# Changes on cylindrospermopsin concentration and characterization of decomposition products in fish muscle (*Oreochromis niloticus*) by boiling and steaming

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## Abstract

The occurrence of cylindrospermopsin (CYN) in toxic blooms of cyanobacteria is increasing in both frequency and distribution over the world. CYN persistence in water potentiates its accumulation in a wide range of aquatic animals destined to human consumption representing a serious problem. The aim of this work was to study for the first time the influence of cooking (boiling and steaming) for 1 or 2 min, on the stability of CYN in muscle of fish (*Oreochromis niloticus*) contaminated under laboratory conditions, also analyzing the waters in which samples were cooked. CYN was extracted and quantification was carried out by a developed and validated UPLC-MS/MS method. The results show that concentrations of CYN in fish are dependent on the cooking method, being the steaming for 2 min the most effective in reducing CYN levels (26%), followed by boiling for 2 min (18%), and significant differences have been found between the two periods assayed (1 min vs. 2 min). CYN was also detected in waters in which fish muscles were cooked in the range 0.10-0.28 µg/L. Moreover, characteristic decomposition products depending on the type of cooking were detected for the first time among the results of these treatments. The present findings emphasize the need for further studies to evaluate the influence of cooking in the presence of CYN in fish for a more realistic risk evaluation for the human health.

**Keywords:** Cylindrospermopsin, Fish, Steaming, Boiling, decomposition products.

## 1. Introduction

Cyanotoxins, produced by cyanobacteria, are increasingly perceived as a global water-quality issue growing in scope and persistence (Loftin et al., 2016). Potential human illness and mortality may occur following direct consumption or indirect exposure to contaminated organisms or toxins in the environment (Jasim and Saththasivam, 2016). In this group, an important emergent toxin is Cylindrospermopsin (CYN), produced by several cyanobacteria such as *Cylindrospermopsis raciborskii* (Ohtani, Moore, & Runnegar, 1992), *Umezakia natans* (Harada et al., 1994), *Anabaena bergii*, *Aphanizomenon ovalisporum*, *Aphanizomenon flos-aquae* and *Raphidiopsis curvata* (Falconer, 2006). Structurally, CYN ($C_{15}H_{21}N_5O_7S$, M=415.43) is an alkaloid consisting of a tricyclic guanidine moiety combined with hydroxymethyluracil (Ohtani et al., 1992). It has hepatotoxic, general cytotoxic and neurotoxic effects, affecting plants, several aquatic organisms and mammals with different degrees of damage (Prieto, Campos, Cameán & Vasconcelos, 2011; Puerto et al., 2011; Gutiérrez-Praena et al., 2014; Guzmán-Guillén et al., 2015a; He et al., 2016). In the normal pH range of natural waters, it is a zwitterion, making it highly water-soluble, as much as 90% of total CYN is found outside the cells (He et al., 2016). The relatively high stability of CYN to light and over a wide range of pH and temperature (21-100°C), although decomposition of CYN occurs after incubation at high temperatures combined with alkaline pH, might have significant consequences for aquatic environments, (Chiswell et al., 1999; Adamski et al, 2016a).

The high levels and persistence of CYN in waters can potentiate its accumulation in a wide range of aquatic animals (Gutiérrez-Praena, Jos, Pichardo, Moreno & Cameán, 2013; Freitas et al., 2016). CYN has been detected in lake samples showing extracellular superficial values ranging from 2.6 to 126 µg/L, exceeding the recommended safety value of 1 µg/L in drinking water, and in tissues from *Salmo trutta* trouts (up to 2.7 ng/g) (Messineo, Melchiorre, Di Corcia, Gallo & Bruno, 2010). CYN was also found to contaminate crayfish (*Cherax quadricarinatus*, up to 4.3 µg/g) and fish (*Melanotaenia eachamensis*, up to 1.2 µg/g) from a small Australian aquaculture pond (Saker & Eaglesham, 1999), as well as freshwater mussels (*Anodonta cygnea*, up to 2.52 µg/g) in a 16-day exposure study (Saker, Metcalf, Codd, & Vasconcelos, 2004).

Berry, Jaja-Chimedza, Dávalos-Lind, & Lind (2012) showed accumulation of CYN (0.09-1.26 µg/kg) in several species of finfish (*Oreochromis aureus*, *Dorosoma mexicana*, *Bramocharax cabelleroi*, *Heterandria jonesii*, *Vieja* sp., *Cichlasoma* sp. and *Rhamidia* sp.) caught and consumed locally in the Catemaco Lake. Therefore, this fact constitutes a serious concern, especially if the organisms can be destined to human consumption (Messineo et al., 2010).

Based on the potential risks for human health, a provisional tolerable daily intake (TDI) of 0.03 µg CYN/kg of body weight has been proposed by Humpage & Falconer (2003). Most evaluations of the risk associated with ingestion of seafood products are performed on the product uncooked, as sold or captured (Domingo, 2011; Chiocchetti, Piedra, Vélez, & Devesa, 2016). However, fish is normally processed and/or cooked before consumption, and these practices can alter the concentration of CYN available in food (Freitas et al., 2016). In addition, one important concern for public health is that cyanotoxins-contaminated fish, either raw or cooked, look normal and undetectable by taste, appearance and smell (Wong, Hung, Lee, Mokb, & Kam, 2009). In this sense, studies of the influence of cooking on food are required to achieve a more accurate knowledge of the actual intake and reducing the risk for the consumer in the last step of the food chain.

With respect to the effects of cooking on the increase or decrease of contaminants in food (especially fish and meat), the studies are mainly focused on a number of chemical pollutants, such as metals (Laparra, Vélez, Montoro, Barberá, & Farré, 2003), polychlorinated biphenyls, polycyclic aromatic hydrocarbons -PAHs-, hexachlororobenzene (HCB) (Perelló, Martí-Cid, Llobet, Castell, & Domingo, 2009), dioxins (Hori et al., 2005), as has been reviewed by Domingo (2011) and Chiocchetti et al. (2016). It seems that these changes depend mainly on the cooking conditions including time, temperature, and medium of cooking, with a clear general tendency to increase metal concentrations after cooking (Domingo, 2011). Concerning cyanotoxins, studies are scarce, and they have shown different patterns of variation for microcystins (MCs), nodularin (NOD), paralytic shellfish toxins (PSP-toxins) and CYN in clams, mussels, scallops, prawns and fish (Van Buynder et al., 2001; Morais, Augusto, Carvalho, Vale, & Vasconcelos, 2008; Bruno et al., 2009; Wong et al., 2009; Zhang, Xie, & Chen, 2010; Guzmán-Guillén, Prieto, Moreno, Soria, & Cameán, 2011; Freitas, Azevedo, Carvalho, Campos, & Vasconcelos, 2014; Freitas et al., 2016).

112 It has been previously demonstrated that boiling fish muscle was able to reduce
113 levels of unconjugated MCs (MC-LR, -RR and -YR) in cooked tilapia fish in a range
114 between 34% and 59% (Guzmán-Guillén et al., 2011). On the contrary, MCs mean
115 concentrations were significantly higher in muscle of bighead carp after boiling, and in
116 clams boiled for 5 and 15 min, compared to unboiled controls (Zhang et al., 2010;
117 Freitas et al., 2014). Concerning CYN, only Freitas et al. (2016) carried out a recent
118 study on changes on CYN concentration in edible mussels after boiling or steaming,
119 showing no significant alterations in toxin concentration due to the cooking processes,
120 although the toxin was found in the cooking water. Therefore, the exact incidence of
121 different cooking and eating practices on cyanotoxin levels and exposure remain
122 uncertain (Testai et al., 2016).

123 Previous studies concerning cooking processes of aquatic organisms have
124 determined MCs by ELISA (Morais et al., 2008; Bruno et al., 2009), High Performance
125 Liquid Chromatography-Diode-Array (HPLC-DAD) (Metcalf & Codd, 2000), or Liquid
126 Chromatography-Mass Spectrometry (LC-MS) (Zhang et al., 2010; Guzmán-Guillén et
127 al., 2011; Freitas et al., 2014), as LC-MS has been proved suitable for determination of
128 MCs in natural blooms, cyanobacterial strain cultures and biological samples (Cameán,
129 Moreno, Ruíz, & Picó, 2004; Ruíz, Cameán, Moreno, & Picó, 2005). PSP-toxins have
130 been analyzed by HPLC-fluorescence (Wong et al., 2009), and NOD and CYN by LC-
131 tandem Mass Spectrometry (LC-MS/MS) (Van Buynder et al., 2001; Freitas et al.,
132 2016). However, these have not been conducted by Ultra-performance liquid
133 chromatography tandem mass spectrometry (UPLC-MS/MS), which has become the
134 technique of choice for analysis of different cyanotoxins (several variants of MCs,
135 NOD, CYN, anatoxin-a, homo-anatoxin and domoid acid) in water and complex
136 matrices, and offers excellent specificity and sensitivity for their detection and
137 quantification (Oehrle, Southwell, & Westrick, 2010; Adamski et al., 2016a,b; Greer et
138 al.., 2016; Pekar et al., 2016).

