

# Endocrine Disruptors Flavorings Found in Brazilian Fishes and Seafood

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## 1 Introduction

Musk fragrances are applied to a large diversity of products like Personal Care Products (PCPs) as parfum, eau-de-toilette, shampoos, lotion, soaps, etc; as well as to household sanitizing as detergents (Cruz & Barceló, 2015; Ebele, Abdallah & Harrad, 2017; Li et al., 2018). Polycyclic musks, in particular galaxolide (HHCB) and tonalide (AHTN) are widely used in many countries. However, due their high lipophilicity, they tend to bioaccumulate, affecting the biota at high trophic levels, and may interfere with the marine animal endocrine systems. There are no legal limits established in seafood, but the European Chemical Agency (ECHA) provided human risk assessment reports for HHCB, AHTN, MX and MK, with oral non-observed adverse effect levels (NOAEL) of 150 mg kg<sup>-1</sup> bw day<sup>-1</sup>, 5 mg kg<sup>-1</sup> bw day<sup>-1</sup>, 7.5 mg kg<sup>-1</sup> bw day<sup>-1</sup>, and 2.5 mg kg<sup>-1</sup> bw day<sup>-1</sup>, respectively (ECHA 2009). Regarding other musks such as cashmeran, celestolide (ADBI), phantolide, traseolide, or HHCB-Lactone, due to the lack of toxicological information, threshold values for risk characterization have not been established yet (Cruz & Barceló, 2015; Montesdeoca-Esponda et al., 2018; Cunha et al., 2022). The present study aimed to determinate and quantify the musks fragrances (HHCB, AHTN and ADBI), in mullet, parati and seabass fishes (*Mugil curema*, *Centropomus spp.*, *Mugil spp.*, respectively), and mussel samples (*Anacardina brasiliiana* and *Mytilus edulis*), caught in Sepetiba Bay and Parnaíba River, Brazil

## 2 Materials and Methods

### 2.1 Analytical Parameters

The sample analysis was performed by gas chromatograph Agilent 7890B (Agilent Technologies, USA) coupled to an Agilent 7000C triple quadrupole mass spectrometer (Agilent Technologies, USA), in electron ionization mode (EI), using a C<sub>18</sub> capillary column, 30 m × 0.25 mm × 0.25 mm (Agilent Technologies, USA). The oven was programmed to start at 150 °C, to hold for 1.5 min, and a ramp at 40 °C.min<sup>-1</sup> to 250 °C; ramp again at 7 °C min<sup>-1</sup> to 320 °C and hold for 3 min, with a total run time of 23.5 min. The carrier gas was Helium, the flow rate was 1.3 mL min<sup>-1</sup>, and the collision cell gases were nitrogen (1.5 mL.min<sup>-1</sup>) and helium (2.25 mL min<sup>-1</sup>). The temperatures of the transfer line, ion source, 1<sup>st</sup> and 2<sup>nd</sup> quadrupole were 250 °C, 320 °C, 150 °C and 150 °C, respectively.

Full scan mass spectra and MS/MS spectra (Agilent model 7890B) were optimized by infusing individual 50 mg/L standard solutions aiming to obtain the maximum number of transitions available for each analyte. The positive ion mode was chosen, in order to achieve the high sensitivity and low baseline noise. The most abundant Selected Reaction Monitoring (SRM) was used for quantification (Q) and the second SRM was used for confirmation purposes (q), according to 2002/657/EC (Antignac et al., 2003). The data processing was performed by MassHunter quantitative analysis software (v. B.02.03). The optimized GC-MS/MS parameters for the target compounds are listed in Table 1.

Table 1: Optimized GC-MS/MS parameters for the musk analysis compounds

Target compounds	Transition <i>m/z</i>		Collision energy (kV)
ADBI celestolide	244>	229.2	10
	229>	173.1	5
HHCB galaxolide	258.2>	215	10
	243.2>	213.2	
AHTN tonalide	258.2>	243.5	5
	242.9>	187.2	

## 2.2 Extraction Method

Fishes and seafood samples were treated by a matrix dispersive solid phase (MSPD) extraction method. The technique consists of some steps as following: ~500 mg of each sample (dry weight basis, dw) was weighed in amber glass vials (15 mL bottles), 100 mg of magnesium sulfate, 100 mg of acidified silica were added to the flasks which were shaken for 1 min. After one night (~15 h), the extracting solvent 3.0 mL of acetonitrile:toluene (4:1, v/v, pH 3.0) was added, and the mixture was taken to a platform mixer at 420 rpm (~21 °C, D-72379 Hechingen, Edmund Bühler GmbH, Germany). Centrifugation was performed at room temperature for 3 min at 1700 rpm. In the first step, 20 µL of triphenyl phosphate TPP (5 ppm) was added as the internal standard. The used acidified silica was prepared with 18.3 mL of concentrated sulfuric acid, after being purified with methanol and dichloromethane, both in the same amount of the silica, 40 g, and evaporated in an oven ~60 °C for 2 h.

