Research Article

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Behavior of diclofenac from contaminated fish after cooking and *in vitro* digestion

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Abstract

BACKGROUND: Seafood consumers are widely exposed to diclofenac due to the high contamination levels often present in aquatic organisms. It is a potential risk to public health due its endocrine disruptor properties. Limited information is available about diclofenac behavior after food digestion to enable a more realistic scenario of consumer exposure. This study aimed to evaluate cooking effects on diclofenac levels, and determine diclofenac bioaccessibility by an *in vitro* digestion assay, using commercial fish species (seabass and white mullet) as models. The production of the main metabolite 4′-hydroxydiclofenac was also investigated. Fish hamburgers were spiked at two levels (150 and 1000 ng g^{-1}) and submitted to three culinary treatments (roasting, steaming and grilling).

RESULTS: The loss of water seems to increase the diclofenac levels after cooking, except in seabass with higher levels. The high bioaccessibility of diclofenac (59.1–98.3%) observed in both fish species indicates that consumers' intestines are more susceptible to absorption, which can be worrisome depending on the level of contamination. Contamination levels did not affect the diclofenac bioaccessibility in both species. Seabass, the fattest species, exhibited a higher bioaccessibility of diclofenac compared to white mullet. Overall, cooking decreased diclofenac bioaccessibility by up to 40% in seabass and 25% in white mullet. The main metabolite 4′-hydroxydiclofenac was not detected after cooking or digestion.

CONCLUSION: Thus, consumption of cooked fish, preferentially grilled seabass and steamed or baked white mullet are more advisable. This study highlights the importance to consider bioaccessibility and cooking in hazard characterization studies.

Supporting information may be found in the online version of this article.

Keywords: LC–MS/MS; seafood; bioaccessibility; pharmaceutical; metabolite; cooking

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INTRODUCTION

Seafood is a rich source of essential nutrients, such as amino acids, vitamins, long-chain polyunsaturated n-3 fatty acids, and trace elements, all of which are associated with several human health benefits.[1,2] However, seafood may also contain environmental contaminants, contributing to consumer exposure to detrimental compounds, such as pharmaceuticals widely applied in human health.[3,4] Some of them have been detected in different seafood species, such as bezafibrate, carbamazepine, chloramphenicol, diclofenac, furosemide, gemfibrozil, ibuprofen, indapamide, ketoprofen, naproxen and simvastatin.[5–7]

Diclofenac is a non-steroidal anti-inflammatory drug (NSAID) used worldwide for painful and inflammatory rheumatic conditions, as well as in certain non-rheumatic conditions. It is the most consumed drug worldwide, with an annual consumption of about 1443 t worldwide, of which 28.7% are in the European Union (EU) and 39.5% in Asia.[8] Since diclofenac is only partially removed during wastewater treatment,[9] it is one of the most persistent, abundant, and commonly found pharmaceuticals in aquatic environments[10] being considered an emerging micropollutant of concern.[11] Among the most abundant pharmaceutically active compounds (PhACs) detected in seafood, diclofenac has presented levels up to 1812 ng g^{-1} in snoeks from South Africa.[12] In Malaysia and Spain, diclofenac was the most abundant compound in fish and gastropods (7.41–10.46 ng g^{-1}), and in mussels (28.3–30.3 ng mL^{-1}), respectively.[13,14] Several studies have demonstrated that diclofenac has endocrine disruptor properties, causing physiological and behavioral effects on fish even at low concentrations.[15–18] The vulture populations in Pakistan and India nearly faced extinction after feeding on cattle carcasses contaminated by diclofenac and its residues.[19,20] Due to its potential risk to public health, diclofenac has been identified as a priority substance for regulation at EU level via the Water Framework Directive and environmental monitoring by member states.[21] The maximum allowed concentration in freshwater is 0.10 μg L^{-1}, and 0.01 μg L^{-1} in marine waters.[21,22] Maximum residual limits (MRLs) have been established for bovine and porcine (5 μg kg^{-1} muscle) European Commission Regulation (ECR),[23] but no MRLs have been settled for seafood. The acceptable daily intake (ADI) for diclofenac is 1.4 μg kg d^{-1}, considering 25–100 mg d^{-1} as a therapeutic dose for adult pain treatments.[24,25] In turn, the Committee for Veterinary Medicinal Products (CVMP) has established an overall pharmacological-toxicological ADI of 0.5 μg kg^{-1} body weight (bw) (30 μg per person).[26]

