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Ultrasound and supercritical fluids as useful tools to recover nutrients and bioactive compounds from aquaculture and marine side streams

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Y para que así conste, expiden y firman el presente certificado

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“Sé como el Sol por gracia y misericordia. Sea como la noche para cubrir las faltas de los demás. Sea como agua corriente por la generosidad. Sea como la muerte para la rabia y la ira. Sea como la Tierra por la modestia. Aparece como eres. Sé como apareces"

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List of abbreviations:

A. flavus	*Aspergillus flavus*
A. parasiticus	*Aspergillus parasiticus*
A_0	Initial absorbance
AAL	AAL toxins
AAPH	Dihydrochloride
ABTS	2,2′-azino-bis-3-ethylbenzothiazoline-6-Sulfonic acid
ACE	Angiotensin-converting enzyme
A_f	Final absorbance
AFs	Aflatoxins
ANOVA	Analysis of variance
AUC	Area under curve
B. nivea	Boehmeria nivea
BEA	Beauvericin
BSE	Bovine spongiform encephalopathy
CE	Collision energy
CFU	Colony forming unit
$CHCl_3$	Chloroform
CIT	Citrinin
CO_2	Carbon dioxide
CP	Cold plasma
CXP	Cell exit potential
DES	Deep eutectic solvents
DHA	Docosahexaenoic Acid
DLLME	Dispersive liquid-liquid microextraction
DMSO	Dimethyl sulfoxide
DON	Deoxynivalenol,
DP	Desclustring potential
DPPH	2,2-diphenyl-1-picryl-hydrazyl-hydrate
DPP-IV	Dipeptidyl peptidase IV
DTT	Dithiotheitol
ENNs	Enniatins
EPA	Eicosapentaenoic Acid
EU	European Union
F. graminarum	*Fusarium graminarum*
FAO	Food and Agriculture Organization
GAE	Gallic acid equivalant
GAG	Glycosamino glycane
GRAS	Generally recognized as safe
HIV	Human immunodeficiency
HPP	High-pressure processing
HPU	High power ultrasound
ISP	Isoelectric solubilization precipitation
K_2HPO_4	Potassium phosphate monobasic
K_2S2O_8	Potassium perusulfate
LC	Liquid Chromatography

LC-PUFAs	Long-chain omega-3 fatty acids
LC-MS/MS-QTRAP	Liquid chromatography coupled to tandem mass spectrometry with time of flight
LC-MS/MS-IT	Liquid chromatography ion trap tandem mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LPU	Low power ultrasound
LSD	Least significant difference
MAE	Microwave-assisted extraction
max	maximum
MeOH	Methanol
min	minimum
MPa	Megapascal
MQ	Milli-Q
MT	Million tons
MUFA	Monounsaturated fatty acids
MW	Molecular weight
N. fisheri	*Nassarius fisheri*
Na_2CO_3	Sodium carbonate
Na_2HPO_4	sodium phosfate
NADES	Natural deep eutectic solvents
NaOH	Sodium hydroxyd
NAS	North Athlantic Shrimps
NIV	Nivaleno
ORAC	Oxygen radical antioxidant capacity
OTA	Ochratoxin A
P. oxalicum	*Penicillium oxalicum*
P. tricornutum	*Phaedoactylum tricornutum*
P_a	Acoustic pressure
PAT	Patulin
P_{amax}	Acoustic pressure maximal
Pc	Critical pressure
PEF	Pulsed electric fields
Ppb	Part per billion
PUFA s	Polyunsaturated fatty acids
RSD	Relative standard deviation
RSM	Response surface methodology
SDEE	SC-dimethyl ether
SDGs	Sustainable Development Goals
SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
SFA	Saturated fatty acids
SFE	Supercritical fluid extraction
SWH	Subcritical water hydrolysis
TAG	Triacylglycerol
Tc	Critical temperature
T-cell	
TE	Trolox equivalent
TNFα	Tumor necrosis factor alpha

TPC	Total phenolic compounds
UAE	Ultrasound assisted extraction
UP	Ultrasonic prob
USN	Ultrasound
ZEA	Zearalenone

