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Evaluation of heavy metals, mycotoxins and mineral bioaccessibility through *in vitro* static digestion models of rainbow trout (*Oncorhynchus mykiss*) and sole (*Dover sole*) side stream extracts obtained by pressurized liquid extraction

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ABSTRACT

The recovery of antioxidants and minerals as well as the content of contaminants of rainbow trout and sole side streams (head, skin and viscera) extracts obtained by pressurized liquid extraction (PLE) were evaluated. Then, the effect of the gastrointestinal digestion was tested. No mycotoxins were detected in the extracts, while heavy metals contents (mg/kg) were up to 2.9 (As), 0.054 (Cd), 0.16 (Hg) and 0.073 (Pb), being below maximum legislated limits. A positive effect of PLE was found for the antioxidant capacity recovery, being the oxygen radical capacity of sole head and skin extracts significantly enhanced after digestion (~3.8 times). PLE significantly increased Mg, Fe, Zn, Se and P ($K_{PLE} > 1$) contents of rainbow trout side streams, Zn (K_{PLE}5.97) and Fe (K_{PLE} 2.80) of head sole and Mg, Se and P of all samples. Moreover, Mg, Ca and Fe bioaccessibility was lower in all sole extracts compared to rainbow trout.

1. Introduction

In recent years, global seafood production has shown a continuous increase. According to the forecast of FAO (Food and Agriculture Organization of the United Nations), fish production will reach 195 million tons around 2025 (Food and Agriculture Organization of the United Nations, 2018). In many countries, the fishery is the mainstay of the economy and an important source of animal protein, unsaturated fatty acids (UFAs) as well as many bioactive components (Rubio-Rodríguez et al., 2012; Sarker, 2020). Many fish side streams such as head, skin, backbones, etc., are generated during the fish production process. These side streams often contain high-added-value compounds that can be used by humans, such as proteins, bioactive peptides, minerals and so on, which exert potential bioactive functions such as antioxidant, anti-inflammatory, microbiota modulation, etc. (Marti-Quijal et al., 2020). The discarding of fish side streams not only cause a waste of resources but also has a negative impact on the environment and is not conducive to the sustainable development of ecology (Marti-Quijal et al., 2020; Zamorano-Apodaca et al., 2020). In order to reduce the waste of side streams and environmental pollution, researchers try to recover high-added-value bioactive components from fish side streams and serve them as functional ingredients in food, medicine, cosmetics and other industries.

Previously, our research team applied pressurized liquid extraction (PLE) as an innovative, green and efficient approach to recover high-added value compounds from fish side streams and obtained compounds with high antioxidant capacity (Wang et al., 2021). The main principle of PLE is the combination of temperature and pressure to extract high-added-value compounds from solid and semi-solid samples, which can be a useful extraction technique by retaining the activity of target compounds and reducing the extraction time. It is a feasible tool to increase the extraction rate of high-added-value compounds, as well as to ensure the *in vitro* bioactivity of the extracts (Wang et al., 2021). However, there is a lack of information about the bioaccessibility of the

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antioxidants and some important compounds such as minerals. Bioaccessibility is an important assay as it is often used to assess diet health risks and to provide reliable information about bioactive compound utilization (Leufroy et al., 2012; Li & Wang, 2019).

Mineral elements play an important role in human metabolism. For example, iron (Fe) participates in the transport of oxygen and energy supply in the body; calcium (Ca) is important for maintaining bone health; magnesium (Mg) and phosphorus (P) also have the potential to regulate protein activity and prevent some diseases (Jha et al., 2021; Mir-Marqués et al., 2016).

Environmental contaminants such as mycotoxins and heavy metals can enter the food production chain causing adverse human health problems. Mycotoxins are metabolites related with cancer induction, mutagenicity, estrogenicity as well as gastrointestinal, urogenital, vascular, kidney and nervous disorders which have detected in some fish species (Pallarés et al., 2019; Tolosa et al., 2014; Tolosa et al., 2021). Moreover, heavy metals are non-degradable pollutants in nature, which in the aquatic environment mainly come from the crust, sediments and wastes produced by human activities, etc. (Habib et al., 2022). These compounds will enter the fish body through the gills and other organs and accumulate in different parts, which has become a concern of people. The concentrations and types of heavy metals contained in different fish vary widely. Among heavy metals, the most common detected are arsenic (As), cadmium (Cd), nickel (Ni), lead (Pb) and mercury (Hg) (Soltani et al., 2019).

In this study, we aimed to determine the heavy metal content and mycotoxins of the extracts (before and after PLE and control treatments) and then, through an *in vitro* static digestion model to assess the effect of digestion on the antioxidant capacity and the bioaccessibility of minerals (Ca, Mg, P, Fe, Zn and Se) of PLE and control extracts obtained from rainbow trout and sole side streams.

2. Materials and methods

2.1. Reagents

Potassium chloride (KCl), sodium chloride (NaCl), magnesium chloride hexahydrate ($MgCl_2(H_2O)_6$), potassium dihydrogen phosphate (KH_2PO_4), acetonitrile and methanol HPLC grade, absolute ethanol, chloroform, titrisol concentrated standards of macro/trace elements (Mg, Ca, P, Fe, Zn, Se), heavy metals solution standards and nitric acid (HNO_3) were purchased from Merck (Darmstadt, Germany); hydrochloric acid (37 %) was obtained from Scharlau (Barcelona, Spain); ammonium formate (99 %), formic acid (≥95 %) and mycotoxins standards including (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, ENNA, ENNA1, ENNB, ENNB1) were purchased from Sigma-Aldrich (St. Louis, MO, USA); ammonium carbonate ($(NH_4)_2CO_3$), sodium bicarbonate ($NaHCO_3$) and calcium chloride dihydrate ($CaCl_2(H_2O)_2$) were purchased from Sigma-Aldrich (St. Louis, MO, USA); the enzymes pepsin (975 units per protein, porcine), pancreatin (8 × USP specifications, porcine) and porcine bile extract were also supplied by Sigma-Aldrich (St. Louis, MO, USA); Trizma® base, ABTS (2,2′-azinobis (3-ethylbenzothiazoline 6-sulfonic acid)), Trolox® (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) and fluorescein sodium salt were obtained from Sigma-Aldrich (St. Louis, MO, USA); AAPH (2,2′-azobis (2-amidinopropane)) (Acros Organics), sodium phosphate dibasic, potassium dihydrogen phosphate and potassium sulphate were obtained from VWR International Eurolab S. L. (Barcelona, Spain); ethyl acetate was obtained from Alfa Aesar (Karlsruhe, Germany); disodium phosphate and sodium dihydrogen phosphate were obtained from Panreac; deionized water was obtained in the Milli-Q SP® reagent water system (Millipore Corporation, Bedford, MA, USA); syringe nylon filters (13 mm diameter and 0.22 μm pore size) were purchased from Membrane Solutions (Plano, TX, USA); nylon filters (0.45 μm) were obtained from Scharlau. Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the method of Minekus (Minekus et al., 2014).

