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Supercritical CO_2 and subcritical water technologies for the production of bioactive extracts from sardine (*Sardina pilchardus*) waste

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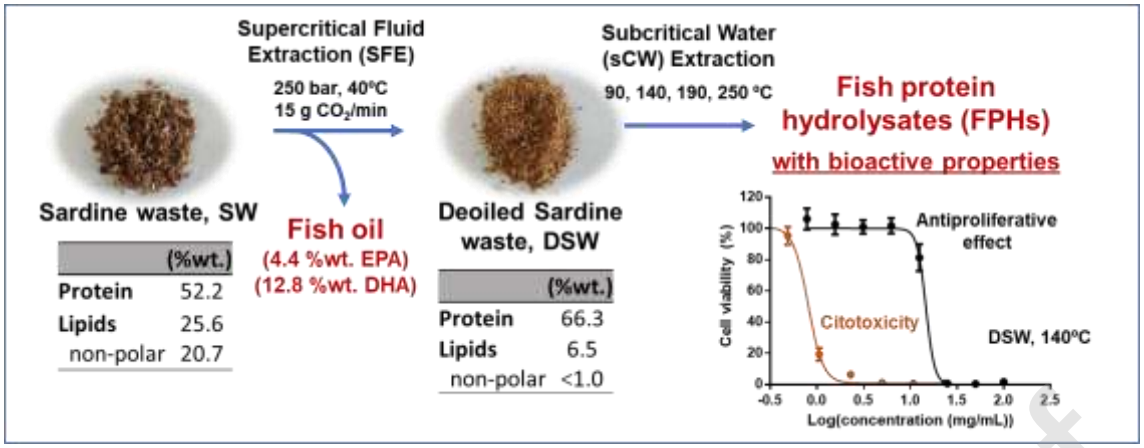
Graphical Abstract



Highlights

- Fractionation of sardine waste from a canning industry was investigated
- Fish oil rich in omega-3 was obtained by supercritical fluid extraction
- Fish protein hydrolysates were produced by subcritical water hydrolysis
- Physical, biochemical, and bioactive properties of the extracts were studied
- Antiproliferative effect of the extracts against adenocarcinoma cells was tested

Abstract

The valorization of sardine (*Sardina pilchardus*) waste (SW) from a canning facility has been investigated within a biorefining approach. Sequential fractionation of SW into its constituents has been carried out using green solvents such as supercritical carbon dioxide ($SCCO_2$) and subcritical water (sCW). The lipid fraction has been isolated through supercritical fluid extraction (SFE) with $SCCO_2$ at 250 bar and 40ºC, yielding 20.3 ± 0.2 g oil/100 g SW with up to 17.2 %wt. omega-3 polyunsaturated fatty acids (PUFAs). Aiming at the protein fraction, sCW extraction/hydrolysis has been carried out at different temperatures (90, 140, 190 and 250ºC), using both SW and defatted sardine waste (DSW) from SFE experiments. Previous

defatting increased protein recovery and purity. Bioactive properties of the fish protein hydrolysates (FPHs) obtained were affected by the extraction temperature. The highest antioxidant activity and *in vitro* antiproliferative effect were found in the extracts obtained at 250ºC.

Keywords

Sardine waste; Subcritical water; Fish protein hydrolysate; Biorefinery; Antioxidant activity; Antiproliferative activity

1. Introduction

According to FAO, the world fisheries and aquaculture production was 171 million tonnes in 2016, being more than 150 million tonnes destined to human consumption [1]. Despite improvements in fish processing and distribution, fish waste generated between landing and consumption accounts for an estimated 27 % of landed fish. Processing industries discard between 20 to 75 % of the fish, depending on the fish species and level of processing [2]. Fish canning of oily species such as tuna, mackerel and sardine-type species is an important industrial sector worldwide and also in Portugal [3]. The large amount of fish waste generated pose important economic and environmental problems for the sector due to its high organic load, consisting mainly of protein and lipids. To date, fish waste is generally considered low value and used to produce animal feed, fish silage, and fertilizers when is not disposed of by burning or discarding in the land or sea [2]. However, studies published in the last decades have illustrated fish waste as a remarkable source of vital bioactive molecules, such as proteins, peptides, amino acids, lipids (omega-3 polyunsaturated fatty acids, PUFAs), enzymes (pepsin, trypsin), vitamins (A, D, E) and biopolymers [2,4,5]. These compounds can be used in nutraceutical, biomedical, pharmaceutical and cosmetic applications, with a much higher

market value [4]. In consequence, fish waste represents a valuable resource for an integrated and product-based biorefinery process [6], obtaining different materials and building blocks with the potential to serve as renewable feedstocks for several industrial sectors.

Fish waste is considered a good source of high quality fish oil, rich in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), two omega-3 PUFAs with applications in nutraceutical, pharmaceutics and cosmetic industries [7]. Conventional extraction of lipids from natural materials uses hazardous organic solvents and is typically energy intensive and time consuming. Supercritical carbon dioxide ($SCCO_2$) extraction is an environmentally friendly alternative, compatible with the label 'natural', since at the end of the process only recyclable CO_2 and a solvent-free extract are produced, leaving an also solvent-free and non-degraded raffinate [8]. $SCCO_2$ presents high diffusivity, low viscosity and low surface tension, improving mass transfer. Through manipulation of pressure and temperature, $SCCO_2$ density can be adjusted to allow efficient extraction and separation of the oil fraction and lipophilic bioactive solutes [7–10].

Fish waste also contains significant amount of proteins, nutritionally superior when compared to those of plant sources and less restricted due to religious concerns and transmissible diseases than those of bovine or porcine origin [11]. Fish protein hydrolysates (FPHs) with enhanced physicochemical and biological properties can be obtained through different extraction and hydrolysis methods [2]. Chemical hydrolysis with either a strong acid or alkali is the most common, inexpensive, and simple method to produce FPHs [12]. However, the method requires of several pre-treatment steps and uses high temperatures for long times, which complicate the control of product quality and functionality. Enzymatic extraction in acidic media has emerged as an alternative due to the milder conditions and the higher specificity. However, enzyme-based methods are complicated, costly, and even more time-consuming than chemical extraction, which limits its implementation at industrial scale [13]. Advanced cost-

effective processing technologies need to be developed to produce high quality FPHs with specific functionalities for specific product applications.

Subcritical water (sCW) has attracted interest as a green solvent for waste and biomass conversion [14]. sCW extraction and hydrolysis processes use water at 100-300 °C and pressure above saturation value but less than critical, just to maintain water liquid. At these conditions, polarity decreases with temperature, allowing solvent tunability for selective extraction of moderately polar to non-polar substances. High temperatures also break hydrogen bonds, facilitating sCW to penetrate into solid matrixes due to the lower viscosity and higher diffusivity. Moreover, the ionic product (K_W) is 3 orders of magnitude greater than that of water at ambient conditions. This drives the formation of hydronium and hydroxide ions and allows sCW to act as acid or base catalyst, which supports hydrolysis of proteins and amino acids [14,15] with no need of additional catalysts suchs as acids or enzymes.