ABSTRACT

Aquaculture products and marine side streams are found to be a great source of diverse groups of compounds with several biological activities, thus attracting the interest of food, pharmaceutic, and cosmetic industries, among others. To benefit from them, more specifically the fish side streams and microalgae, as a potential source of diverse antioxidant compounds, proteins, carbohydrates, pigments, phenolic compounds etc., the extraction itself plays a crucial role, essentially when it is assisted by a green and sustainable technology. The conventional extraction techniques involve the use of organic solvents and require long extraction times while the innovative green extraction technologies, avoid the challenges related to the conventional extraction methods and are environmentally sustainable. In addition, compared to conventional methods, innovative green extraction technologies maximize the extraction yields. In this line, ultrasound-assisted and supercritical fluid extraction technologies are among the new technologies widely used for the extraction of valuable compounds from fish and marine microalgae. Moreover, these technologies are widely used for food mycotoxins extraction and decontamination.

The objective of the present Doctoral Thesis is the optimization of the **ultrasound-assisted extraction** (UAE) technology for the extraction of nutrients and antioxidant bioactive compounds fish (i.e. sea bass) side streams and microalgae (i.e. *Phaeodactylum tricornutum*), in addition to evaluating mycotoxins´ contamination in fish extracts. For this purpose, UAE conditions have been optimized using a response surface methodology (RSM) with the dependent variables: time (0.5–30 min), pH (5.5–8.5), and temperature (20–50 °C).

Regarding sea bass side streams (head, skin, bones and viscera), the results obtained after analyzing the extracts obtained revealed a high percentage of proteins recovery and a high antioxidant activity present in these side streams. The highest values were obtained for viscera, when the time and temperature increased up to 30 min and 50 °C. The RSM study showed that the optimal values to obtain the highest protein percentage and antioxidant capacity for the head were 25 min,

20 °C and pH=5.5, while for the skin side streams the optimal parameters were 30 min, 35 °C and pH=8.5, for bones, 30 min of extraction at 20 °C and 8.5 pH and for the viscera 26 min of UAE at 50 °C with the same pH of 8.5 were the optimal conditions. The experimental values obtained to achieve the highest proteins and antioxidant values from fish side streams were close to those expected, thus confirming the validity of the employed model to establish the optimal UAE conditions. Furthermore, the analysis of the mycotoxins content in the extracts using LC-MS/MS-QTRAP showed the absence of the analyzed mycotoxins in all the extracts.

As for the microalgae *P. tricornutum*, the maximum extraction yield of nutrients, bioactive compounds and antioxidant capacity were achieved after 30 min of extraction at 50 ºC and a pH of 8.5. The evaluation of the carotenoids and total phenolic compounds showed that both antioxidant bioactive compounds were positively affected by the ultrasound extraction time, whereas the carbohydrates extraction was positively affected by the temperature. The antioxidant capacity, measured by 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), was strongly modulated by the extraction time, while for the antioxidant capacity measured by oxygen radical antioxidant capacity (ORAC) assay, the temperature was the most significant factor followed by the extraction time.

Keywords: Aquaculture; fish side streams; nutrients; antioxidants; ultrasound; supercritical fluid extraction.

RESUMEN

Los productos provenientes de la acuacultura, así como de los subproductos marinos contienen una cantidad muy importante y diversa de compuestos con diferentes actividades biológicas, atrayendo así el interés de las industrias alimentaria, farmacéutica y cosmética, entre otras. Para beneficiarse de éstos, especialmente subproductos del pescado y microalgas, como fuente potencial de diversos compuestos antioxidantes, proteínas, carbohidratos, pigmentos, compuestos fenólicos, etc., la extracción en sí juega un papel crucial, fundamentalmente cuando se trata de tecnologías verdes y sostenibles. Las técnicas de extracción convencionales implican el uso de disolventes orgánicos y requieren tiempos de extracción prolongados. Las tecnologías innovadoras de extracción ecológicas evitan los desafíos relacionados con los métodos de extracción convencionales y son ambientalmente sostenibles. Además, en comparación con los métodos convencionales, las tecnologías innovadoras de extracción ecológicas maximizan los rendimientos de extracción. En esta línea, las tecnologías de extracción de fluidos supercríticos y ultrasonidos se encuentran entre las nuevas tecnologías ampliamente utilizadas para la extracción de compuestos con un potencial valor añadido a partir de subproductos de pescado y microalgas. Además, estas tecnologías se utilizan ampliamente para la extracción y descontaminación de micotoxinas alimentarias.

