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Decomposition of Microcystin-LR, Microcystin-RR, and Microcystin-YR in Water Samples Submitted to in Vitro Dissolution Tests

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The presence of cyanobacterial toxins (microcystins) in waters and food increases the risk of toxicity to animal and human health. These toxins can degrade in the human gastrointestinal tract before they are absorbed. To evaluate this possible degradation, water samples spiked with known concentrations of microcystins MC-LR, MC-RR, and MC-YR, which are the toxins most commonly produced by such toxic cyanobacteria as *Microcystis aeruginosa*, *Oscillatoria* spp., and *Nostoc* spp., were submitted to a dissolution test that used gastric and intestinal fluids according to U.S. Pharmacopeia conditions. HPLC with UV detection was used to determine the toxins before and after treatments. This study revealed enzymatic alterations in gastric conditions for all the toxins assayed. MC-RR was the toxin most affected: its range of inactivation was 49−64%. The percentage of degradation for MC-YR and MC-LR was around 30%. However, none was degraded by intestinal digestion.

KEYWORDS: Microcystins; MC-LR; MC-RR; MC-YR; gastrointestinal fluids; dissolution test

INTRODUCTION

Microcystins (MCs) are cyclic peptide hepatotoxins and tumor promoters produced by freshwater cyanobacteria (blue-green algae). Currently, more than 70 different microcystins are known to be produced from at least five genera worldwide: *Anabaena*, *Microcystis*, *Oscillatoria (Planktothrix)*, *Nostoc*, and *Anabaenopsis* (*1*). Microcystins have been shown to be acutely toxic to animals. The LD_{50} by intraperitoneal injection (i.p.) of MC-LR is about 50 μg/kg bodyweight. This toxin is 30−100 times less toxic via oral ingestion than via i.p. injection (*2*). The toxicity of microcystins can vary, but the most toxic are potent hepatotoxins, which appear to inhibit the activity of protein phosphatases 1 and 2A. This inhibition causes protein phosphorylation to increase throughout the cell and activates the cascade of caspases, which results in apoptotic necrosis of hepatocytes (*3*−*5*).

The World Health Organization (WHO) has established that the tolerable daily intake (TDI) of MC-LR by humans is 0.04 μg kg^{-1} body weight day^{-1} (*6*) and that the maximum concentration of MC-LR in drinking water is 1 μg L^{-1} (*7*). The latter guideline is based on the daily water consumption for a 60 kg human.

Characterizing the hazards and assessing the risks of cyanobacterial toxins for human health necessarily involves identifying the exposure routes. These include the oral route of drinking and recreational water (*8*, *9*). An additional exposure route may be the consumption of foods such as fish (*10*, *11*), mussels (*12*, *13*), and crop plants (*14*−*16*), which have been exposed to toxin-producing cyanobacterial cells or toxins released into the water. Therefore, evaluating the risk to human health of consuming these foods is particularly interesting.

Microcystins, being cyclic peptides, are extremely stable and resistant to chemical hydrolysis or oxidation near neutral pH. At high temperatures (40 °C), and high or low pH, hydrolysis is slow: 90% breakdown was achieved in about 10 weeks at pH 1 and more than 12 weeks at pH 9 (*17*).

Little work has been undertaken on the enzymatic hydrolysis of microcystins, and the results are contradictory (*18*, *19*). The resistance or degradation of toxicants or chemical substances under the conditions of the stomach—acidic pH, enzyme action, or even the effects of the intestinal flora—is of vital importance, so toxicants can hydrolyze in the stomach or intestine to new compounds whose toxicity is completely unlike that of the parent compound.

In vitro assays have been applied to compare the bioavailability of minerals from rich dietary sources and as an initial step prior to the in vivo assays of some natural components of food, such as phenolic compounds in wines (*21*), orange juice (*22*), and pomegranate juice (*23*). Recently, the bioaccessibility

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of total arsenic and inorganic arsenic contents from cooking edible seaweed has been examined by an in vitro digestion method; the in vitro methods provide an effective approximation of in vivo situations and have the advantage that reproducibility is good, as it is possible to control conditions better than in in vivo tests (*24*). U.S. Pharmacopeia (USP) (*25*) proposes the dissolution test (with gastrointestinal fluids) as a necessary prior step for evaluating the bioavailability of medicines in vivo.

