1 month, respectively) (Fig. 3b). In the case of tench, only the longest freezing period (1 month) was effective in decreasing MCs concentrations, being 5.27% for MC-LR, 37.4% for MC-RR and 42.0% for MC-YR (Fig. 4b). These results are in agreement with those previously obtained in mussels by Morais et al. (2008), who also observed an important decrease in MCs concentrations at the same periods of time (48 h, 7 days and 1 month). However, in the case of tench this response was only observed after 1 month. Different results were reported by Freitas et al. (2014) in clams, depending on the freezing time. In this case, these authors also observed a decrease after 48 h,

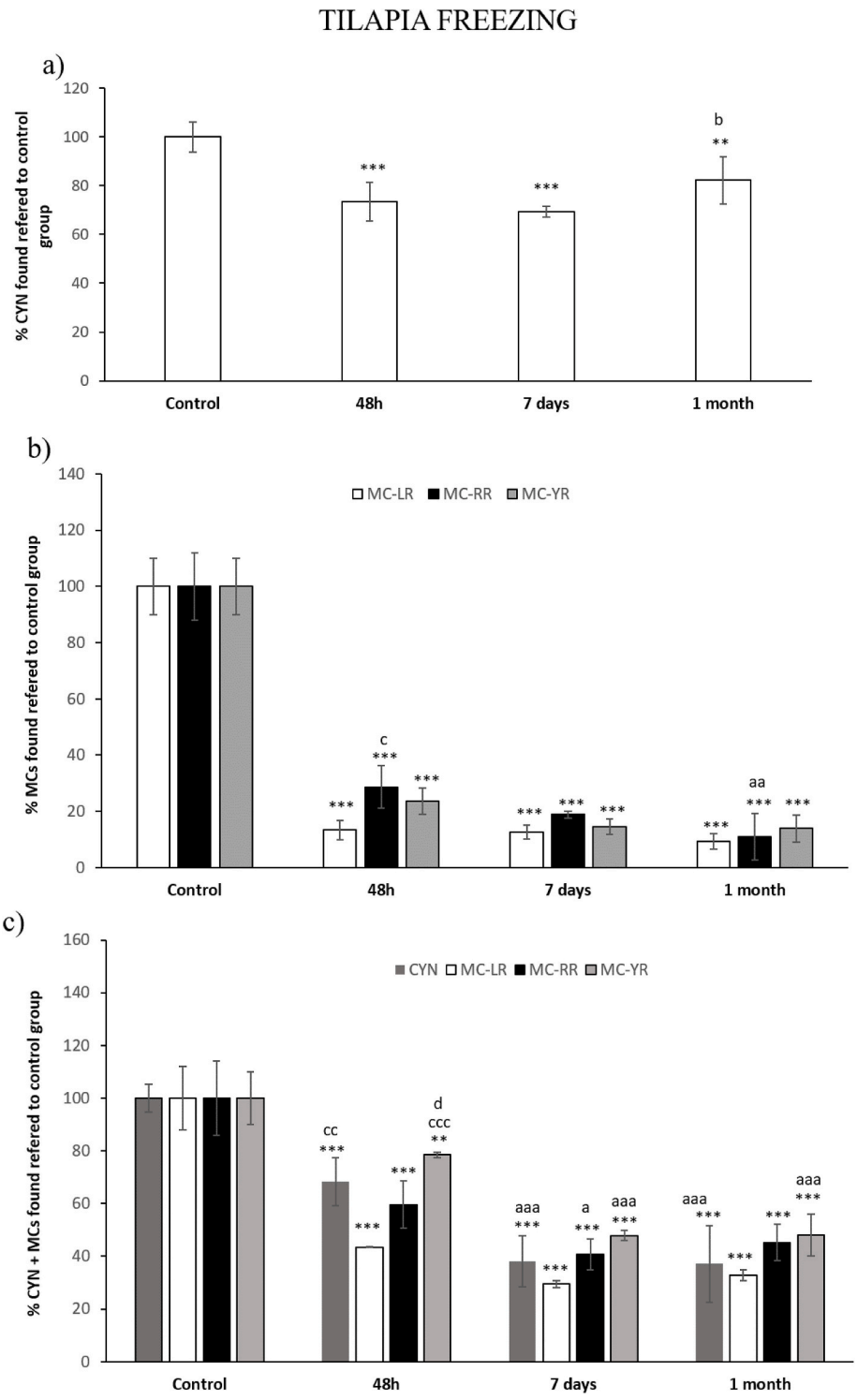

Fig. 3. Effects of freezing on contaminated tilapia fish muscle (*Oreochromis niloticus*) spiked with 500 μL of a standard solution containing 1.5 μg/mL of each toxin (CYN, MCs and CYN + MCs) and frozen at −20 °C for 48 h, 7 days and 1 month. CYN (a), MCs (b) and CYN + MCs (c). Values are expressed as the mean ± SD (N = 5). The significant levels observed are **p < 0.01 and ***p < 0.001 compared to the control group, [a] p < 0.05, [aa]p<0.01 and [aaa]p<0.001 compared to 48 h, [b] p < 0.5 compared to 7 days, [c] < 0.05; [cc]p<0.01 and [ccc]p<0.001 when comparing to MC-LR within the same period, [d]p < 0.05 when comparing to MC-RR within the same period.

similarly to our results in tilapia. By contrast, they showed a significant higher concentration in MC-LR after 7 days and 1 month, whereas in our study a significant decrease of MC-LR was detected after 7 days in tilapia and 1 month in tilapia and tench. In our work, it is worth highlighting that the highest differences in the freezing process derived from the storage time and the toxin congener were observed in tench, compared to tilapia. Thus, a longer freezing time was necessary in tench in comparison to tilapia to produce a decrease in the content of the MCs. Moreover, MC-LR showed to be the most unstable toxin mainly in tench compared to MC-RR and MC-YR. For all this, both the MCs congener and the fish species must be considered in order to perform a correct risk assessment, although no direct relationship was observed between the nature of the different cyanotoxins and fish composition in our study.

The co-occurrence and increase of multiple variants and/or classes of cyanotoxins, such as CYN and MCs, is a growing area of research (Metcalf and Codd, 2020). However, there is no research works focused on the effect of storage techniques on the concentration of these cyanotoxins mixtures so far, that allow us to approach a more realistic scenario. This is the first study that assesses the effect of food storage (refrigeration and freezing) on distinct contaminated fish species with