El objetivo de la presente Tesis Doctoral es la optimización de la tecnología de extracción asistida por ultrasonidos (UAE) para la extracción de nutrientes y compuestos bioactivos antioxidantes A partir de subproductos de pescado (ej: lubina) y microalgas (ej: *Phaeodactylum tricornutum*), además de evaluar la contaminación con micotoxinas´ en extractos de pescado. Para este propósito, las condiciones de los UAE se han optimizado utilizando una metodología de superficie de respuesta (RSM) con las variables dependientes: tiempo (0,5–30 min), pH (5,5–8,5) y temperatura (20–50 ° C).

En cuanto a los subproductos de pescado (cabeza, piel, espinas y vísceras), los resultados obtenidos tras el análisis de los extractos obtenidos revelaron un alto porcentaje de recuperación de proteínas y una alta actividad antioxidante presente en éstos. Los valores más altos se obtuvieron

para las vísceras, cuando el tiempo y la temperatura aumentaron hasta 30 min y 50 °C. El estudio de RSM mostró que los valores óptimos para obtener el mayor porcentaje de proteína y capacidad antioxidante para la cabeza fueron 25 min, 20 °C y pH = 5.5, mientras que para los subproductos provenientes de la piel los parámetros óptimos se obtuvieron tras aplicar 30 min, 35 °C y pH = 8.5, para espinas fueron necesarios 30 min de extracción a 20 °C y 8.5 pH y para las vísceras se necesitaron 26 min de UAE a 50 °C con el mismo pH de 8.5 para conseguir las condiciones óptimas. Los valores experimentales obtenidos para lograr los valores más altos de proteínas y antioxidantes de los subproductos de pescado fueron cercanos a los esperados, confirmando así la validez del modelo empleado para establecer las condiciones óptimas de los UAE. Además, el análisis del contenido de micotoxinas en los extractos mediante LC-MS/MS-QTRAP mostró la ausencia de las micotoxinas analizadas en todos los extractos.

En cuanto a la microalga *P. tricornutum*, el máximo rendimiento de extracción de nutrientes, compuestos bioactivos y capacidad antioxidante se logró tras aplicar 30 min de extracción a 50 ºC y un pH de 8.5. La evaluación de los carotenoides y los compuestos fenólicos totales mostró que ambos compuestos bioactivos antioxidantes se vieron afectados positivamente por el tiempo de extracción por ultrasonidos, mientras que la extracción de carbohidratos se vió afectada positivamente por la temperatura. La capacidad antioxidante, medida por el ácido 2,2′-azino-bis-3-etilbenzotiazolina-6-sulfónico (ABTS), se vió influenciada de forma significativa por el tiempo de extracción, mientras que en el caso de la capacidad antioxidante medida por el ensayo de capacidad antioxidante de radicales de oxígeno (ORAC), la temperatura fue el factor más significativo seguido por el tiempo de extracción.

Palabras clave: Acuacultura; subproductos de pescado; nutrientes; antioxidantes; ultrasonidos; extracción con fluidos supercríticos.

1. INTRODUCTION

1. Introduction

World aquaculture production attained a high record of 114.5 million tons (MT) in live weight in 2018 (FAO, 2020) (**Figure 1**). The total production consisted of 82.1 MT of aquatic animals, 32.4 MT of aquatic algae and 26 000 T of ornamental seashells and pearls. Moreover, the global fish production is estimated to be increased ≈179 MT, of which 46 % (82 MT) are derived from aquaculture production.