139 Besides, in most of the above-mentioned studies, the structure and properties of
140 the toxin decomposition products have not been described. Only Adamski et al. (2016a,
141 b) have investigated the stability of CYN from a *C. raciborskii* culture under the
142 influence of pH, temperature and irradiation, characterizing its forming products by
143 UPLC-MS/MS. Considering the relevant toxic properties of CYN, it would be of
144 interest to investigate the mechanism of its decomposition in intoxicated fish after

cooking. For this purpose, orbitrap can provide a much higher mass resolution and mass precision compared to triple quadrupole mass analyzers (MS/MS) (Gerssen, Mulder, & Boer, 2011).

In view of these reports, the aim of the present study was to investigate for the first time the influence of common practices for cooking of fish meat and obtention of fish soups around the world, such as boiling and steaming, on CYN concentration in muscle of fish (*Oreochromis niloticus*) contaminated under laboratory conditions, assaying two different periods of time (1 and 2 min). Moreover, a precise characterization of CYN by-products in fish samples under the influence of cooking was conducted.

## 2. Materials and Methods

### *2.1 Chemicals and reagents*

Cylindrospermopsin standard (purity 95%) was supplied by Alexis Corporation (Lausen, Switzerland). Standard solutions of CYN were prepared in Milli-Q water (100 µg/mL) and diluted as required for their use as working solutions (1-100 µg/L). All chemicals and reagents used in this study were analytical grade materials. HPLC-grade methanol, dichloromethane, acetonitrile, and trifluoroaceticacid (TFA) were purchased from Merck (Darmstadt, Germany). Deionized water (418 MΩ/cm resistivity) was obtained from a Milli-Q water purification system (Millipore, Bedford, 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. For UHPLC-MS/MS analyses reagents were of LC-MS grade. Water and acetonitrile were purchased from VWR International (Fontenay-sous-Bois, France) and formic acid was purchased from Fluka (Stainheim, Germany).

### *2.2 Experimental setup*

Tilapia fish (*Oreochromis niloticus*) were supplied by Valenciana de Acuicultura (fish hatchery of Valencia, Spain) and were transferred to the laboratory where they were acclimatized and were kept during 15 days in two aquariums (8 individuals/aquarium) with 96 L of tap-water, and the temperature was kept constant (21 ± 2°C). Fish were fed daily (0.3 g/day) only with commercial fish food (Dibaq S.L.,

Segovia, Spain). After acclimation, they were sacrificed, dissected and each muscle sample was cut into approximately 4 g portions. Five fish muscle samples per cooking method (n=5) were spiked with 500 µL of a stock solution containing 100 µg CYN/L (equivalent to 50 ng CYN/g dry weight -d.w.-), directly injecting the toxin into the muscle. This concentration was considered relevant from a naturally environmental point of view and considering the accumulation of CYN found in aquatic organisms in other studies (Seifert, McGregor, Eaglesham, Wickramasinghe, & Shaw, 2007; Kinnear, 2010; Freitas et al., 2016).

*2.3 Cooking of fish samples*

Following spiking of samples with the toxin, they were cooked by boiling and steaming, for 1 and 2 min. The experimental control group was CYN-fortified fish muscle which was not processed, being directly extracted for toxin quantification. The assays were always carried out by quintuplicate (n=5).

To test the boiling method, the fish muscle sample was introduced into pots of stainless steel with cool tap water (50 mL). In the case of steaming, the sample was put into a metal food steamer where the food is kept separate from the boiling water but has direct contact with the steam. Both for boiling and steaming, the fish muscle was introduced into the pot or onto the food steamer, respectively, with cool water, heated to boiling (100°C) and then continued to boil for 1 and 2 min. The remaining water in which fish muscle was cooked was also analyzed. Water evaporation was prevented in this study, by the use of a top cover, in order to simulate the usual household cooking procedure. No salt, spices or any other additional ingredients were added to the samples. Values are expressed as ng CYN/g dw or µg CYN/L, representing the unconjugated fraction of CYN.

Muscle fish weights were recorded before (approximately 4 g) and after cooking. The final mean weights of fish muscle after boiling were 3.78 ± 0.21 g (1 min) and 3.68 ± 0.14 g (2 min), and after steaming, means were 3.85 ± 0.12 g (1 min) and 3.91 ± 0.19 g (2 min). All samples were frozen at -80ºC and lyophilized (Cryodos 80 model, Telstar, Tarrasa, Spain) before CYN was extracted. Once the lyophilization process was finished, the samples were weighed, and the final mean lyophilized weight was 0.86 ± 0.08 g dw. Thus, the mean loss of weight due to the cooking and lyophilization processes was approximately 78.5 %.

208 *2.4 Extraction of CYN from tissues and waters and clean up*

209 After cooking and lyophilization of the samples, CYN was extracted from fish
210 muscles and from water samples used for boiling and steaming. Extraction and clean up
211 procedures were performed according to the validated methods of Guzmán-Guillén et
212 al. (2015b) in muscle samples, and Guzmán-Guillén, Prieto, González, Soria-Díaz, &
213 Caméan (2012) in water samples, with some modifications, applying a UPLC-MS/MS
214 method in the present study, instead of the former LC-MS/MS system, reducing costs
215 and time of analysis (5 min vs. 20 min run, with a retention time of 1.30 min vs. 9.55
216 min) (Guzmán-Guillén et al., 2015b). Regression equations were obtained for this
217 method by linear regression of two 7-point calibration curves calculated from CYN
218 standards prepared in blank extracts from lyophilized muscle (2; 10; 15; 20; 40; 60 and
219 100 ng/g dw) and from blank boiling water (processed water) (1; 5; 7.5; 10; 20; 30 and
220 50 µg/L). Limits of detection (LOD) and quantitation (LOQ) were estimated according
221 to the equation $Y_{LOD\ or\ LOQ} = Y_{blank} + nS_{blank}$, where $Y_{blank}$ and $S_{blank}$ are the average value
222 of 10 blank signals and its corresponding standard deviation, and *n* is a constant (3 for
223 LOD and 10 for LOQ). These values are then converted into concentration by using the
224 calibration functions obtained before. Recoveries and precision (within-day and
225 between-day) were also assessed by calculating the relative standard deviation (RSD).
226 This was carried out by spiking before extraction, in triplicate (n=3) within the same
227 day, and on three different days, blank extracts from lyophilized muscle or from
228 processed waters at three levels of CYN concentration (5, 20 and 50 µg/L, equivalent to
229 10, 40 and 100 ng CYN/g dry muscle).

230 *2.5 Analytical determination*

231 Chromatographic separation was performed using a UPLC Acquity (Waters)
232 coupled to Xevo TQS-micro (Waters) consisting of a triple quadrupole mass
233 spectrometer equipped with an electrospray ion source operated in positive mode.
234 UPLC analyses were performed on a 50 x 2.1 mm Acquity BEH C18 1.7 µm column.
235 The flow rate was 0.45 mL $min^{-1}$. Chromatographic separation was performed using a
236 binary gradient consisting of (A) water, and (B) methanol. Both eluents contained 0.1%
237 formic acid (v/v). Injection volume was 5 µL. The elution profile was: 0 % B (0.8 min),
238 linear gradient to 90% B (2.2 min), 90 %B (1 min) and finally 100 % B (1 min).
239 Multiple Reaction Monitoring (MRM) experiment was applied where the parent ions

and fragments ions were monitored at Q1 and Q3, respectively. The transitions for the analyte CYN are: 416.2/194.0, and 416.2/176.0. The transition 416.2/194.0 was chosen for quantitation of CYN and the transition 416.2/176.0 as confirmatory. For UPLC-ESI-MS/MS analyses, the mass spectrometer was set to the following optimised tune parameters: Capillary voltage: 3.0 kV, Source Temperature: 500ºC and source Gas flow: 1000 L/h.

*2.6 Characterization of CYN decomposition products after boiling and steaming by UHPLC-MS/MS Orbitrap*

In order to characterize the possible decomposition products of unconjugated CYN by the effects of cooking, the same cooking procedures were reproduced for 2 min, with two fish muscle samples per cooking method by injecting into the muscle 500 µL containing 80 µg CYN/mL (equivalent to 40 µg CYN/g d.w.), as unconjugated CYN contents detected after spiking with 50 ng CYN/g d.w. were too low to be analyzed by Orbitrap. This quantity was the same that Adamski et al. (2016b) used in their experiment to identify CYN by-products in a *C. raciborskii* culture. Afterwards, cooking of samples and extraction of CYN from them were carried out as described in sections *2.3* and *2.4*, respectively.

All analysis were performed using a Thermo Scientific liquid chromatography system consisting of a binary UHPLC Dionex Ultimate 3000 RS, connected to a quadrupole-orbitrap Qexactive hybrid mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), which was equipped with a heated-electrospray ionization probe (HESI-II). Xcalibur software was used for instrument control, data acquisition and data analysis. Trace Finder 3.3 software was used for data analysis also. Chromatographic separations were performed using an Acquity UPLC BEH (bridged ethyl hybrid) C18 column (2.1 × 100 mm, 1.7 µm) (Waters). The column was maintained at 40°C and eluted under the following conditions: 100% of eluent A over 2 min and gradient elution from 100% to 30% of eluent A over 10 min at a flow rate of 0.3 mL $min^{-1}$. Eluent A was water/formic acid (0.1%, v/v), and eluent B was acetonitrile/formic acid (0.1%, v/v). Injection volume was 5µL.

