SUPERCRITICAL FLUID EXTRACTION PRIOR PULSED ELECTRIC FIELDS TO IMPROVE HIGH-ADDED-VALUE COMPOUNDS RECOVERY FROM SALMON SIDE STREAMS: EVALUATION OF PROTEIN, BIOACTIVE PEPTIDES AND MINERALS

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Supercritical Fluid Extraction prior Pulsed Electric Fields to improve high-added-value compounds recovery from salmon side streams: Evaluation of protein, bioactive peptides and minerals

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Abstract

A sequential extraction process combining supercritical fluid extraction (SFE: 25.0 MPa, 75 min, 50.0 ºC, and 10.0 mL/min flow) with pulsed electric fields (PEF: 3.0 kV/cm, 300 kJ/kg for head/backbones, 124.8 kJ/kg for viscera) or conventional extraction (CE: 60 min, 300 rpm) was optimized to enhance the recovery of high-added-value compounds, including proteins, bioactive peptides, minerals, heavy metals, and total antioxidant capacity (TAC) from salmon side streams. Protein levels increased notably with PEF, particularly in viscera liquid extracts, reaching 15.5 g/100g. Bioactive peptides displayed anti-inflammatory, immunomodulatory, anticancer, hypolipidemic, and antithrombotic potential. Viscera liquid extracts showed high TAC (402.8 and 3263.6 μM). ICP-MS analysis revealed PEF treatment helps solid matrices retaining more minerals and heavy metals. Principal component analysis (PCA) on proteins, peptides, minerals, and heavy metals elucidated PEF's impact, identifying two main components affecting the salmon matrix and extract composition.

Keywords: pulsed electric field (PEF); salmon solid matrices and liquid extracts; protein; bioactivity peptides; mineral and heavy metal.

1. Introduction

Although the consumption of fresh fish is decreasing due to its high price for the consumer, the market for frozen filleted fish has increased exponentially in recent years, implying the generation of a high amount of side streams (Nelluri, Kumar Rout, Kumar Tammineni, Joshi, & Sivaranjani, 2024). According to FAO, capture fisheries production reached 91.2 million tons in 2021, and fish production is expected to reach up to 194 million tons by 2026 (FAO, 2023; Ganjeh, Saraiva, Pinto, Casal, & Silva, 2023).

The increasing number of fish side streams generated poses a serious environmental threat, requiring effective treatment and proper disposal (Nelluri et al., 2024). These side streams represent a great opportunity since they are an important source of high-added-value compounds such as proteins and bioactive peptides, essential fatty acids as well as minerals among others, although traditionally they have hardly been exploited or have been used for low-income purposes (Wang, Zhou, Selma-Royo, Simal-Gandara, Collado, & Barba, 2021). Moreover, when fish side streams are used to effectively recover high-added-value compounds such as proteins, omega-3 fatty acids, vitamins, and minerals, they are supporting sustainability by increasing resource consumption and reducing waste by improving their nutritional value, which is in full correspondence with the principles of a circular economy (Rathod et al., 2024).

Atlantic salmon (*Salmo salar*) has an interesting nutritional composition and flavor, which may explain the remarkable growth in the global marketing demand. Rich in high-biological value proteins and polyunsaturated fatty acids (PUFA) (such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) (Asensio-Grau, Calvo-Lerma, Heredia, & Andrés, 2021; de la Fuente et al., 2022). Moreover, it is a potential source of compounds with umami activity, especially Arg-Val and Ser-Asn, which are

the key compounds contributing to the umami taste (Dong, Zhang, Wang, Huang, Wang, & Qin, 2024). The generation of fish side streams represents between 9-12%, 12-18%, and 9-15% of the total produced, being head, viscera, and backbones as the most important ones (Villamil, Váquiro, & Solanilla, 2017). Thus, it represents a matrix of high interest to be valorized in the form of high-added-value compounds´ recovery.

Conventional methods used to recover compounds from fish side streams involve the use of high temperatures and long extraction times. In order to obtain more eco-sustainable processes for the recovery of these compounds, our group has carried out previous studies with the aim of evaluating the use of innovative technologies such as PEF (Zhou, Wang, Berrada, Zhu, Grimi, & Barba, 2022), pressurized liquid extraction (Wang, Zhou, Pallarés, et al., 2021) or microwaves (de la Fuente et al., 2022), among others, for the extraction of compounds from fish side streams, obtaining promising results.

However, in the case of protein recovery at the industrial level, it involves a prior defatting stage to facilitate its extraction (Pellerin & Doyen, 2024). Previous studies have used PEF or conventional extraction without defatting the sample, which considerably limited protein extraction efficiency (Martí-Quijal, Castagnini, Ruiz, & Barba, 2023). For this reason, the present study will focus on evaluating the influence of defatting to facilitate the subsequent extraction of proteins and other non-lipophilic compounds assisted PEF and compared with the conventional extraction.

The defatting stage at the industrial level is usually carried out using hexane (Walters, Lima Ribeiro, Hosseinian, & Tsopmo, 2018) involving a high consumption of toxic solvents, such as defatting is an important method for producing high-quality food from oats (Doehlert, Moreau, Welti, Roth, & McMullen, 2010; Senarathna & Malalgoda, 2024). Therefore, what is intended in this work is, in addition to promoting

protein extraction, implementing a more eco-sustainable process for defatting the sample such as supercritical fluid extraction.

Supercritical fluid extraction (SFE) is an environmentally friendly technology that eliminates the need for harsh chemicals, unlike conventional solvent extraction, and it is renowned for extracting active ingredients from foods and natural products (Xia et al., 2022). Previous researchers have concluded that SFE involves two main steps: the solubilization of bioactive compounds from the solid matrix and their separation in supercritical carbon dioxide extraction (SC-CO_2) solvent (Pagano, Campone, Celano, Piccinelli, & Rastrelli, 2021). SFE technologies have been used to extract polyunsaturated fatty acids (PUFAs) from animal tissues, particularly from fish oil derived from various species (Haq, Ahmed, Cho, & Chun, 2017; Hidalgo-Vázquez et al., 2022). This technology based on the use of CO_2 in a supercritical state has the main advantages with green and safe extraction (Amador-Luna, Herrero, Domínguez-Rodríguez, Ibáñez, & Montero, 2024), economical (Molino et al., 2020); and efficient in extraction (Haq et al., 2017).

On the other hand, the use of PEF, a technology involving placing a product between two electrodes, typically submerged in an aqueous solution, and exposing it to electric fields, as a technique to improve the efficiency of extraction of compounds is well-known and has been recently applied for the extraction of biomolecules from fish side streams obtaining good results (Martí-Quijal et al., 2023; Wang, Zhou, Collado, & Barba, 2021b). That is why in the present study the use of defatting using SFE combined with extraction either assisted by PEF or without PEF (conventional) is proposed for the recovery of proteins, bioactive peptides, minerals, and antioxidant capacity. In addition, to verify the safety of the extracts obtained, the content of heavy metals in the samples under study will also be evaluated.

This approach, which has seldom been investigated, provides scientific guidance for the recovery of biomolecules with different nutritional and biological activities from salmon side streams. It also tries to establish an effective method for the fish side streams industry, promoting the sustainable utilization of fish side streams.