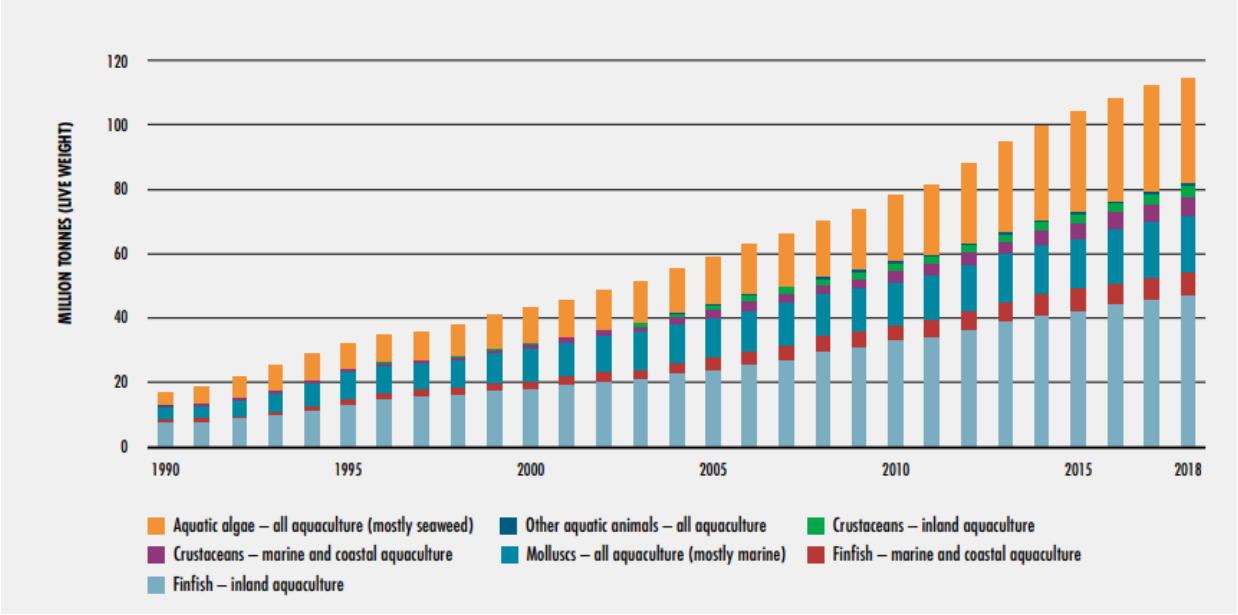


Figure 1: World aquaculture products of aquatic animals and algae 1990-2018 (FAO, 2020).

Out of the total, 156 MT of these resources were used for human consumption, equivalent to an estimated annual supply of 20.5 kg per capita. The remaining 22 MT were destined for non-food uses, mainly to produce fish meal and fish oil (FAO, 2020). Besides, China remained as the main fish producer, accounting for 35% of global fish production. Excluding China, a significant share of production in 2018 came from Asia (34%), followed by America (14%), Europe (10%), Africa (7%) and Oceania (1%) (FAO, 2020). In addition, aquaculture provides a great diversity of species such as common carp (*Cyprinus carpio*) (8%), Nile tilapia (*Oreochromis niloticus*) (8%), bighead carp

(*Hypophthalmichthys nobilis)* (7%), catla (*Catla catla*) (6%), Atlantic salmon (*Salmo salar*) (4%), and rainbow trout (*Oncorhynchus mykiss*) (2%) which were the main species produced (FAO, 2018).

On the other hand, aquaculture is the main source of edible aquatic plants, accounting for 97% of the total production in 2018. The volume of global farmed algae has increased by about 55% in the last two decades. Of the total million tons of algae from aquaculture in 2018, seaweeds represented 32.4 MT while only 87,000 tons were recorded for microalgae, although this last value is understood because of missing data from important producers and farmed algae for scientific purposes that are not included (FAO, 2020). The farming of microalgae fits into the widely accepted definition of aquaculture. However, microalgae cultivation tends to be tightly regulated and monitored at the national or local level separately from aquaculture. A recently conducted national aquaculture census in one of the top 20 aquaculture producing countries covered microalgae farming, but it is yet to be part of the national aquaculture data collection and reporting system. Furthermore, farming of microalgae such as *Spirulina* spp., *Chlorella* spp., *Haematococcus pluvialis* and *Nannochloropsis* spp., ranging in scale from backyard to large-scale commercial production, is well established in many countries for the production of human nutrition supplements and other uses (FAO, 2020).