MS detection of the CYN decomposition products observed by Adamski et al. (2016a, b) by the influence of pH, temperature and irradiation was performed in the present study with the Q-Exactive Orbitrap mass spectrometer through the accurate *m/z*

measurement of each molecule, by scanning from 100 to 1000 *m/z*, in positive and full scan (FS) mode at a resolution of 70000 (full width half maximum, FWHM at *m/z* 200). Each compound observed was associated with a chemical formula hypothesis. Then, a relative mass error expressed in ppm was calculated, a result lower than 5 ppm being considered as an acceptable value (Makarov, Denisov, Lange, & Horning, 2006). The Q-Exactive Orbitrap was also used to sustain the identity of CYN decomposition products performing tandem mass spectrometry ($MS^2$) experiments using the parallel reaction monitoring (PRM) method, which was acquired in positive mode and a resolution of 17500, with an isolation window of 1 *m/z*, and normalized collision energy was set at 35 eV. The selected $[M+H]^+$ were 214.15500, 290.08052, 292.09617, 336.16663, 338.18228, 414.10780, 416.12345 and 434.13401. The identity of the compounds was then sustained by the agreement between theoretical and experimental isotopic patterns, and by the consistency with the resulting product ions formed during $MS^2$ analysis. HESI source parameters were as follows: spray voltage 3.5 kV, capillary temperature 320ºC, sheath, auxiliary and sweep gas flow rate ($N_2$) 45, 15 and 2 (arbitrary units) respectively, probe heater temperature 350ºC and S-Lens RF level 50. Considering the areas of the corresponding chromatographic peaks, a relative comparison among different cooking treatments and the control was carried out by selecting the following fragments: *m/z* 194.12879 for the molecular ions $[M+H]^+$ 416.12345 and 336.16663; *m/z* 178.13387 for the molecular ions $[M+H]^+$ 338.18228 and 214.15500; *m/z* 292.09617 for the molecular ion $[M+H]^+$ 434.13401; *m/z* 210.12427 for the molecular ion $[M+H]^+$ 290.08052; *m/z* 212.13935 for the molecular ion $[M+H]^+$ 292.09617, and the fragment ion at *m/z* 192.11314 for the molecular ion $[M+H]^+$ 414.10780.

### *2.7 Statistical Analysis*

Results were subjected to one-way analysis of variance (ANOVA) with the statistical package INSTAT, Graph Pad™, and represent mean ± standard deviation (SD) of 5 samples per group. Differences in mean values between groups were assessed by the Tukey's test and were considered statistically significant at $p < 0.05$ level.

## 3. Results

Concerning UPLC-MS/MS, the LOD and LOQ obtained for the muscle were 1.6 and 3.4 ng/g dw, respectively, compared to the former 2.5 and 7.3 ng/g dw obtained by

LC-MS/MS (Guzmán-Guillén et al., 2015b). In the case of waters, LOD and LOQ were 0.36 and 0.40 µg/L, respectively, compared to the former 0.5 and 0.9 µg/L obtained by LC-MS/MS (Guzmán-Guillén et al., 2012). This contributes to a better sensitivity of the analysis by UPLC-MS/MS compared to LC-MS/MS. The linear ranges were within 2-100 ng CYN/g dw for the muscle (regression equation y = 97.826x – 33.396; $r^2$ = 0.9962), and within 0.4-50 µg/L for waters (regression equation y = 165.43x + 5.0475; $r^2$ = 0.9989). Recoveries were 90, 100 and 100% for muscle, and 96, 94 and 106% for waters, for the CYN spiked levels of 5, 20 and 50 µg/L, respectively, and RSDs were below 20% in all cases. All these values are acceptable in comparison to the recovery and RSD ranges depending on the analyte level (González & Herrador, 2007).

Both cooking treatments applied in the present study produced a significant decrease in unconjugated CYN concentration in fish muscles (Fig. 1). Cooking the fish reduced unconjugated CYN levels by 9 % ($p < 0.05$) and 8 % ($p<0.05$) by boiling and steaming for 1 min, respectively, compared to control fish. Reductions found by cooking for 2 min were of 18% ($p < 0.001$) and 26% ($p < 0.001$) for boiling and steaming, respectively, compared to control fish. Moreover, significant differences have been found between the two periods assayed (1 and 2 min) for boiling ($p < 0.05$) and steaming ($p < 0.001$). Unconjugated CYN was also detected in waters in which fish muscles were cooked, showing values of 0.27 and 0.28 ng/mL in case of boiling for 1 and 2 min, and 0.10 and 0.12 ng/mL in case of steaming for 1 and 2 min, respectively (Fig. 2). CYN was not detected in blank boiling water samples for non-cooked fish. It is important to emphasize that CYN concentration detected in the fish muscles refers to un-bound, free toxin, once the method applied to analyze these samples does not detect CYN bound to proteins.

The mass spectra of control, boiled and steamed samples, and of the cooking waters, revealed the presence of seven similar decomposition products. Their retention times (RT), putative chemical formula, observed *m/z* (experimental, $[M+H]^+$ ions), calculated *m/z* (theoretical, $[M+H]^+$ ions), mass error (ppm) and fragmentation ions with their respective information are presented in Tables 1-3. The decomposition product with *m/z* 290.08052 was detected with RT≈1.51 min (named as C-1). As can be observed, two of the compounds (C-2 and C-3) were detected at two different RT having the same molecular ion $[M+H]^+$, indicating that they may be diastereoisomers (thus named as A and B). The parent ions with *m/z* 292.09617 were detected at ≈2.42

and ≈3.19 min (C-2A and C-2B, respectively.). At ≈2.86 min, slightly before CYN, its epimer 7-epi-CYN was eluted. Moreover, as seen in Fig. 4, a peak was observed at ≈3.25 min, with the putative chemical formula $C_{30}H_{42}N_{10}O_{14}S_2$ and an *m/z* 831.24005 (*m/z* calculated 831.23961; mass error: 0.53 ppm); this is the CYN dimer which was observed in all samples. Besides, the fragment with *m/z* 336.16663 is observed in the mass spectrum of CYN, corresponding to the loss of $SO_3$ from the CYN molecule due to in-source fragmentation (see Fig. 6). Then, two different peaks with the same *m/z* 336.16663 were observed at ≈3.45 min (C-3A) and ≈5.15 min (C-3B), possibly diastereoisomers. Whereas C-3B is observed in all samples, C-3A is only observed in cooked samples, but not in control uncooked samples. Finally, the decomposition product at *m/z* 414.10780 was detected from 4.94 to 5.04 min (C-4). The decomposition products C-1, C-2A, C-2B, 7-epi-CYN, C-3B and C-4 are observed in all samples assayed, non-cooked and cooked fish and waters (Tables 1-3). Figs. 4 and 5 show the MS and $MS^2$ spectra in FS and PRM, respectively, for characteristic decomposition products detected in control uncooked samples and in the water used for steaming. The extracted ion chromatogram for full MS experiments of CYN and its decomposition products in control and steamed fish are shown in Fig. 6.

## 4. Discussion

Generally, estimations of exposure to cyanotoxins as food contaminants have assumed that the available toxin concentrations in raw and cooked food is similar, which is not true (Freitas et al., 2014). Ibelings & Chorus (2007) suggest that in industrially processed seafood, the removal of parts in which cyanotoxins can accumulate prior to processing could be effective in avoiding hazards. But this is impracticable in case of CYN accumulation in muscle of fish, as this is the edible fraction, or in cases where fish are eaten whole. Analysis of the available data by the European Food Safety Authority (EFSA) indicates that additional efforts should be put into elucidating the levels of human exposure to cyanotoxins under different scenarios. In this sense, new research should be conducted to explore the effect of cooking on the concentration of cyanotoxins in seafood (Testai et al., 2016), because these studies are few in the case of MCs (Morais et al., 2008; Bruno et al., 2009; Zhang et al., 2010; Guzmán-Guillén et al., 2011; Freitas et al., 2014) and even scarcer with CYN (Freitas et al., 2016). To our knowledge, this is the first report presenting the influence of some

domestic cooking treatments such as boiling and steaming on unconjugated CYN concentration in fish, characterizing its possible decomposition products.

In the present work, the effectiveness in reducing unconjugated CYN levels in cooked fish followed this descending order, with statistical differences: steaming 2 min > boiling 2 min > boiling 1 min ≈ steaming 1 min, achieving the major reduction in CYN concentration with the longer period assayed (2 min) (Figs. 1 and 3). In this sense, boiling and steaming were able to decrease unconjugated CYN levels in fish muscle up to 18 and 26 % of the initial concentration, respectively. By contrast, in most of the cited studies carried out with the cyanotoxins MCs or CYN (only one study with the latter), boiling or steaming the food containing them (fish or shellfish), results have shown that these cooking treatments did not produce any significant reduction in toxin concentration in the tissues, even causing an increase. Our results could be explained by the high water-solubility of CYN, in direct and continuous contact with the high temperatures of the boiling water and steam, as heat processing seemed to enhance CYN extraction from fish muscles, phenomenon also observed with MCs in fish (Guzmán-Guillén et al., 2011) and with CYN in mussels (Freitas et al., 2016). This fact is reinforced by the results of the analysis of the waters in which fish were cooked, showing a small but sufficient concentration of unconjugated CYN (Fig. 2). Similarly to domestic cooking, in this study evaporation of water was prevented, and therefore lower CYN levels may have been detected in the cooking waters, compared to a situation where water is evaporated and the toxin could be more concentrated. In any case, the risks of consumption could be reduced by discarding the waters in which CYN-contaminated fish is cooked. Generally, the variation in the content of toxic agents during cooking is related to some extent to product weight losses or to a possible solubilisation of the toxicant to the liquid used for cooking (Chiocchetti et al., 2016). In this sense, some authors have observed increased toxicants concentrations in food due to weight changes after the heating process (Hori et al., 2005). In the present work the original sample weights have been taken into account, so decreases found in CYN concentrations can be associated with the cooking process itself. Moreover, it has been suggested that a decrease in CYN detection in samples could be due to the binding to enzymes (Freitas et al., 2016). Recently, varying percentages of unbound CYN were detected with incubation with different animal and plant proteins (Esterhuizen-Londt and Pflugmacher, 2016). In the present work, the decrease found in CYN levels could

be attributed to the cooking process itself, and this could not be explained as an increase of the conjugated fraction of the toxin, as no binding is expected in samples.