The extent to which microcystins are absorbed and whether they are degraded in the intestinal tract also needs to be elucidated. As digestion is an initial step that involves changes in pH and the activity of proteolytic enzymes, possible alterations to microcystins caused by these changes should also be evaluated.

The aim of the present study is to determine whether gastrointestinal fluids decompose the microcystins MC-LR, MC-RR, and MC-YR present in natural waters and to evaluate their stability under gastric and intestinal conditions. This study was carried out at two MC concentration levels in water reservoirs, and the possible effects of changes in gastrointestinal pH were investigated. High-performance liquid chromatography (HPLC) with diode array detection has been proposed in the literature as being suitable for determining MCs in waters (*26*−*28*).

MATERIALS AND METHODS

Reagents. The enzymes used in the test solutions were pepsin, from hog stomach (Fluka 77163, Fluka Chemische, Buchs, Switzerland), and pancreatin, from porcine pancreas (Sigma P-1500, Sigma-Aldrich Química, Madrid, Spain).

Microcystins-LR (99.0%), -RR (98.7%), and -YR (95.7%) were purchased from Calbiochem (La Jolla, CA). Standard stock solutions of each toxin (500 μg/L) in methanol were used to spike the waters, and working standard solutions (20 and 40 μg/L MCs) were prepared from these stock solutions by dilution in methanol prior to analysis.

Gastric and intestinal fluids were prepared as follows (*25*). Gastric fluid (simulated): 2.0 g of sodium chloride and 3.2 g of pepsin were dissolved in 7.0 mL of hydrochloric acid in water to a final volume of 1000 mL. This test solution has a pH of about 1.2.

Intestinal fluid (simulated): 6.8 g of monobasic potassium phosphate was dissolved in 250 mL of water, mixed, and diluted with 190 mL of 0.2 N sodium hydroxide and 400 mL of water. Then, 10.0 g of pancreatin was added and mixed, and the resulting solution was adjusted with 0.2 N sodium hydroxide to a pH of 7.5 ×b1 0.1 and diluted with water to 1000 mL.

All solvents and chemicals used in this study were HPLC or analytical grade. Distilled, deionized water (Milli-Q Water System, Millipore Corporation, Bedford, MA) was used to prepare all aqueous solutions.

Samples. Water samples (10 L) were collected from the surface near the shore at several points along the Guadiana River and were mixed. They were spiked with two levels of MCs. Two criteria were taken into account to choose the final concentrations: (1) the levels of extracellular microcystins found in eutrophicated waters containing toxic blooms of cyanobacteria and (2) the range of linearity and detection and the quantification limits of the chromatographic method.

Instruments. Samples were submitted to gastric and intestinal fluids in a USP Turugrau apparatus automatized dissolution test using the paddle method at 37 °C and 100 rpm (*25*).

The LC system used to analyze gastric and intestinal digestion samples was a Varian 9012 equipped with an Ultraviolet Detector Varian 9050. Chromatographic data were processed with Star Chromatography Workstation 4.5 (Varian Technologies). Chromatographic separation of MCs was performed on a 250 mm × 4.6 mm i.d., 5 μm LiChrosphere C18 column purchased from Merck (Darmstadt, Germany).

In Vitro Gastrointestinal Digestion Method. A gastrointestinal digestion study was performed with the technique described by USP (*25*) and which has been used by Martínez-Ortega et al. (*21*). The technique consisted of a pepsin-HCl digestion first for 30 min (to simulate gastric digestion) and then a pancreatin digestion for 2 h, both at 37 °C (to simulate intestine conditions).