1.1. Fish side streams

1.1.1. Nutritional value and bioactive compounds

Fish is a highly valuable food source rich in proteins, amino acids, unsaturated fatty acids (mainly omega-3), minerals and vitamins which are essential for a healthy and balanced nutrition (Kundam et al., 2019) (**Figure 2**). Several factors can affect the average of the fish composition such as the species and the age of fish among others, but mostly fish are characterized by an important content of moisture (50–80%), proteins (15–30%) and lipids (0%–25%) (Caldeira et al., 2018). Although, the represented percentages are not so important in numbers, fish is also a great source of micronutrients such as minerals and vitamins, especially calcium, potassium and magnesium for minerals (Munekata et al., 2020) and vitamin A and D (Pateiro et al., 2020).

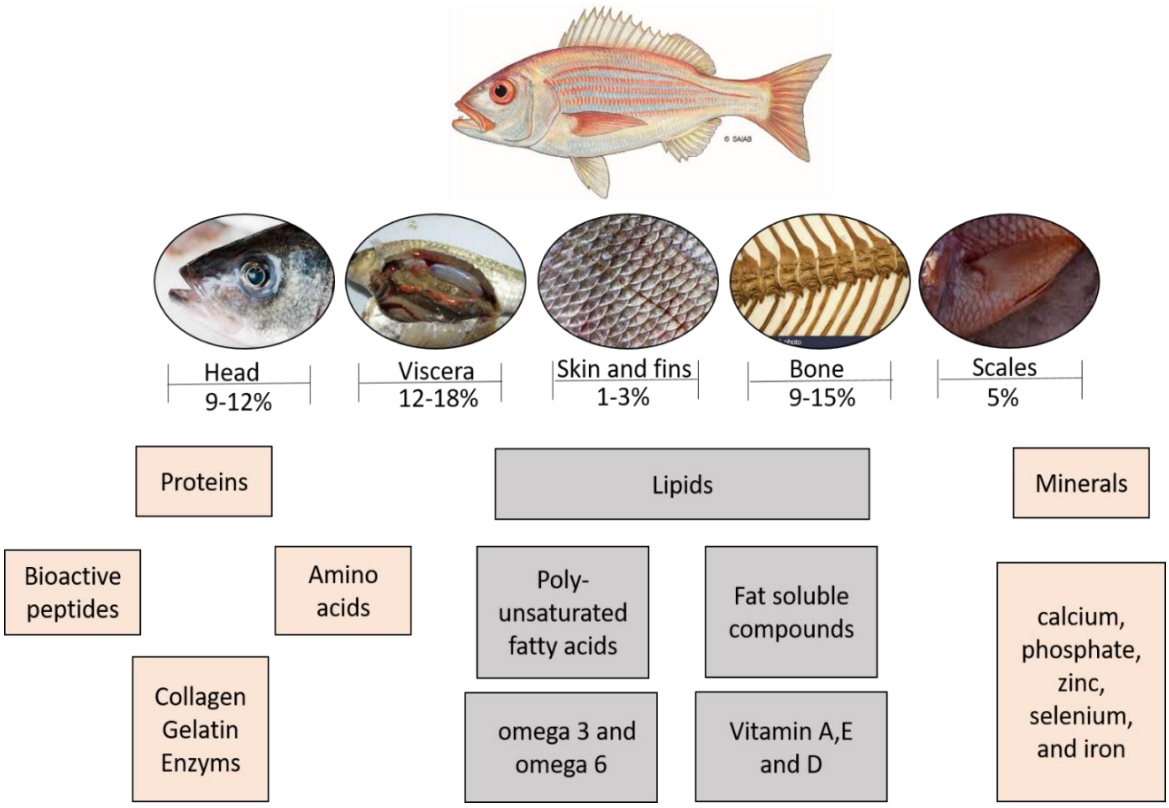


Figure 2: Percentages of different fish by-products according to the whole fish weight, with the different valuable compounds that they can generate (Villamil, Váquiro, & Solanilla, 2017).

The processing of fish generates considerable amounts of side streams such as head, skin, scales, bones and viscera that remain underused and/or unexploited. The Food and Agriculture Organization (FAO) reported that 9.1 MT of fish waste are estimated to be discarded yearly (FAO, 2020). Generally, these side streams are incorporated into animal feeding or biofuels, or incinerated and discarded, resulting in a negative impact on the environment as well as an increase in energy consumption and in the financial cost. Moreover, up to 20-80% of valuable waste is produced by fish processing in fish industries and it all depends on the fish type and the level of processing (Al Khawli, Martí-Quijal, et al., 2020). Consequently, in the last decade, a considerable attention has been paid to the use of fish side streams for pharmaceutical, cosmeceutical and nutraceutical applications in industries due to their high quality nutrients and bioactive compounds (Marc Antonyak et al., 2018).