The assessment of human exposure to contaminants through seafood consumption can be overestimated or underestimated since the effects of cooking practices and bioaccessibility are usually neglected.[27,28] Despite a lack of studies focusing on the evaluation of food processing conditions for PhACs in seafood, cooking has been observed as a mitigation strategy to reduce exposure levels to these contaminants.[2] Therefore, it is crucial to quantify the contaminant levels released from seafood that can reach target organs through systemic absorption. The bioaccessible fraction available for absorption by the intestinal epithelium after the digestion process depends on the food matrix, the type of processing and storage, as well as on the compound chemistry.[29,30] Considering the high diclofenac levels found in seafood, it is important to understand the behavior of diclofenac from food preparation to its digestion in order to direct care to ensure safe consumption of seafood by the world population. *In vitro* digestion models (static and dynamic) simulating the physiological conditions of gastrointestinal tracts are used to assess bioaccessibility of contaminants via food consumption.[31,32] These methodologies have reduced cost, are fast and reproducible, being considered as suitable tools to study the behavior of xenobiotics after oral ingestion.[2] Seafood is mainly consumed after cooking, but most bioaccessibility studies are performed in raw samples, whereas some cooking methods have shown reduction of several antibiotics in chicken meat.[33–36] Recently, Hao *et al.*[37] investigated diclofenac bioaccessibility in raw fish commercialized in China. However, to the best of our knowledge, there are no reports concerning the impact of cooking on diclofenac bioaccessibility.

Its primary and major metabolite, 4′-hydroxydiclofenac, which is generated by CYP2C9,[17] is also the most abundant and frequent metabolite detected in the environment and biota. In Spanish wastewater treatment plants, 4′-hydroxydiclofenac presented much higher concentrations than diclofenac.[38] This metabolite has been detected in bile of trout exposed to contaminated waters, which increases the chronic human exposure to this product.[39] As the 4′-hydroxydiclofenac has exhibited significant estrogenic, anti-androgenic, (anti)-glucocorticoid and anti-thyroid hormonal activities,[17] its formation after human digestion deserves to be studied in order to aim for food safety.

In this context, this study aimed to evaluate the fate of diclofenac present in seafood after cooking (roasting, steaming, and grilling) and *in vitro* digestion, using seabass (aquaculture specie) and white mullet (from fishing) as fish models. Furthermore, the presence of 4′-hydroxydiclofenac metabolite derived from cooking the seafood was also investigated. To the best of our knowledge, this is the first time that the influence of cooking and bioaccessibility on fish fillets diclofenac levels has been investigated.

MATERIAL AND METHODS

Sampling, sample fortification and culinary treatments

Farmed seabass (*Dicentrarchus labrax*, $n = 3$) samples from Turkey were purchased at the market in Porto, Portugal, while white mullet (*Mugil curema*, $n = 3$) samples were captured by fisherman from Porto, in September 2020. Both species are highly consumed in many countries and have different fat content. Before the experiment, the samples were analyzed, and no diclofenac levels were detected. For each species, 500 g (*w/w*) of muscle (without bones or skin) were taken and homogenized with a grinder (Retsch Grindomix GM2000; Retsch, Germany) using polypropylene cups and stainless-steel knives at 3600 × *g* until visual disruption of the tissue.

To ensure the presence of diclofenac in both fractions (bioaccessible and non-bioaccessible) following the digestion simulation, half of the samples were fortified with diclofenac sodium salt (99.5% purity) at two concentrations (150 and 1000 ng g^{-1}). The levels chosen to take into account the dilution factor that occurs along *in vitro* digestion and the limit of quantification (LOQ) of the liquid chromatography–tandem mass spectrometry (LC–MS/MS) method. After homogenization, the fortified samples were chilled at 5 °C for 1 h.

Control and fortified samples were divided into two parts: raw and cooked. For cooked samples, three fortified and three non-fortified hamburgers of 10 g (3.5 cm diameter × 1 cm height) were prepared for each treatment. The culinary practices were: (i) steaming in a teflon pan at 105 °C during 10 min after boiling; (ii) roasting (Rational Combi-master CM6, Landsberg am Lech,

Germany) for 5 min at 200 °C; and (iii) grilling at 155 °C for 3 min (90 s each side), in a domestic grill (Flama Sketch) with 2000 W power. In all procedures, the temperature inside the burger reached 70 °C and was measured using a rod-type thermometer. Moreover, all hamburgers were weighed before and after cooking to calculate the water loss.

Nutritional composition

Moisture, lipid, and protein content of raw and cooked fish samples were evaluated according to the standard methods of the Association of Official Analytical Chemists,[40] three replicate measurements (Table 1). Briefly, moisture (%) was calculated in a halogen moisture analyzer HR73 (Mettler Toledo, Barcelona, Spain) using 3.5 g of samples, inserted in a capsule along with sand and sodium sulfate (Na_2SO_4) and macerated until dry. Lipid content (%) was calculated by gravimetry after overnight extraction in a Soxhlet apparatus using petroleum ether. Proteins (%) were quantified by the Kjeldahl method, using 1 g of sample.