2.2. Sample collection and processing

The rainbow trout and sole used in the experiments were deceased at the time of purchase from a local market of Valencia (Spain) during 2021. Fish were sectioned in the laboratory to obtain three side streams: head, skin, and viscera, then fish side streams were stored at −20 ℃ for further experiments.

2.3. Extraction technologies

The PLE extraction process was carried out following the same procedure as previously described by Wang et al. (2021). For this, an ASE-200 Accelerated Solvent Extractor (Sunnyvale, CA, USA) located at the Faculty of Pharmacy of the University of Valencia was used. The fresh fish side streams were freeze-dried and mixed with diatomaceous earth in a certain proportion (head, skin and viscera: 1.0:2.0, 2.0:2.0 and 1.5:3.0 g/g, respectively). Then, the mixture was added to a PLE extraction chamber, and the operating conditions were set at: preheating time 1 min, heating time 5 min, flushing volume 60 %, and nitrogen purge 60 s. Finally, the extracts were stored at −20 °C for subsequent analysis.

For the different fish side streams, a control group without PLE was set up. For that, the fish side stream samples after freeze-dying (Lyo-Quest, Telstar, Spain) were extracted by stirring with distilled water for 15 min under certain conditions (head: 55 °C + pH 5.2, skin: 45 °C + pH 6.5, viscera: 50 °C + pH 6.8) to obtain the extracts. The extracts were centrifuged at 4000 × rpm at 4 °C for 15 min (Centrifuge 5810R, Eppendorf, Germany), and the supernatants collected, which were stored at −20 °C for analysis.

In order to evaluate the extract efficiency of PLE, the efficiency coefficient K_{PLE} is introduced:

$$K_{PLE} = \frac{M}{Mc} \quad (1)$$

M and Mc refer to the mineral content in PLE-assisted treatment and no-PLE-assisted group, respectively.

2.4. Mineral profile and heavy metals determination

The contents of heavy metals (As, Cd, Hg and Pb) and mineral profile (Mg, Ca, P, Fe, Zn and Se) in fish side stream were determined according to a previous study (de la Fuente et al., 2021). Specifically, different fish side streams were placed in a microwave oven (MARS, CEM, Vertex, Spain) for mineralization. Three hundred milligrams of samples were weighted into a Teflon digester, then HNO_3 (14 M, 4 mL) and H_2O_2 (30 % *v/v*, 1 mL) was added, and the samples were digested at 800 W, 180 ℃ for 15 min. The digested samples were taken out and cooled to room temperature, after removing nitrogen, it was filtered, and the volume was made up with distilled water. The inductively coupled plasma mass spectrometer (ICP-MS) was used for the determination. The conditions were set as follows: carrier gas flow (1.07 L/min), helium (He) as reactant gas, high-frequency emission power (1550 W), Ar gas flow (15.0 L/min), nebulizer pump speed (0.10 rps), and radio frequency matching (1.80 V). For heavy metals, ^{72}Ge, ^{103}Rh and ^{193}Ir were used as internal standard solutions to correct the fluctuation and drift of the instrument signal. The 0 ~ 1000 μg/L standard calibration curve was used for the quantitative analysis of As, Cd and Pb, and the 0 ~ 100 μg/L standard curve was used for the quantitative analysis of Hg. For mineral profiles, ^{45}Sc and ^{72}Ge were used as an internal standard solution, and the standards of 0 ~ 10000 μg/L were utilized for quantitative analysis of minerals. The value of the correlation coefficient was R ≥ 0.9999 and each calibration point had an RSD value of ≤ 5%. At the end of the sample sequence analysis, a calibration pattern was analyzed obtaining an average between the reference and the obtained value around RSD ≤

5%.

2.5. Mycotoxins analysis in fish side streams

2.5.1. Dispersive liquid–liquid microextraction procedure (DLLME)

The DLLME method was employed to extract mycotoxins from the samples according to a previous study carried out by our team (Khawli et al., 2021). The extraction was carried out employing the mixture of dispersant and extractant solvents (950 μL AcN/620 μL EtOAc) in a first step. After that, the top organic phase was transferred to another tube. Then, in a second step, the mixture of dispersant and extractant solvents (950 μL MeOH/620 μL $CHCl_3$) was added to the remaining residue. The organic phase at the bottom of the tube was separated and placed with the previously transferred organic phase. Then the mixture of two organic phases was dried and the residue was reconstituted with 1 mL of 20 mM ammonium formate (MeOH/ACN: 50/50 *v/v*) before injection into the LC-MS/MS-IT system.

2.5.2. LC-MS/MS-IT determination

Mycotoxins were determined using an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA) equipped with a 3200 QTRAP system (Applied Biosystems, AB Sciex, Foster City, CA) with Turbo Ion Spray (ESI) electrospray ionization. The instrumental parameters are detailed in previously work (Khawli et al., 2021).

2.5.3. Method validation

The DLLME method was previously validated by our team for fish side streams (Khawli et al., 2021). The method was characterized in terms of recoveries, repeatability (intraday precision), reproducibility (interday precision), matrix effects, linearity, limits of detection (LOD), and limits of quantification (LOQ). The results obtained revealed the suitability of the method to be applied for mycotoxins determination. In that regard, the recoveries obtained at level of 10 × LOQ were between 68 and 120%. Moreover, no significant signal suppression/enhancement (SSE) was observed and the LODs ranged from 0.05 to 5 μg/L and LOQs from 0.2 to 17 μg/L, respectively.