2. Materials and methods

2.1. Samples

The frozen-dry Atlantic salmon (*Salmo salar*) was provided by the Department of Nutrition and Feed Technology of the Norwegian Institute of Food, Fisheries, and Aquaculture Research (Nofima) (Bergen, Norway). The Atlantic salmon (*Salmo salar*) was processed in a local laboratory, where the head, backbones, and viscera were separated. These salmon heads, backbones, and viscera were subsequently freeze-dried and crushed into the sample powder using the Pulverisette 11(Fritsch, Germany). The prepared salmon samples were then stored at -20.0 °C for future experimental analyses.

2.2. Chemicals and Reagents

2,2′-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid (ABTS), 2,2′-azobis (2-amidinopropane) dihydrochloride (APPH), and sodium nitrate were also from Sigma-Aldrich. The purity of all reagent compounds is above 99%. Deionized water was obtained from the Milli-Q SP Reagent Water System (Millipore Corporation, Bedford, MA, USA).

2.3. Treatments

For supercritical fluid extraction (SFE) extraction, a JASCO system (JASCO, Tokio, Japan), located at the laboratory of ALISOST team at the Faculty of Pharmacy and Food

Sciences of the Universitat de València (València, Spain) was used. The extraction condition for SFE to remove the lipid compounds from the freeze-dried salmon side streams (head, backbone, and viscera) were: 25.0 MPa, 75.0 min, 50.0ºC, 10.0 mL/min flow (1.0 mL/min of ethanol absolute + 9.0 mL/min of CO_2) (Fig. 1).For the PEF pretreatment, a PEF-Cellcrack III (German Institute of Food Technologies (DIL)) equipment (ELEA, Quakenbrück, Osnabrück, Germany) located at the laboratory of ALISOST team at the Faculty of Pharmacy and Food Sciences of the Universitat de València (València, Spain) was used. The conductivity was maintained between 1000 and 2000 µS/cm. A sample of 1.5 g of defatted salmon side streams was mixed with 150.0 g of deionized water and the PEF-specific energies were 3.0 kV/cm and 300.0 kJ/kg for the head or backbone, and 3.0 kV/cm 124.8 kJ/kg for the viscera (Martí-Quijal et al., 2023; Wang, Zhou, Pallarés, et al., 2021), separately. The conventional extraction (CE) after SFE was 1.5 g of salmon side streams (head, backbone, and viscera removed fat) with 150.0 g deionized water magnetic stirring at 300*x*rpm for 60 min.

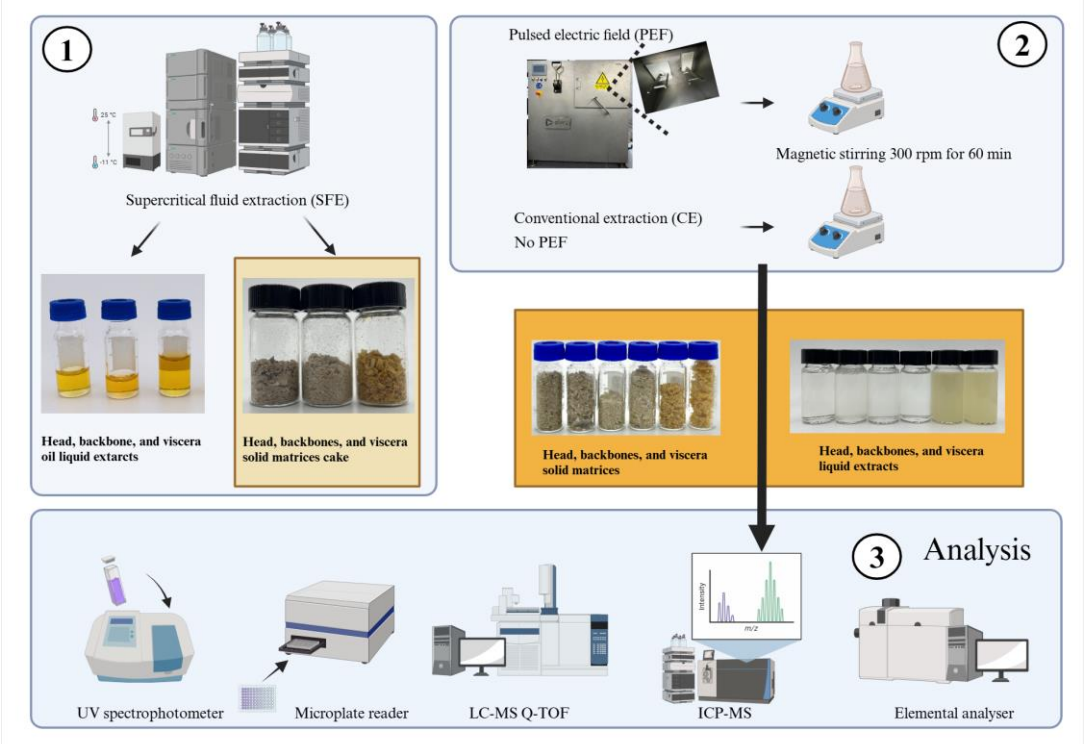

Fig. 1. The process for supercritical fluid extraction (SFE) before pulsed electric fields

(PEF) or conventional extraction (CE) to improve high-added-value compounds recovery from salmon side streams (Created in BioRender).

2.4. Determination of protein content

All the solid matrices and liquid extracts were freeze-dried (Model 10N, Gedilab S.L., Spain) at -61.0 ºC for 72 h and then crushed into the sample powder using the Pulverisette 11(Fritsch, Germany). for 3 min. A Flashsmart Elemental Analyzer (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) was applied to determine the protein content referenced to the previous Dumas method (Serrano, Rincón, & García-Olmo, 2013). The weighed powers were wrapped in tin foil and burned in high purity in the combustion reactor, and the helium was carried as the gas. The total nitrogen values were obtained after the determination in the Elemental Analyzer. The total protein content was determined using the total nitrogen values and a protein-nitrogen conversion factor of 6.25, appropriate for fish and fish side streams (de la Fuente, Pallarés, Berrada, & Barba, 2021).

2.5. Bioactivity peptide tests

The liquid chromatography-tandem mass spectrometry (LC-MS/MS) was applied to determine the bioactive peptides present in the samples studied, according to the method previously described (de la Fuente et al., 2021; Martí-Quijal et al., 2023). After all the sequences were identified, only the possibility of 1 was chosen to continue the potential bioactivity peptides analysis in the BIOPEP-UWM database (Minkiewicz, Iwaniak, & Darewicz, 2019). In this research, bioactive peptides with five different bioactivities (anti-inflammatory, immunomodulatory, anticancer, hypolipidemic, and antithrombotic) have been isolated in the solid matrices and liquid extracts obtained

from salmon side streams.