Bioaccessibility assay

Reagents

The four digestive juices (saliva, gastric juice, duodenal juice and bile) were prepared with potassium chloride (KCl, 99.5% purity; Merck, Darmstadt, Geramny), potassium thiocyanate (KSCN; P2713; Sigma, St Louis, MO, USA), monosodium phosphate (NaH_2PO_4, 99.5%; Merck), Na_2SO_4 (90% purity; Merck), sodium chloride (NaCl, 99.5% purity; Merck), sodium bicarbonate ($NaHCO_3$, 99.5% purity; Merck), calcium chloride dihydrate ($CaCl_2{\cdot}2H_2O$; C3881; Sigma), ammonium chloride (NH_4Cl, 99.5% purity; Riedel-de Haen, Seelze, Germany), potassium dihydrogen phosphate (KH_2PO_4, 99.5% purity; Merck), magnesium chloride ($MgCl_2$, 99.5% purity; Riedel-de Haen), hydrochloric acid (HCl, 37% purity *w/w*; Merck); urea (U5128; Sigma), glucose (G5400; Sigma), glucuronic acid (G5269; Sigma), D-(+)-glucosamine hydrochloride (G4875; Sigma), uric acid (U2625; Sigma), albumin from bovine serum (A7906; Sigma), α-amylase from *Aspergillus oryzae* (86250; Sigma), mucin from porcine stomach (M2378; Sigma), pepsin from porcine stomach mucosa (P7125; Sigma), lipase from porcine pancreas, type II (L3126; Sigma), pancreatin from porcine pancreas (P8096; Sigma), trypsin from porcine pancreas (T6567; Sigma), α-chymotrypsin from bovine pancreas (C4129; Sigma) and bile porcine extract (B8631; Sigma). Sodium hydroxide (NaOH, 106498, Merck) and HCl were also used for pH adjustment.

In vitro *digestion protocol*

A prior decontamination of the material with warm (60 °C) nitric acid (Merck, Darmstadt, Germany) at 20% during 24 h was performed.[41] Organic and inorganic solutions were prepared to produce *in vitro* saliva, gastric juice, duodenal juice, and bile. The composition of each *in vitro* digestive juices as well as the *in vitro* digestion protocol used in this work were based on the work of Marmelo *et al.*[42] Briefly, 1 g of fish was used in the digestion process that was performed in triplicate. Negative controls without samples were performed in each batch of digestion. To simulate the oral phase, 4 mL of saliva (pH 7 ± 0.2) were added to samples, which were digested at 37 °C using a Rotary Tube Mixer with Disc (LSCI, Portugal) in an incubator (Select 400 W) for 5 min. In the gastric phase, 8 mL of gastric juice was added to the previous mixture and incubated for 2 h at pH 2.0 ± 0.2. For the intestinal phase, 8 mL of duodenal fluid and 4 mL of bile fluid were subsequently added and incubated for 2 h at pH 7.0 ± 0.2. The pH was adjusted using NaOH (1 mol L^{-1}) or HCl (1 mol L^{-1}). Afterwards, to stop the digestion reactions, samples were placed on ice and centrifuged (2750 × *g*; 10 °C; 10 min) for the separation of bioaccessible and non-bioaccessible fractions. The final volume of bioaccessible fractions was around 25 mL and non-bioaccessible fractions averaged 0.55 g.

Total protein levels were measured before the assay and after *in vitro* digestion in bioaccessible and non-bioaccessible fractions using an FP-528 DSP LECO nitrogen analyzer [LECO, St Joseph, MO, USA; limit of detection (LOD) = 0.84 mg N], calibrated with ethylenediaminetetraacetic acid (EDTA) according to the Dumas method.[43] Both fractions are used to calculate the method recovery and the percentage of protein in the bioaccessible fraction was calculated according to the following equation:

$$\text{BIO}\,(\%) = \frac{\text{BIO}}{\text{BD}} \times 100, \quad (1)$$

where BIO corresponds to the diclofenac levels detected in the bioaccessible fraction (BIO), and BD in the sample before digestion.

Table 1. Nutritional composition and average concentrations of diclofenac (ng g^{-1} in dry and lipid weight, dw) in white mullet and seabass from a Portuguese market

Samples	Moisture (%)	Protein (%)	Lipid (%)	Concentration of diclofenac (ng g^{-1} dw)		
				C	L1	L2
Seabass						
Raw	62.5	20.5	10.7	ND	300.7	3069
Roasted	58.4	28.9	13.3	ND	636.1	2229
Grilled	56.0	28.1	14.4	ND	486.2	2757
Steamed	58.3	28.4	12.9	<LOQ	455.4	2103
White mullet						
Raw	68.2	21.9	5.9	<LOQ	555.1	3672
Oven	60.1	30.7	6.5	<LOQ	682.4	4003
Grilled	64.3	26.4	6.7	<LOQ	593.2	4229
Steamed	61.8	28.6	7.5	<LOQ	594.7	41 634

Note: C, control; L1, fortified level 1 (150 ng g^{-1}); L2, fortified level 2 (1000 ng g^{-1}), < LOQ (2 ng g^{-1}). ND, not detected; LOQ, limit of quantification.