Yoshida et al. [16] reported the sCW extraction/hydrolysis of fish meat from horse mackerel in a batch reactor. Analysis of the aqueous phase obtained revealed sequential production of organic acids, amino acids, and fish oil when increasing hydrolysis temperature from 200 to 400 ºC; thus, a temperature-based fractionation process was proposed for industrial valorisation of fish waste. Other authors have also investigated the sCW extraction/hydrolysis of fish waste from different origins, such as white croaker [17,18], bonito [17], squid viscera[19], mackerel [20], or shrimp [13], and studied the physicochemical, biochemical, and bioactive properties of the FPHs obtained. In some cases, the sCW process was also combined with a previous $SCCO_2$ defatting step [21,22]. However, most of the mentioned studies were performed in batch-mode and focusing on the production of amino acids, rather than intermediate peptides. Recently, Marcet et al. [23] compared the sCW extraction and hydrolysis of proteins with the chemical and enzymatic techniques for the recovery of peptides and free amino acids from animal and vegetal food wastes, concluding that sCW allows higher efficiency and flexibility

with no further addition of reagents. This is at the cost of higher equipment and energy expenditures, although energy consumption can be minimized by correct design and optimization of process parameters.

In this work, the utility and feasibility of an integrated biorefinery process for the valorisation of sardine waste from a canning facility has been evaluated. For this, the sCW extraction and hydrolysis process has been coupled with a pre-defatting step with $SCCO_2$, aimed at the specific recovery of the lipid fraction of sardine waste, rich in omega-3 polyunsaturated fatty acids. The sCW process was performed in a packed-bed reactor operating in semi-continuous mode since it allows the fractionation of the target bioactive compounds present in the sardine waste through continuous temperature variation. The potential effect of the sCW temperature and the prior $SCCO_2$ defatting on the extraction/hydrolysis yield and the extract composition was investigated, trying to assess the potential advantage of using $SCCO_2$ in a first step to selectively extract the lipid fraction from sardine waste, and later, to use sCW to hydrolyse its protein fraction. Furthermore, the biological activity of the extracts in terms of antioxidant and antiproliferative effects was also determined.

2. Experimental section

2.1. Materials

Sardine (*Sardina pilchardus*) waste (SW), consisting of heads, spines, and viscera generated in a fish canning facility, was provided by Conservas A Poveira S.A. (Portugal) and used as raw material. Each batch of fish waste was received fresh, frozen with liquid nitrogen and lyophilized for 3 days. After drying, the sample was milled to ca. 5 mm using a blender (Kenwood CH580). Prior to experiments, the ground sardine powder was stored in airtight plastic bags at -20 °C.

Lowry reagent, 2N Folin-Ciocalteu's reagent, Bovine Serum Albumin (BSA) standard, phenol (99%), D(+)-glucose monohydrate, and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich. Amino acid analysis kit was purchased from Phenomenex (Torrance, CA, USA). Human colorectal adenocarcinoma cell lines, Caco-2 and HT-29, were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Germany) and American Type Culture Collection (USA), respectively. Cell culture medium (Dulbecco's Modified Eagle Medium, DMEM) and supplements (heat-inactivated Fetal Bovine Serum, FBS, and non-essential aminoacids, NEAA) were purchased from Invitrogen (Gibco, Invitrogen Corporation, Paisley, U.K.); and CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) was obtained from Promega (Madison, WI, USA). All other reagents and solvents used in the present study were of analytical grade and purchased from available suppliers.

2.2. Supercritical Fluid Extraction (SFE) of oil from sardine waste

$SCCO_2$ extraction of oil from SW was carried out in a lab-scale apparatus. Firstly, CO_2 taken from a cylinder is liquefied in the cooling section, which is immersed in a chilled water/ethylene glycol bath. Subsequently, the liquid CO_2 is pumped with a pneumatic pump (Williams P250V300) at the desired flow rate, which was measured by a mass flow meter (Rheonik RHM 007). Then, CO_2 is pre-heated to the desired temperature by a heating wire and passes through the tubular extractor (316SS; 570 mm length, 24 mm I.D.; HiP). Temperature in the extractor is maintained by means of an electrical resistance and a series of thermocouples connected to a digital controller. Pressure in the extractor is controlled with a back-pressure regulator (BPR) (Tescom Europe, model 26-1700, Selmsdorf, Germany) and measured with a digital pressure meter. The depressurized stream is collected into an electrically-heated separator (Swagelok 316L-HDF4-500) where precipitation of extracted substances takes place. The exit of the separator is connected to the cooling section, allowing solvent recirculation.

In a typical experiment, ca. 50 g of fish waste were charged into the extractor. Based on previous experiments [7] and equipment specifications, $SCCO_2$ extraction conditions were set at 250 bar and 40 ºC, whereas separation was achieved at 50 bar and 50 ºC. Similarly, CO_2 mass flow was set at 15 g/min. Extracted oil was periodically removed from the separator along the experiment and weighted in order to follow the extraction kinetics. The experiment was terminated when constant weight of extract was achieved.

The raffinate obtained after extraction, consisting of defatted sardine waste (DSW), was recovered and kept at -20ºC until proximate composition analysis and further sCW extraction/hydrolysis.

2.3. Subcritical water (sCW) extraction and hydrolysis

The sCW extraction/hydrolysis of fish waste was performed in a lab-scale apparatus described in previous publications [15]. Briefly, it consists of a distilled water reservoir, a high-pressure pump (Knauer 1800), and a high-pressure reactor (HiP) with 510 mm length, 50 mm O.D., and 26 mm I.D., placed in an electrical oven (Nabertherm P330). Pressure of the system is controlled by a Tescom BPR. Water mass flow and total mass of water fed to the system was recorded in a Rheonik RHM 007 mass flow meter.

In a typical experiment, the high-pressure reactor was filled with ca. 60 g of either SW or DSW, kept between porous discs. Subsequently, the reactor was placed in the electrical oven and connected to the high-pressure system, the pump was turned on at the selected flow rate (ca. 10 mL/min), and the BPR was set at 100 bar. No fluid left the reactor while pressure increased. When pressure reached 100 bar, the pre-heating wires and the electrical oven were turned on, and extract collection started, setting time t = 0. In order to assess the influence of temperature on the composition and bioactive properties of the FPHs, samples were collected during 30 min at target temperatures of 90, 140, 190 and 250 ºC. Between samples, temperature was

increased at a heating rate of approximately 1ºC/min for a total experiment time length of 6 h. Samples were subsequently frozen with liquid nitrogen and lyophilized for 3 days in a Labconco freeze-dryer to determine the extraction yield.