CYN is stable at temperatures ranging from 4-50 ºC for up to 5 weeks in the dark; after 4 weeks at 50 ºC, 83% of initial (1 mg/L) toxin concentration remained (Chiswell et al., 1999). Moreover, Adamski et al. (2016a) demonstrated that boiling (100°C) does not result in decomposition of CYN in acidic and neutral conditions (pH 3, 5 and 7), whereas under alkaline conditions (pH 10 and 12) it reduces CYN concentration within 4 h to 47% and 18% of the initial value, respectively. In our experiment, the water boiling temperatures were higher than 100°C, which could explain the decrease in CYN concentration for the cooking processes. Besides, significantly higher pH values for steamed samples (7.9-8.9) were recorded than for the boiled ones (6.7-6.8). According to the results from Adamski et al. (2016a), this may be also the reason why higher decreases in CYN concentration were found in steamed samples, compared to the boiled ones.

Although a reduction in unconjugated CYN concentration up to 26% in cooked samples was observed in the present study, Freitas et al. (2016) showed in cooked mussels that boiling and steaming did not produce significant differences in CYN concentration. Differences could be attributed to the matrix (fish vs. mussels), as previously suggested by Domingo (2011). Nevertheless, in agreement with our results, they found CYN in the cooking water, with higher values for boiled than for steamed samples, suggesting that heat processing can be used to reduce the availability of this toxin in the flesh if water is discarded.

In the present study, a total of seven decomposition products were detected in control and cooked fish samples, six of them in all samples and one only in cooked samples, apart from the dimer observed in all samples. The dimer could be characteristic of the matrix studied (fish muscle), as Adamski et al. (2016 a, b) did not mentioned this compound. The decomposition products with *m/z* 292.09617, 416.12345 and 336.16663 agree with that previously observed by Adamski et al. (2016a) under high temperature. However, as a novelty, diastereoisomers for two of those three decomposition products were detected, thus naming them as C-2A, C-2B, 7-epi-CYN, C-3A and C-3B. Moreover, two more compounds were observed, agreeing with two decomposition products observed by Adamski et al., (2016b) under irradiation

conditions (*m/z* 290.08052 and 414.10780, C-1 and C-4, respectively). Therefore, our results suggest that boiling and steaming could also yield some fragments that Adamski et al. (2016b) observed after irradiation of CYN.

Based on the fragmentation spectra by PRM obtained for all samples in the present study, every characteristic fragment from the coincident parent ions with Adamski et al. (2016a, b) have been confirmed in the present work, enabling a correct identification of the parent ions. The fragmentation pathways for these compounds have been proposed by Adamski et al. (2016a, b). The decomposition product C-1 with an *m/z* of 290.08052 (RT≈1.51 min) could derive from the cleavage of the uracil ring, with insertion of an oxygen atom. Its fragmentation spectra showed two fragments in all samples: one with *m/z* of 210.12370, another with *m/z* of 192.11314, by the loss of $SO_3$ and $H_2O$. For this parent ion, the relation among areas in the control (uncooked) sample and in the cooked ones is similar, so this would not be the main fragmentation pathway under the influence of the cooking methods assayed. The parent ions C-2A and C-2B, both with *m/z* 292.09617 and different RT, are a reduced form of C-1, and follow a similar fragmentation pathway (Adamski et al., 2016b), giving the fragments at *m/z* 212.13935, 194.12879 and 176.11822 in all samples, although there are cases where, after cooking, the last fragment is not observed, maybe because of its low intensity in relation to the fragment at *m/z* 194.12879. In any case, among all the decomposition products observed in steamed samples, C-2 has been the less detected, indicating that the decrease found in CYN concentration in this study by steaming does not seem to be related to the formation of this ion. 7-epi-CYN, an epimer of CYN, was eluted with a RT≈2.86 min, slightly before CYN (RT≈3.25 min), and its five fragmentation ions were observed in all samples. This product has an S configuration for its hydroxyl group at C-7, in contrast to an R configuration in CYN (Adamski et al., 2016b). Two different peaks, possibly diastereoisomers, with the same *m/z* (336.16663) were observed at ≈3.45 min (C-3A) and ≈5.15 min (C-3B). In this case, the sulphate group of the CYN molecule is substituted by a hydroxyl group; the subsequent loss of the uracil group and $H_2O$ yield the characteristic fragments at *m/z* 194.12879 and 176.11822 (Adamski et al., 2016a). Since C-3A is not present in uncooked control samples, it can be assumed that it is characteristic of the cooking procedure itself (Table 1). By comparing the relative areas of this fragment in steamed and boiled muscle, a steamed/boiled ratio of 1.3 is obtained, agreeing with the higher degradation of CYN shown by the steaming process

compared to boiling. However, C-3B is observed in all samples, but it is not increased in the cooked samples compared to the uncooked control samples. Again, in some cases, the fragment with *m/z* 176.11822 might not have been detected because of its low intensity in relation to the fragment at *m/z* 194.12879. Finally, the decomposition product at *m/z* 414.10780 (C-4, RT from 4.94 to 5.04 min) may come from the hydroxylation with dehydration of the tricyclic guanidine moiety (Adamski et al., 2016b). Loss of the uracil ring from this parent ion results in the ion at *m/z* 272.06995, whereas loss of the sulphate group and $H_2O$ result in the ions at *m/z* 334.15098 and 192.11314, all of them detected in the present study. Of all the decomposition products observed by steaming, C-4 is the most detected, based on its relative abundance, suggesting that this might be one of the decomposition pathways when CYN degradation occurs (Fig. 5).

Concerning other cyanotoxins, cooking (e.g. boiling) may alter MCs concentration in aquatic edible organisms, although very contrasting results are available (Testai et al., 2016), showing various degrees of decrease (Bruno et al., 2009; Guzmán-Guillén et al., 2011), no effects (Morais et al., 2008), or even an increase (Zhang et al., 2010; Freitas et al., 2014). Bruno et al. (2009) showed that baking had no effect on MCs concentration, but stir-frying and braising resulted in 36 and 82% decrease, respectively, measured by ELISA. Moreover, 25-50% reductions in unconjugated MCs levels in cooked tilapia (by boiling and microwaving) were detected by LC-MS, being more effective boiling than microwaving (Guzmán-Guillén et al., 2011). Morais et al. (2008) reported no effect on MCs availability in mussels after boiling for 5 or 30 min. On the other hand, other authors showed an increase after boiling in MCs concentration up to 300% in fish (Zhang et al., 2010) and by 57-213% in clams (Freitas et al., 2014). Freitas et al. (2014) suggested that boiling was the most effective method for a better MC-LR extraction from clams. However, it is worth noting that they analyzed boiled clams together with the boiling water, so MC-LR levels reflect the combination of the contents in both matrices. It cannot be assumed a certain effect of the boiling treatment in their study and, therefore, their results may be not comparable to ours in this point. Besides, the chemical differences between MCs and CYN may be taken into account, as CYN is highly hydrophilic, thus a higher transfer of this toxin to water may be expected in our study.

Van Buynder et al. (2001) suggested that NOD is not destroyed by boiling, but simply redistributes between prawn viscera, flesh and water used for cooking. Similar to our results, steaming induced significant losses (11-24%) of PSP-toxins from several organs of scallops contributing to obtention of PSP-toxins-contaminated soups (Wong et al., 2009). Some uncertainties are related to the saxitoxins stability during cooking, since the toxins are only partially destroyed by high temperatures of cooking and are, at least in part, poured into the cooking fluids, which may still be ingested, for example when eating soups (Testai et al., 2016). A similar process may be occurring with CYN, with a possible solubilisation of the toxin into the liquid used for cooking or exuded by the cooked fish muscle.

Although data for CYN safety limits in tissues are scarce, a maximum allowable intake of 18 µg CYN/day can be calculated from the TDI of 0.03 µg/kg/d proposed by Humpage & Falconer (2003) for a 60 kg adult. Therefore, a derived health alert level in fish flesh of approximately 158 µg/kg wet weight is obtained based upon consumption over a 14-d period of 1600 g of fish (Ibelings & Chorus, 2007). Considering these values, none of the concentrations of CYN found in fish muscle after cooking in the present study would exceed the maximum allowable values indicated, thus suggesting there would be no potential human risk.