Diclofenac analysis

Chemicals and reagents

Diclofenac sodium salt (99.5% purity), 4′-hydroxydiclofenac (> 99% purity) and internal standard diclofenac-acetylphenyl ($^{13}C_6$, 99.99% purity) sodium salt were acquired from Sigma-Aldrich (West Chester, PA, USA). Acetonitrile (MeCN) LC–MS grade was obtained from Honeywell (Seelze, Germany), while formic acid (purity > 99%) was obtained from Merck (Darmstadt, Germany). The sorbents anhydrous magnesium sulfate (anhydrous $MgSO_4$; 99.5% purity) was supplied by Sigma-Aldrich (West Chester, PA, USA); NaCl (99.5% purity) by AppliChem Panreac ITW Co. (Barcelona, Spain); QuECheRS sorbents Supel™ QuE Z-Sep$^+$ and primary secondary amine (PSA) were acquired from Supelco (Bellefont, PA, USA); EMR-Lipid was from Agilent Technologies (Santa Clara, CA, USA) and C18 from Waters (Milford, MA, USA). Air and nitrogen (Gasin, Portugal) for LC analyses were > 99.99% pure.

Sample extraction

Diclofenac and its metabolite 4′-hydroxydiclofenac were determined using the method of Cunha *et al.*[44] in raw and cooked samples of white mullet and seabass as well as in their bioaccessible and non-accessible fractions after *in vitro* digestion.

Solid matrices. For extraction of diclofenac in the raw and cooked fish, 1 g of wet sample was spiked at 100 ng g^{-1} of internal standard and extracted with 5 mL of MeCN with 1% formic acid (pH ≅ 4.5) in the presence of 2 g anhydrous $MgSO_4$ and 0.5 g of NaCl. Then, 1 mL of extract was cleaned with 0.5 g EMR sorbent previously activated with 2.5 mL Milli-Q water. Finally, the extract was dried under a nitrogen stream and reconstituted in 0.5 mL of methanol with 0.1% formic acid (phase B).

Bioaccessible fraction. For extraction of diclofenac in bioaccessible fraction, 5 mL of bioaccessible fraction was spiked with 75 ng g^{-1} internal standard and extracted with 2 mL of MeCN with 1% formic acid. In the presence of 800 mg of $MgSO_4$ and 200 mg of NaCl. Then, 1.5 mL of extract was cleaned with 50 mg C18 and 100 mg Z-Sep. Afterwards, the extract was dried under a nitrogen stream and reconstituted with 0.5 mL of phase B.

Non-bioaccessible fraction. The pellets were spiked with 75 ng g^{-1} internal standard and extracted with 1 mL of MeCN with 1% formic acid and 50 mg PSA. Then, dried under a nitrogen stream and reconstituted with 0.5 mL of phase B.

Liquid chromatography–tandem mass spectrometry (LC–MS/MS)

An ultrahigh-performance liquid chromatography coupled to a triple quadrupole tandem mass spectrometry (UHPLC-QQQ-MS/MS, Perkin Elmer Mod QSIGHT 420; Perkin Elmer, Waltham, MA, USA) was used for diclofenac determination. The injection volume was 5 µL and the auto-sampler was kept at ambient temperature (±25 °C). A reversed-phase column (Kinetex 1.7 µm C18, 50 mm × 2.1 mm) allowed chromatographic separation of the compounds. The mobile phase consisted of a mixture A: water with formic acid 10 mmol L^{-1} and mixture B: acetonitrile with formic acid 10 mmol L^{-1}. Its elution was performed in a total run time of 12.5 min. The gradient was programmed with 95% of water acidified (0.1% formic acid; *v/v*) held for 10 min followed by an isocratic elution of 100% of methanol acidified (0.1% formic acid; *v/v*) until the end of the run. The column temperature was maintained at 40 °C and the flow rate was 300 µL min^{-1}. Mass analysis was carried out in electrospray negative mode (ESI$^-$) with multiple reaction monitoring (MRM) and the following MS parameters: voltage ES, −4000 V; entrance voltage, 18 V; collision cell lens, 6 V; dry gas, 120 mL min^{-1}; nebulizer gas (nitrogen), 350 mL min^{-1}; source temperature, 350 °C. For diclofenac and 4′-hydroxydiclofenac two transitions were used *294–250*; 294–252 and *310–266*; 310–230, respectively, the transition shown in italic text is used for quantification. The corresponding cone voltage (in volts) and collision energy (in kilovolts) were optimized for maximum intensity. For both diclofenac transitions, 16 V/12 kV were applied, while 10 V/10 kV (310–266) and 25 V/30 kV (310–230) were used for 4′-hydroxydiclofenac transitions. The retention times were 6.86 min for 4′-hydroxydiclofenac and 7.91 min for diclofenac (Fig. 1). Simplicity 3Q was the software used to collect data.