To account for eventual mass losses and off-gases, the hydrolysis yield of each experiment was also determined using the following formula:

$$\text{Hydrolysis yield } (\%) = \frac{W - W_1}{W} \cdot 100 \quad (1)$$

Where W is the initial weight of fish waste placed in the reactor, and W_1 represents the weight of dried solid residue obtained after an experiment.

2.4. Proximate composition analysis

Protein content was determined through modified Lowry method [24]. Absorbance at 750 nm was read in a spectrophotometer (Thermoscientific Genesys 50) and protein concentration in the samples was calculated based on a calibration curve made with BSA standard at concentrations ranging from 40 to 400 μg/mL. Blank runs were also performed with distilled water instead of the protein sample. Total lipid content was measured through the Bligh & Dyer [25] method. Soxhlet extraction with *n*-hexane was also performed in order to estimate the amount of non-polar lipids, more easily extractable with $SCCO_2$, following the method described by Pedras et al.[15]. Total sugars were determined by phenol-sulphuric acid colorimetric method [15]. Absorbance of the samples was measured at 490 nm in a spectrophotometer (Thermoscientific Genesys 50). Carbohydrate content was expressed as glucose equivalents by means of a calibration curve built with D(+)-glucose monohydrate solutions ranging from 5 to 100 mg/L. Ash content was gravimetrically analysed through calcination in a muffle at 550 ºC during 4 h. Major minerals and heavy metals present in the calcinated residue were measured by Inductively Coupled Plasma - Atomic Emission

Spectroscopy (ICP-AES) using a Horiba Jobin Yvon ULTIMA sequential ICP and a monochromator with a Czerny Turner spectrometer. Argon was used to generate the plasma. Moisture was determined in a thermogravimetric balance (Kern DAB 100-3) at 105 ºC.

2.5. Fatty acid profile

The fish oil and the FPH extracts were analysed by gas chromatography to determine their fatty acid profile following AOAC method [26]. Fatty acid methyl esters (FAMEs) were prepared and then analysed in a Thermo Scientific gas chromatograph (Trace ULTRA Series) with autosampler and flame ionization detector (GC-FID). A Phenomenex ZB 5HT Inferno (30 m x 0.32 mm i.d.) coated with a 0.10 μm thickness film of 5% diphenyl-95% dimethylpolysiloxane was used for separation. Injections were made in split-splitless mode (SSL), using hydrogen as the carrier gas (1 mL/min). All the data was processed with software Chrom-Card. Most of the fatty acids were identified by comparison of their retention times with those of chromatographic standards (Sigma Aldrich).

2.6. Amino acid profile

The amino acid profile of fish waste was determined using a gas chromatograph (Hewlett-Packard HP 6890 Series GC System) equipped with a flame ionization detector (FID), a Zebron ZB-AAA 10 m × 0.25 mm capillary column (Phenomenex), and an Agilent Technologies 7683B series automatic injector. Oven temperature was varied from 110 ºC to 320 ºC, at 32 ºC/min. Typically, 2 μL of sample were injected, using a split ratio of 1:2. The injector temperature was kept at 250 °C, while the detector temperature was at 320 °C. Helium (1 mL/min) was used as carrier gas.

Sample preparation consisted of an acidic digestion with 6M HCl for 24 h at 110 ºC. Since tryptophan is destroyed at acid conditions, fish waste was also submitted to alkaline digestion with 4.2M NaOH for 24 h at 110 ºC. After proper neutralization and dilution of the amino acid

extracts, 100 µL of solution were taken and derivatised following the Phenomenex EZ:faast assay kit specifications [27]. Immediately afterwards, samples were submitted to GC analysis. Amino acid content was expressed as mg/g of freeze-dried extract. A series of amino acid standards (Sigma Aldrich) were used for method calibration, and norvaline (200 µmol/L) was used as internal standard.

Free amino acid content of FPHs was determined following the same method. Freeze-dried FPH were redissolved in milliQ water (20-30 mg/mL) and derivatized following the assay kit specifications [27]. Immediately afterwards, samples were submitted to GC analysis.

2.7. pH and Maillard reaction product measurement

The pH levels of FPH extracts were read at room temperature using a Crison Basic 20 pHmeter. Technical buffer solutions of pH 4.01, 7.00, and 9.1 were used to calibrate the equipment before the measurement.

The browning of FPHs and formation of Maillard reaction products were determined following a method described elsewhere [13] with some modifications. Briefly, FPH were diluted 5 times using distilled water, and the absorbance (Abs) was measured at 294 and 420 nm using a spectrophotometer (Thermoscientific Genesys 50). Then, the transformation of UV-absorbing compounds was calculated using the following equation:

$$\text{Formation of UV-absorbing compounds} = \frac{\text{Abs } 294 \text{ nm}}{\text{Abs } 420 \text{ nm}} \quad (2)$$

2.8. Bioactive properties of hydrolysates

DPPH radical scavenging activity was measured using a previously described method [15]. To evaluate the inhibition of the free radical by each sample, the following equation was used:

$$\%\ \text{inhibition} = \frac{\text{Abs}_{\text{DPPH}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{DPPH}}} \cdot 100 \quad (3)$$

where Abs_{DPPH} is the absorbance of the blank and Abs_{sample} is the absorbance of the ABTS radical scavenging activity. To evaluate the antioxidant activity of FPH extracts, the half maximum effective concentration (EC_{50}) was calculated from the inhibition curves obtained.

Human colorectal adenocarcinoma cell lines, Caco-2 and HT-29, were cultured following previously described methods [28]. Firstly, cytotoxicity effects were assessed through MTS assay using confluent and non-differentiated Caco-2 cells as a model of the human intestinal epithelium. Briefly, cells were seeded in 96-well plates at a density of $2x10^4$ cells/well and allowed to grow for 7 days. After 7 days, the cells were incubated for 24 h with the extracts diluted in culture medium (DMEM + 0.5% FBS + 1% NEAA). The range of maximum concentrations tested was 7.5-100 mg/mL. Cells incubated with only culture medium were considered as control. After 24 h of treatment, the medium was removed and cell viability was measured according to a previously described method [28]. Three independent experiments were performed in a microplate spectrophotometer at 490 nm (EPOCH, 219 Bio-Tek, USA). Cell viability was expressed in terms of percentage of viable cells relative to the control and half maximal effective concentration (EC_{50}) values were calculated from dose-response curves, as the concentration of sample necessary to decrease cell proliferation by 50 %.

Antiproliferative effect of extracts was evaluated in human colorectal adenocarcinoma HT-29 cells following a previously described method [28]. After 24 h culture, cells were incubated with non-cytotoxic concentrations of the FPH extracts diluted in culture medium (DMEM + 0.5% FBS). The range of maximum concentrations of FPH extracts tested was 7.5-50 mg/mL. Cells incubated with only culture medium were considered as control. Cell proliferation was measured using MTS reagent, as mentioned above. Results were expressed in terms of percentage of viable cells in comparison to the control and EC_{50} values were calculated from dose-response curves.