2.6. Evaluation of bioaccessibility of fish by-product extracts

2.6.1. In vitro simulated gastrointestinal digestion

The static *in vitro* digestion process was performed according to the standardized methodology proposed by INFOGEST (Minekus et al., 2014). The *in vitro* static digestion simulation includes three stages: chewing, gastric phase, and intestinal phase. To mimic the digestive processes in the oral cavity, stomach and intestine, simulated saliva fluid (SSF, pH = 7), simulated gastric fluid (SGF, pH = 3) and simulated intestinal fluid (SIF, pH = 7) were prepared.

Oral stage (chewing): the oral simulated digestion stage was carried out without α-amylase, 2.5 mL of fish side streams extracts were mixed with 2 mL of SSF and shaken quickly for 1 min. Then, 12.5 mL of $CaCl_2$ was added, and the volume was made up to 5 mL with distilled water, the mixture was shaken in a water bath at 37 ℃ for 2 min.

Gastric stage: 4.55 mL of SGF was added to the above mixture and vortexed for 1 min. Then, 8 mg pepsin (2000 U/mL) from porcine gastric mucosa and 2.5 μL $CaCl_2$ were added to the solution and vortexed for 1 min. The pH was adjusted to 3 with NaOH (1 M)/HCl (6 M), and the volume up to 10 mL with distilled water, before incubating at 37 ℃ for 2 h.

Intestinal stage: 5.5 mL of SIF was added to the above solution and vortex 1 min. Then 2.5 mL pancreatin (800 U/mL) from porcine pancreas, 1.25 mL porcine bile extract (0.16 M) and 20 μL $CaCl_2$ were added, and the mixture was vortexed for 1 min. The pH was adjusted to 7 and the volume completed up to 20 mL. Then, the solution was incubated at 37 °C for 2 h and centrifuged at 4000 rpm for 40 min at 4 °C, and the supernatant (bioaccessible fraction (BF)) was collected for antioxidant capacity and mineral profile analysis. In parallel was prepared a blank group, containing all the digestive juices, and replacing the fish by-product extracts with deionized water.

For bioaccessibility estimation, the digestion blank value needs to be subtracted from the sample value to eliminate the interference caused by reagents such as digestive enzymes. The results can be calculated as the ratio of the concentration of bioactive compounds in the digested sample to the original samples, as following equation (2):

$$Bioaccessibility\ (\%) = \left(\frac{content\ in\ BF}{original\ content}\right) \times 100 \quad (2)$$

2.6.2. Antioxidant capacity

Total antioxidant capacity was evaluated by oxygen radical absorption capacity test (ORAC) and Trolox equivalent antioxidant capacity assay (TEAC) according to a previous study (Wang et al., 2021).

For the evaluation of the oxygen radical absorption capacity (ORAC), Trolox was used as the antioxidant standard and phosphate buffer 148 (pH 7.0 ~ 7.4) as the blank group. 50 μL samples/Trolox standard and 50 μL fluorescein sodium salt was added to a 96-well microplate. After 10 min incubation at 37 °C in the dark, 25 μL of AAPH was added to initiate the oxidation reaction. A microplate reader was employed to measure the absorbance at 520 nm, and 45 cycles were set, of 60 s each cycle. Five parallels were set for each group of samples and repeated three times, and the antioxidant capacity of the samples was calculated according to the formula:

$$ORAC(trolox) = \frac{A_{sample} - A_{blank}}{A_{trolox} - A_{blank}} \quad (3)$$

For the Trolox equivalent antioxidant capacity (TEAC) assay, 25 mL of ABTS and 440 μL sodium thiosulfate were mixed to obtain a working solution, which was kept at room temperature for 12 ~ 16 h in the dark. Then, the working solution was diluted with 96 % ethanol to maintain the absorbance between 0.700 ± 0.020. In the experiment, different concentrations of Trolox were used as the standard, 2 mL of working solution was mixed with 0.1 mL of sample/Trolox, and the absorbance was measured at 734 nm after 3 min reaction in the dark at room temperature.

2.7. Statistical analysis

All experiments were performed in triplicate. GraphPad Prism (GraphPad Software Company, La Jolla, CA, USA) and Statgraphics (version 5.1, Statpoint Technologies Inc., Warrenton, VA) were used for graph plotting and analysis of results, respectively. One-way analysis of variance (ANOVA) and Duncan's multiple-level difference test was used for determining the significant differences among samples. For each analysis, a significance level of 5 % was assumed. The error bars presented on the figures correspond to the standard deviations, and letters were used to label the significance of the difference.

3. Results and discussion

3.1. Heavy metal content and mycotoxins in the fish side streams

Heavy metals have been explored as one of the main pollutants in the water environment. Table 1 shows the contents of As, Cd, Hg, and Pb in the fish side streams selected in this study. In the six side streams involved in this study, the As content was in the range of about 2.0 ~ 2.9 mg/kg, of which rainbow trout skin presented the lowest concentration (1.949 ± 0.030 mg/kg).

Regarding Cd, the contents in the viscera of both fish (0.046 ± 0.001 mg/kg in rainbow trout and 0.054 ± 0.003 mg/kg in sole, respectively) were higher than in the other side streams being the head byproduct those with lower content, 0.008 ± 0.001 mg/kg (rainbow trout head) and 0.018 ± 0.007 mg/kg (sole head). All results were in general below the maximum allowed limit (0.050 mg/kg) (Regulation (CE) 1881/

Table 1
Heavy metals content in different fish side streams.