Quality assurance and quality control (QA/QC)

Blanks in each batch were prepared in the same conditions as samples and replicates. The variability criteria among samples were standard deviation (SD) < 20% and relative standard deviation (RSD) < 10%. The methods were validated for each species studied in agreement with internationally recognized principles (SANTE/11312/2021)[45] through the assessment of linearity, precision, accuracy, and sensitivity parameters. Matrix-matched calibrations with nine levels calibration curve were used to compensate for matrix effects. Due to different levels of internal standard in the pretreatment samples, distinct ranges were adopted in each matrix-matched calibration. For seafood and pellet samples, levels ranged from 0 to 1000 ng g^{-1} (0, 2, 5, 20,

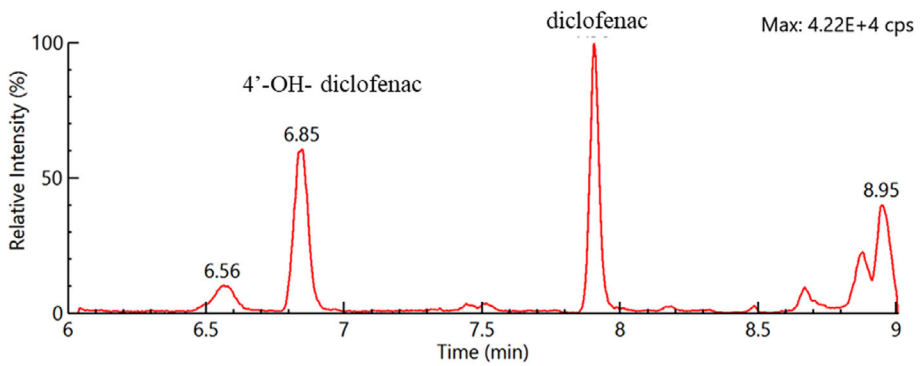

Figure 1. Total ion chromatogram (TIC) of white mullet sample spiked with 100 ng g^{-1} of 4′-hydroxydiclofenac (4′-OH-diclofenac, 6.85 min) and diclofenac (7.91 min). The total run time was 12.5 min although the chromatogram was recorded only up to 9 min.

50, 100, 250, 500, 750, 1000 ng g^{-1}), while for bioaccessible samples were up to 100 ng g^{-1} (0, 1, 2, 5, 10, 25, 50, 75, 100 ng g^{-1}). Calibration curves used for pellets were prepared in MeCN with 1% formic acid in a higher range due to the small amount of available sample. The linearity (R^2) was above 0.98 for both compounds in all matrices and the linear equations are shown in the Supporting Information Table S1. For evaluation of extraction yield, white mullet and seabass samples were fortified at three concentrations (50, 175 and 500 ng g^{-1} for solid samples; 2, 20 and 50 ng g^{-1} for bioaccessible ones) for both diclofenac and 4′-hydroxydiclofenac and five replicates were performed. Spikes were made before sample extraction and at the end of extraction, the extraction yield ranged between 60% and 120%. LOQ was set as the lowest calibration level tested with a signal-to-noise ratio of 10:1. Diclofenac presented 2 ng g^{-1} *w/w* as LOQ, while 4′-hydroxydiclofenac presented 5 ng g^{-1} *w/w* in all matrices.

Concerning bioaccessibility assay, more than 75% of proteins were digested in all samples analyzed, which support the efficiency of the digestion process. Furthermore, diclofenac recoveries ranged from 70% to 114.9%.

Data analysis

Concentrations in ng g^{-1} were expressed in wet, dry and lipid weight, but wet weight was considered for statistical purposes. Prism 6 for Windows (GraphPad Software, Inc. San Diego, CA, USA) was the software used for statistical analysis with 95% of confidence limit. Limited statistical analyses were possible since sample size was reduced. Shapiro–Wilk was applied to test normality and consequently, non-parametric tests were used for comparisons. Mann–Whitney test was applied to compare diclofenac bioaccessibility between species and between the two spiking levels. Kruskal–Wallis was used to assess if there was an influence of culinary procedures on bioaccessibility, whereas Dunn's multiple comparison's test indicated which ones differentiate from each other. Bioaccessibility and non-bioaccessibility percentages were calculated considering these fractions in relation to mean concentrations of diclofenac in solid samples before *in vitro* digestion.