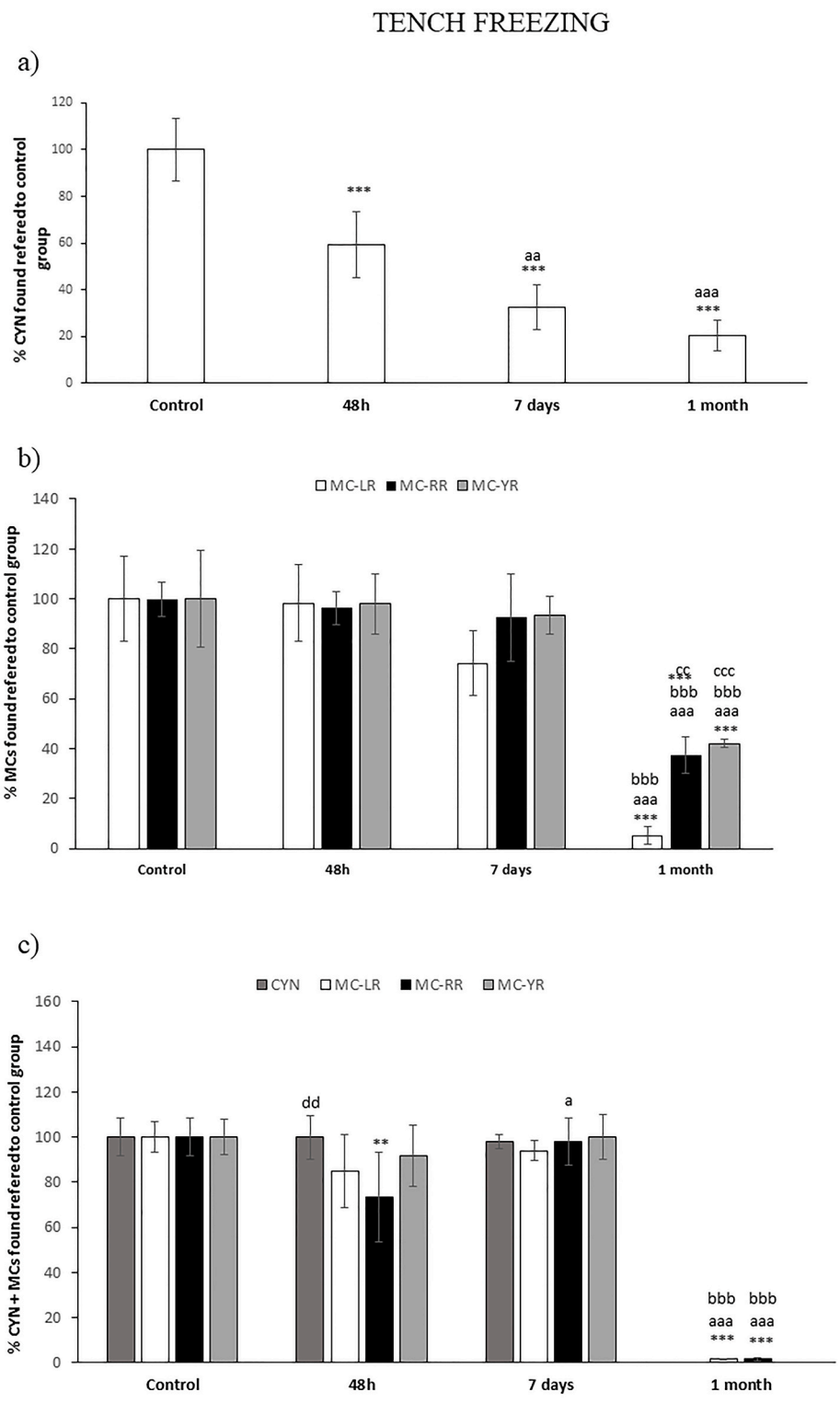

Fig. 4. Effects of freezing on contaminated tench fish muscle (*Tinca tinca*) spiked with 500 μL of a standard solution containing 1.5 μg/mL of each toxin (CYN, MCs and CYN + MCs) and frozen at −20 °C for 48 h, 7 days and 1 month. CYN (a), MCs (b) and CYN + MCs (c). Values are expressed as the mean ± SD (N = 5). The significant levels observed are $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to the control group, ^{a} $p < 0.05$, $^{aa}p<0.01$ and $^{aaa}p<0.001$ compared to 48 h, ^{bbb} $p < 0.001$ compared to 7 days, $^{cc}p<0.01$ and $^{ccc}p<0.001$ when comparing to MC-LR within the same period, $^{dd}p<0.01$ when comparing to MC-RR within the same period.

the mixture of CYN and MCs (MC-LR, MC-RR and MC-YR). Similarly to the results obtained in absence of MCs, CYN did not show any significant differences in its concentrations in the presence of MCs both in tilapia (Fig. 1c) and tench (Fig. 2c) for the three refrigeration periods assessed. In tilapia, in the case of MC-LR, only a significant reduction was observed after 7 days, regardless of the presence or absence of CYN (32.8% versus 34.2% for 7 days, respectively). MC-RR showed a slight reduction after 7 days (73.5%) in the CYN + MCs mixture, while MC-YR concentration was not affected in comparison to its control group. In tench, there was only a reduction in MC-LR concentration (54.9%) in the presence of CYN after 7 days of refrigeration, evidencing a lower effectiveness of the process for reducing the concentration of this toxin in co-occurrence with CYN. In fact, refrigeration did not decrease the concentration of MC-RR and MC-YR in the presence of CYN for any of the periods evaluated. Possible interactions between MCs and CYN, mainly in the case of MC-RR (the most hydrophilic together with CYN), could be occurring during refrigeration process, that increase toxins stability in the samples.