2.6. Inductively coupled plasma spectrometer detector (ICP-MS) in mineral and heavy metals tests

The measurement of ICP-MS for mineral and heavy metal tests has been carried out according to (Wang, Zhou, Pallarés, Castagnini, Carmen Collado, & Barba, 2023). Fish side streams were mineralized in a microwave oven (MARS, CEM, Vertex, Spain) and three hundred milligrams of samples were digested in a Teflon vessel with HNO_3 (14 M) and H_2O_2 (30% v/v) at 800 W and 180 ℃ for 15 minutes to remove nitrogen. After cooling, the samples were filtered and diluted with distilled water and then conducted by ICP-MS.

2.7. Total Antioxidative Capacity (TAC) tests

2.7.1. Trolox equivalent antioxidant capacity (TEAC) assay

The $ABTS^+$ free scavenging capacity was tested of the absorbance (0.70 ± 0.02 at 734 nm) in the TEAC assay referenced to the previous method (de la Fuente, Aspevik, Barba, Kousoulaki, & Berrada, 2023). 440.0 μL potassium persulfate solution (140 mM) and 15.0 mL ABTS (7 mM) were mixed and stored in darkness for 12-16 h before use as the mother solution. The working solutions (0.70 ± 0.02 at 734 nm) were prepared with the mother solution and the absolute ethanol. 100 μL of diluted samples was combined with 2.5 mL and absorbance was measured after a 3 min reaction period. The standard curve was prepared to calculate the $ABTS^+$ free scavenging capacity for salmon liquid extracts obtained from the PEF treatment with removing the fat previously.

2.7.2. Oxygen radical absorbance capacity (ORAC) assay

The Trolox equivalent antioxidant capacity was measured at the wavelengths of 528 ±

20 nm referenced to the previous method (de la Fuente et al., 2023), using the microplate reader. Fifty microliters of phosphate buffer or diluted liquid extracts, 50 μL fluorescein, and μL Trolox (1 mM) were added to the 96-well plate. The 96-well plates were incubated at 37.0 ℃ for 10.0 mins, followed by the 25 μL AAPH (221.25mM) added. Besides, the measurements are operated during the 37℃ for 180 minutes to investigate the oxygen radical scavenger activity.

2.8. Statistical Analysis

All tests were conducted in triplicate, and the average values are presented as mean ± SD. The data were analyzed using one-way and three-way analysis of variance (ANOVA) and Duncan's multiple range test to identify significant differences ($P < 0.05$). Graphs were created using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA), while statistical analyses, including principal component analysis (PCA) to explore the connection between various variables and principal parameters, were performed using SPSS 20.0 (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Protein content

The protein contents of solid matrices and liquid extracts obtained from salmon side streams after SFE+pulsed electric fields (PEF) treatment were evaluated and compared with SFE+conventional extraction (CE) and the results are presented in Fig. 2. As can be observed in the figure, the lowest and highest protein contents for heads were 9.7 g/100 g (SFE+CE-liquid) and 90.3 g/100g (SFE+CE-solid), respectively, while for backbones, the lowest and highest protein values were 13.6 g/100g (SFE+CE-liquid) and 86.3 g/100g (SFE+CE-solid), respectively. Finally, for viscera, the lowest value

was 33.0 g/100g (SFE+PEF-solid) while the highest value (67.1 g/100g) was found for SFE+PEF-liquid. Significant differences ($P < 0.05$) were obtained in the protein contents of the three side streams, independently of the treatments (SFE+PEF or SFE+CE) and the matrices evaluated (solid matrices or liquid extracts).

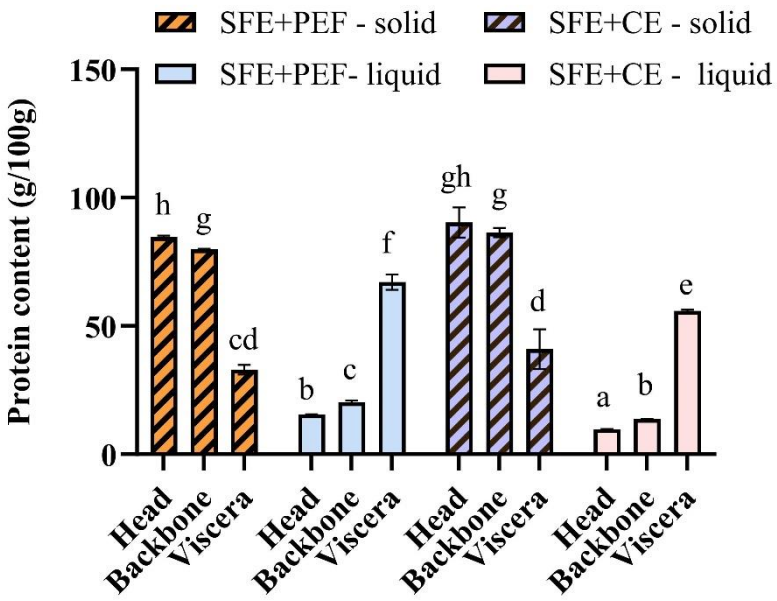

Fig. 2. Protein contents of solid matrices (SFE+PEF-solid; SFE+CE-solid) and liquid extracts (SFE+PEF-liquid; SFE+CE-liquid) obtained from supercritical fluid extraction defatted salmon head, backbones and viscera and subsequent PEF (SFE+PEF) or conventional extraction (SFE+CE).

It should also be noted that, for liquid extracts, the protein values were lower for head and backbones compared to the solid matrices, while the opposite trend was found for viscera. This fact can be attributed to the different tissue structures of the different side streams, being easier to extract the proteins from the initial SFE solid sample into liquid for viscera. Moreover, interestingly, no significant differences ($P>0.05$) were found for protein recovery between SFE+PEF-solid and SFE+CE-solid, while significant differences ($P<0.05$) were found between SFE+PEF-liquid and SFE+CE-

liquid, independently of the side stream evaluated. These results are in agreement with previous findings of our team which reported that PEF pretreatment promoted the protein extraction from the side streams into the liquid. For example, in the study of (Wang, Zhou, Collado, & Barba, 2021a), they observed how PEF-assisted treatment significantly increased ($P < 0.05$) the protein extract efficiency that both rainbow trout skin and sole skin reached around 80% followed by the viscera and head.

Recent findings underscore the potential for achieving high protein yields through optimized extraction conditions, including ultrasound-assisted extraction, chemical solvents, and isoelectric solubilization precipitation (ISP). A novel sequential extraction process using ISP increased protein recovery significantly, from 49% with 0.1M HCl and 64% with 0.1M NaOH to 98.6% with a 0.1M HCl/NaOH sequential extraction (Álvarez, Lélu, Lynch, & Tiwari, 2018). Optimal ultrasound-assisted extraction conditions, identified using response surface methodology, included a 14 g/kg NaOH concentration, 428 W ultrasonic power, and a 52-minute extraction time, yielding a 64.89% protein recovery (Wu, Jia, Wen, Yu, Zhao, & Hu, 2021). Ultrasound at 60% amplitude for ten minutes in a 0.1M NaOH solution achieved a 94% protein recovery in a single step (Álvarez et al., 2018). These high protein yields typically require acidic or alkaline aids. Whereas our study employs defatted salmon by-products with PEF or non-PEF treatments and magnetic stirring with water, demonstrating an environmentally friendly approach to protein extraction. Especially, the high contents in the solid matrices also display the potential to be utilized in food consumption and health promotion.