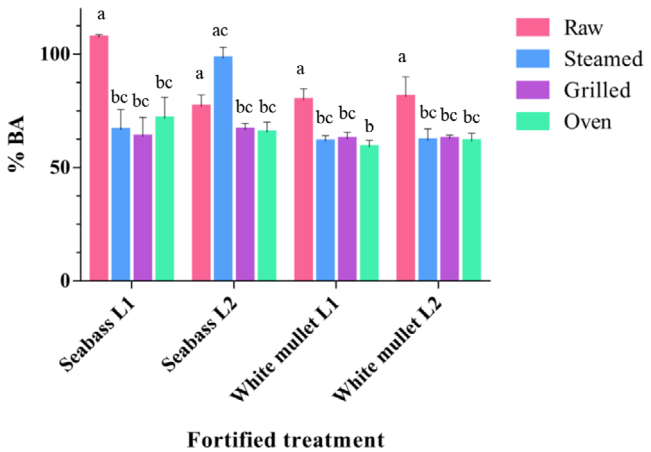

Figure 2. Bioaccessibility percentages (BA%) in each fortification level, relative standard deviation (RSD) and mean in each culinary treatment of fish muscles. L1, fortified level 1 (150 ng g^{-1}); L2, fortified level 2 (1000 ng g^{-1}), < LOQ (2 ng g^{-1}). Different letters indicate significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Influence of cooking practices on fish nutritional composition and diclofenac level

The proximate composition of raw and cooked samples is shown in Table 1. Our results for nutritional composition were similar to those reported in the literature.[46] In general, wild white mullet presented higher moisture and lower lipid content, whereas seabass from aquaculture presented higher lipid content. Possibly, the origin of samples (wild × farmed) is influencing the results since white mullet usually has more fat than seabass. Cooking induced a water loss (7–10% in seabass; 6–12% in mullet), with a consequent enhanced relative protein content (36% in seabass; 20–40% in mullet) and lipid content (20–34% in seabass; 10–27% in mullet). Roasting resulted in a higher water loss for white mullet (6.5%) and grilling for seabass (8.1%) (Table 1).

Control samples of both species did not reveal diclofenac levels above LOQ (Table 1). The culinary practices have shown to influence diclofenac concentrations in white mullet and seabass (raw and cooked) (Table 1). For both seabass and mullet samples with low-fortified levels, all cooking practices promote an increase in diclofenac levels (Table 1; $P > 0.05$). Similar results were reported for steamed mussels, with a significant increase in diclofenac levels (from 1.3 in raw samples to 37.6 in cooked samples).[47] According to the authors, this observation may be justified due to the reconversion of acyl glucuronides to the parent compound after such thermal treatment, in addition to the water loss. At high-fortified level, mullet also showed an increase in diclofenac concentration with the water loss (L2, 9–15%) (Table 1). Contrarily, seabass presented a reduction on diclofenac concentration after cooking (L2, 10–31%; Table 1). *et al.*[36] Sobral also found reduced spiked levels of amoxicillin and tetracyclines in chicken breast muscle after cooking, although the levels were at initial levels. Molecular rearrangement and dechlorination reactions are the probable causes that justify such reduction.[36] Changes in morphology, movements and color alterations of diclofenac crystals were seen starting at around 149 °C.[48] Since higher temperatures were applied in our culinary treatments, our results suggest a possible degradation of the compound.

4′-Hydroxydiclofenac was not detected in any sample before or after cooking, which indicates that culinary practices did not imply the formation of this hydroxyl metabolite.

Diclofenac bioaccessibility in seafood consumed in Portugal

Protein digestibility was above 74.6% on average in raw, grilled, steamed, and roasting white mullet and seabass samples. This high percentage indicates that almost all proteins were hydrolyzed and released to bioaccessible fraction, thus validating the *in vitro* digestion procedure used. The metabolite 4′-hydroxydiclofenac was not detected either in bioaccessible or non-bioaccessible fractions of seabass and white mullet, regardless of the cooking procedure. The main routes of diclofenac degradation are through co-metabolism by fungi and bacteria, as well as through direct metabolism by cytochrome P-450.[22,49,50] Since this assay did not consider the human gut microbiota, it is not possible to ensure that there is not a 4′-hydroxydiclofenac formation during the digestive process.