Aliquots of 125 mL of natural water samples were independently spiked with the three toxins studied (MC-LR, MC-RR, and MC-YR) at two concentration levels, 20 and 40 μg/L. They were also treated with an equal volume of gastric fluid for 30 min. Other 125 mL aliquots of water samples were treated in an equal volume of intestinal fluid for 2 h. Consequently, final concentrations of toxins in simulated media were 10 and 20 μg/L. This process was performed in triplicate. Blanks were prepared from 125 mL of a natural water sample without spiking MCs and 125 mL of gastric or intestinal fluids and submitted to the digestive process.

To study the possible effects of pH changes in the gastrointestinal tract, another two 125 mL aliquots of spiked water samples were diluted with 125 mL solutions with a pH of 1.2 and 7.5. They were prepared as test solutions without enzymes and subjected to simulated media for 30 min (gastric conditions) and 2 h (intestinal conditions). The assays were performed in triplicate.

Sample Preparation and Analysis of MCs by HPLC. Water samples from the Guadiana River, which had been spiked and submitted to the digestion process or pH action, were loaded and concentrated with the solid-phase extraction procedure (SPE) using Empore C18 Disks (3 M, St. Paul, MN) and in the conditions suggested by Aguete et al. (*28*). Briefly, 250 mL of the total solutions (125 mL water + 125 mL gastric or intestinal fluids) were passed through C18 disks under vacuum. The toxins were eluted with MeOH-TFA 0.1% and concentrated under nitrogen, and the residue was dissolved in 2.5 mL of methanol for HPLC analysis. The final concentrations of MCs in the extracts were then concentrated 50 times more than the original amount spiked in natural water samples. Previous studies carried out with the C18 disks showed recovery values of 107, 103, and 79% for MC-RR, MC-YR, and MC-LR, respectively, when these toxins were spiked in reservoir water at a concentration of 1 μg/L (*29*).

To study how the pH affects the extraction of MCs, the same Empore disks under the conditions described previously were used to concentrate microcystins with standard solutions (125 mL) prepared with 125 mL of simulated media (gastric or intestinal) at the corresponding pH (1.2 or 7.5). These standards were known as stgastric and stintestinal. Thus, natural samples were compared to these solutions, and the differences between them were due only to how the samples had been treated and not to the effect of the pH during the extraction procedure.

Moreover, solutions spiked with deionized water were included in each treatment so that the extent of the matrix effects could be studied (**Figure 1**). As can be seen, the matrix effects were minimal, and the chromatograms show no interfering peaks. However, as natural waters are variable (they depend on climatic conditions, sediments, etc.) all the experiments were carried out using water samples from the Guadiana River.

Microcystins -LR, -RR, and -YR were determined in water samples after gastric and intestinal treatment by HPLC/UV. Chromatographic separation was performed under isocratic conditions with a mobile phase consisting of 38% MeCN in water with 0.05% TFA. The wavelength was set at 240 nm, and the flow rate was 1 mL min^{-1}. The method had been previously evaluated and optimized (*28*).

Statistical Analyses. There were three replicates for each analyzed sample. The mean values of the different groups were compared using the Student's *t*-test. The statistical package STATISTICA 99, from Statsoft (*30*), was used for all the calculations.

RESULTS AND DISCUSSION

This is the first paper in which MCs have been submitted to the in vitro dissolution test, according to the USP 23 assay (*25*), so that the possible decomposition caused by enzymes or pH conditions in the gastrointestinal tract can be assessed. **Figure 2A** shows an example of the chromatograms obtained for natural water spiked at a final concentration of 20 μg/L of MC-RR toxin, after gastric (c) and acid pH (d) treatments, a blank for extra peaks in the treatment (b), and the Stgastric solution (a)