In general, in view of these results, the potential human exposure to CYN due to consumption of contaminated fish based on the amount of toxin in raw fish could be over-estimated. However, transference of CYN from the tissue to the cooking waters might be taken into account, and this water should be removed to avoid its consumption. Further studies are necessary in this regard in order to not under- or overestimate the potential threat of aquatic food contaminated with cyanotoxins to human health. These studies could confirm first if the reported reductions in the levels of CYN are correlated basically with the specific cooking process and, second, with the different cooking conditions (such as time, temperature, and medium of cooking). Furthermore, some authors reported that the influence of cooking on the levels of diverse contaminants depends not only on the particular cooking process, but even more on the specific food item, probably due to the variable lipid content of the foodstuffs or to the efficient lipid removal (Domingo, 2011). Therefore, new studies carried out with different fish species would help to elucidate if the variations found in CYN concentration in the present work are species-specific too.

## 5. Conclusions

In conclusion, this study sheds new light on the issue of the influence of some cooking practices (boiling and steaming) on the levels of unconjugated CYN in edible organisms, being the first one conducted in fish. The results show that concentrations of CYN in fish are dependent on the cooking method, being the steaming for 2 min the most suitable, followed by boiling for 2 min, for a significant reduction of CYN levels in the fish muscles. Moreover, the decrease in CYN levels in cooked samples leads to changes in several and characteristic decomposition products depending on the type of cooking, observed for the first time under their influence. Thus, C-3A (*m/z* 336.16663) was exclusive in cooked samples, and C-4 (*m/z* 414.10780) is the most relevant compound found in steamed fish muscles. Therefore, for estimating real intakes of CYN, it is necessary to take into consideration levels in both the cooked and uncooked food. These findings emphasize the need for further studies to evaluate the influence of cooking in the presence of CYN in several fish species for a more realistic risk evaluation for the human health, and to investigate the possible decomposition products obtained when CYN degradation occurs under cooking conditions.

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**Figure Legends**

**Fig. 1.** Concentration of CYN (ng CYN/g dry weight-d.w.-) in fish muscles (*Oreochromis niloticus*) spiked with 500 µL of a CYN standard solution containing 100 µg CYN/L (equivalent to 50 ng CYN/g dw) and submitted to either no treatment or to different cooking treatments for 1 and 2 min (boiling and steaming). Values are expressed as the mean ± SD ($n$ = 5). The significant levels observed are $^{*}p < 0.05$ or $^{***}p < 0.001$ in comparison to control group (spiked and non-cooked fish), $^{\#}p < 0.05$ or $^{\#\#\#}p < 0.001$ when comparing 1 min vs 2 min within the same cooking method, $^{\alpha}p < 0.05$ when comparing steaming vs boiling within the same period, $^{\&\&\&}p < 0.001$ in comparison to boiling for 1 min, $^{\pm}p < 0.05$ in comparison to steaming for 1 min.

**Fig. 2.** Concentration of CYN (ng CYN/mL) detected in collected water samples from the boiling and steaming treatments assayed. Values are expressed as the mean ± SD ($n$ = 5). The significant levels observed are $^{*}p < 0.05$ or $^{**}p < 0.01$ between waters for boiling and steaming within the same period of time.

**Fig. 3.** UPLC-MS/MS chromatograms showing CYN retention time and peak areas of a CYN-spiked control muscle sample (50 ng CYN/g dw) and not cooked (A), a CYN-

spiked (50 ng CYN/g dw) muscle sample submitted to steaming for 1 min (B) and a CYN-spiked (50 ng CYN/g dw) muscle sample submitted to steaming for 2 min (C). Chromatograms are presented from 0-3 min.

**Fig. 4.** Mass spectra of CYN ($C_{15}H_{21}N_5O_7S$) and the CYN dimer ($C_{30}H_{42}N_{10}O_{14}S_2$) in control (uncooked) muscle of tilapia fish (*Oreochromis niloticus*), eluting at 3.25 min, recorded (a) without fragmentation, in FS mode and (b) with fragmentation in PRM mode at 35 eV.

**Fig. 5.** Mass spectra of the ion 414.10780 with the chemical formula $C_{15}H_{19}N_5O_7S$ in the water used for steaming muscle of tilapia fish (*Oreochromis niloticus*), eluting at 5.03 min, recorded (a) without fragmentation, in FS mode and (b) with fragmentation in PRM mode at 35 eV.

**Fig. 6.** Extracted ion chromatogram (XIC) for full MS experiments of CYN and its decomposition products in control (uncooked) muscle of tilapia fish (*Oreochromis niloticus*) (a) and in steamed muscle of tilapia fish (*Oreochromis niloticus*) for 2 min (b).

Table 1. CYN decomposition products in control (uncooked) muscle of tilapia fish (*Oreochromis niloticus*).

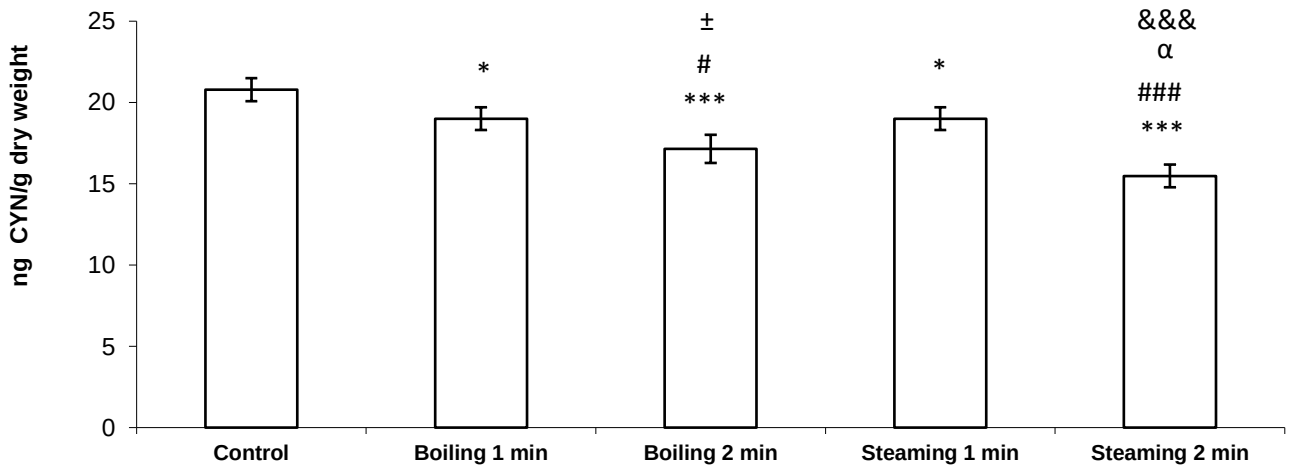
| Decomposition products | Retention Time (min) | Putative Chemical formula | $[M+H]^+$ ion Full Scan (FS) | | | Fragments Parallel Reaction Monitoring (PRM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *m/z* obs. | *m/z* calc. | Mass error (ppm) | Fragment formula | *m/z* obs. | *m/z* calc. | Mass error (ppm) |
| C-1 | 1.52 | $C_{10}H_{15}N_3O_5S$ | 290.08109 | 290.08052 | 1.96 | $C_{10}H_{15}N_3O_2$ | 210.12415 | 210.12370 | 2.10 |
| | | | | | | $C_{10}H_{13}N_3O$ | 192.11348 | 192.11314 | 1.77 |
| C-2A | 2.42 | $C_{10}H_{17}N_3O_5S$ | 292.09665 | 292.09617 | 1.65 | $C_{10}H_{17}N_3O_2$ | 212.13972 | 212.13935 | 1.75 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12921 | 194.12879 | 2.18 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11832 | 176.11822 | 0.53 |
| C-2B | 3.79 | $C_{10}H_{17}N_3O_5S$ | 292.09686 | 292.09617 | 2.38 | $C_{10}H_{17}N_3O_2$ | 212.13985 | 212.13935 | 2.33 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12917 | 194.12879 | 1.94 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11858 | 176.11822 | 1.99 |
| 7-epi-CYN | 2.86 | $C_{15}H_{21}N_5O_7S$ | 416.12384 | 416.12345 | 0.94 | $C_{15}H_{21}N_5O_4$ | 336.16751 | 336.16663 | 2.62 |
| | | | | | | $C_{15}H_{19}N_5O_3$ | 318.15689 | 318.15607 | 2.60 |
| | | | | | | $C_{10}H_{15}N_3O_4S$ | 274.08612 | 274.08560 | 1.89 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12924 | 194.12879 | 2.33 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11865 | 176.11822 | 2.43 |
| CYN | 3.25 | $C_{15}H_{21}N_5O_7S$ | 416.12387 | 416.12345 | 1.02 | $C_{15}H_{21}N_5O_4$ | 336.16730 | 336.16663 | 1.98 |
| | | | | | | $C_{15}H_{19}N_5O_3$ | 318.15674 | 318.15607 | 2.11 |
| | | | | | | $C_{10}H_{15}N_3O_4S$ | 274.08618 | 274.08560 | 2.11 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12920 | 194.12879 | 2.10 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11848 | 176.11822 | 1.48 |
| C-3A | ND | $C_{15}H_{21}N_5O_4$ | ND | 336.16663 | | $C_{10}H_{15}N_3O$ | ND | 194.12879 | |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-3B | 5.07 | $C_{15}H_{21}N_5O_4$ | 336.16705 | 336.16663 | 1.26 | $C_{10}H_{15}N_3O$ | 194.12924 | 194.12879 | 2.33 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-4 | 4.94 | $C_{15}H_{19}N_5O_7S$ | 414.10822 | 414.10780 | 1.01 | $C_{15}H_{19}N_5O_4$ | 334.15043 | 334.15098 | 1.34 |
| | | | | | | $C_{10}H_{13}N_3O_4S$ | 272.07056 | 272.06995 | 2.22 |
| | | | | | | $C_{10}H_{13}N_3O$ | 192.11351 | 192.11314 | 1.93 |