Diclofenac bioaccessibility was significantly different between species ($P = 0.0144$), with an average of 66.4 ± 8.8% in white mullet and 77.1 ± 16.5% in seabass. Similar diclofenac bioaccessibility

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values (average 78.4%) were found in market fishes from Shanghai ($n = 70$; seven species).[37]

Overall, diclofenac levels released after digestion were higher in seabass than in white mullet (Fig. 2). Bioaccessible percentages ($n = 16$) ranged from 59.10% to 107.30%, with the highest bioaccessibility for low (L1) fortified seabass and the lowest for low (L1) fortified white mullet. Hao *et al.*[37] also found different pharmaceutical bioaccessibility values according to the species, ranging from 18.8% in cuttlefish to 99.6% in bream. Diclofenac presented the highest mean bioaccessible concentration among six pharmaceuticals (sulfadiazine, sulfapyridine, roxithromycin, tylosin and carbamazepine).[37] Additionally, the authors also highlighted that higher bioaccessibility of pharmaceuticals was found in freshwater fishes compared to marine fishes.

The most relevant difference between seabass and white mullet was the muscle lipid content, 107 g kg^{-1} in seabass and 59 g kg^{-1} in mullet (Table 1). In general, the lower lipid content in fish is correlated with the high bioaccessibility of pharmaceuticals.[37] The bioaccessibility of pharmaceuticals in freshwater fish is higher than that in marine fish, possibly because of the lower lipid content in freshwater fish.[37] High lipid levels could impair the pharmaceuticals released from the food matrix.[51] In this study, however, diclofenac seems to show the opposite behavior, being more bioaccessible in the fattest species. As diclofenac is a highly lipophilic molecule (Kow = 4.51), it is expected to be released with fish fat and emulsified by bile.[52] Indeed, amphiphilic bile components, such as bile salts can increase the solubility of poor soluble drug contaminants[53] and consequently promote their uptake. Another point could be related to the high affinity between diclofenac and bile since this compound is metabolized by bile salts.[54] These factors may explain the higher bioaccessibility observed in species with higher lipid content. Consequently, diclofenac could easily reach the blood by crossing lipid cell membranes in the intestine.[2]

For the validation of the diclofenac bioaccessibility method, fortified samples were used. Spiking levels (150 and 1000 ng g^{-1}) did not influence diclofenac bioaccessibility in the studied species ($P = 0.5292$). In both species and levels, diclofenac bioaccessibility percentage showed a reduced variation (63–76%) for cooked fish, with exception of the L2 steamed seabass (98%). Statistical analysis showed differences in diclofenac bioaccessibility among cooking treatments of seabass and white mullet ($P = 0.0002$). Raw white mullet (81.3%) and L1 raw seabass (> 100%) showed the highest bioaccessibility (Fig. 2). Dunn's multiple comparison test revealed that all cooking procedures decrease diclofenac bioaccessibility in fish, except in L2 steamed seabass (Fig. 2). Considering species separately regardless of the fortification level, the highest mean diclofenac bioaccessibility of 92% in raw seabass differed significantly only from grilled one ($P = 0.0315$; Fig. 3). Conversely, raw white mullet had higher bioaccessibility values than those roasted and steamed ($P < 0.0029$; Fig. 3). Raw samples showed higher bioaccessibility compared to cooked ones, for example, towards polycyclic aromatic hydrocarbons (PAHs),[55] polybrominated diphenyl ethers (PBDEs) and their methoxylated metabolites[56] and mercury.[57] Meat doneness (cooking time) seems to improve PAHs bioaccessibility in grilled meat,[58] as observed here for steamed seabass (Fig. 2).

Cooking methods have also been observed to have a significant influence on the bioaccessibility of other contaminants, such as an increased in bioaccessibility of polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins/furans after frying (200–300 °C, 5 min) and boiling (100 °C, 5 min);[59] or a decrease

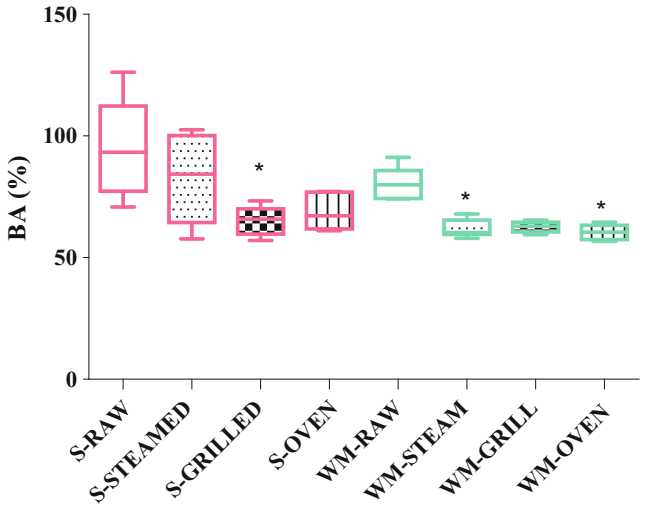

Figure 3. Bioaccessibility percentages (BA%) in seabass (S) and white mullet (WM) in each treatment (median, 25–75%, minimum and maximum). Asterisks indicates significant differences ($P < 0.05$) of cooked samples in relation to raw ones in each species.