2.9. Statistical analysis

SFE and sCW extraction/hydrolysis experiments were duplicated. Analytical data are expressed as mean ± standard deviation (SD) of triplicates. One-way analysis of variance was performed using Statgraphics software (version XVII for Windows). GraphPad Prism 6 software was used to calculate the EC_{50} values.

3. Results and discussion

3.1. SFE of oil from sardine waste. Effects on sardine waste composition

The raw material was firstly submitted to SFE with $SCCO_2$ at 25 MPa and 40 ºC in a semi-continuous lab-scale apparatus, in order to simultaneously obtain the lipid fraction and the defatted fish waste for subsequent sCW extraction/hydrolysis experiments.

The SFE extraction kinetics are shown in Figure 1. The extraction curve presents a slight *S* shape which might indicate that internal diffusion is controlling the process from the beginning of the extraction. Similar behaviour was observed by Rubio-Rodríguez et al. [29] for the SFE of hake offcuts; they found that the model by Kandiah and Spiro [30] was the most adequate to describe the extraction kinetics (Eq. 4).

$$\frac{C}{C_0} = 1 - \left[f_1 \exp\left(-k_1 t\right) + f_2 \exp\left(-k_2 t\right) \right] \quad (4)$$

where C_0 is the initial concentration of oil in the sardine waste (g oil/g insoluble solid), C is the oil concentration at a given time, and f_1 and f_2 are the fractions of solute extracted with rate constants k_1 and k_2, respectively.

Fitting of the model by Kandiah and Spiro [30] to the SFE extraction kinetics of SW was performed by non-linear regression with Statgraphics program and Marquardt algorithm.

Estimated model parameters were f_1 = 0.284, f_2 = 0.716 with 0.0355 and 0.0121 min^{-1} for k_1 and k_2 rate constants, respectively. Adjusted R^2 value was 0.998.

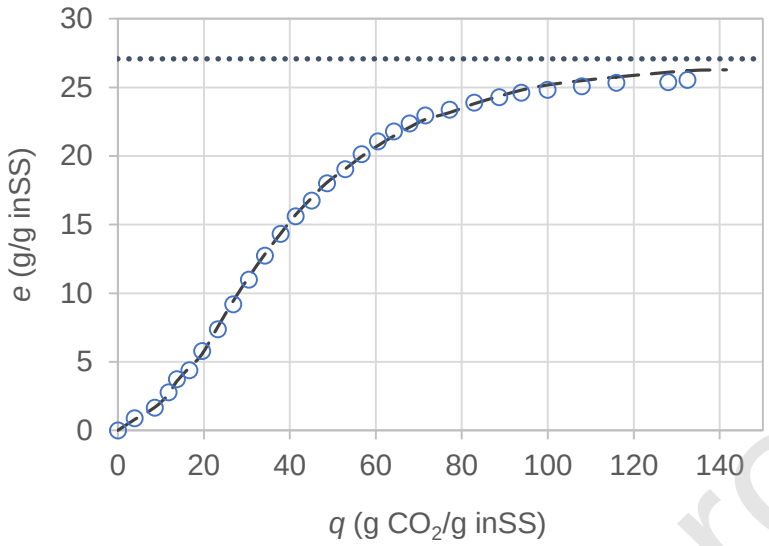


Figure 1. Extraction kinetics of oil from sardine waste with $SCCO_2$ at 250 bar and 40 ºC. Dashed line represents the extraction curve obtained with the fitted model by Kandiah and Spiro [30], and dotted line represents the amount of oil extracted by Soxhlet extraction with hexane.

SFE extraction yield at 250 bar and 40 ºC reached 20.3 ± 0.2 g/100 g raw material, which accounts for 79.3 % of total oil according to Bligh & Dyer assay, and 95.3 % of non-polar lipids present in the sample, according to Soxhlet extraction with n-hexane. GC-FID analysis of the fatty acid profile of the extracted oil confirmed the presence of omega-3 PUFAs and other fatty acids, such as palmitic acid (30.1 ± 0.7 %wt.), oleic acid (14.3 ± 0.2 %), and DHA (12.8 ± 0.1 %). Minor components were cetoleic acid (7.9 ± 0.4 %), EPA (4.4 ± 0.1 %), and gadoleic acid (3.1 ± 0.1 %).

The composition of SW and DSW after extraction with $SCCO_2$ is shown in Table 1. Since $SCCO_2$ is a selective solvent for less polar compounds, the SFE process was able to significantly decrease ($p < 0.05$) the lipid content of DSW, although some lipids (6.5 ± 0.3 % wt.) were still detected. Amphiphilic compounds present in fish waste such as phospholipids

are not easily extracted by $SCCO_2$, due to its more polar nature. Proteins are not soluble in $SCCO_2$, so its concentration in DSW increased from 52.2 ± 0.4 %wt. up to 66.3 ± 0.7 %. Slight accumulations of ash and total sugars were also observed for the same reason. Some water could have been stripped off during extraction, although initial moisture was low and its variation unimportant.

Table 1. Proximate compositions of sardine waste and defatted sardine waste.

Compound (%wt.)	Sardine waste, SW	Defatted sardine waste, DSW
Protein	52.2 ± 0.4[b]	66.3 ± 0.7[a]
Lipids		
Total	25.6 ± 0.5[a]	6.5 ± 0.3[b]
Non-polar	21.3 ± 0.5[a]	< 1.0[b]
Ash	16.6 ± 0.9[a]	18.5 ± 0.7[a]
Total sugars	2.9 ± 0.2[a]	3.1 ± 0.1[a]
Moisture	5.9 ± 0.5[a]	5.6 ± 0.3[a]

[a, b]: Values with different superscript letters in the same row differ significantly ($p < 0.05$).

3.2. sCW extraction/hydrolysis

Semi-continuous sCW extraction/hydrolysis of SW and DSW has been performed in a packed-bed reactor, allowing the production of different extracts through continuous temperature variation. The same tubular packed-bed reactor can be used both for SFE defatting and subsequent sCW extraction/hydrolysis, providing a one-pot, integrated process with practical advantages. Environmental and socio-economic benefits of this kind of $SCCO_2$ and sCW-based biorefinery strategies, compared to conventional extraction methods, have been also confirmed for different food and forest wastes [31].

The results obtained in the sCW extraction/hydrolysis experiments at different temperatures are shown in Table 2. In terms of total extraction yield, experiments using DSW as feed

achieved slightly higher values than those performed with SW at each temperature, although differences are within experimental error in many cases. On the other hand, protein purity is substantially higher in the case of the FPH extracts obtained from DSW. Similar results have been reported in the literature for fish residues of different nature, such as squid viscera [21], mackerel skin [22], and shrimp waste [13], as well as for other proteinaceous wastes such as rice bran and soybean meal [32]. The beneficial effect of prior $SCCO_2$ defatting on the protein yield has been also observed before in the case of squid viscera [21] and mackerel skin [22], and might be attributed to increased accessibility of sCW to extractable solids due to the lower content of hydrophobic interferences.