With respect to freezing, different changes in cyanotoxins response were observed when they were alone or in the CYN + MCs mixture depending on the fish species. In the case of tilapia, a similar response was observed in CYN and MCs both individually and simultaneously in the mixture, for all periods of time assessed (Fig. 3c). A progressive significant reduction was observed for CYN in the presence of MCs in tilapia (48 h: 68.3%; 7 days: 38.1% and 1 month: 37.1%) and this pattern was also observed for the three MCs in the presence of CYN with important decreases (MC-LR: 43.4%, 29.4% and 32.9%; MC-RR: 59.6%; 40.8% and 45.1%; MC-YR: 78.5%, 47.9% and 48.0% for 48 h, 7 days and 1 month, respectively). In tench, in the case of CYN alone, a significant and progressive decrease on toxin levels was detected for all periods evaluated. Moreover, that decrease was not detected in the presence of MCs even after 1 month of freezing. In the case of MCs, a similar behavior was observed in the different experimental groups (MCs alone and CYN + MCs mixture). After 1 month of freezing, all the MCs suffered an important reduction of their concentrations MC-LR: 1.6%, MC-RR: 1.5% and MC-YR was not detected in presence of CYN (Fig. 4c).

To our knowledge, this is the first study evaluating the effects that refrigeration and freezing have on the concentration of cyanotoxins mixtures, so there are no previous data to compare these findings with. Our results showed that it is necessary and important to know the type of cyanotoxins present in the contaminated fish to predict the level of exposure to these toxins after refrigeration and freezing processes, especially in the last case. In general, these processes produce a higher reduction in MCs concentrations when they were evaluated individually (CYN or MCs) in comparison to the CYN + MCs mixture. However, this depends on the type of conservation process (refrigeration or freezing) and the species of fish employed.

With respect to the time of food storage necessary to decrease cyanotoxins concentrations, it has been observed that in the case of refrigeration, MCs showed higher reductions after 7 days, being this period of time the minimum advisable in terms of food safety. CYN was not affected for any refrigeration period assessed, being necessary a freezing process to decrease its concentration. Moreover, the time of freezing is not relevant in tilapia, but time-dependent differences must be considered in tench, mainly in the case of MCs, where longer periods of time (1 month) are needed to ensure the effectiveness of this storage process on their reduction, in order to guarantee the safety for consumers.

4. Conclusions

This is the first study that assesses the effect of refrigeration and freezing on different fish species such as tilapia and tench contaminated with CYN and MCs (MC-LR, MC-RR and MC-YR) alone or with their combination, providing new light on this issue. In general, these food conservation procedures produce significant differences on cyanotoxins concentrations in contaminated fish depending on different factors, such as type of cyanotoxin, alone or combined, and fish species. In this way, free CYN concentration is only decreased by freezing, while in the case of free MCs, refrigeration is also effective. Moreover, the reduction of free MCs concentrations contained in the mixtures is lower in comparison to individual MCs. The fish species is a factor to take into account since toxins are more stable in tench than in tilapia. In general, the longest periods of time assayed (7 days for refrigeration, and 1 month for freezing) are more effective to reduce free cyanotoxins concentrations in fish, and freezing is shown as the most effective food storage technique. These findings highlight the need to assess the influence of food storage processes on the presence of cyanotoxins in fish species for a more realistic human health risk assessment.

CRediT authorship contribution statement

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. **Remedios Guzmán-Guillén:** 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. **Ángeles Jos:** Conceptualization, Funding acquisition, Investigation, 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.

Acknowledgements

The authors wish to thank the Spanish Ministerio de Ciencia e Innovación (PID2019-104890RB-I00 MICIN/ AEI/10.13039/ 501100011033) for the financial support, and the Mass Spectrometry Facility of Centro de Investigación, Tecnología e Innovación from Universidad de Sevilla (CITIUS), for providing technical assistance. Leticia Diez-Quijada Jiménez thanks to the Ministerio de Economía, Industria y Competitividad of Spain for the grant BES-2016-078773 and the VI PPIT-US for the granting of a Postdoctoral Bridges Aid and its funding.

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