Table 2. CYN decomposition products in boiled muscle of tilapia fish (*Oreochromis niloticus*) for 2 min.

| Decomposition products | Retention Time (min) | Putative Chemical formula | $[M+H]^+$ ion Full Scan (FS) | | | Fragments Parallel Reaction Monitoring (PRM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *m/z* obs. | *m/z* calc. | Mass error (ppm) | Fragment formula | *m/z* obs. | *m/z* calc. | Mass error (ppm) |
| C-1 | 1.52 | $C_{10}H_{15}N_3O_5S$ | 290.08069 | 290.08052 | 0.59 | $C_{10}H_{15}N_3O_2$ | 210.12408 | 210.12370 | 1.81 |
| | | | | | | $C_{10}H_{13}N_3O$ | 192.11363 | 192.11314 | 2.57 |
| C-2A | 2.42 | $C_{10}H_{17}N_3O_5S$ | 292.09659 | 292.09617 | 1.44 | $C_{10}H_{17}N_3O_2$ | 212.13969 | 212.13935 | 1.61 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12889 | 194.12879 | 0.53 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-2B | 3.78 | $C_{10}H_{17}N_3O_5S$ | 292.09665 | 292.09617 | 1.65 | $C_{10}H_{17}N_3O_2$ | 212.13965 | 212.13935 | 1.39 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12918 | 194.12879 | 2.02 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11830 | 176.11822 | 0.44 |
| 7-epi-CYN | 2.84 | $C_{15}H_{21}N_5O_7S$ | 416.12366 | 416.12345 | 0.51 | $C_{15}H_{21}N_5O_4$ | 336.16895 | 336.16663 | 6.89 |
| | | | | | | $C_{15}H_{19}N_5O_3$ | 318.15778 | 318.15607 | 5.37 |
| | | | | | | $C_{10}H_{15}N_3O_4S$ | 274.08606 | 274.08560 | 1.67 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12918 | 194.12879 | 2.02 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| CYN | 3.22 | $C_{15}H_{21}N_5O_7S$ | 416.12427 | 416.12345 | 1.98 | $C_{15}H_{21}N_5O_4$ | 336.16718 | 336.16663 | 1.62 |
| | | | | | | $C_{15}H_{19}N_5O_3$ | 318.15665 | 318.15607 | 1.83 |
| | | | | | | $C_{10}H_{15}N_3O_4S$ | 274.08609 | 274.08560 | 1.78 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12914 | 194.12879 | 1.78 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11867 | 176.11822 | 2.52 |
| C-3A | 3.45 | $C_{15}H_{21}N_5O_4$ | 336.16699 | 336.16663 | 1.08 | $C_{10}H_{15}N_3O$ | 194.12926 | 194.12879 | 2.41 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-3B | 5.06 | $C_{15}H_{21}N_5O_4$ | 336.16788 | 336.16663 | 3.71 | $C_{10}H_{15}N_3O$ | 194.12912 | 194.12879 | 1.71 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-4 | 4.94 | $C_{15}H_{19}N_5O_7S$ | 414.10831 | 414.10780 | 1.24 | $C_{15}H_{19}N_5O_4$ | 334.15112 | 334.15098 | 0.43 |
| | | | | | | $C_{10}H_{13}N_3O_4S$ | 272.07047 | 272.06995 | 1.88 |
| | | | | | | $C_{10}H_{13}N_3O$ | 192.11337 | 192.11314 | 1.22 |

Table 3. CYN decomposition products in steamed muscle of tilapia fish (*Oreochromis niloticus*) for 2 min.