The hydrolysis yield is 30 % and 20 % higher than the final extraction yield at 250 ºC for SW and DSW, respectively. This indicates that some organic compounds have been decomposed to volatile carbon (e.g. carbon dioxide) due to the high temperatures and hydrolytic conditions of the sCW experiments [33,34]. Similar values for the hydrolysis yield have been obtained for raw and defatted mackerel skin after sCW hydrolysis at 240 ºC (84.25 ± 0.46 and 86.15 ± 0.49 %, respectively) [22] and for shrimp waste at 200 ºC (88.55 ± 2.15 %) [13]. In view of these results, proper control of reaction parameters such as temperature and residence time is necessary in order to avoid decomposition of extracted compounds and maximize extraction yield.

Table 2. Extraction yield, hydrolysis yield, and proximate compositions of the extracts involved in the sCW experiments.

	Extraction yield (g/100 g feed)	Hydrolysis yield (%)	Composition (g/100 g)			
			Protein	Lipids	Ash	Total sugars
Sardine waste, SW						
FPH90	18.0 ± 0.1		61.6 ± 3.1	8.1 ± 1.5	27.3 ± 0.9	3.04 ± 0.05
FPH140	27.0 ± 2.3		70.0 ± 0.5	18.6 ± 0.7	9.5 ± 0.5	1.89 ± 0.05
FPH190	38.6 ± 3.2		73.2 ± 1.2	22.7 ± 2.6	3.0 ± 0.7	1.10 ± 0.05
FPH250	58.5 ± 0.4	87.4 ± 3.0	57.5 ± 1.8	37.1 ± 4.4	3.5 ±1.9	1.89 ± 0.05

Raffinate	12.6 ± 1.5		5.9 ± 0.5	54.7 ± 3.4	39.4 ± 2.9	n.d.
Defatted sardine waste, DSW						
FPH90	21.5 ± 0.3		77.0 ± 0.6	0.56 ± 0.05	19.8 ± 1.5	2.62 ± 0.05
FPH140	29.1 ± 0.2		91.3 ± 1.4	2.2 ± 0.3	4.5 ± 0.5	1.98 ± 0.05
FPH190	45.7 ± 2.8		87.5 ± 2.7	7.2 ± 2.8	3.9 ± 0.5	1.30 ± 0.05
FPH250	61.7 ± 2.0	81.5 ± 2.0	85.2 ± 0.6	9.5 ± 1.8	3.9 ± 0.2	1.34 ± 0.05
Raffinate	18.5 ± 1.0		4.0 ± 0.4	36.2 ± 3.3	59.8 ± 2.9	n.d.

n.d.: not detected.

FPH extracts obtained after sCW extraction/hydrolysis at different temperatures are mostly composed by protein (Figure 2). At each extraction temperature, FPH extracts from DSW contained more protein than those of non-defatted SW. Figure 2 also shows that protein content in FPH extracts first increases with temperature (up to 190 ºC in the case of SW and 140 ºC in the case of DSW), and then decreases. This behaviour might respond to (1) the enhanced extraction of lipids at high temperatures, and (2) the degradation of protein compounds to organic acids and volatile carbon. The lipid extraction effect is more apparent in the extracts from SW. Lipid content of FPH250 from SW was almost 40 %wt., which lead to a viscous semi-solid aspect after lyophilisation. In the case of the extracts from DSW, the lipid content is much lower and differences in protein content in the range 140-250 ºC might be linked to protein degradation with temperature. The highest protein content in FPH extracts were 73.2 ± 1.2 g/100 g extract for SW at 190 ºC and 91.3 ±1.4 g/100 g extract for DSW at 140 ºC. In general, at ambient temperature, protein has low solubility in water due to strong aggregation through hydrophobic interactions. However, the solubility of protein in water increased with temperature, which is likely due to the increased hydrolysis rate caused by the higher dissociation constant or ion product of sCW.

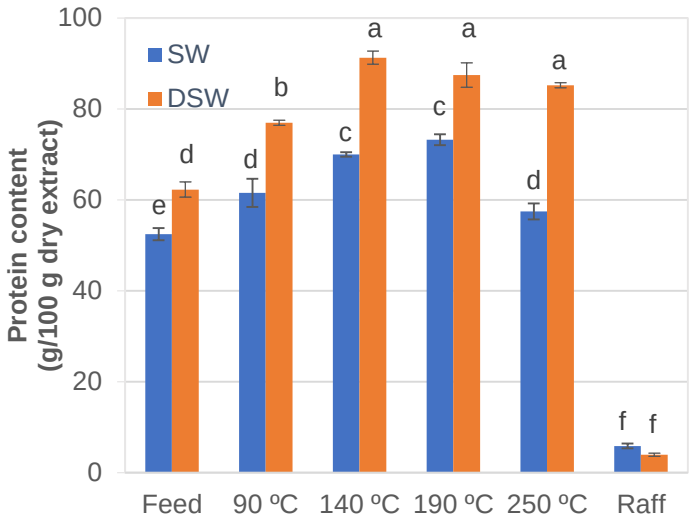


Figure 2. Protein content of the streams involved in the sCW experiments at different temperatures. SW: sardine waste; DSW: defatted sardine waste. Different letters above columns indicate statistically significant differences among samples ($p < 0.05$).

Regarding non-protein compounds, sugars appeared as minor constituents of the extracts since fish tissue in general is poor in these compounds (from 1.10 ± 0.1 to 3.04 ± 0.1 g/100 g). However, the ash content indicates an important presence of minerals at low extraction temperatures (up to 90 ºC). Table 3 shows the levels of major minerals in SW and DSW as well as in the FPHs obtained. The absence of heavy metals (As, Cd, Cr, Hg) in all the analysed samples was confirmed, which opens the utilization of FPHs from sardine waste in human food and pharmaceutical applications. Furthermore, we can see from the results that the water-soluble Na and K based salts were extracted in the early stages of the experiments at temperatures up to 90 ºC. On the contrary, Ca, P, Mg and Si accumulated in the raffinate, meaning that hydroxyapatite and other mineralized structures were not significantly affected by sCW up to 250 ºC. The mineral composition of FPH extracts is an important quality parameter, since an excessive content of certain salts might have negative effects when added to nutraceutical or pharmaceutical formulations (e.g., unpleasant flavours or inappropriate nutritional profiles). The results obtained in this work clearly show that for a scale-up of the

sCW process, a leaching step should be performed prior to lyophilization of sardine waste to remove the soluble salts.

Table 3. Results obtained in the atomic emission spectrometry (AES) analysis of the streams involved in the sCW experiments.