Head, skin, bone and viscera from fish are promising sources of bioactive compounds which can accomplish multiple medical and health benefits through their biological activity (i.e. antioxidant, anti-inflammatory, anticancer, anti-aging and antihypertension activities, among others). For example, fish skin is considered as a source of antioxidant, immunodulatory, antiproliferative and angiotensin-converting enzyme (ACE) inhibitory compounds that can contribute to the improvement of human health (Zamora-Sillero et al., 2018). Moreover, fish viscera, including gut, liver and stomach provide a valuable source of enzymes that produce various bioactive peptides which have demonstrated various properties such as antioxidant, antihypertensive and antimicrobial properties (Ucak et al., 2021). Numerous studies have been conducted to extract bioactive compounds from diverse side streams, having some of them different biological activities. Some of these studies are listed in **Table 1**.

1.1.1.1. Proteins and derived compounds

In fact, the nutritional value of most fish proteins is equivalent or higher than that of casein. In addition, the quality of proteins from fish is higher than terrestrial animal meat (Le Gouic et al., 2019). For instance, fish side streams are an excellent source of proteins that contain all the essential and non-essential amino acids (Shahidi & Ambigaipalan, 2018). Up to 10-20% of fish proteins can be present in fish side streams being source of collagen, gelatin and bioactive peptides which can be used to produce beneficial bio-products for human health and many industries (Al Khawli et al., 2019).

1.1.1.1.1 Collagen and gelatin

Collagen is the most abundant single protein present in fish, corresponding to 25% of the total protein (Caldeira et al., 2018). It is organized in a fibrillar arrangement, and it is mainly found in the extracellular matrix of fish, contributing in many physiological functions of tissues, bones, skin, head, cartilage, tendons, and muscles (Silva et al., 2014).

Table 1: Biological activities obtained from different by-products of various species

Side streams	Source	Biological activities	References
		Peptides	
Skin	Seabass (*Lates calcarifer*)	Antioxidant, immunomodulatory, antiproliferative	(Sae-leaw et al., 2016)
	Tilapia (*Oreochromis niloticus*)	Antidiabetic	(Wang et al., 2015)
Head	Bluefin leatherjacket (*Navodon septentrionalis*)	Antioxidant	(Chi et al., 2015)
	Tilapia (*Oreochromis niloticus*)	Antimicrobial	(Robert et al., 2015)
	sardinelle (*Sardinella aurita*)	Antioxidant	(Bougatef et al., 2010)
Bone	*Rastrelliger kanagurta*	Antioxidant	(Sheriff et al., 2014)
	Alaska Pollack (*Theragra chalcogramma*)	Ca-binding	(Jung et al., 2006)
	Tuna	Antioxidant	(Je et al., 2007)
Viscera	BLack scabbard fish (*Aphanopus carbo*)	Antioxidant	(Batista et al., 2010)
	Black Pomfret, *Parastromateus niger*	Antioxidant	(Jai ganesh et al., 2011)
	Smooth hound (*Mustelus mustelus*)	Antioxidant, anti-ACE, antibacterial activities	(Abdelhedi et al., 2016)
		PUFA	
Viscera	Atlantic cod (*Gadus Morrhua L.*)	Antibacterial	(Ilievska et al., 2016)
	Sardine (*Sardinops sagax*)	Nitric oxide inhibitory Tumor necrosis factor alpha (TNFα) inhibitory Anti-inflammatory	(Ahmad et al., 2019).
Head	Salmon	Antimicrobial	(Inguglia et al., 2020)
		Nitric oxide inhibitory TNFα inhibitory Anti-inflammatory	(Ahmad et al., 2019).