A cleanup steps was performed, in which the supernatant (1 mL) was subjected to a small aluminum oxide column (100 mg, preactivated with 500 µL of the extracting solvent acetonitrile:toluene), then eluted with 500 µL of the same extracting solvent. After the cleaning steps, the supernatant was evaporated under a nitrogen stream at 40 °C. Then, 70 µL of toluene was added to the vial and shaken. The extract was then transferred to a 2 mL amber bottle containing a 100 µL insert and analyzed immediately by GC-MS/MS.

## 2.3 Validation

The method was validated by linearity, recovery, repeatability, limit of detection (LOD) and quantification (LOQ) studies, applied to a lean fish matrix (*Mugil curema*), to a fatty fish matrix (*Salmon salar*) and a bivalve matrix (*Mytilus edulis*). Linearity was evaluated by the recovery test, and for this purpose, an analytical curve was obtained with spiked matrices, at concentrations among 0.1 µg kg<sup>-1</sup> dw and 8.4 µg kg<sup>-1</sup> dw of each analytes (5 to 8 different concentrations) for all matrices, followed by extraction and GC-MS/MS.

Recovery test was performed after a blank of reagent, a blank of matrix, and three matrices be spiked before extraction, at the respective concentrations of 0.1 µg kg<sup>-1</sup> dw, 0.8 µg kg<sup>-1</sup> dw and 3.2 µg kg<sup>-1</sup> dw of the analytes. Determination of the relative standard deviation (RSD) was obtained from five injections of these matrices spiked in three concentrations, to evaluate repeatability. The limit of detection (LOD) and limit of quantification (LOQ) were calculated, by the signal/noise ratio adopting 3:1 and 10:1, respectively, using the relative standard deviation (RSD) of ten injections from the reagent blank. Concentrations were calculated based on the calibration curve by the linear regression model versus ratios of peak areas of the analytes and internal standard peak area (TTP).

## 2.4 Determination of musks in samples

Seabass (*Centropomus spp.*, n = 16), parati (*Mugil curema*, n = 17), mullet (*Mugil spp.*, n = 39), shellfish (*Anomalocardia brasiliiana*, n = 19 pools) and blue mussel (*Mytilus edulis*, n = 6 pools) caught from Brazilian Sepetiba Bay and Parnaiba River were pre-treated by MSPD and analysed by GC-MS/MS.

## 3 Results

Linearity test, represented by linear coefficient of determination ( $r^2$ ) was equal to or greater than 0.99 for ADBI and HHCB in fatty fish and bivalves, and for AHTN-tonalide in lean fish. The values of  $r^2$  are presented in table 2.

Table 2: Linear coefficient of determination ( $r^2$ ) of the different analytes in three different matrices

	Fatty fish	Lean fish	Bivalve
ADBI celestolide	0.998	0.988	0.996
HHCB galaxolide	0.995	0.964	0.992
AHTN tonalide	0.965	0.991	0.984

Regarding the intermediate precision test, the relative standard deviations (RSD) of five replicates were less than 20% in fish for almost all analytes at different concentrations, except for ADBI in fatty fish (0.2 µg kg<sup>-1</sup> dw). In bivalves, the RSD was higher than 20%. Table 3 below presents the RSD values obtained from repeatability studies.

Table 3: RSD obtained from repeatability studies in three musks concentration of spiked in lean fish, fatty fish and bivalve

µg kg <sup>-1</sup> dw	Lean fish			Fatty fish			Bivalve		
	0.1	0.8	3.2	0.2	1.2	2.7	0.2	1.2	4.0
ADBI celestolide	3.6	1.7	9.3	62.1	16.0	10.3	49.3	22.4	14.2
HHCB galaxolide	2.9	2.2	3.1	0.0	15.1	12.5	24.9	32.7	83.0
AHTN tonalide	2.5	1.6	5.3	30.7	8.9	5.6	92.9	19.0	10.1