	Macrominerals (mg/g, dry basis)					
	Na	**K**	**Ca**	**P**	**Mg**	**Si**
Sardine waste, SW						
Feed	11.8	7.81	23.5	13.3	2.08	0.038
FPH90	130.4	50.1	6.90	-	4.22	0.091
FPH140	0.77	0.27	1.42	-	0.56	0.28
FPH190	0.19	0.033	0.068	-	0.19	0.048
FPH250	0.61	0.11	0.063	-	0.10	0.12
Raffinate	0.12	0.50	86.1	54.9	3.26	0.55
Defatted sardine waste, DSW						
Feed	13.1	6.99	25.6	15.5	1.64	0.042
FPH90	102.1	51.4	5.71	-	4.50	0.10
FPH140	8.67	4.11	1.43	-	0.64	0.20
FPH190	3.48	1.49	0.50	-	0.22	0.10
FPH250	1.94	0.54	0.30	-	0.24	0.16
Raffinate	0.24	0.25	76.2	44.9	5.88	0.99

The second major impurity of FPH extracts were lipid compounds, which were mostly found in the FPH extracts from SW and at high extraction temperatures. Lipid miscibility in sCW was likely favoured by the decrease in solvent polarity and the hydrolysis of triacylglycerides to form free fatty acids, both temperature-driven phenomena. Fatty acid profile of the extracts obtained at 250 ºC was analysed through GC-FID, finding mostly medium-chain fatty acids such as myristic acid (16.9-24.3 %), palmitic acid (44.4-57.2 %), stearic acid (7.3-14.5 %) and oleic acid (7.5-7.7 %).

3.3. pH and Maillard reaction products

Figure 3 illustrates the evolution of pH and Maillard reaction products with temperature. The pH variation during sCW extraction/hydrolysis might indicate protein conversion and production of alkaline compounds at high temperatures. In general terms, extraction temperature did not have great influence on the pH of extracts in the range from 90 to 250 ºC. Slightly decrease in pH from 5.32 ± 0.1 to 4.82 ± 0.1 and from 5.72 ± 0.1 to 5.11 ± 0.1 for SW and DSW, respectively, was observed when increasing temperature from 90 to 190 ºC. At these temperatures, pH values of the extracts from SW were lower than those of DSW. Further temperature increment up to 250 ºC promoted a pH rise to initial or even higher values (5.62 ± 0.1 and 5.51 ± 0.1 for SW and DSW, respectively). pH increase with temperature might be associated with the degradation of organic acids and acidic compounds that previously decreased pH, as well as with the formation of salts and generation of alkaline compounds such as methylamine and other amines due to glycine decomposition [35].

Maillard reaction products are formed through consecutive reaction of amino acids and reducing sugars, rearrangement, cross-linking and aggregation to produce brown-coloured and UV-absorbing bioactive polymers [36]. In the FPH extracts, absorbance at 294 nm indicates the formation of UV-absorbing Maillard reaction products and that at 420 nm indicates the browning intensity [13].

Figure 3 shows the ratio of Abs420 to Abs294, illustrating the effect of sCW extraction temperature on the transformation of Maillard reaction intermediates into brown polymers. Absorbance ratio of FPH extracts increases with temperature, indicating an enhancement of polymerization reactions and a higher browning intensity in the FPH extracts obtained at higher temperatures. FPHs obtained from DSW presented higher absorbance ratios than those obtained from SW at each temperature, although differences are not statistically significant in

some cases. Maillard reaction is favoured by temperature and by high concentrations of reactive compounds, such as proteins in the case of FPHs from DSW.

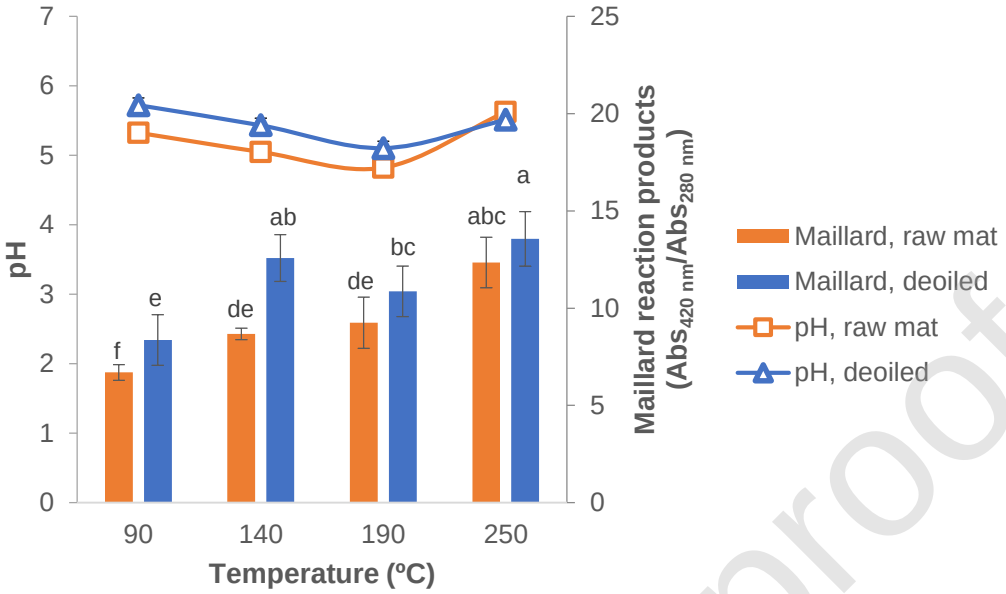


Figure 3. Evolution of pH and Maillard reaction products index of the FPH extracts with temperature of the sCW experiments. Different letters above columns indicate statistically significant difference ($p < 0.05$).

3.4. Amino acid profile of FPHs

Total and free amino acid contents of FPHs obtained in the sCW experiments are shown in Tables 4 and 5. The amino acid profile of the raw material is also shown in Table 4 for comparison purposes. Results obtained are similar to those reported in the literature for this fish species [37,38]. Differences in the content of some amino acids (mainly Met, Lys, His, Asp, Glu, and Val) can be noticed, although available published data [37,38] report the amino acid profile of the whole fish, whereas the sardine waste used in this work consist only in the head, spines and viscera of the fish. Arginine cannot be detected by this method of analysis. Since Asparagine and Glutamine are not stable under analysis conditions [27], they were quantified together with Aspartic acid and Glutamic acid, and denoted as Asp and Glu, respectively.

The results from Table 4 show that Gly, Val, Leu, Met, Glu and His were the most abundant amino acids both in raw material and in the FPH extracts. In relative terms, the total amino acid profile of the FPH extracts do not show substantial variations with temperature. Prior $SCCO_2$ defatting did not significantly affect the amino acid profile of the extracts either. As a general trend, the extraction of low molecular weight amino acids such as Ala, Gly, Val and Leu increased with temperature from 90 to 190 ºC, whereas the content of high molecular weight amino acids such as Met, Glu, Phe and Trp is higher at mild temperatures and then decreases. This has been also observed in sCW extraction/hydrolysis of white croaker [18], and defatted squid viscera [19,21], and might be explained by differences in thermosensitivity between amino acids, depending on their size and molecular structure, and conversion of complex amino acids into more simple intermediaries such as Ala and Gly [33,34].