Actually, there are several types of collagens, but collagen type I is the most frequent form in fish side streams. It is found in the connective tissues, skin, muscles, bones (Caldeira et al., 2018) and

cornea of fish (Raman & Gopakumar, 2018). In fact, collagen has been obtained from the skin of different fish types (Chi et al., 2014; Pei et al., 2010). Furthermore, the hydrolysis of fish collagen facilitates the generation of active peptides. Some of these collagen-derived peptides reveal interesting antioxidant and antimicrobial activities against different strains of bacteria (Ennaas et al., 2015), in addition to showing effective antihypertensive activity through ACE inhibitory properties (Alemán et al., 2013).

On the other hand, the irreversible thermal denaturation of collagen generates polypeptides called gelatin (Qiu et al., 2019). Gelatin can rearrange and stabilize its tridimensional structure forming a gel. Thus, it can be used to improve the elasticity, consistency and stability of foods as well as to produce edible and biodegradable films which can increase the shelf life of food products (Caldeira et al., 2018). Besides, gelatin obtained from fish had a higher antioxidant activity than the synthetics one (Ishak & Sarbon, 2018).

Many fish species are rich in gelatin where skin is its main source (Irwandi et al., 2009). For instance, gelatin can be obtained from both sea bass (*Lates calcarifer*) (Sae-leaw et al., 2016) and pacific cod (*G. macrocephalus*) (Ngo et al., 2016) skin. Moreover, it can be also extracted from scales of Bighead carp (*Hypophthalmichthys nobilis*) (Huang et al., 2017), bones of Black tilapia gelatin (Zakaria et al., 2015), and head of mackerel (*Scomber scombrus)* (Khiari, Rico, Martin-Diana, 2011). As for collagen, gelatin can be hydrolyzed to obtain peptides with important biological activities.

For instance, gelatin hydrolysates from salmon showed a remarkable bioactivity, including antioxidant, antiproliferative and immunomodulatory effects in cell culture systems (Sae-leaw et al., 2016). Additionally, peptides and free amino acid isolated from Atlantic salmon (*Salmo salar*) skin, bone and muscle through the extraction of gelatin hydrolysates were shown to have great biological activities such as angiotensin-converting enzyme inhibitory, dipeptidyl peptidase IV (DPP-IV) inhibitory and antioxidant activies (Neves et al., 2017).

Finally, it has been proven that both collagen and gelatin have a wide range of applications in the food related sector and the health sector, specifically in cosmetics, likewise in the pharmaceutical industry and in the medical care area (including plastic surgery, orthopedics, ophthalmology and dentistry) (Silva et al., 2014).

1.1.1.1.2 Bioactive peptides

Bioactive peptides are naturally present in the whole fish and incorporated in fish protein, they consist of a short sequence of 2-20 amino acids (Ucak et al., 2021). These peptides are inactive within the native protein and only become active, after being liberated by the digestion process *in vivo* (proteolysis) or by the enzymatic hydrolysis process in vitro, which is considered the best process in order to obtain bioactive properties (Zamora-Sillero et al., 2018). Still, the bioactive properties of the peptides depend on their amino acid composition and sequence. Thus, they can have an important role in human health promotion and aid in the prevention and treatment of many noncommunicable diseases. These properties include anti-cancer, anti-diabetic, antioxidant, anti-inflammatory, anti-aging, and ACE inhibitory activities (Le Gouic et al., 2019).

In fact, many research have shown that head, viscera, skin and bones from different fish species are a good source of bioactive peptides. For example, three peptides were purified from the head of *Bluefin leatherjacket*, showing an excellent antioxidant activity measured with ABTS and 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) radical scavenging assays (Chi et al., 2015). In addition, a remarkable antioxidant activity was observed after the purification of seven peptides from the combination of head and viscera of *sardinelle* hydrolysates (Bougatef et al., 2010). In this sense, three peptides isolated from grass carp skin hydrolysates exhibited high scavenging activity on DPPH, hydroxyl and ABTS radicals (Cai et al., 2015).

In another study, eleven peptides from salmon skin collagen were isolated after enzymatic hydrolysis, which exhibited a significant ACE inhibitory activity and might be considered as antihypertensive agents (Gu et al., 2011). Additionally, Guo et al. (2013) showed that a tripeptide

isolated from the skin collagen of Alaska Pollock, after treatment with commercial enzymes, demonstrated high iron-chelating activity (Guo et al., 2013). In addition, some peptides hydrolysates with both immunomodulatory and anti-proliferative activities were extracted from skin gelatin of seabass (*Lates calcarifier*) (Sae-leaw et al., 2016) and from unicorn leatherjacket (Karnjanapratum et al., 2016).