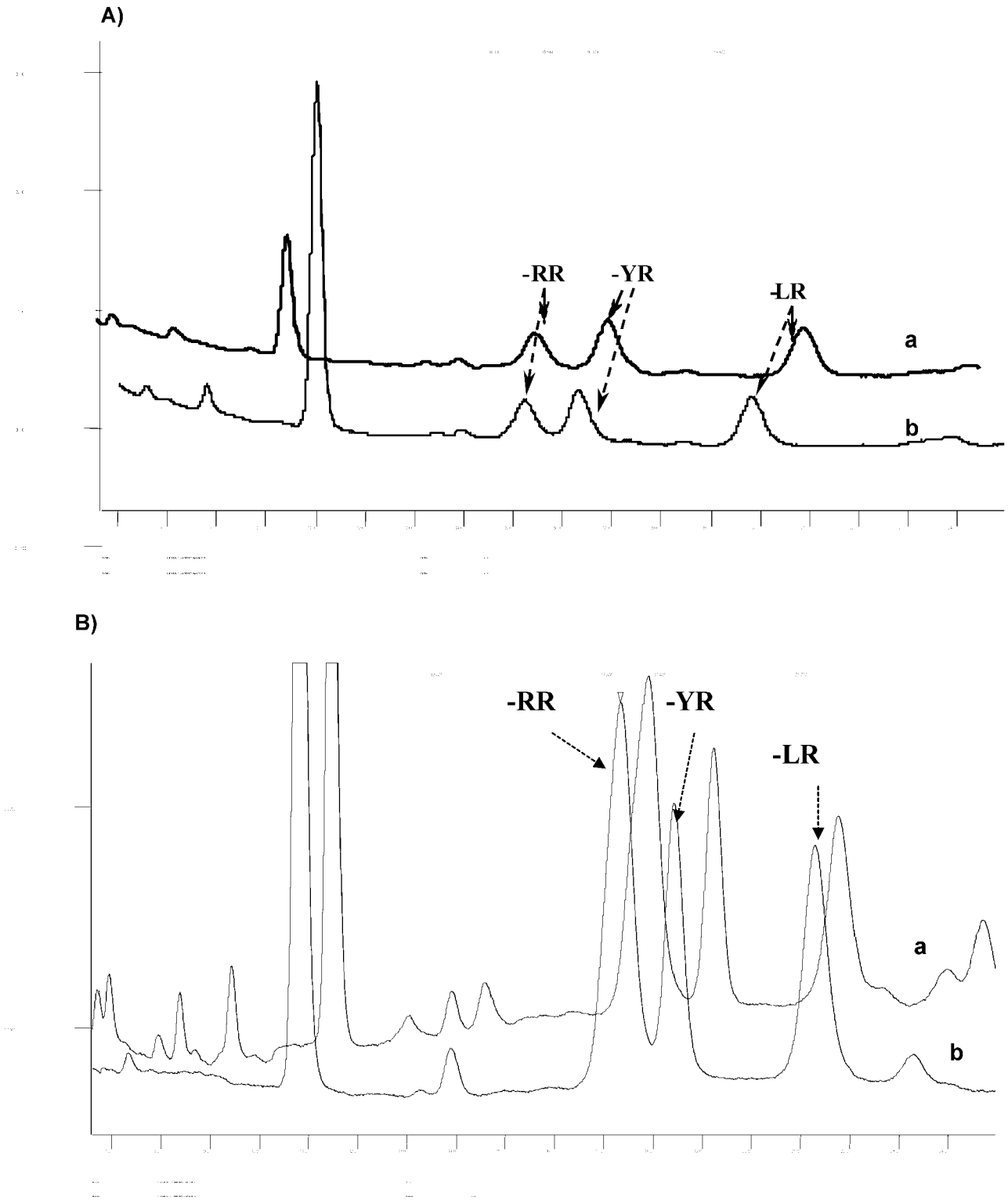

Figure 1. (**A**) Chromatogram of matrix effects in gastric treatment. (a) Natural water from the Guadiana River spiked with 10 μg/L toxins and (b) deionized water contaminated with 10 μg/L toxins. (**B**) Matrix effects in intestinal media. (a) Natural water spiked with toxins (10 μg/L) and (b) deionized water contaminated with toxins (10 μg/L).

(see sample preparation). Similarly, **Figure 2B** shows the results obtained after intestinal (c) and 7.5 pH (d) treatments of MC-YR, using a blank (b) and stintestinal solution (a).