Comparing SW and DSW in terms of total amino acid content, it can be seen that prior defatting with $SCCO_2$ results in higher amino acid content at each temperature, which is in consonance with the higher protein yield obtained from DSW compared to that of SW. The sum of total amino acids also serves as a quantification of the protein content in the FPH extracts, although the Lowry method tends to detect higher protein content [39]. Total amino acid content of the FPH extracts obtained from both SW and DSW increased with temperature up to 190 ºC, and then decreased with further temperature increase. In the literature, it has been reported that amino acid yield increases with temperature up to a certain point where amino acid decomposition into organic acids and volatile carbon starts to predominate over extraction [33,34]. Maximum amino acid yields have been found in the temperature range 220-260 ºC, depending on the raw material [13,17–22]. In this work, a slightly lower optimal temperature for amino acid yield has been obtained (190 ºC), which might be attributed to differences in the amino acid profile of the feed and/or the mode of operation, with a semi-batch reactor and long residence times that might have enhanced amino acid decomposition.

Table 4. Total amino acid profile of the raw material and the FPH extracts obtained by sCW extraction/hydrolysis of sardine waste at different temperatures.

	AMINO ACID PROFILE								
	Raw material (mg/g)	Sardine waste, SW (mg/g dry extract)				Defatted sardine waste, DSW (mg/g dry extract)			
Amino acid		**FPH90**	**FPH140**	**FPH190**	**FPH250**	**FPH90**	**FPH140**	**FPH190**	**FPH250**
Alanine (Ala)	22.2	25.9	31.6	32.1	22	39.5	51.7	53.8	48.1
Aspartic acid[a] (Asp)	18.9	9.4	8.1	12.3	6.2	10.6	12.2	13.1	11.3
Cysteine (Cys)	1.7	2.5	n.d.	n.d.	n.d.	2.8	n.d.	n.d.	n.d.
Glutamic acid[b] (Glu)	35.1	80.2	51.6	55.3	52.7	68.2	84.5	69.4	74.5
Glycine (Gly)	32	40.1	46.6	46.8	32.8	59.6	77.7	82.2	74.7
Histidine (His)	33.2	38.5	41.9	50.7	33.1	63.3	69.6	79	72.7
Hydroxyproline (Hyp)	8.1	12.2	10.3	18.8	8.5	14.4	15.3	18	16.4
Isoleucine (Ile)	24	27.6	32.8	37	23	44.2	53.4	57.2	50.8
Leucine (Leu)	35.9	39.7	44	49.9	31.8	57.2	73.3	82.4	73.2
Lysine (Lys)	5.2	4.6	5.3	5.8	5.1	6.3	6.9	7.2	6.9
Methionine (Met)	35.2	68.9	66.3	69	45.3	84.7	68.2	67.8	48.8
Phenylalanine (Phe)	23.4	24.8	28	12.4	10.9	39.4	27.2	12.2	16.1
Proline (Pro)	24.5	29.7	33.3	36.7	22.4	46.2	55.5	63.3	50.2
Serine (Ser)	12.6	28	17.8	20.4	22.7	22.9	28.3	20.8	26.8
Threonine (Thr)	17.7	21.5	23.8	28.2	17.4	35.4	38.4	44.6	36.7
Tryptophan (Trp)	30.1	28.1	26.7	27.6	22.8	31.1	24.8	26	23.7
Tyrosine (Tyr)	9.6	9.2	10.8	11.2	6.9	13	16.2	19.3	14.8
Valine (Val)	41.7	49.1	56.6	63.9	40.7	79.9	91.5	102.2	93.2
Total (mg/g)	**411.2**	**540.0**	**535.5**	**578.1**	**404.3**	**718.7**	**794.7**	**818.5**	**738.9**

Experimental uncertainty, $u = \pm 0.1$ mg/g extract; n.d.: not detected.

[a] Represents the sum of aspartic acid and asparagine.

[b] Represents the sum of glutamic acid and glutamine.

Table 5. Free amino acid content of the FPH extracts obtained by sCW extraction/hydrolysis of sardine waste at different temperatures.

	FREE AMINO ACID CONTENT							
	Sardine waste, SW (mg/g dry extract)				Defatted sardine waste, DSW (mg/g dry extract)			
Amino acid	**FPH90**	**FPH140**	**FPH190**	**FPH250**	**FPH90**	**FPH140**	**FPH190**	**FPH250**
Alanine (Ala)	2.9	16	13	13.7	3.6	9.6	8.3	10.1
Aspartic acid[a] (Asp)	2.1	3.2	3.3	2.8	3.7	4.2	4.3	4.5
Cysteine (Cys)	1.2	n.d.	n.d.	n.d.	1.2	n.d.	n.d.	n.d.
Glutamic acid[b] (Glu)	13.3	14.3	1.4	1	12.1	12.9	1.2	0.3
Glycine (Gly)	1.1	5.3	11.2	11.2	1.8	3.8	7.3	8.8
Histidine (His)	2.1	11.6	2.6	2.3	2.4	18.2	2.9	2.4
Hydroxyproline (Hyp)	0.2	3.7	1.8	3.4	4.8	3.8	0.9	2.4
Isoleucine (Ile)	2.1	12.1	3.8	3.7	3.7	8.3	1.8	5.7
Leucine (Leu)	2.6	21.7	7.1	3	4.3	16.4	9.7	2.9
Lysine (Lys)	3.2	4.2	3.6	4	4.7	4.9	1.2	2
Methionine (Met)	11.2	8.9	3.3	2.6	14.8	7.3	1.2	3.3
Phenylalanine (Phe)	11.7	13.9	3.8	3.1	12.5	11.5	1.3	5.5
Proline (Pro)	0.6	5.7	4.7	3.5	1.2	3.7	2	4.7
Serine (Ser)	2.4	1.6	2.9	1.2	2.7	1.1	0.8	0.7
Threonine (Thr)	2.1	8.2	2	1.4	3.4	6	1.6	1.4
Tryptophan (Trp)	5.3	4.1	1.5	2.4	7	3.9	0.4	1.8
Tyrosine (Tyr)	1.7	3.1	4.4	4.5	1.4	11.2	1.7	3.9
Valine (Val)	3.5	23.6	10.9	6	4.8	15.5	18.7	2.6
Total (mg/g)	**69.3**	**161.2**	**81.3**	**69.8**	**90.1**	**142.3**	**65.3**	**63**
Ratio free / total	0.13	0.30	0.14	0.17	0.12	0.18	0.08	0.09

Experimental uncertainty, $u = \pm 0.1$ mg/g extract; n.d.: not detected.

[a] Represents the sum of aspartic acid and asparagine.

[b] Represents the sum of glutamic acid and glutamine.

