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

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ABSTRACT

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

1. Introduction

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

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

Major routes of human exposure are through ingestion of CYN contaminated drinking water, inhalation while showering, dietary intake via consumption of cyanotoxins in contaminated foods as fish, mollusks, vegetables and algal dietary supplements (Jasim and Saththasivam, 2017; Testai et al., 2016). The accumulation capacity of CYN in different food items has been demonstrated and reviewed several times, mainly in bivalves, crustaceans, gastropods and fish (Gutiérrez-Praena et al., 2013), although the data reported at the moment are still limited according to the European Food Safety Authority

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(EFSA) (Testai et al., 2016). Moreover, few studies are focused on the bioaccumulation and its effects in agricultural crops (Silva and Vasconcelos, 2010; Prieto et al., 2011; Kittler et al., 2012; Corbel et al., 2014). Recently, Cordeiro-Araujo et al. (2017) described the bioaccumulation of CYN in lettuce (*Lactuca sativa L.*) and arugula (*Eruca sativa Mill.*), where its content decreased at the highest concentration assayed. The results indicated that irrigation of both vegetables, lettuce and arugula, with CYN-contaminated water at low concentrations, may constitute a potential human exposure route. This bioaccumulation may be possible due to the absorption of toxins by vegetables, if surface water contaminated with cyanotoxins is used for agricultural purposes, thus representing risks in terms of food safety for human consumers (Prieto et al., 2018).

Lots of vegetables, such as lettuce, spinach, cabbage, and sprouts are minimally processed and they are usually consumed fresh. Moreover, their consumption has been intensified over the last decade due to their high nutritional value, changes in social eating habits, and easy accessibility (Ngnitcho et al., 2017). Although some recommendations and limit values have been already mentioned in order to prevent or manage the possible human health effects induced by CYN exposure under different scenarios, no suggestions have been proposed in the case of vegetables, even when the risks have been demonstrated (Cordeiro-Araujo et al., 2017).

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

To the extent of our knowledge, only two recent works have evaluated the effects on CYN bioaccessibility after the application of an *in vitro* digestion model in food matrices, specifically in mussels and fish (Freitas et al., 2016; Maisanaba et al., 2017). However, there are no available studies focused on CYN degradation in vegetables after being submitted to the different digestion phases.

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

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

2. Materials and methods

2.1. Chemical and reagents

Cylindrospermopsin standard (MW = 415.43 g/mol; 95% purity) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in Milli-Q water (1000 μg mL^{-1}) and diluted as required for their use as working solutions (0.005–5 μg mL^{-1}). All chemicals and reagents used in this study were analytical grade materials. For the simulated digestion: formic acid (HCOOH), potassium chloride (KCl), potassium thiocyanate (KSCN), monosodium phosphate (NaH_2PO_4), sodium sulfate ($NaSO_4$), sodium chloride (NaCl), sodium bicarbonate ($NaHCO_3$), urea, α-amylase, hydrochloric acid (HCl), pepsin, pancreatin and bile salts were obtained from Sigma–Aldrich (St. Louis, MO, USA). MRS broth for the bacterial strains growth was supplied by Oxoid (Madrid, Spain).

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

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

The selected bacterial strains and their growth conditions were described by Maisanaba et al. (2017). The bacterial strains used could be found in the large intestine, being a suitable representation of the real conditions in humans. LAB include a large number of bacterial genera, being *Lactobacillus* and *Bifidobacterium*, two of the best known and most predominant in the large intestine. Eight commercial probiotic strains were used, namely, *Lactobacillus casei* CECT 4180, *Lb. casei rhamnosus* CECT 278T, *Lb. plantarum* CECT 220, Lb. *delbur sub bulgaricus* CECT 4005, *Lb. Salivarus* CECT 4305, *Lb. johnsoni* CECT 289 and *Bifidobacterium breve* CECT 4839T and *B. bifidum* CECT 870T. All of them were obtained from the Spanish Type Culture Collection (CECT, Valencia, Spain), in sterile 18% glycerol. For longer survival and higher quantitative retrieval of the cultures, they were stored at −80 °C. When needed, recovery of strains was undertaken by two consecutive subcultures in appropriate growth media prior to use (Laparra and Sanz, 2009; Meca et al., 2012).

2.3. Experimental setup

Commercial fresh lettuce and spinach were bought in a supermarket and transferred to the laboratory. In this work, uncooked lettuce, uncooked spinach and boiled spinach were tested as different matrices. Then, 6 leaves per type of edible vegetable (uncooked lettuce, or uncooked spinach or boiled spinach) (n = 6, average weight per leaf: 5.03 ± 0.08 g fresh weight (f.w.)) were homogenously spiked with 250 μL of a stock solution containing 20 μg CYN mL^{-1} (equivalent to 1 μg CYN g^{-1} f. w.). For spiking the samples, the volume of the CYN stock solution (250 μL) were pipetted over the whole surface of the leaves, as spread as possible, inside of a tube in horizontal position. Then, leaves were left at room temperature until the total absorption and complete absorption of the previously spiked CYN volume. The