in the bioaccessibility of DDTs and PBDEs in raw fish samples after cooking.[60] Diclofenac has high binding affinity with different proteins (e.g., serum albumin, glutathione *S*-transferase, cellulose binding protein, maltose binding protein).[61,62] Changes in the protein structures and loss of highly digestible proteins during cooking hampers the access of enzymes and consequently, contribute to a lower bioaccessibility of some compounds.[42,63,64] After digestion, proteins in the bioaccessible fraction were lower in cooked samples, indicating less digestibility of proteins after cooking likely due to their denaturation.[65] As observed, diclofenac bioaccessibility in seabass presented a decrease of 37% for steamed; 40% for grilled and 32% for roasting treatments; while white mullet presented a decrease of 22% for steamed; 21% for grilled and 25% for roasting treatments (L1 fortified treatments, Fig. 2), compared with raw samples. For high-fortified levels (1000 ng g^{-1}), the same behavior was observed for both species, with the exception of steamed seabass (Fig. 2). Nonetheless, in some cases, cooking could improve overall digestibility. During protein denaturation and the unfolding of the amino acid chains, the affinity between digestive enzymes and protein can increase and the compounds linked to them can become bioaccessible.[66] This may explain the higher bioaccessibility of diclofenac in steamed seabass (Fig. 2). In addition, the steam introduces moisture into the food, which may affect the solubility of certain compounds. Increased water content might enhance the dissolution and subsequent absorption of bioactive components. The composition of the specie (lipid, fiber proteins) and microbial environment may impact the breakdown of certain compounds, affecting the bioacessibility. However, further research is needed to provide deeper insights into the observed variations in bioaccessibility.

In contrast, the lipophilicity of the compound does not seem to play a significant role in the bioaccessibility of cooked samples as they had higher lipid content and lower bioaccessibility. Nevertheless, setting clear parameters that influence such differences is difficult since different chemical and nutritional changes may be induced by cooking. In addition, the scarcity of data on diclofenac bioaccessibility prevents comparisons in order to define patterns.

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In the present study, diclofenac had a high bioaccessibility in the studied species. It indicates that most contamination levels present in seafood are available to be absorbed in the human intestine, which can be a concern to human health. However, the use of spiked samples for cooking and *in vitro* digestion tests can present differences from residual samples because of the binding forms of drugs *in vivo*. Studies on behavior of relevant pharmaceuticals in commercially available seafood using naturally contaminated samples is totally encouraged. A higher number of samples should also be considered in the next studies in order to strengthen statistical analysis.

Notwithstanding, it is important to include bioaccessibility in studies of risk assessment of diclofenac through dietary exposure to avoid overestimation of risks for consumers. For example, Mello *et al.*[7] reported an estimated daily intake of diclofenac in white mullets and snook from Brazil of 0.42 and 1.07 ng kg^{-1} bw per day, respectively. Such values would be lower if the bioaccessible fraction was included instead of total contamination values. The culinary practices must also be considered in the risk assessment of diclofenac, since the levels are generally higher in cooked products, whereas bioaccessibility is lower, and most people eat these species cooked. Therefore, the risk of exceeding diclofenac ADI is reduced through the consumption of cooked fish. For seabass consumers, grilling is the safest treatment in terms of bioaccessibility of diclofenac, while steam and oven cooking seems the best way to consume white mullet.

CONCLUSION

This study fills some gaps on the influence of cooking and bioaccessibility on risk assessment of diclofenac ingested from seabass and white mullet. Cooking did not strongly influence diclofenac levels in seabass and white mullet. After digestion, diclofenac bioaccessibility showed high levels in both species. These results confirm the importance of monitoring and regulating this compound in animal food, since most of the diclofenac present can potentially be absorbed by consumers. Future risk assessment studies should consider the bioaccessibility of diclofenac, in order to have a more realistic scenario without overestimation of risks for consumers through dietary exposure. Moreover, the risk associated with cooked seabass and white mullet consumption is lower than raw ones since bioaccessibility of diclofenac also is lower. However, this percentage still is relevant, which ratifies the importance of monitoring this compound in seafood. Thus, it is more advisable to consume cooked fish, preferentially grilled seabass and steamed or baked white mullet. Although the main human metabolite has not been detected in this study, future investigations should assess diclofenac metabolite levels during the cooking of contaminated fish, as well as their bioaccessibility, including the action of the microbiota present in the end of the digestive tract in order to achieve a full picture of the diclofenac behavior once ingested.

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CONFLICT OF INTEREST

The authors have no conflicts of interest, either real or potential, associated with this work.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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