Free amino acid profile of the FPH extracts (Table 5) is similar to previously reported profiles from different fishery sources [13,19–22]. Differences are important in some cases [13,21], and are likely conditioned by the initial amino acid profile of the raw material, the sCW extraction/hydrolysis conditions and/or the mode of operation, as well as by the analysis method. In this work, free amino acid content increased with temperature up to 140 ºC and then decreased in the range 190-250 ºC for both types of extracts. Higher temperatures possibly caused decomposition of free amino acids into organic acids and other products. At elevated temperatures, the dissociation (K_w) and dielectric constants of water increase, leading to increased amino acid hydrolysis [34]. Similar to the total amino acid profile, high molecular weight amino acids such as Met, Glu, Phe and Trp are the most abundant at low temperature (90 ºC) and then decrease, whereas simpler amino acids such as Ala and Gly increase with temperature up to 250 ºC. This behaviour confirms the hypothesis that two main consecutive reactions are happening during sCW extraction/hydrolysis: (1) production of amino acids from hydrolysis of proteins, and (2) decomposition of amino acids into other products such as simpler amino acids, organic acids, and ultimately, volatile carbon and water [18]. Moreover, the ratio of free to total amino acids indicates that proteins in sardine waste are majoritarily broken down into peptide units rather than directly into amino acid monomers. The highest ratios were observed in the FPH extracts collected at 140 ºC, being 0.30 and 0.18 for SW and DSW, respectively. These values decrease at higher temperatures, indicating that free amino acids ceased to accumulate due to excessive hydrolysis and decomposition [18].

3.5. Bioactive properties of FPHs

Table 6 summarizes measured bioactive properties of the FPHs obtained from SW and DSW at each target temperature. The antioxidant activity was evaluated through the

action of FPHs on DPPH radical reduction. Results are expressed as half maximal effective concentration (EC_{50}). The extract obtained from DSW at 190 ºC (FPH190) showed the highest antioxidant activity for DPPH assay, with the lowest EC_{50} value (4.1 ± 0.3 mg extract/mg DPPH). The EC_{50} DPPH values correlated rather well with the free amino acid content of each sample, although some studies suggest that certain sequences of amino acids present in the FPH extracts and interactions among them must also influence the radical scavenging activity [40]. Moreover, other compounds generated in Maillard, caramelization and thermo-oxidation reactions might influence the antioxidant activity of FPHs [22].

Table 6. Summary of the bioactive properties of the FPH extracts obtained by sCW extraction/hydrolysis of sardine waste at different temperatures.

	DPPH, EC_{50} (mg extract/mg DPPH)	**Cytotoxicity effect, EC_{50} (mg/mL)**	**Antiproliferative activity, EC_{50} (mg/mL)**
Sardine waste, SW			
FPH90	2.61 ± 0.13^{c}	9.63 ± 1.73^{cd}	6.44 ± 0.27^{b}
FPH140	3.18 ± 0.18^{b}	7.84 ± 0.57^{d}	2.05 ± 0.11^{d}
FPH190	3.45 ± 0.20^{ab}	11.7 ± 2.13^{c}	1.23 ± 0.04^{f}
FPH250	3.56 ± 0.18^{a}	7.17 ± 0.05^{e}	0.77 ± 0.05^{g}
Defatted sardine waste, DSW			
FPH90	1.65 ± 0.13^{d}	16.3 ± 1.42^{b}	8.09 ± 0.75^{a}
FPH140	0.81 ± 0.13^{e}	16.7 ± 1.98^{b}	3.59 ± 0.20^{c}
FPH190	0.9 ± 0.08^{e}	24.4 ± 3.04^{a}	6.98 ± 0.27^{b}
FPH250	1.68 ± 0.13^{d}	7.20 ± 0.06^{e}	1.58 ± 0.13^{e}

$^{a, b\ldots}$: Values with different superscript letters in the same column differ significantly ($p < 0.05$).

Before antiproliferative assays, FPH extracts were tested for their toxicity using confluent and undifferentiated Caco-2 cells as a model for crypt enterocytes [28]. Results obtained after are shown in Table 6. Comparison of EC_{50} values showed that FPH extracts from

SW are more toxic to Caco-2 cell line than the ones obtained from DSW. The cytotoxic effect of the FPH extracts also increased with extraction temperature. Certain lipid compounds, specially free fatty acids and lipid oxidation products, have been reported to exert cytotoxic effects [41]; thus, the higher lipid content of FPHs from SW and the increased extraction of lipid compounds at high extraction temperatures might offer an explanation to this behaviour.

In order to evaluate the antiproliferative effect of FPH extracts, a human colorectal adenocarcinoma cell line (HT-29) was subjected to treatment with non-cytotoxic concentrations of extracts for 24h. For each FPH extract, the effective concentration that decreases 50% of cell viability (EC_{50} values) was calculated from dose-response curves. Results obtained (Table 6) suggest a stronger antiproliferative effect against HT-29 cells of the FPH extracts derived from SW, with lower EC_{50} values than the extracts from DSW at each temperature. The antiproliferative effect of the FPH extracts increase with the extraction temperature, which might be related to the protein content but also to the formation of Maillard reaction products and the presence of lipid compounds.

In the literature, FPHs obtained from enzymatic hydrolysis of fish waste of different nature are reported to inhibit proliferation of human cancer cells *in vitro* [42]. However, up to our knowledge, no anticancer activities have been reported for FPH extracts obtained by sCW extraction/hydrolysis so far. Furthermore, the analysis of each EC_{50} pair for cytotoxicity and antiproliferative effects showed that all extracts were capable of inhibiting HT-29 cell proliferation while being non-cytotoxic for Caco-2 cells at the same concentrations (i.e., EC_{50} for HT-29 is lower than EC_{50} in Caco-2). Moreover, the differences between EC_{50} values in Caco-2 and HT-29 cells are large, revealing a wide range of applicability and suggesting that FPH extracts from sardine waste may be promising for biomedical applications.

Conclusions

The results presented in this study demonstrate that biorefining strategies using green solvents such as supercritical carbon dioxide ($SCCO_2$) and subcritical water (sCW) represent a suitable alternative for the valorisation of waste streams such as sardine (*S. pilchardus*) heads, viscera and spines. Sequential supercritical fluid extraction (SFE) and sCW extraction and hydrolysis offer the possibility of obtaining value-added fractions of different nature, each of them with promising applications in the food, pharmaceutical, and cosmetic industries. Sardine waste has been fractionated in fish oil rich in omega-3 PUFAs, and a fish protein hydrolysate (FPH) fraction rich in peptides and amino acids. As expected, the previous extraction of the lipid fraction increased the purity of FPH extracts, and extraction temperature positively affected the extraction yield and the bioactive properties of the FPH extracts. FPH extracts have been obtained in an environmentally friendly way, but also able to produce functional materials, since good antioxidant activity and antiproliferative effect against HT-29 adenocarcinoma cells have been observed in the bioactivity assays.

Declaration of interests

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.

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