3.2. Bioactive peptides

Bioactive peptides are composed of short chains of amino acids, consisting of 2 to 20

residues and having a low molecular weight of less than 6 kDa, produced through the process of proteolysis (Chalamaiah, Yu, & Wu, 2018; Sila & Bougatef, 2016). Normally, the mechanisms responsible for these functional properties are influenced by the structural characteristics of bioactive peptides, including their amino acid composition, types of amino acid at the C- or N- terminus, hydrophobicity, molecular weight, and net charge (Kurnianto, Defri, Syahbanu, & Aulia, 2024). The anti-inflammatory, immunomodulatory, anticancer, hypolipidemic, and antithrombotic activity sequences have been identified in this study for both solid matrices and liquid extracts, showing the quantities for each activity sequence of various salmon side streams (removing the fat previously) in Fig. 3 (A-D).

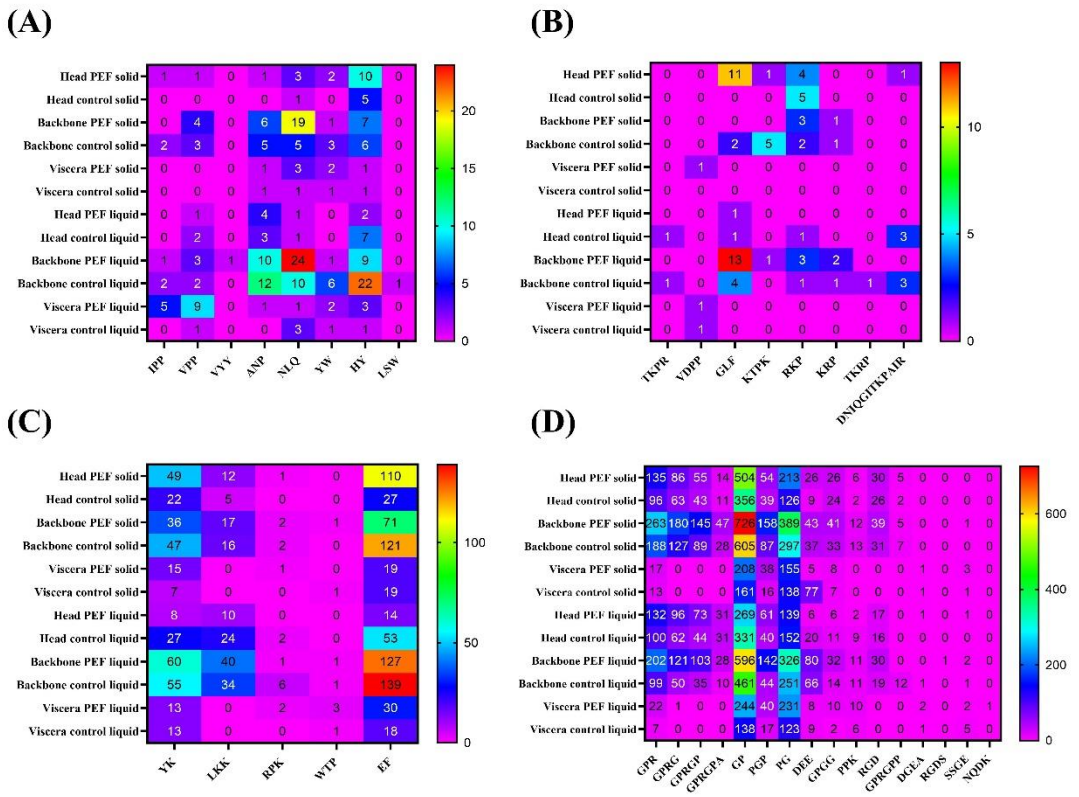

Fig. 3. Bioactive peptides bioactivity sequences determined from the solid matrices and liquid extracts obtained from the pulsed electric fields (PEF) treated after removing fat assisted by supercritical fluid extraction. (A), anti-inflammatory activity sequence; (B),

immunomodulating activity sequence; (C), anticancer and hypolipidemic activity sequence; (D), antithrombotic activity sequence.

Inflammation plays a crucial role in mammalian physiology, underlying a wide variety of physiological and pathological processes (Medzhitov, 2008). Fig. 3 (A) shows the anti-inflammatory sequences (IPP, VPP, VYY, ANP, NLQ, YW, HY, and LSW) identified from salmon solid matrices and liquid extracts after SFE. The HY sequence is the most commonly found for anti-inflammatory activity, with the SFE+CE backbone presenting the highest sequence number (22). As for the anti-inflammatory activity, most of the anti-inflammatory activity sequences were isolated in the liquid extracts and not in the solid matrices. In addition, the salmon side streams (solid and liquid) in SFE+PEF have been found to have more anti-inflammatory activity sequences compared with the SFE+CE. Recent research found three fish roe-derived extracts (sardine, horse mackerel, and sea bass) have been obtained, and it determined, that those aqueous extracts are characteristic with no cytotoxic concentration and exhibit potent anti-inflammatory and antioxidant bioactivity (Guedes, Vieira, Reis, Ferreira, & Neves, 2022). Similarly, these results are in full agreement with those obtained by our team's previous studies, which also found that the sole-PEF-skin extracts presented a significant anti-inflammatory potential by inhibiting the TNF-α activity around 35%, the anti-inflammatory potential of the rainbow trout and sole fish side streams are determined in the NF-κB action inducted by TNF-α (Wang, Zhou, Pallarés, et al., 2021). The anti-inflammatory experiment of this fish extract directly confirmed that the extract of fish side streams has anti-inflammatory activity, which can corroborate the results of the anti-inflammatory active peptides in this experiment.

On the other hand, immunomodulatory compounds protect the body from invading

pathogens and play a significant role in health (Chalamaiah et al., 2018). The first immunomodulatory peptide was discovered in the enzymatic hydrolysate of human casein (Parker et al., 1984). Eight types of immunomodulatory activity sequences (TKRR, VDPP, GLF, KTPK, RKP, KRP, TKRP, and DNIQGITKPAIR) have been identified in our salmon side streams (both in solid matrices and liquid extracts), with GLF being the most prevalent, especially in backbone-SFE+PEF-liquid (19 in total). Moreover, the backbone showed the highest amount of immunomodulatory bioactive peptides if both SFE+PEF and SFE+CE, solid and liquid matrices, were considered, compared to head and viscera, being backbone liquid extracts, either with or without PEF treatment, those with more immunomodulatory bioactive peptides. Regarding head, the most immunomodulatory activity sequences were found in the solid matrices compared with the head liquid extracts, showing the head- SFE+PEF-solid the highest total number (17). Viscera presented the lowest amount of immunomodulatory bioactive peptides either in the solid matrices (1) or liquid extracts (2). A previous study explored the immunomodulatory activity of protein hydrolysate derived from tilapia with the protein substrates hydrolyzed by *Vilibacillus halodenitrificans* SK1-3-7 proteinase, observing that tilapia hydrolysate enhanced the innate immunity by promoting IL-1β and COX-2 (Toopcham, Mes, Wichers, & Yongsawatdigul, 2017).