		As (mg/kg)	Cd (mg/kg)	Hg (mg/kg)	Pb (mg/kg)
Rainbow trout	Head	2.121 ± 0.031^{c}	0.008 ± 0.001^{a}	0.159 ± 0.005^{f}	0.012 ± 0.001^{a}
	Skin	1.949 ± 0.030^{a}	0.010 ± 0.001^{a}	0.110 ± 0.003^{d}	0.032 ± 0.001^{c}
	Viscera	2.040 ± 0.020^{b}	0.046 ± 0.001^{c}	0.099 ± 0.003^{c}	0.065 ± 0.001^{d}
Sole	Head	2.431 ± 0.041^{d}	0.018 ± 0.007^{b}	0.079 ± 0.002^{b}	0.073 ± 0.002^{e}
	Skin	2.852 ± 0.039^{e}	0.022 ± 0.002^{b}	0.050 ± 0.002^{a}	0.025 ± 0.001^{b}
	Viscera	2.070 ± 0.014^{bc}	0.054 ± 0.003^{d}	0.126 ± 0.006^{e}	0.072 ± 0.001^{e}

*None of the extract surpass the EU regulation maximum levels of the heavy metals for human consumption: Cd (0.050 mg/kg); Hg (0.5 mg/kg); Pb (0.3 mg/kg) (Regulation (CE) 1881/2006). One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. For one type of heavy metal, different letter in the same column indicates statistically significant differences ($p < 0.05$).

2006). In rainbow trout side streams, the Hg content in decreasing amount was as follows: head (0.159 ± 0.005) > skin (0.110 ± 0.003) > viscera (0.099 ± 0.003), while for sole, the order was as follows: viscera (0.126 ± 0.006) > head (0.079 ± 0.002) > skin (0.050 ± 0.002). Among them, the highest content (0.159 ± 0.005 mg/kg) was observed in rainbow trout head. In all cases, Hg contents were below the maximum allowable limit (0.5 mg/kg).

Pb levels were also measured. For all side streams, the content of Pb ranged from 0.012 ~ 0.073 mg/kg, obtaining the highest levels in sole head and viscera (about 0.073 mg/kg), however, the levels were also below the maximum allowable limit (0.3 mg/kg). According to the information available in the literature, in farmed rainbow trout liver, As, Cd and Hg were reported at similar levels, ranging from 0.74 to 5.23 mg/kg, 0 to 0.61 mg/kg and 0.097–2.23 mg/kg respectively, however higher levels were reported for Pb (0.713–6.18 mg/kg) by these authors, this concentration exceeded the legislation limits (Fallah et al., 2011). In another study performed in sole from the North-Eastern Mediterranean Sea, Pb and Cd levels were detected in the liver in the range of 0.05–0.37 mg/kg and 0.02–0.06 mg/kg, respectively (Kılıç et al., 2021). These results are in line with those observed in the present study.

Regarding mycotoxins, none of the studied was detected in the fish extracts obtained from rainbow trout and sole side streams under PLE-assisted treatments. Due to the low solubility of mycotoxins in water, it is not to expect an important transference of mycotoxins from the fish side streams to their resulted extracts. The mycotoxins carry-over from feed to edible fish tissues depends on several factors such as the mycotoxin structure and the animal species. Similar to this study, in a previous one AFs, OTA, FUS-X, STG, FBs, ENNs, and BEA were not found in trout samples (Tolosa et al., 2019). Contrary to these results ENNs were detected in sea bass (*Dicentrarchus labrax*) and sea bream (*Sparus aurata*) species in another study (Tolosa et al., 2014).

3.2. Evaluation of antioxidant capacity in fish side streams extracts

Several changes have been observed in the oxygen free radical absorption capacity (ORAC) and $ABTS^{+}$ scavenging capacity (TEAC) of fish side streams extracts (PLE-assisted and no-PLE) before and after *in vitro* digestion (Table 2). Changes in ORAC and TEAC of fish side streams extracts (PLE-assisted and no-PLE) before and after *in vitro* digestion were observed. In previous studies (Wang et al., 2021), we have confirmed that the extracts from PLE-assisted treatment have higher protein content and antioxidant capacity compared to samples without PLE-assisted treatment, however, *in vitro* digestion caused a different effect on the extract's antioxidant capacity.

Table 2
Total antioxidant capacity (ORAC and TEAC) in control (no PLE-assisted) and PLE-assisted treatment extracts from fish side streams (head, skin and viscera) before and after *in vitro* digestion.

	ORAC (μM TE)		TEAC (μM TE)	
	Before digestion	After digestion	Before digestion	After digestion
Rainbow trout				
Head-PLE	2147.56 ± 24.88^{c}	3779.36 ± 20.80^{c*}	940.71 ± 12.14^{d}	995.92 ± 4.39^{f*}
Head-control	1862.30 ± 20.56^{a}	1913.90 ± 9.17^{b*}	358.71 ± 7.14^{a}	346.15 ± 3.07^{b*}
Skin-PLE	8559.76 ± 18.57^{f}	4607.27 ± 8.43^{f*}	1247.86 ± 5.42^{f}	761.46 ± 6.85^{d*}
Skin-control	6313.88 ± 24.40^{e}	4217.93 ± 13.48^{e*}	543.01 ± 2.11^{b}	269.23 ± 3.88^{a*}
Viscera-PLE	4152.41 ± 29.74^{d}	1517.03 ± 12.74^{a*}	713.71 ± 8.57^{c}	899.99 ± 5.84^{e*}
Viscera-control	2060.48 ± 11.22^{b}	4091.94 ± 12.11^{d*}	976.02 ± 9.87^{e}	684.62 ± 8.46^{c*}
Sole				
Head-PLE	1489.20 ± 18.31^{c}	4599.27 ± 16.57^{f*}	790.71 ± 3.57^{f}	149.23 ± 3.07^{a*}
Head-control	722.90 ± 17.10^{a}	1286.93 ± 16.31^{a*}	376.93 ± 9.64^{c}	307.69 ± 18.46^{b*}
Skin-PLE	1583.20 ± 19.30^{d}	2192.54 ± 18.22^{b*}	501.43 ± 25.00^{d}	430.77 ± 7.69^{c*}
Skin-control	1168.71 ± 18.88^{b}	4462.82 ± 12.37^{e*}	210.86 ± 12.43^{a}	ND
Viscera-PLE	4330.90 ± 15.41^{f}	2999.20 ± 14.64^{d*}	287.14 ± 13.14^{b}	553.84 ± 7.93^{d*}
Viscera-control	2724.41 ± 11.43^{e}	2651.24 ± 15.75^{c*}	554.79 ± 16.07^{e}	607.69 ± 15.38^{e*}

ORAC: oxygen radical absorbance capacity; TEAC: Trolox equivalent antioxidant capacity; Results are expressed as mean ± standard deviation. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different lowercase letters indicate statistically significant results in the same column. (*) indicates that the antioxidant capacity of the digested samples changed significantly compared to before and after digestion in the same row. Among them, antioxidant capacity before digestion has been published (Wang et al., 2021) and was used here to compare with the digested groups. ND: not detected (the value of antioxidant capacity is<0).