To quantify the changes in MC concentrations (mg/L) in natural spiked samples after gastric and intestinal treatments, the peak areas obtained for each toxin were compared to stgastric and stintestinal solutions. **Table 1** shows the experimental concentrations expressed as the mean of triplicate determinations and their standard deviations. **Table 2** shows the percentages of degradation (%) undergone by the MCs after the enzymatic and pH treatments.

The concentrations obtained after each treatment and stgastric and stintestinal were compared by the unpaired, two-tailed Student's *t*-test. The differences were considered to be significant at $p < 0.05$. The test led to significant differences only in the gastric treatment.

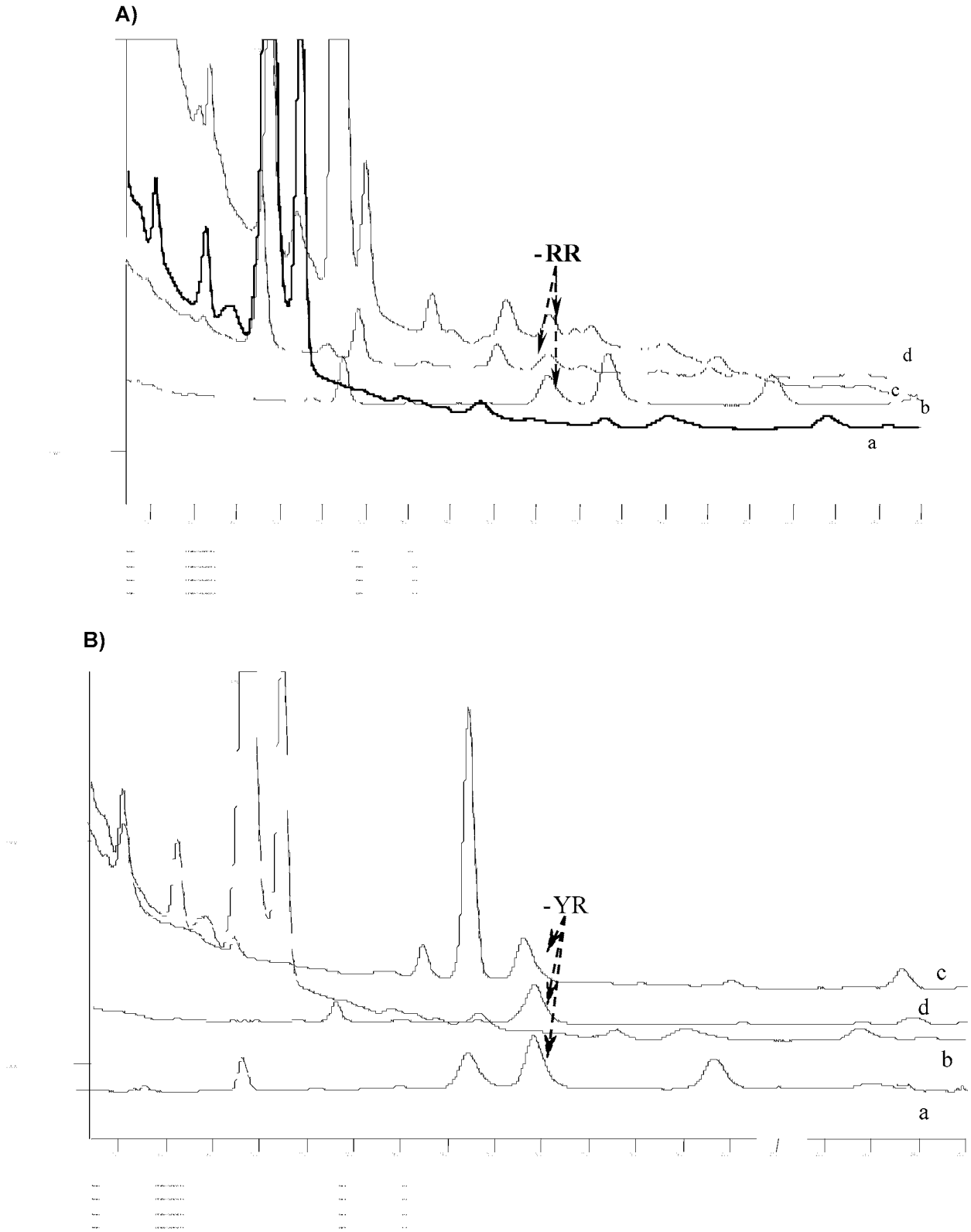

Figure 2. (**A)** Chromatograms (240 nm) of a Stgastric solution (2 mg/L) (a), a blank for extra peaks in the treatment (b), and a water sample spiked with 20 μg/L MC-RR after gastric assay (c), and after acidic assay (d). (**B)** Chromatograms (240 nm) of a Stintestinal solution (2 mg/L) (a), a blank for extra peaks in the treatment (b), and a water sample spiked with 20 μg/L of MC-YR after intestinal assay (c), and after 7.5 pH assay (d).

When natural waters spiked with MCs were submitted to gastric conditions, the MC-RR toxin was affected more than MC-YR and -LR. It decreased by 49−64% (**Figure 2A**), whereas both MC-YR and -LR degraded by 30%. Similarly, the acidic treatment (pH 1.2) changed the stability of the three toxins, which degraded by up to 45−50% (**Figure 2A**; **Table 1**). The alterations in all cases were similar at the two concentrations assayed, although the percentages of degradation were higher at lower concentrations (10 μg/L) (**Table 1**).