The potential anticancer mechanism for the food-derived peptides through the body (scavenging reactive oxygen species (ROS) and reducing oxidative damage (Kaushik, Kaushik, & Parvez, 2022)) and the gut (modulating the immune response to reduce the risk of inflammation-related cancers (Norouzi, Mirmohammadi, & Houshdar Tehrani, 2022)) (Yan et al., 2024) has been described. Most of the anticancer sequences have been identified in backbone-solid (141) and backbone-liquid (198), showing PEF treatment a higher number compared to SFE+CE. Especially, the highest

number sequence of YK (60) was identified in the backbone- SFE+PEF-liquid, being YK the most commonly found and remaining the highest amounts in our samples. Moreover, the quantities found in the SFE+PEF solid matrices (head, backbone, and viscera) presented more sequences compared with the SFE+CE, while the liquid extracts showed the opposite trend.

Only one hypolipidemic activity sequence (EF) has been identified in our solid matrices and liquid extracts, being the profile sequences: i) head-SFE+PEF-solid (110), ii) head-SFE+CE-solid (27), iii) head-SFE+PEF-liquid (14), and iv) head-SFE+CE-liquid (53).

On the other hand, food-derived antithrombotic peptides, as potential ingredients in health-promoting functional foods targeting thrombus, have attracted increasing attention because of their high biological activities, low toxicity, and ease of metabolism in the human body (Cheng, Tu, Liu, Zhao, & Du, 2019). The antithrombotic activity is a significant biological activity found in salmon side streams, after removing the fat), obtaining 16 various types of sequences (GPR, GPRG, GPRGP, GPRGPA, GP, PGP, PG, DEE, GPGG, PPK, RGD, GPRGPP, DGEA, RGDS, SSGE, and NQDK) in our samples. The highest (726) and second highest quantities (605) found were GP sequences found in the backbone-SFE+PEF-solid and backbone-SFE+CE-solid, respectively. On the other hand, significant quantities of antithrombotic sequences were also found in the salmon backbones. Specifically, the GP was notably present at 726 in backbone-PEF-solid, 605 in backbone control solid, and 596 in backbone-PEF-liquid.

In the research, the five biological activities with the activity sequence have been determined and isolated for the salmon side streams (removing the fat previously assisted by SFE), being backbones those which exhibited excellent antithrombotic activities.

3.3. Analysis of mineral profiles

Macro and micro minerals are the essential nutrients to maintain the human body's daily physiological functions, such as calcium (Ca), magnesium (Mg), potassium (K), sodium (Na), and phosphorous (P) for macro mineral and iron (Fe), zinc (Zn), copper (Cu) as micro minerals (de la Fuente et al., 2023; Jha, Panda, Kishore, Mathew, & C.N, 2021). Microminerals (Cu, Fe, Mn, Se, and Zn) play an important role in the structural fraction of enzymes, in the formation of erythrocyte cells (cobalt (Co), I, and Fe), in the regulation of glucose levels of activation of antioxidant enzymes (Mo), and may be involved in the various processes of the immune system (Cu, selenium (Se), and Zn) (Gharibzahedi & Jafari, 2017).

The mineral profiles of salmon head, backbones, and viscera in the samples defatted with SFE and subsequently pretreated with or without PEF treatment were investigated (Figure 4). Besides, the three-way ANOVA analysis (treatment (PEF or CE); matrix (solid matrices or liquid extracts); and salmon side stream (head, backbone, or viscera)) was performed for each mineral.

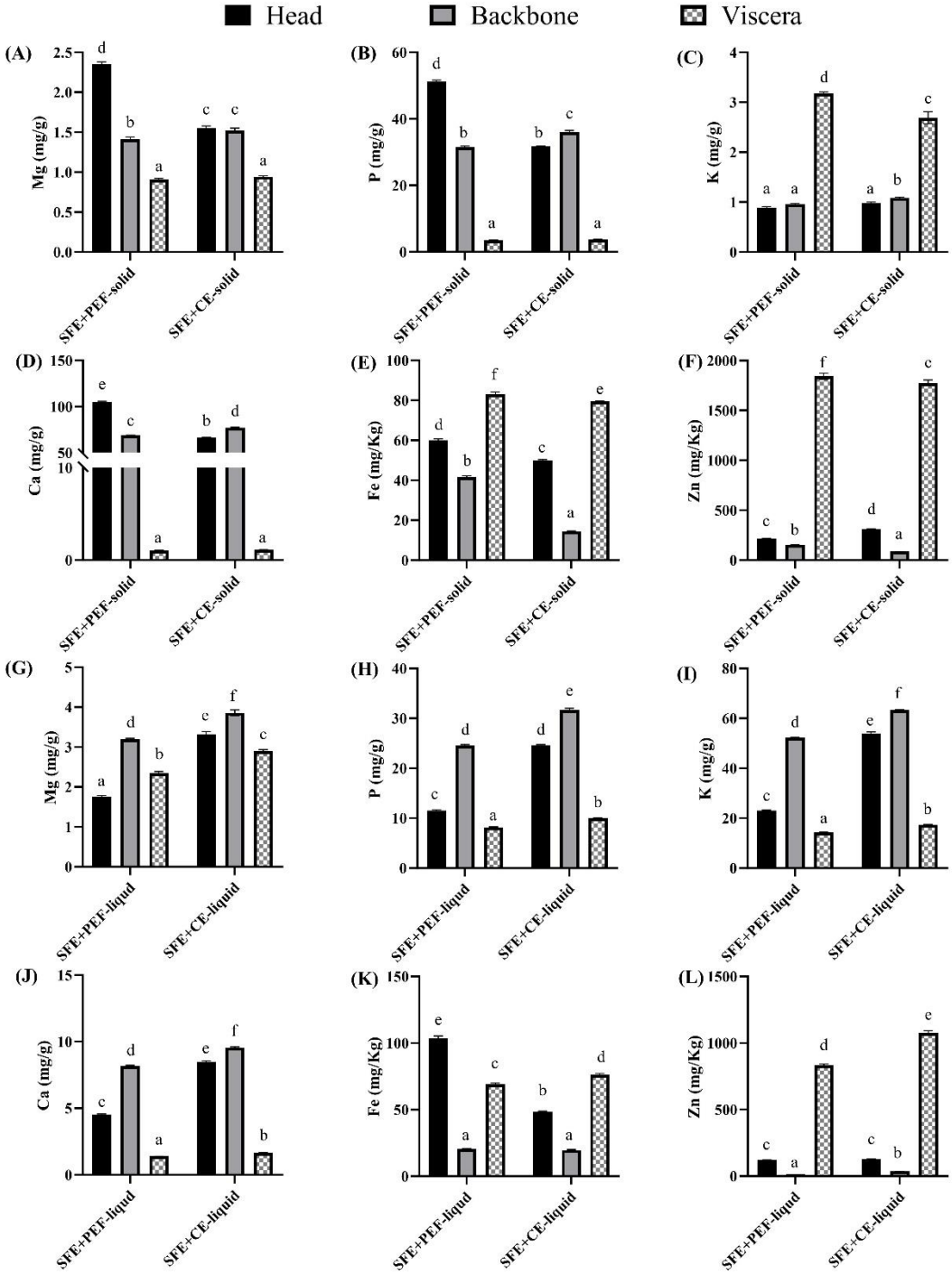

Fig. 4. The mineral profiles of solid matrices and liquid extracts obtained from the pulsed electric fields (PEF) treated after removing fat by supercritical fluid extraction. (A-F) for the solid matrices; (G-L) for the liquid extracts.