Firstly, for rainbow trout, digestion improved the oxygen radical absorption capacity of head extract, and the PLE-assisted effect was more obvious (~1.8 times), while $ABTS^{+}$ scavenging capacity did not change significantly before and after the digestion process. After *in vitro* digestion, the oxygen radical capacity and $ABTS^{+}$ scavenging capacity of skin extracts showed a decreasing trend (up to ~ 2 times less). Regarding the extracts obtained from sole side streams, the oxygen radical capacity (ORAC) of head and skin extracts was significantly enhanced after digestion (up to ~ 3.8 times). The difference is that in head extract, PLE-assisted showed a more positive effect. Moreover, digestion also enhances the $ABTS^{+}$ scavenging capacity of viscera extract, with a more obvious increase assisted by PLE compared (1.9 times) with the control group (1.1 times). As it can be seen, PLE-assisted treatment shows a certain positive effect on antioxidant capacity in some extracts.

According to the change in the oxidative capacity of the fish side streams extracts before and after digestion, it can be speculated that the changes in the antioxidant capacity of the extracts before and after digestion were mainly attributed to the bioactive components. In previous studies, the protein content of the extracts has been determined. Digestion process promotes the degradation of proteins into low molecular weight peptides in the stomach and intestine, which are further degraded into amino acids by the small intestinal epithelial cells and enter the body fluid circulation. These fish protein-derived peptides have often antioxidant capacity, so the changes in the antioxidant capacity before and after digestion may be attributed to the different sequences of peptides contained in the different extracts. Likewise, the

effect of digestion on the antioxidant properties of fish-derived protein has also been reported in the bibliography. De la Fuente et al. (2021) used PLE to obtain protein extracts with high antioxidant capacity from salmon side stream, among them, viscera extracts exhibited excellent antioxidant properties, which can be attributed to the small peptides (glycine-proline-proline and glycine-alanine-alanine). Moreover, Ahn et al. (2014) evaluated salmon side streams proteolytic peptides and their antioxidant capacity after *in vitro* digestion. The digested octapeptides showed high oxygen radical scavenging capacity and reduced oxidative stress-induced DNA damage. Vásquez et al. (2022) also explored the antioxidant properties of *Oncorhynchus mykiss* viscera hydrolysate. The oxygen radical absorption capacity of the viscera hydrolysate obtained by Alcalase® did not change significantly, but the hydrolysis increased the $ABTS^{+}$ radical action scavenging capacity and reduced the hydroxyl radical antioxidant capacity, which is also related to the bioactive peptides. In another study, Mirzapour-Kouhdasht et al. (2021) observed that peptide concentration of all fractions obtained from barred mackerel side streams increased after *in vitro* gastrointestinal digestion while antioxidant activities were significantly decreased. These authors attributed this fact to the digestion of 3–10 kDa peptides, which present a high activity, to lower molecular weight peptides (<3 kDa) (Mirzapour-Kouhdasht et al., 2021).

3.3. Mineral content in fish side streams extracts

Minerals, as micronutrients play an important role in human health, they can participate in a variety of enzymatic reactions and the anabolism of nutrients (Lall, 2022). In this study, the mineral content of fish side streams extracts is shown in Fig. 1~2.

As can be seen from Fig. 1, PLE-assisted treatment can significantly increase the content of various minerals in the rainbow trout side streams extracts, including Mg, Fe, Zn, Se and P ($K_{PLE} > 1$). For the head extract, the PLE-assisted treatment had the most significant effect on Fe and Zn contents, with K_{PLE} of 3.14 and 3.91, followed by P (K_{PLE}2.21) > Mg (K_{PLE}1.38) > Se (K_{PLE}1.25). Contrary, the content of Ca in the PLE-assisted treatment was half of that in the control group (K_{PLE} 0.47). Similarly, in the skin extract, PLE-assisted treatment increased the content of Fe and Zn (Fe: K_{PLE} 4.15, Zn: K_{PLE} 4.38), moreover, the effect was higher than in head extracts. PLE-assisted treatment also increased the content of Mg, Se and P in comparison with the control group (Mg: K_{PLE} 1.12, Se: K_{SE} 1.68, P: K_{PLE} 2.01), however, Ca content was about 50 % of the control group. A comparable tendency was observed in viscera extracts, PLE-assisted treatment showed a greater effect on Fe and Se contents with K_{PLE} of 2.20 and 1.94, respectively. In addition, the PLE-assisted treatment also increased the Mg, Zn and P contents in the viscera extracts (Mg: K_{PLE} 1.56, Zn: K_{PLE} 1.10, P: K_{PLE} 1.17), while the content of Ca was slightly lower than in the control group ($K_{PLE} = 0.83$), as was observed in head and skin side streams.

The results show that PLE-assisted treatment can significantly increase the content of Zn in the sole head extract (Fig. 2), which is about 6 times that of the control group (K_{PLE} 5.97), and it also has a significant effect on the content of Fe (K_{PLE} 2.80), Se (K_{PLE} 2.97), Mg (K_{PLE} 1.71) and P (K_{PLE} 2.10). However, the content of Ca in the PLE-assisted treatment extract was lower than in the control group ((K_{PLE} 0.69). PLE-assisted treatment increased the contents of Mg, Zn and P in skin extracts (Mg: K_{PLE} 1.22, Zn: K_{PLE} 1.27, P: K_{PLE} 1.36), but the contents of Ca, Fe and Se were lower than in the control group (Ca: K_{PLE} 0.53. Fe: K_{PLE} 0.72, Se: K_{PLE} 0.61). In the viscera extracts, the content of Ca in the PLE-assisted treatment group was lower than in the control group (K_{PLE} 0.92), while the contents of other minerals were higher than in the control group. Thus, PLE-assisted treatment had a significant positive effect on mineral recovery from fish side streams.