Accuracy study by the standard recovery in 0.1  $\mu\text{g kg}^{-1}$  dw concentration to lean fish, the majority of analytes showed an extractive yield out of the accepted margin (70% to 120%). For this reason, the recovery of the other matrices was analyzed from 0.2  $\mu\text{g kg}^{-1}$  dw. In the other concentrations, almost all compounds showed a yield among 70% and 120%, except AHTN in 3.2  $\mu\text{g kg}^{-1}$  dw. For the fatty fish, almost all analytes presented an extractive yield in the accepted range, except HHCB in 1.2  $\mu\text{g kg}^{-1}$  dw. Regarding to bivalve, the musks presented an extractive yield up the accepted range in 0.2  $\mu\text{g kg}^{-1}$  dw, and the found were satisfactory in 0.12  $\mu\text{g kg}^{-1}$  dw concentration, except to AHTN. The limits of detection were among 0.1  $\mu\text{g kg}^{-1}$  dw and 2.0  $\mu\text{g kg}^{-1}$  dw, and the limits of quantification were between 0.2  $\mu\text{g kg}^{-1}$  dw and 6.2  $\mu\text{g kg}^{-1}$  dw. Table 4, below, shows the LOD and LOQ values in the three matrices.

Table 4: LOD and LOQ of musk analysis in lean fish, fatty fish and bivalve

	Lean fish ( $\mu\text{g kg}^{-1}$ dw)		Fatty fish ( $\mu\text{g kg}^{-1}$ dw)		Bivalve ( $\mu\text{g kg}^{-1}$ dw)	
	LOD	LOQ	LOD	LOQ	LOD	LOQ
ADBI celestolide	0.1	0.3	0.1	0.4	0.1	0.2
HHCB galaxolide	0.9	2.7	0.2	0.5	2.0	6.2
AHTN tonalide	0.1	0.2	0.4	1.3	1.7	5.1

The presence of musks was found in at least one of the analyzed matrices. ADBI was observed in fourteen samples of mullet and twelve samples of bivalves, but all values were below the LOQ. HHCB was found in fourteen samples of Parati, four of them with values above the LOQ (among 5.28  $\mu\text{g kg}^{-1}$  and 26.0  $\mu\text{g kg}^{-1}$ ). HHCB was also determinate in ten seabass samples, with one of them (27.2  $\mu\text{g kg}^{-1}$ ) above the LOQ value, in two mullet samples ( $< \text{LOQ}$ ) and in one shellfish ( $< \text{LOQ}$ ).

AHTN was observed in fifteen Parati samples, thirteen of them above the LOQ (0.22  $\mu\text{g kg}^{-1}$  to 12.92  $\mu\text{g kg}^{-1}$ ). AHTN was also found and in twelve seabass samples, one of them (9.44  $\mu\text{g kg}^{-1}$ ) with a value above the LOQ. Table 5, below, presents the analyte values found in the analyzed samples.

Table 5: Concentrations of musks founded in analyzed samples ( $\mu\text{g kg}^{-1}$ )

Compounds	Class	Species	Concentration ( $\mu\text{g kg}^{-1}$ )
ADBI (celestolide)	Polycyclic Musk	Mussel: 1	$< \text{LOQ}$
		Shellfish: 12	$< \text{LOQ}$
		Mullet: 14	$< \text{LOQ}$
HHCB (galaxolide)	Polycyclic Musk	Parati: 14	$< \text{LOQ}$ a 26.0
		Seabass: 10	$< \text{LOQ}$ a 27.2
		Mullet: 2	$< \text{LOQ}$
		Shellfish: 1	$< \text{LOQ}$
AHTN (tonalide)	Polycyclic Musk	Parati: 15	$< \text{LOQ}$ a 12.9
		Seabass: 12	$< \text{LOQ}$ a 9.4

#### 4 Discussion

Several scientific studies have confirmed the presence of musks in fishes, crustaceans, and mussels of different origins and localization as Union European. Cunha et al., (2018) found a total of seven polycyclic musks, which the highest degree was 156  $\mu\text{g kg}^{-1}$  from mackerel. HHCB is found as the most prevalent and abundant musk in seafood, according to several studies (Cunha et al. 2015, 2018; Gadelha et al., 2019; Rocha et al. 2018). Lower levels of musks were obtained in industrial samples than from other origins (Cunha et al., 2018, 2022; Castro et al., 2018).

#### 5 Conclusion

The application of the validated method enabled the determination of three musks compounds in the fatty fish, lean fish and bivalve matrices. The values of HHCB and AHTN in lean fish samples stands out. There is a lack of studies concerning these PCPs on marine biota, as well as their routes and destinations in the environment, so further studies are recommended.

## 6 References

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