The main three sources of variation for Mg were matrix (solid matrices or liquid

extracts), salmon side streams×treatment (PEF or CE), and salmon side streams (head, backbone, or viscera), which accounted for the 60.0%, 11.35, and 10.2% of the total variation, respectively. As can be observed in the type of matrix Fig 4(A-G), PEF treatment tends to increase Mg retention in the solid matrices compared to the CE but decreases it in the liquid extracts. In solid matrices, the head (SFE+PEF or SFE+CE) presented the highest values, followed by backbone and viscera. For instance, the highest and lowest amount of Mg in the solid matrices were found in the head-SFE+PEF-solid (2.4 mg/g) and viscera-SFE+PEF-solid (0.9 mg/g), respectively, while in the liquid extracts the highest Mg values were found in the backbone-SFE-liquid (3.9 mg/g) while head-SFE+PEF-liquid (1.8 mg/g) had the lowest values.

The main three sources of variation for P were salmon side streams (head, backbone, or viscera), salmon side streams×matrix, and matrix (solid matrices or liquid extracts), which accounted for 62.4%, 17.2%, and 7.5% of the total variation, respectively. When P was evaluated, it was found that PEF treatment resulted in higher P retention in solid matrices but lower values in the liquid extracts (Figure 4(B-H)). Regarding the viscera, both for solid matrices and liquid extracts, the SFE+PEF and SFE+CE exhibited the lowest values compared with the head and backbone. In this line, in the solid matrices, the highest and lowest content were found for head-SFE+PEF-solid (51.3 mg/g) and viscera-SFE+PEF-solid (3.6 mg/g), while in the liquid extracts, backbone-SFE+CE-liquid has the highest P contents (31.7 mg/g) while viscera-SFE+PEF-liquid present the lowest P values (8.2 mg/g).

The main three sources of variation for K were matrix (solid matrices or liquid extracts), salmon side streams×matrix, and salmon side streams (head, backbone, or viscera), which accounted for 62.3%, 15.8%, and 13.1% of the total variation, respectively. For K, PEF treatment decreased K content in solid matrices but increased

it in the liquid extracts (Fig. 4(C-I)), observing the highest and lowest contents in the viscera-SFE+PEF-solid (3.2 mg/g) and head-SFE+PEF-solid (0.9 mg/g), respectively, while in the liquid extract, backbone-SFE+CE-liquid presented the highest values (63.4 mg/g) while viscera-SFE+CE-liquid had the lowest (14.3 mg/g).

On the hand, the main three sources of variation for Ca were matrix (solid matrices or liquid extracts), salmon side streams (head, backbone, or viscera), and salmon side streams×matrix, which accounted for 42.6%, 30.1%, and 22.4% of the total variation, respectively. On the other hand, PEF treatment led to higher Ca content in solid matrices, with a noticeable reduction in liquid extracts (Fig. 4(D-J)). For instance, the highest recovery of Ca in solid matrices was from the head-SFE+PEF-solid (104.9 mg/g) and lowest from viscera-SFE+PEF-solid (1.0 mg/g). Regarding liquid extracts, backbone-SFE+CE l-liquid had the highest values (9.6 mg/g) while viscera-SFE+PEF-liquid presented the lowest amount (1.4 mg/g).

The main three sources of variation for Fe were salmon side streams (head, backbone, or viscera), salmon side streams×treatment, and treatment (PEF or CE), which accounted for 70.9%, 8.0%, and 7.5% of the total variation respectively. When the impact of PEF and CE on Fe was evaluated, it was found that PEF treatment reduced Fe content in solid matrices but enhanced it in the liquid extracts (Fig. 4(E-K)). The highest and lowest FE contents were obtained in the viscera-SFE+PEF-solid (83.0 mg/g) and backbone-SFE+CE-solid (24.5 mg/g), respectively, while in the liquid extract, the head-SFE+PEF-liquid presented the highest values (103.6 mg/g) and backbone-SFE+CE-liquid the lowest (19.7 mg/g).

Finally, the main three sources of variation for Zn were salmon side streams (head, backbone, or viscera), matrix (solid matrices or liquid extracts), and salmon side streams×matrix, which accounted for 84.0%, 7.8%, and 7.3% of the total variation,

respectively. Zn values after PEF and CE extraction were also assessed, observing that PEF treatment significantly increases Zn content in solid matrices while reducing it in the liquid extracts. The high content of Zn was presented in the SFE+PEF or SFE+CE for solid matrices or liquid extracts, much higher than the head and backbone (Figure 4(F-L)). For instance, the highest and lowest Zn contents were found in the viscera-SFE+PEF-solid (1843.0 mg/g) and backbone-SFE+CE-solid (85.6 mg/g), respectively. For liquid extracts, viscera-SFE+CE-liquid presented the highest values (1077.0 mg/g) while backbone-SFE+PEF-liquid had the lowest values (14.1 mg/g).

These results suggest that PEF treatment helps solid matrices retain more minerals. While SFE+PEF treatment did not significantly enhance the mineral profiles of the salmon head and viscera, more minerals remained in the solid matrices after extraction. In contrast, for the salmon backbone, SFE+PEF treatment resulted in lower mineral content in the solid matrices, implying that more minerals were extracted into the liquid part. Besides, one conclusion in these studies, is that minerals´ extraction differs according to the type of salmon side stream (head, backbone, and viscera); treatment (PEF or CE); and type of matrix (solid matrices or liquid extracts). It was important because according to the target mineral one technique or another can be used by the food, pharmaceutical or cosmetic industry to extract each mineral.

3.4. Analysis of heavy metal profiles

The heavy metal profiles for the salmon side streams (head, backbone, and viscera) with removed lipid components in solid matrices and liquid extracts have been determined to ensure food safety. In this study, three different heavy metals (As, Hg, and Pb) were evaluated as they are some of the most prominent heavy metals found in fish samples (Fig. 5). The profiles consistently show that most heavy metals are

concentrated in the viscera, both in PEF-treated and control samples, compared to the head and backbone. The highest value (0.78 mg/kg) was observed for As in the viscera-SFE+PEF-solid. Some previous research found maximum permitted levels for As, Hg, and Pb in wet fish tissue at 13.5 mg/kg, 0.5 mg/kg, and 0.3 mg/kg according to the (Kandyliari et al., 2021; Wang et al., 2023). All results for both solid matrices and liquid extracts in this study are below these limits, indicating that the salmon side streams subjected to PEF treatment are safe for human consumption in terms of heavy metals. Besides, the three-way ANOVA including 3 factors: treatment (PEF or CE); matrix (solid matrices or liquid extracts); and salmon side stream (head, backbone, or viscera) has been analyzed for As, Hg, and Pb, respectively.