There is scarce information available in the literature about the impact of non-thermal technologies on essential minerals. Emerging technologies do not affect minerals directly but induce changes in the physical properties and structure of the associated macromolecules (Roselló-Soto et al., 2019). The mineral content in fish is related to various factors such as species, growing environment, food, etc. At the same time, there are interactions between some mineral elements, resulting in antagonistic or synergistic effects, thus affecting the recovery of minerals. In addition, minerals such as Fe and Zn can exist in combination with proteins. In this regard, in previous work, Abdollahi et al., (2021) explored the effect of mechanical separation and pH-shift processes on the mineral content of protein recovered from fish

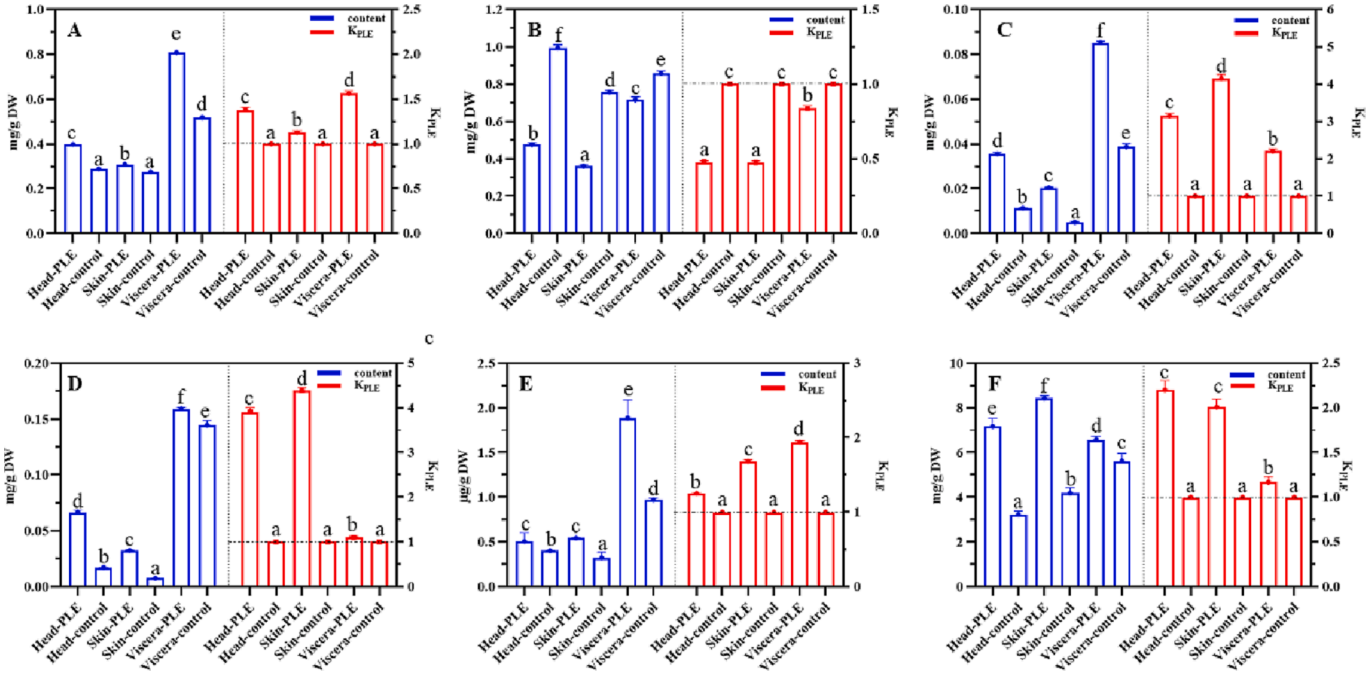

Fig. 1. Mineral content in rainbow trout side streams extracts (PLE-assisted treatment and control group) and the efficiency coefficient K_{PLE}: A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences ($p < 0.05$) for each mineral.

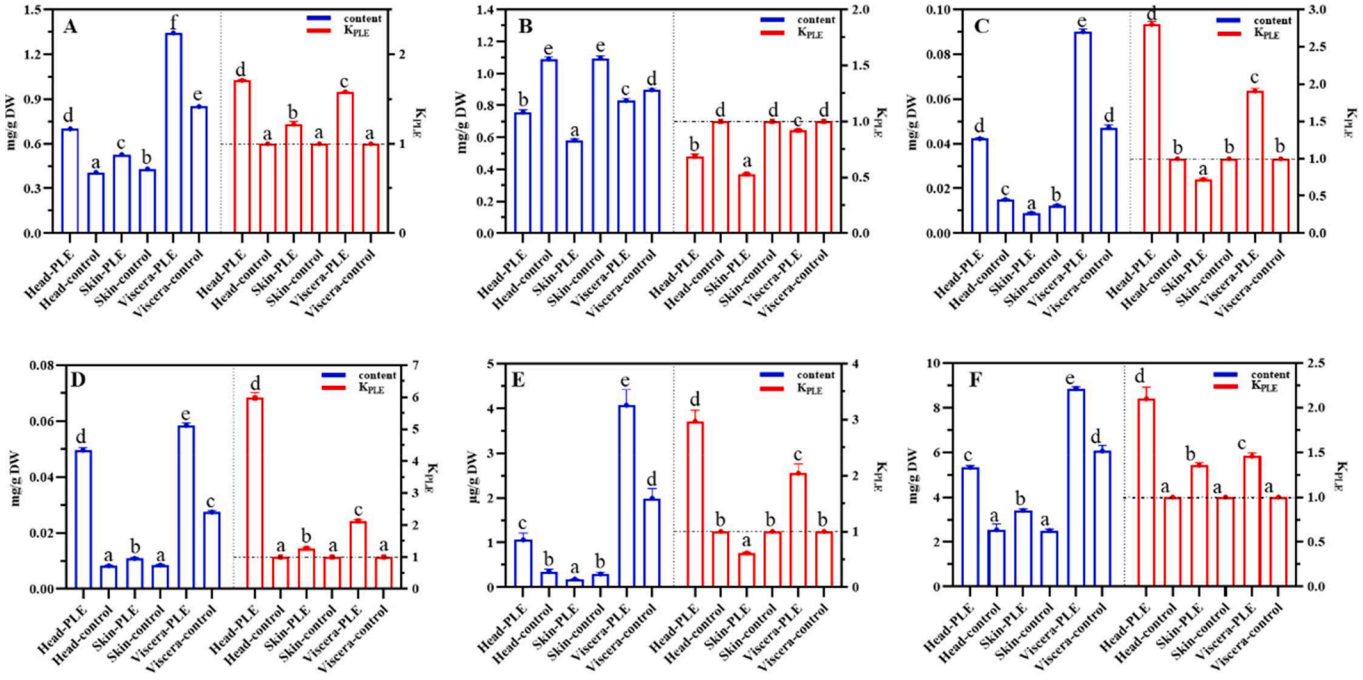

Fig. 2. Mineral content in sole side streams extracts (PLE-assisted treatment and control group) and the efficiency coefficient K_{PLE}: A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences ($p < 0.05$) for each mineral.