| Decomposition products | Retention Time (min) | Putative Chemical formula | $[M+H]^+$ ion Full Scan (FS) | | | Fragments Parallel Reaction Monitoring (PRM) | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | *m/z* obs. | *m/z* calc. | Mass error (ppm) | Fragments formula | *m/z* obs. | *m/z* calc. | Mass error (ppm) |
| C-1 | 1.52 | $C_{10}H_{15}N_3O_5S$ | 290.08109 | 290.08052 | 1.96 | $C_{10}H_{15}N_3O_2$ | 210.12408 | 210.12370 | 1.81 |
| | | | | | | $C_{10}H_{13}N_3O$ | 192.11333 | 192.11314 | 0.98 |
| C-2A | 2.42 | $C_{10}H_{17}N_3O_5S$ | 292.09665 | 292.09617 | 1.65 | $C_{10}H_{17}N_3O_2$ | 212.13972 | 212.13935 | 1.75 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12941 | 194.12879 | 3.20 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11913 | 176.11822 | 5.12 |
| C-2B | 3.77 | $C_{10}H_{17}N_3O_5S$ | 292.09662 | 292.09617 | 1.54 | $C_{10}H_{17}N_3O_2$ | 212.13968 | 212.13935 | 1.53 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12917 | 194.12879 | 1.94 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| 7-epi-CYN | 2.86 | $C_{15}H_{21}N_5O_7S$ | 416.12415 | 416.12345 | 1.68 | $C_{15}H_{21}N_5O_4$ | 336.16641 | 336.16663 | -0.65 |
| | | | | | | $C_{15}H_{19}N_5O_3$ | 318.15735 | 318.15607 | 4.03 |
| | | | | | | $C_{10}H_{15}N_3O_4S$ | 274.08575 | 274.08560 | 0.55 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12926 | 194.12879 | 2.41 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11894 | 176.11822 | 4.08 |
| CYN | 3.21 | $C_{15}H_{21}N_5O_7S$ | 416.12415 | 416.12345 | 1.68 | $C_{15}H_{21}N_5O_4$ | 336.16730 | 336.16663 | 1.98 |
| | | | | | | $C_{15}H_{19}N_5O_3$ | 318.15680 | 318.15607 | 2.30 |
| | | | | | | $C_{10}H_{15}N_3O_4S$ | 274.08624 | 274.08560 | 2.33 |
| | | | | | | $C_{10}H_{15}N_3O$ | 194.12920 | 194.12879 | 2.10 |
| | | | | | | $C_{10}H_{13}N_3$ | 176.11847 | 176.11822 | 1.39 |
| C-3A | 3.41 | $C_{15}H_{21}N_5O_4$ | 336.16681 | 336.16663 | 0.53 | $C_{10}H_{15}N_3O$ | 194.12915 | 194.12879 | 1.86 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-3B | 5.15 | $C_{15}H_{21}N_5O_4$ | 336.16815 | 336.16663 | 4.53 | $C_{10}H_{15}N_3O$ | 194.12920 | 194.12879 | 2.10 |
| | | | | | | $C_{10}H_{13}N_3$ | ND | 176.11822 | |
| C-4 | 5.04 | $C_{15}H_{19}N_5O_7S$ | 414.10785 | 414.10780 | 0.13 | $C_{15}H_{19}N_5O_4$ | 334.15109 | 334.15098 | 0.33 |
| | | | | | | $C_{10}H_{13}N_3O_4S$ | 272.07080 | 272.06995 | 3.12 |