Regarding As, the three main sources of variation were matrix, salmon side streams×matrix, and salmon side streams, accounting for 62.2%, 16.1%, and 11.1% of total variation, respectively. The higher profile of As was obtained in the liquid extracts and less values after SFE+PEF. For As, the highest and lowest values in solid matrices were obtained in the viscera-SFE+PEF-solid (0.9 mg/kg) and backbone-SFE+CE-solid (0.2 mg/kg). Wherever, the highest and lowest values in liquid extracts were obtained in the head-SFE+ CE -liquid (11.0 mg/kg) and viscera-SFE+PEF-liquid (2.2 mg/kg), respectively.

On the other hand, the three main sources of variation for Hg were salmon side streams×matrix, salmon side streams, and salmon side streams×treatment, which accounted for 78.3%, 10.4%, and 5.0% of total variation, respectively. The SFE+PEF promoted the value of Hg extracting into the liquid extracts for the head and backbone but was not presented to enhance the trend for viscera-liquid. For Hg, the highest and lowest values in solid matrices were obtained in the viscera-SFE+PEF-solid (105.0 μg/kg) and head-SFE+PEF-solid (37.0 μg/kg). Regarding the liquid extracts, the

highest and lowest values were obtained in the head-SFE+ CE-liquid (95.0 μg/kg) and viscera-SFE+PEF-liquid (42.0 μg/kg g), respectively.

Finally, the three main sources of variation for Pb were salmon side streams×treatment, salmon side streams×matrix, and treatment, accounting for 28.8%, 21.4%, and 18.8% of the total variation, respectively. For Pb, (μg/kg) and viscera-SFE+CE-solid (μg/kg). Regarding the liquid extracts, the highest and lowest values were obtained in the head-SFE+ CE -liquid (μg/kg) and viscera-SFE+PEF-liquid (μg/kg g), respectively. Regarding the head and backbone, the SFE+PEF enhanced the Pb contents extracted into the liquid extracts and decreased the Pb contents extracted into the liquid extracts for viscera. For Pb, the highest and lowest values in solid matrices were obtained in the viscera-SFE+PEF-solid (52.0 μg/kg) and backbone-SFE+CE-solid (16.7 μg/kg). Regarding the liquid extracts, the highest and lowest values were obtained in the backbone-SFE+PEF-liquid (82.0 μg/kg) and backbone-SFE+CE-liquid (9.2 μg/kg g), respectively.

Similarly, previous research (de la Fuente et al., 2023) investigated the heavy metal content in Salmon (*Salmo salar*) and Mackerel (*Scomber scombrus*) protein hydrolysates' head and viscera, obtaining values of As between 4-14 μg/g, Hg <0.03-0.07 μg/g, Cd <0.01-0.03 μg/g, and Pb <0.01 μg/g, respectively. Our study showed a similar pattern in salmon side streams, with As being the most prevalent, followed by Pb and Hg. As was more abundantly extracted into the liquid extracts, SFE+PEF treatment significantly affected the heavy metal content in various side streams.

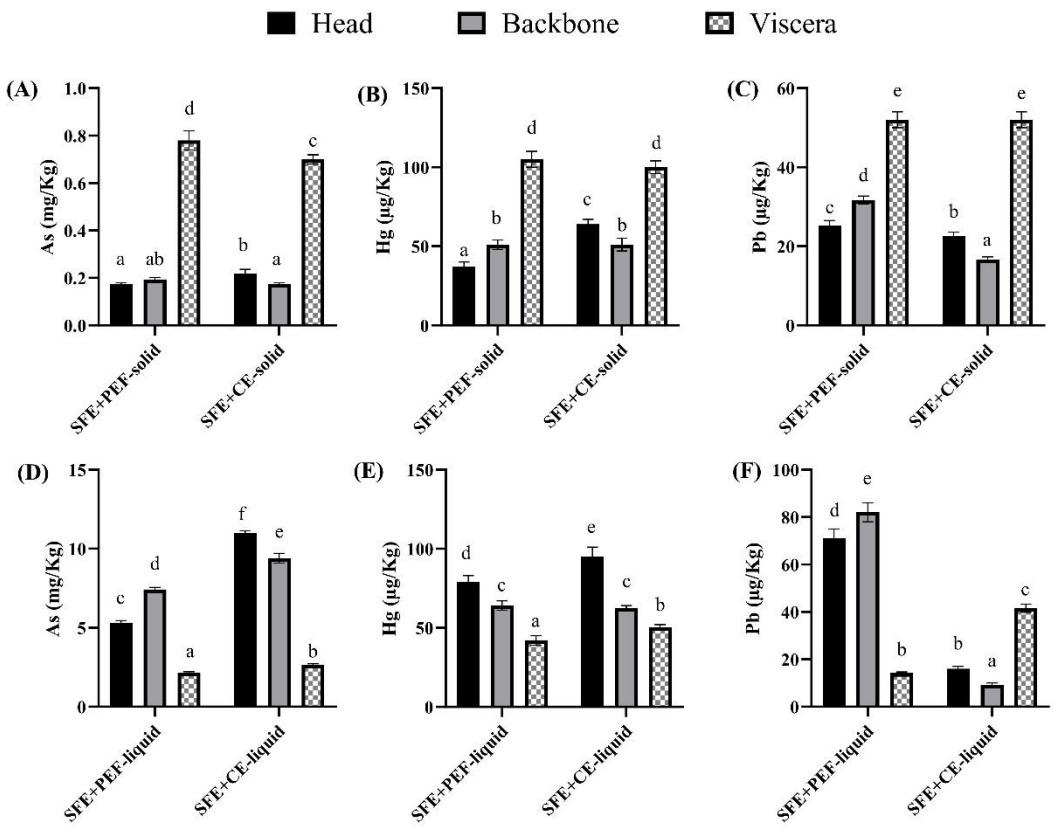

Fig. 5. The heavy metal profiles of solid matrices and liquid extracts obtained from the pulsed electric fields (PEF) treated after removing fat. (A), (B), and (C) for the solid matrices; (D), (E), and (F) for the liquid extracts; nd, not detected.

3.5. Analysis of Trolox equivalent antioxidant capacity (TEAC) and oxygen radical antioxidant capacity (ORAC)

As for the head and the backbone, there are no significant differences in the ABTS and ORAC values were found in the samples, independently if PEF was applied or no as pretreatment. As shown in Fig. (A), the values for SFE+PEF-head, SFE+PEF-backbone, SFE+CE-head, and SFE+CE-backbone are present in 56.3 μM ,18.4 μM, 58.9 μM, and 20.6 μM, respectively.

Regarding viscera, the liquid extracts obtained after PEF pretreatment exhibited the highest antioxidant ability in the *ABTS*+ scavenging ability (402.8 μM) and ORAC

(3263.6 μM) compared with head and backbone with or without PEF pretreatment.

In this line, the enhancement of the antioxidant capacity (DPPH, ABTS, and FRAP) from fish residues (gills, bones, and head) of sea bass and sea bream after the application of PEF pretreatment has been shown by different authors (Franco et al., 2020). Similarity, that both accelerated solvent extraction (ASE) and PEF treatments significantly increased the antioxidant capacity (ORAC and TEAC) of rainbow trout and sole skin and head extracts ($P < 0.05$) by our teams previous research (Wang, Zhou, et al., 2021a).