The three MCs tested appeared to be stable when they were analyzed after intestinal treatment (**Figure 2B**). The percentage of degradation was around 1−14% and considered not to be

Table 1. Experimental Concentrations of Microcystins (mg/L), Expressed as the Mean of Triplicate Determinations ×b1 Standard Deviations[a]

toxin	pH 1.2	gastric	pH 7.5	intestinal
MC-RR				
1 mg/L	0.58 ± 0.11 (*)	0.36 ± 0.08 (***)	1.01 ± 0.25 (ns)	0.86 ± 0.13 (ns)
2 mg/L	0.97 ± 0.25 (**)	1.03 ± 0.3 (**)	1.97 ± 0.10 (ns)	2.1 ± 0.24 (ns)
MC-YR				
1 mg/L	0.37 ± 0.05 (***)	0.65 ± 0.07 (**)	1.02 ± 0.05 (ns)	1.01 ± 0.16 (ns)
2 mg/L	1.42 ± 0.09 (**)	1.48 ± 0.08 (***)	1.89 ± 0.001 (ns)	1.95 ± 0.02 (ns)
MC-LR				
1 mg/L	0.44 ± 0.16 (**)	0.59 ± 0.05 (**)	1.04 ± 0.07[b] (ns)	1.08 ± 0.07[b] (ns)
2 mg/L	1.11 ± 0.19 (**)	1.41 ± 0.8 (***)	2.02 ± 0.01 (ns)	1.97 ± 0.04 (ns)

[a] *p < 0.05, **p < 0.01, ***p < 0.001 and not significant (n.s.) p > 0.05. [b] In this case, there was an increase in the area of these toxins.

Table 2. Percentages of Degradation (%) of Microcystins in the Standard Solutions Submitted to Enzymatic (Gastric and Intestinal) and pH Treatments

toxin	pH 1.2	gastric	pH 7.5	intestinal
MC-RR				
10 μg/L	42	64	+1[a]	14
20 μg/L	52	49	1.5	+5[a]
MC-YR				
10 μg/L	63	35	+2[a]	+1[a]
20 μg/L	29	26	5.5	2.5
MC-LR				
10 μg/L	56	41	+4[a]	+8[a]
20 μg/L	44	29	+1[a]	1.5

[a] In this case, there was an increase in the area of these toxins.

significant (p > 0.05 in all cases). In general, MCs seem to be very stable under intestinal conditions (pH 7.5).