| | | | | | | $C_{10}H_{13}N_3O$ | 192.11346 | 192.11314 | 1.70 |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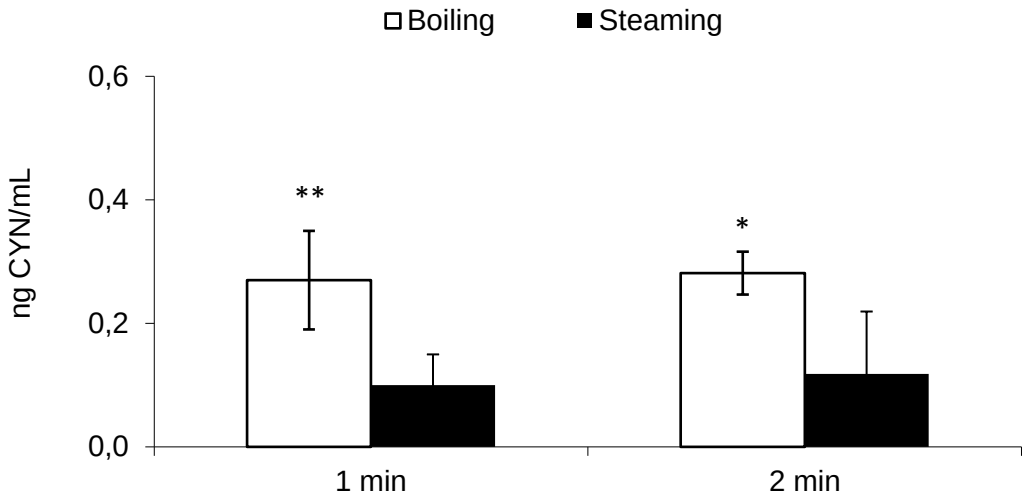
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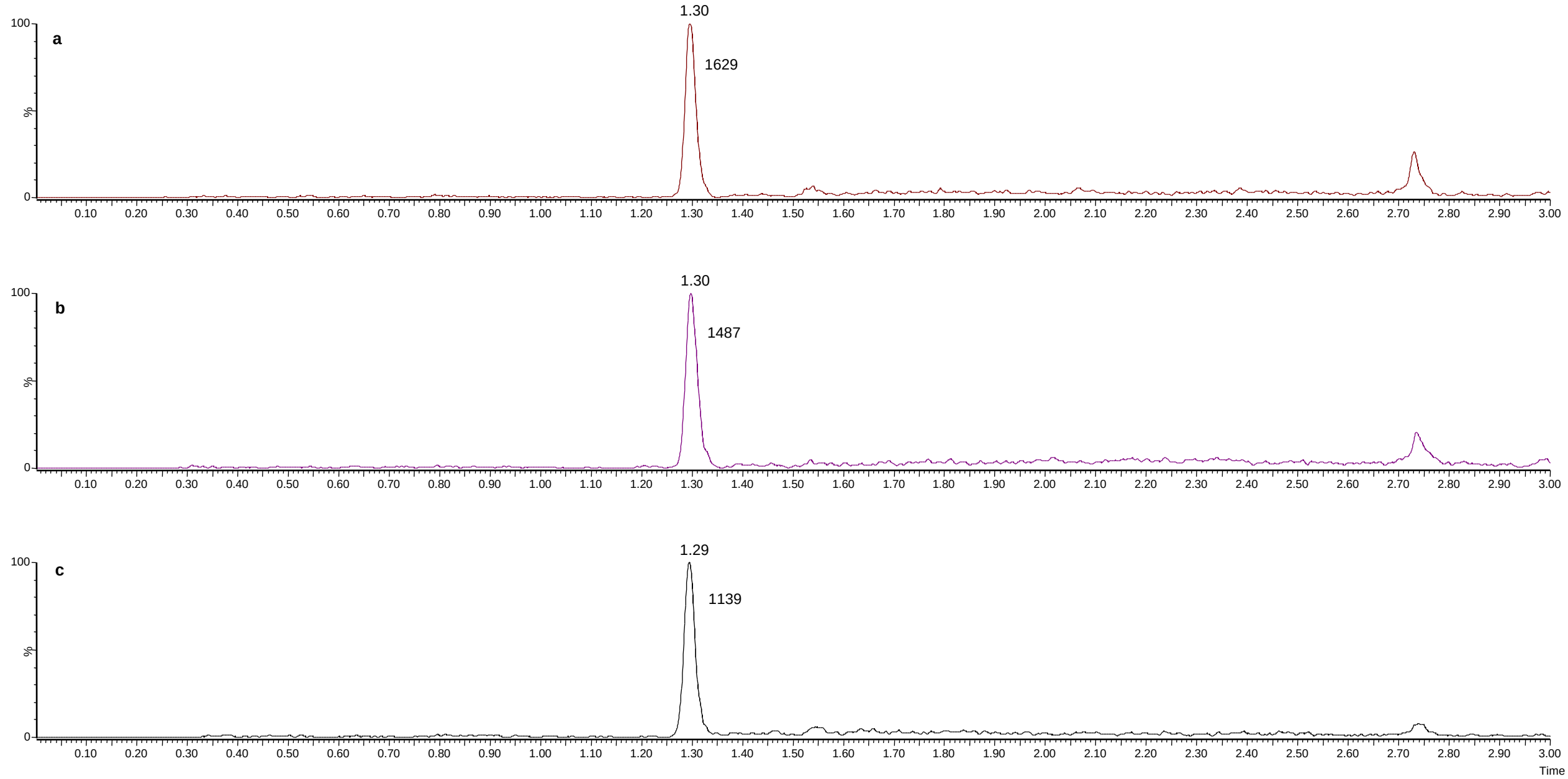
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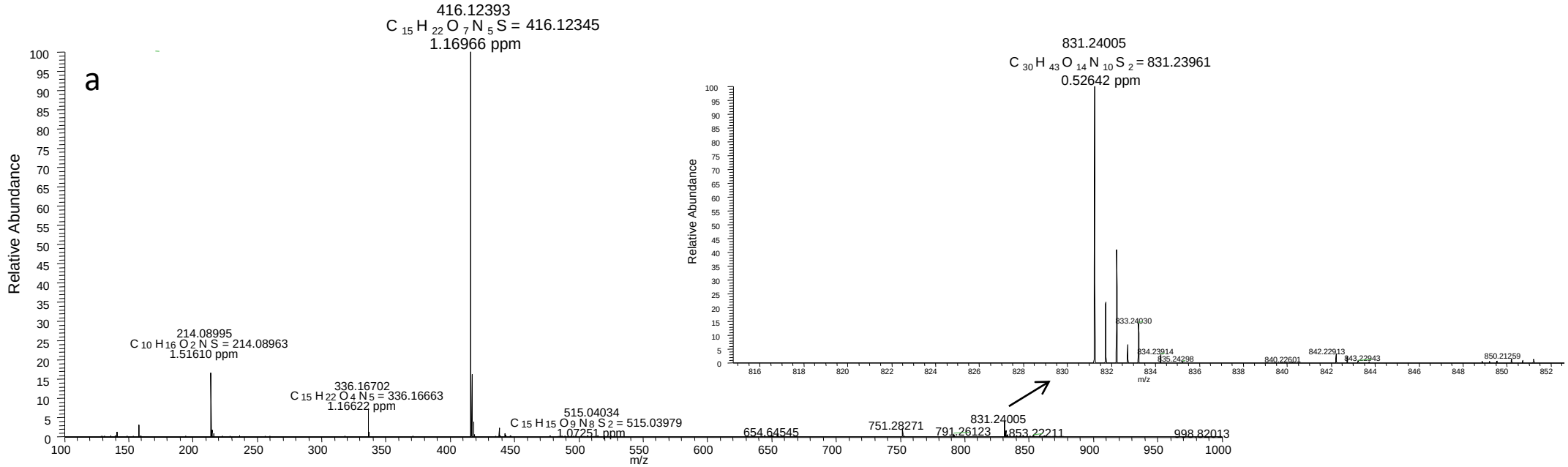
Time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1.16966 ppm
214.08995
C 10 H 16 O 2 N S = 214.08963
1.51610 ppm
336.16702
C 15 H 22 O 4 N 5 = 336.16663
1.16622 ppm
515.04034
C 15 H 15 O 9 N 8 S 2 = 515.03979
1.07251 ppm
654.64545
751.28271
791.26123
831.24005
853.22211
998.82013
Relative Abundance
m/z
831.24005
C 30 H 43 O 14 N 10 S 2 = 831.23961
0.52642 ppm
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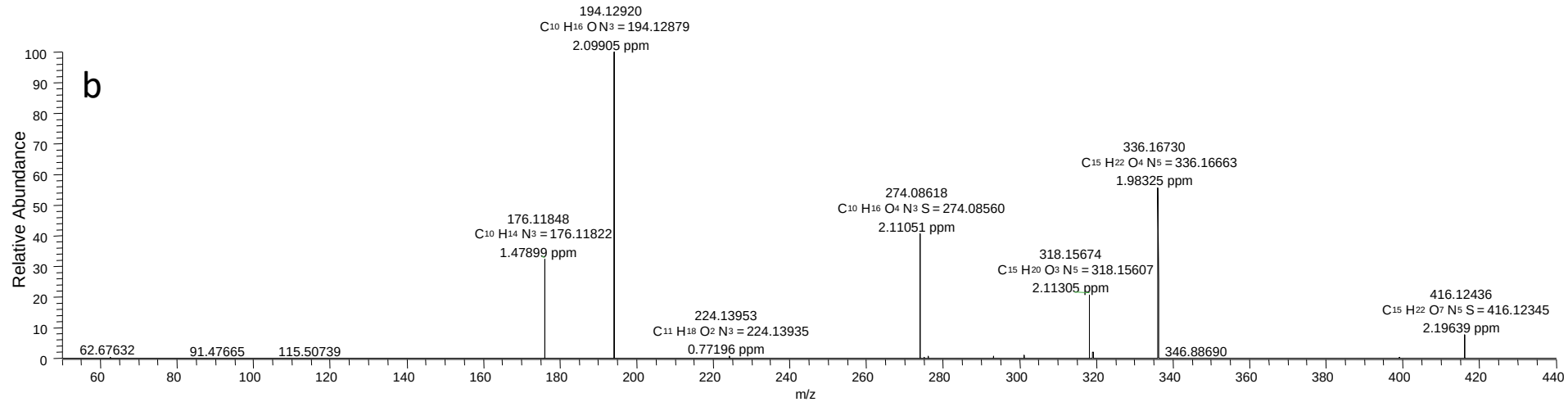
834.23914
835.24298
840.22601
842.22913
843.22943
850.21259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2.09905 ppm
176.11848
C10 H14 N3 = 176.11822
1.47899 ppm
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C11 H18 O2 N3 = 224.13935
0.77196 ppm
274.08618
C10 H16 O4 N3 S = 274.08560
2.11051 ppm
318.15674
C15 H20 O3 N5 = 318.15607
2.11305 ppm
336.16730
C15 H22 O4 N5 = 336.16663
1.98325 ppm
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416.12436
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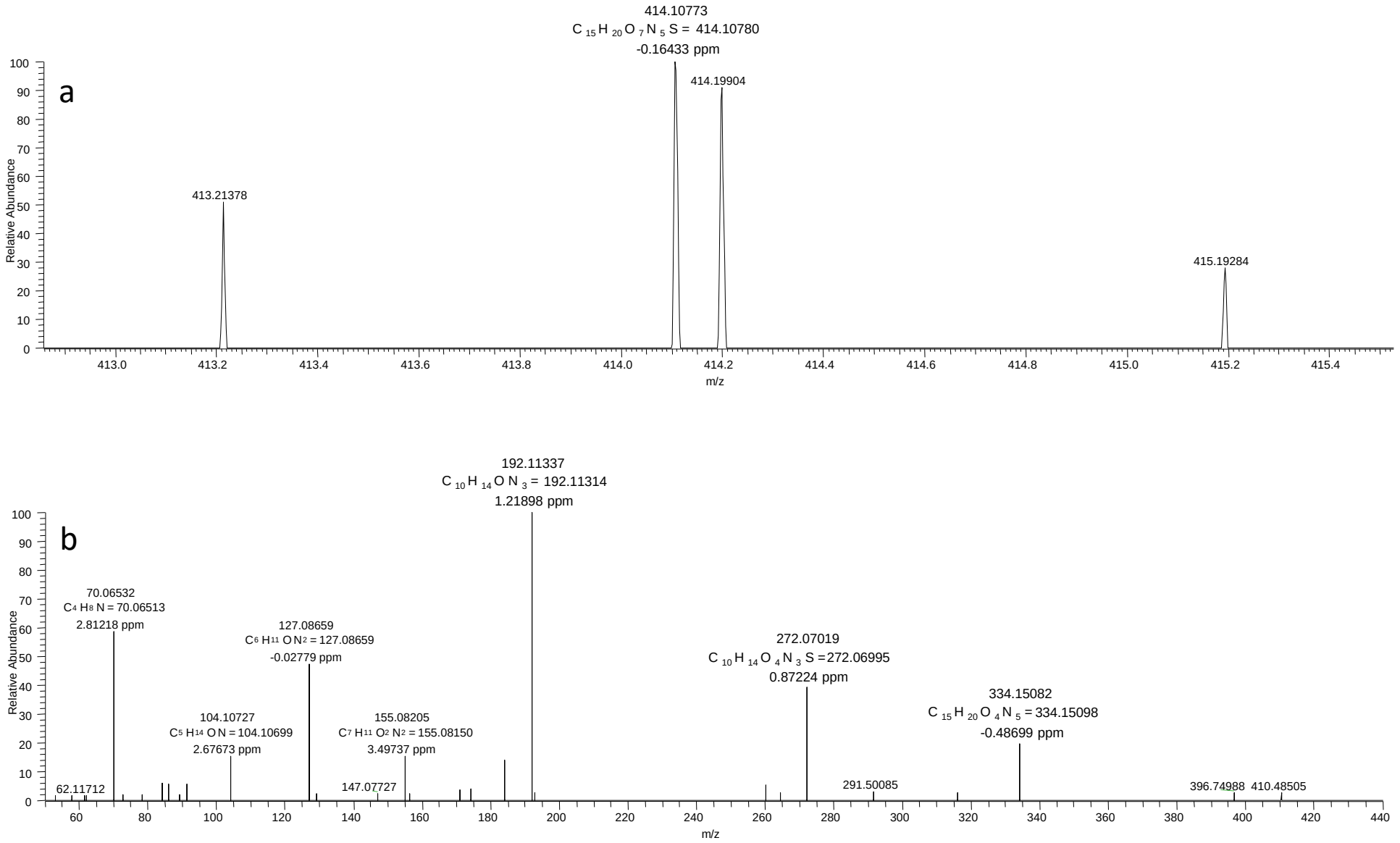
115.50739
Relative Abundance
m/z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414.10773
C 15 H 20 O 7 N 5 S = 414.10780
-0.16433 ppm
414.19904
413.21378
415.19284
Relative Abundance
m/z
b
192.11337
C 10 H 14 O N 3 = 192.11314
1.21898 ppm
70.06532
C4 H8 N = 70.06513
2.81218 ppm
127.08659
C6 H11 O N2 = 127.08659
-0.02779 ppm
272.07019
C 10 H 14 O 4 N 3 S = 272.06995
0.87224 ppm
334.15082
C 15 H 20 O 4 N 5 = 334.15098
-0.48699 ppm
104.10727
C5 H14 O N = 104.10699
2.67673 ppm
155.08205
C7 H11 O2 N2 = 155.08150
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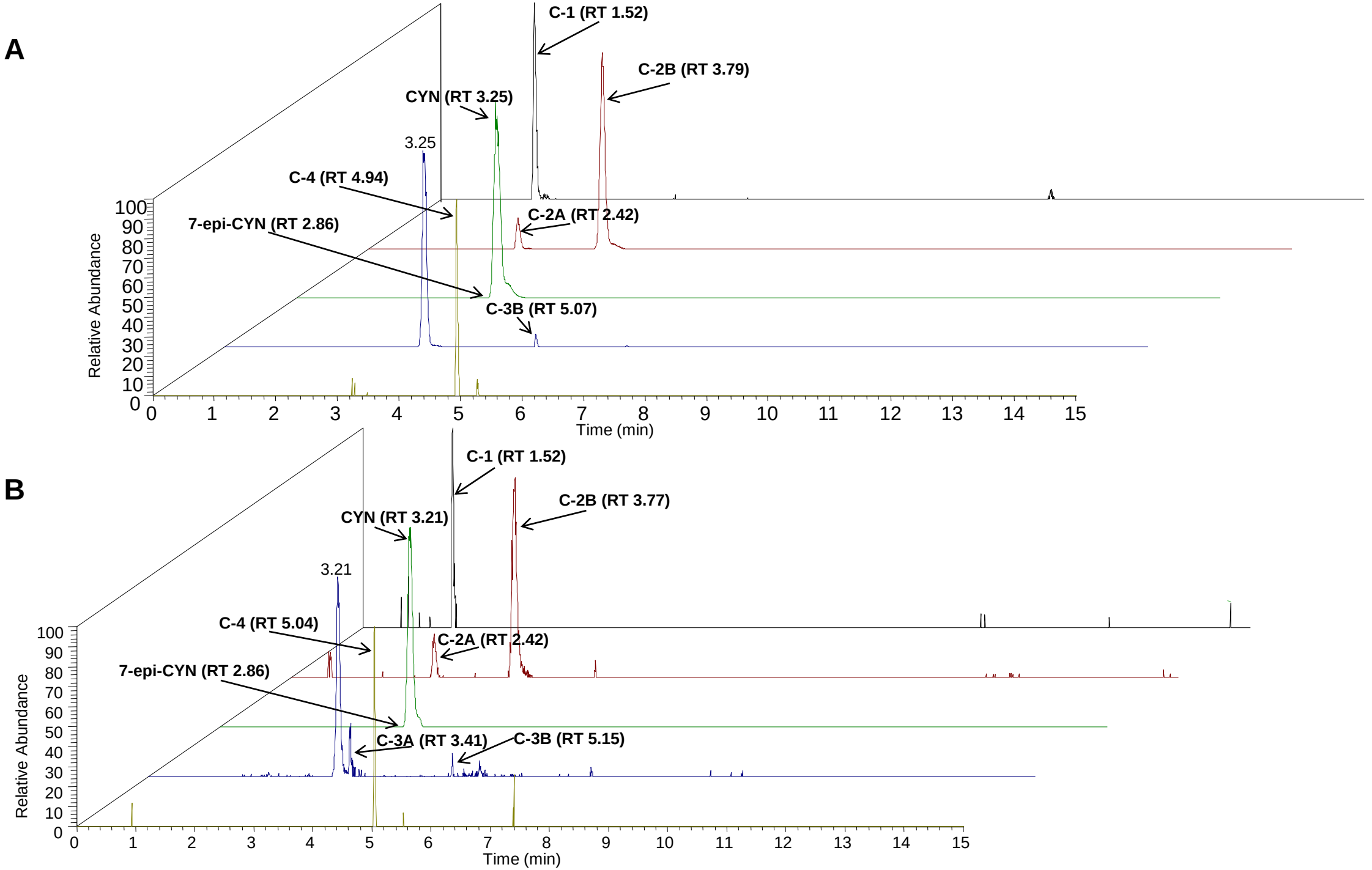
291.50085
396.74988
410.48505
Relative Abundance
m/z


A
C-1 (RT 1.52)
C-2B (RT 3.79)
CYN (RT 3.25)
3.25
C-4 (RT 4.94)
C-2A (RT 2.42)
7-epi-CYN (RT 2.86)
C-3B (RT 5.07)
Relative Abundance
Time (min)
B
C-1 (RT 1.52)
C-2B (RT 3.77)
CYN (RT 3.21)
3.21
C-4 (RT 5.04)
C-2A (RT 2.42)
7-epi-CYN (RT 2.86)
C-3A (RT 3.41)
C-3B (RT 5.15)
Relative Abundance
Time (min)