backbones. These authors observed that mineral content seemed to be dependent on whether the minerals are located, as well as their binding affinity to proteins vs. their water solubility. For instance, minerals enriched in the bone (Ca and Mg), resulting in more effectively removed by the pH-shift process, while the opposite was observed for minerals with high binding affinity to protein (Zn). Thus, factors such as fish species and the composition of input materials define the content of minerals in the final protein-enriched extracts. In the present work, the results obtained have also been influenced by the fish spice (rainbow trout or sole), the extraction technique (such as PLE), as well as the minerals' solubility and binding affinity. In a previous study, we confirmed that PLE-assisted treatments can change the molecular size

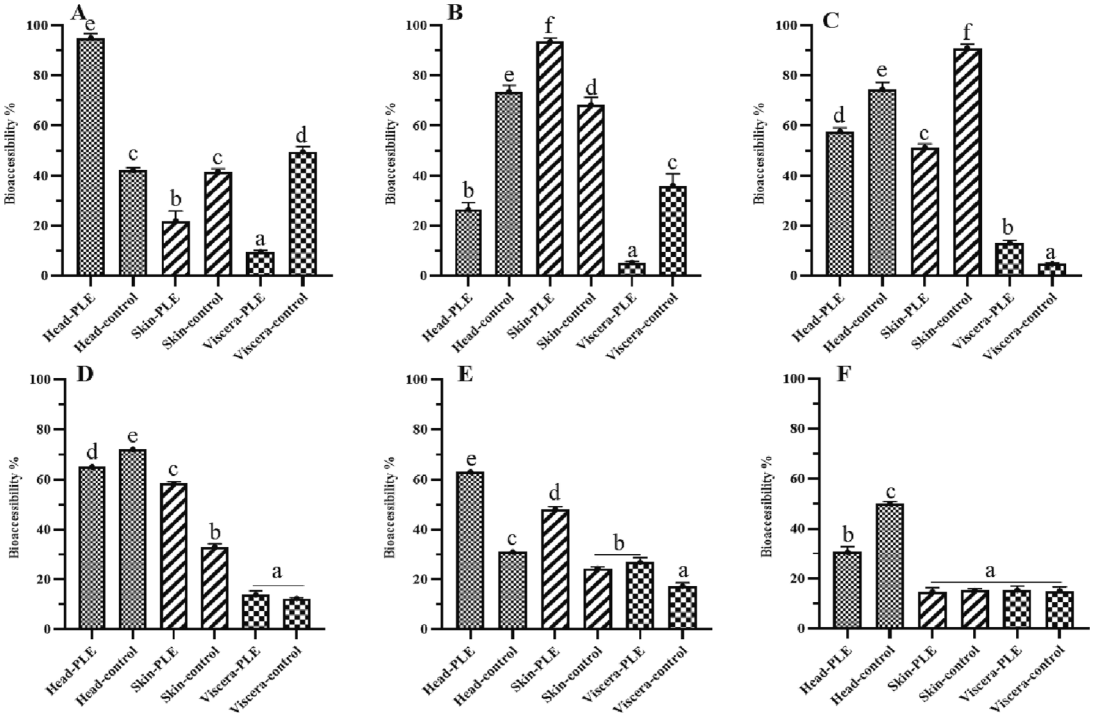

Fig. 3. Bioaccessibility evaluation of minerals in rainbow trout side streams extracts with PLE-assisted treatment. A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences ($p < 0.05$) for each mineral.

distribution of protein in the recovered, which may also be one of the reasons affecting the minerals' recovery (Wang et al., 2021).

3.4. Bioaccessibility of minerals in fish side streams extracts

The bioaccessibility of minerals was assessed by establishing an *in vitro* Minekus digestion protocol. Figs. 3-4 show the bioaccessibility of minerals from fish side streams recoveries in the PLE-assisted treatment and control group. For rainbow trout, the bioaccessibility of different minerals showed large differences, in head extract, PLE-assisted treatment significantly improved the bioaccessibility of Mg and Se, while the bioaccessibility of the other minerals was lower than in the control group. In skin extracts, Ca, Zn and Se showed higher bioaccessibility under PLE-assisted treatment. Moreover, PLE-assisted treatment showed a positive effect on Fe and Se bioaccessibility in viscera extract, however, the bioaccessibility of Mg and Ca in PLE-assisted extracts was significantly lower than in the control.

The bioaccessibility of Mg, Ca and Fe was low in all sole extracts compared to rainbow trout (Fig. 4). Among the skin extract, only Fe presented a slightly higher bioaccessibility in the PLE-assisted treatment group than in the control group, while the other minerals were not significantly affected. In the viscera extract, the bioaccessibility of Zn and Se in the PLE-assisted treatment group was lower than in the control group while the bioaccessibility of Ca was higher. In contrast, the effect of PLE-assisted treatment on Mg, Fe and P content in viscera extract was not significant.

PLE as non-thermal technology has been shown to affect the bioaccessibility of various bioactive compounds, such as phenols and carotenoids, which may be due to the fact that non-thermally assisted treatment could alter the interactions between food matrices or affect the release of the compounds (Cilla et al., 2018; Ribas-Agustí et al., 2019). However, to the best of our knowledge, the effect of PLE-assisted treatment on mineral bioaccessibility in fish side streams has not been reported.