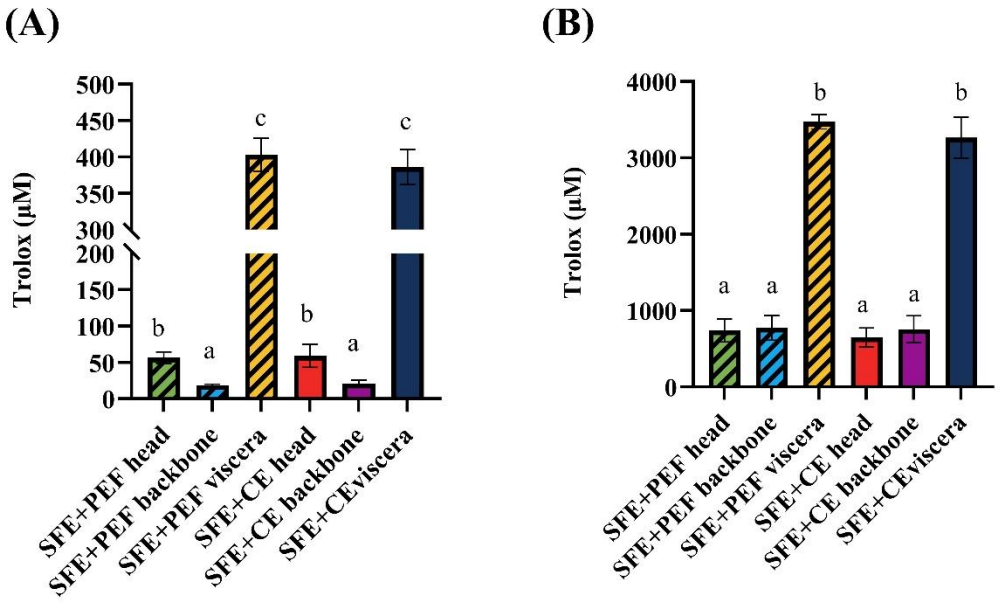

Fig. 6. The ABTS+ scavenging ability (A) and ORAC (B) of liquid extracts obtained from the pulsed electrified field (PEF) treated after removing fat

3.6. Analysis of principal component analysis (PCA)

The PCA results are presented in Fig. 7, highlighting two main principal components of this study. The four variances described 80.3% of the total variance, and the PC1, PC2, PC3, and PC4 describe 38.2%, 19.1%, 12.1%, and 11.1% of the total variance, separately. The first nine principal components' feature values (Liu et al., 2023) are all

greater than 1, and both PC1 and PC2 are higher than 8 which indicates that the two main components' feature values are sufficient to explain the protein, bioactive peptides, mineral, and heavy profiles from the solid matrices, and liquid extracts obtained in the SFE-defatted samples pretreated by PEF.

In Fig. 7 (A), the Pb, Fe, Zn, Cd, Hg, and the bioactive sequence of VDPP, SSGE, DGEA, NQDK, and WTP are separated in the positive area for PC1, indicating that those mineral, heavy metals, and bioactive peptides exhibited a certain correlation between them. A strong positive correlation between Fe and Pb. The PC1 divides all the PEF (solid matrices and liquid extracts) into positive regions in Fig.7 (B), implying a certain promoted correlation among the PEF pretreatment to recover protein, bioactive peptides, and minerals for PC1. This result can be easily found for proteins in the liquid extracts obtained after PEF pretreatment. According to Fig.7 (B), the viscera-PEF-solid, viscera-control-solid, viscera-PEF-liquid, and viscera-control-liquid are located in the positive region of PC1, and all the backbone samples are located in the negative area. Additionally, head-control-solid and head-PEF-liquid are situated in the first quadrant, indicating that these two groups are more prominent in obtaining bioactive protein compounds and minerals than other sea bass side stream groups.

It is easier to find the correlation along the sea bass side streams, PEF treatment, solid matrices, and liquid extracts at the same time according to the PCA loading and score plot in Fig.7. The TEAC and ORAC are not included in the PCA analysis because the antioxidant capacity was determined only for the liquid extracts. Future PCA analyses should focus on solid matrices and liquid extracts separately to better explain the correlations between salmon side streams and PEF pretreatment.

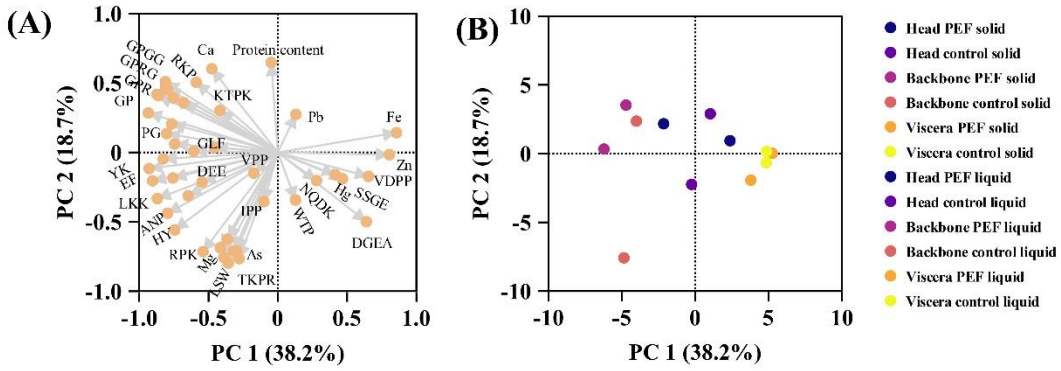

Fig. 7. The PCA loading plot (A) and scores plot (B) of solid matrices and liquid extracts obtained from the pulsed electrified field (PEF) treated after removing fat by supercritical fluid extraction (SFE).

4. Conclusions

In conclusion, the study evaluated a sequential extraction process combining SFE with PEF or CE to enhance the recovery of high-added-value compounds, mainly proteins, bioactive peptides and minerals (Mg, K, Ca, Fe, Zn), from salmon side streams for both solid matrices and liquid extracts. Moreover, the TAC of liquid extracts was also assessed. Compared to SFE+CE, more protein remained in the solid matrices obtained after SFE+PEF. On the other hand, the SFE+PEF matrices obtained from backbone significantly enhanced the bioactivity (anticancer and antithrombotic activity), which may be attributed to the electroporation phenomena. The study of minerals and heavy metals in solid and liquid extracts showed several differences in the recovery of compounds which are mainly dependent on the types of salmon side streams (head, backbone, or viscera) or the treatment (SFE+PEF or SFE+CE) or matrix (solid matrices or liquid extracts) according to the results of three-way ANOVA. Salmon viscera liquid extracts showed remarkable TAC values, with PEF significantly enhancing the TEAC values for heads and backbones. Principal component analysis (PCA) revealed two

main components that clarified the impact of SFE+PEF on the salmon solid matrices and liquid extracts. Overall, SFE+PEF offers a promising approach for optimizing the extraction and enhancement of valuable compounds from salmon side streams, contributing to potential innovations in processing and utilization.

Conflict of Interest

The authors have no conflict of interest to declare.

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