The results obtained under acidic conditions in gastric fluids are in good agreement with those reported by other authors who concluded that these heptapeptide toxins are very stable in natural waters at neutral pH but hydrolyze slowly at high or low pH, such as acidic conditions (pH 1.5) (*18*). Moreover, although proteins undergo total hydrolysis in extreme conditions (concentrated HCl at 100 °C), they also undergo partial hydrolysis in less harsh conditions, such as concentrated HCl at 37 °C, the temperature used in the USP 23 assay.

The effect of intestinal pH on the stability of these toxins was insignificant because the usual pH values recorded for the surface lake waters where cyanobacterial scums usually grow are neutral or alkaline pH (6.3−8.3) (*31*, *32*).

For the three toxins studied, the analysis of variance proves that the significant decrease in their concentration was a consequence of gastric treatment (p < 0.05) (**Table 1**). We can only conclude that MC-RR,-YR, and -LR were affected during the gastric digestion process, in which acids and enzymes act simultaneously. In fact, digestion is a complex process, which commences in the stomach where the acid environment (pH 1−2) denatures the proteins and renders them more susceptible to enzymatic action (*33*).

It must be pointed out that the activity of the enzymes used in the USP in vitro dissolution test is relatively unspecific in comparison with trypsin and chymotrypsin. Pepsin cuts in the middle of a polypeptide chain, severing the peptide bond just beyond the carbonyl group of a residue with an aromatic side chain (*34*). It also hydrolyzes peptide bonds in which the amine group comes from residues such as glycine, glutamic acid, or alanine. Both D-glutamic acid and alanine are present in the general structure of the MCs studied. This wide specificity of pepsin could explain why all the toxins studied were affected.

Although some authors indicate that there is no evidence that MCs are hydrolyzed by peptidases in the stomach and that a significant amount of microcystin-LR passes the intestinal barrier and is absorbed (*6*), some experiments have demonstrated that microcystin-LR is degraded by several gastrointestinal proteases (*19*). Therefore, the degradation in the gastric fluid simulated by MC-RR, -YR, and -LR was consistent with the results obtained by Takenaka (*19*), who treated MC-LR with various proteases (although the assay was different to our experimental conditions) and assumed that MCs are degraded by the gastrointestinal proteases. They reported that this toxin was degraded only by trypsin, an intestinal protease, to form the compound 3-amino-2,6,8-trimethyl-10-phenyldeca-4*E*,6*E*-dienoic acid (DmADDA). The other proteases, such as pepsin and chymotrypsin, did not form this metabolite. The literature provided no evidence of possible changes in MC-RR and -YR caused by intestinal enzymes or similar conditions.

Some toxicants are much less toxic when administered orally and not intravenously because they are broken down by digestive enzymes of the GI tract (*20*). It would be of considerable interest to study the stability and fate of MCs under gastrointestinal conditions because some authors assume that the difference in toxicity of MCs by oral and intraperitoneal routes in some animal species is because they are degraded by various enzymes in the gastrointestinal tract, such as trypsin (*19*). In this respect, our study reveals that in gastric conditions there are enzymatic alterations in all the toxins assayed. MC-RR was the most affected toxin (49−64%), and the percentage of degradation undergone by MC-YR and -LR was around 30%. However, none were degraded by intestinal digestion. These results may partly explain the differences in toxicity observed when the toxins were administered by oral and intraperitoneal routes. The chemical structure of MCs may influence the stability of these toxins in this in vitro dissolution test.

In conclusion, when natural waters containing the most common MCs are treated with the in vitro dissolution test, MC-RR proved to be the most sensitive, and it underwent alterations under gastric and acidic conditions. MC-YR and -LR were also degraded by 30−35%. On the other hand, the MCs studied were resistant to intestinal conditions. These results should be taken into account in toxicokinetic studies so that the real doses of these toxins that act on target organs can be determined. Also, the behavior of MCs in the GI tract should be considered a toxicological risk when humans and animals ingest contaminated waters and food. Consequently, it is important to know the fate of MCs in gastrointestinal conditions as well as the identity of the hydrolysis products from the gastrointestinal decomposition of microcystins and their toxicological significance.

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