In another study performed employing PEF-assisted treatment in beef muscle, no significant decrease was observed in the release of Fe, K, P, Ca, Na and Mg minerals from the muscle after gastrointestinal digestion. In this sense, PEF treatment may induce higher membrane permeability resulting in higher minerals release (Bhat et al., 2019). Thus, minerals bioaccessibility is related to some factors such as food matrix, and cooking method, among others (Jiang et al., 2021). In addition, complex structures formed between minerals and other components can affect their absorption.

4. Conclusions

This study shows that the heavy metals and mycotoxins in fish side streams are lower than the maximum legislative limit. The antioxidant capacity of high-value extracts from rainbow trout and sole side streams using PLE-assisted treatment can be affected by *in vitro* digestion, which can improve the oxygen radical absorption capacity and $ABTS^{+}$ scavenging capacity in some samples. It is obvious that digestion can enhance the oxygen free radical scavenging capacity of the extract sole head and skin by nearly 3.8 times. Then, PLE-assisted treatment also has a certain positive effect on the recovery of minerals, which can significantly increase the content of Mg, Fe, Zn, Se and P in the side stream of rainbow trout, and also can increase the content of Mg, Se and P in sole side streams. The bioaccessibility of minerals in side streams extracts was evaluated after an *in vitro* digestion model, resulting in a bioaccessibility increase in some extracts, however not in all cases was observed a positive effect. It is worth noting that this study still has limitations. The static *in vitro* digestion model used in this study can only

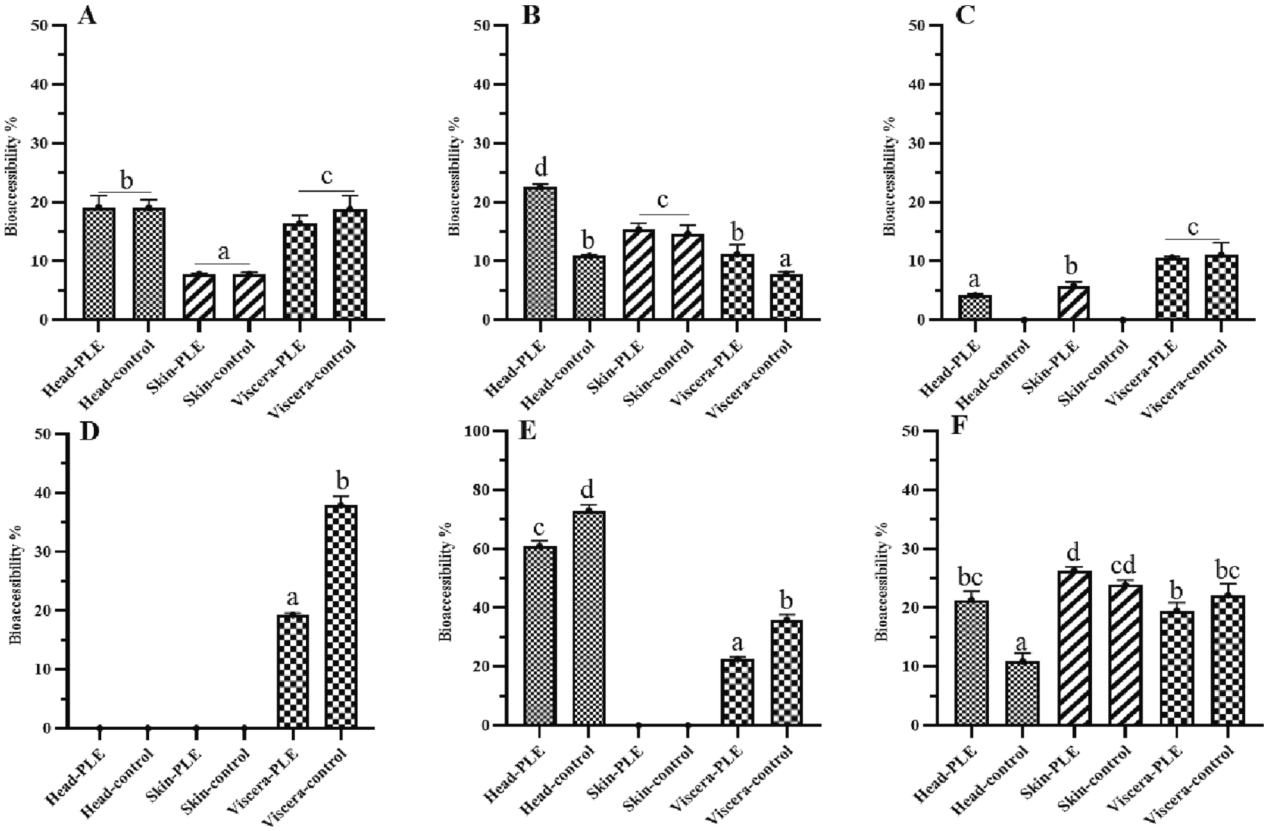

Fig. 4. Bioaccessibility evaluation of minerals in sole side streams extracts with PLE-assisted treatment. A): Mg, B): Ca, C): Fe, D): Zn, E): Se, F): P. One-way ANOVA was performed using Duncan's multiple comparison post-hoc test to assess statistical significance between groups. Different letters in the bars indicate statistically significant differences ($p < 0.05$) for each mineral.

simulate the process of *in vitro* digestion through mechanical and continuous stirring and cannot simulate the complete digestion and absorption process. It still needs to be verified and explored in combination with *in vivo* experiments. Understanding the absorption efficiency and properties of high-value components from fish side streams in the digestion process could help to find a more reasonable method to improve the utilization rate of high-value compounds and make them more valuable.

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6. Institutional Review Board Statement

Not applicable.

7. Informed Consent Statement

Not applicable.

CRediT authorship contribution statement

Min Wang: Investigation, Methodology, Formal analysis, Software, Visualization, Writing – original draft. **Jianjun Zhou:** Investigation, Methodology, Formal analysis, Software, Visualization, Writing – original draft. **Noelia Pallarés:** Conceptualization, Methodology, Writing – review & editing, Supervision. **Juan Manuel Castagnini:** Software, Writing – review & editing, Supervision. **María Carmen Collado:** Resources, Visualization, Supervision, Writing – review & editing. **Francisco J. Barba:** Conceptualization, Methodology, Resources, Visualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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