1.1.1.2 Lipids

Fish oil represents about 2% of the world consumption of fats and oils and is commonly categorized as a functional food with proven beneficial influence on human health and nutrition (Soldo et al., 2019). Fish oil contains the most important ω-3 polyunsaturated fatty acids (PUFAs) (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)), which represent about 30% of fish oil weight (Melgosa et al., 2019). The main sources of omega-3 PUFAs are the fatty fish such as herring, sardine, salmon and mackerel (Kundam et al., 2019).

Notably, fatty acids (FA) are in the whole fish. From the fish side streams, FA can be obtained mainly from the head, viscera, skin and bone of different fish species. For example, the extraction of PUFAs from the bone and skin of Atlantic salmon (Haq et al., 2017), viscera of tilapia (*Oreochromis niloticus*) (Shirahigue et al., 2016), common carp (*Cyprinus carpio L.*) (Lisichkov et al., 2014) and mackerel skin (Sahena et al., 2010) was carried by several authors. Additionally, PUFAs were extracted from sardine heads and tails (Létisse & Comeau, 2008) and form tuna heads (Ferdosh et al., 2016).

Besides, it is well notable that fish oils are a rich source of vitamins (A, D, E) (Fang et al., 2019). Vitamin A is accumulated mostly in fish liver oil. Among the different fish species, halibut, cod and sardine reserve vitamin A and D in their liver (Kundam et al., 2019), while other species such as herring, mackerel, trout and salmon possess vitamin D in their tissues, while the yellow tuna accumulates it in its bones (Talib & Zailani, 2017).

1.1.1.3. Micronutrients

The human body requires vitamins for it to perform its chemical and physiological functions. Fish side streams are a notable source for these vitamins, mainly vitamins A, D and E (Kundam et al., 2019). Vitamin D main function is rickets prevention, and its deficiency is very common worldwide. intriguingly, Vitamin D3 is thoroughly found in fish with concentrations depending on the fish species (Välimaa et al., 2019)

In addition, fish bones are an important source of calcium, zinc, phosphate, iron and selenium (Bruno, Ekorong, et al., 2019). This is due to their richeness in minerals that are mostly inorganic (60%). Minerals were succesfully isolated from numerous fish species. For instance, calcium and phosphore form sea bass bone (*Lates calcarifer*) (Pal et al., 2017). Similarly, calcium, phosphore, magnesium and strontium were isolated from *Catla catla* fish scales (Paul et al., 2017). These minerals, considered as bioactive compounds, are essential components in nutraceutical products targeted to improve health, such as bones, cardiovascular and immunological diseases (Webb, 2015).

1.2. Microalgae

Microalgae consist of a wide range of photosynthetic microorganism living in salty or fresh water. Their shapes are diverse, with a diameter or length varying from a few micrometers to a few hundred micrometers (De Morais et al., 2015). They include the eukaryotic microalgae and the prokaryotic cyanobacteria, well-known as blue-green algae. Generally, the algae that have higher composition of chlorophyll *a* and chlorophyll *b* as in higher plants are called green algae. Accordingly, cyanobacteria are classified as microalgae due to their content of chlorophyll *a* and compounds related to photosynthesis. Microalgae play a fundamental role in aquatic ecosystems whereas they are responsible of approximately 40% of global photosynthesis (De Morais et al., 2015).

Nevertheless, although a lot of research has been conducted on natural products from microalgae, and despite having many advantages over land plants, they are generally considered as not fully explored products when compared to those obtained from terrestrial plants. Among the

many advantages the algae possess, it should be noted their rapid growth, easy cultivation, and the fact that they lack a competition with harvests for agricultural land. Having said that, it is of great value to develop the microalgae for the discovery, acquisition and production of valuable bioactive compounds from the various algal species, mostly the medicinally and pharmaceutically important natural products.

1.2.1. Nutritional value

For a healthy lifestyle, a balanced diet constituting of antioxidants, PUFAs, vitamins and other beneficial compounds is mandatory. Numerous species of microalgae are reported to be rich in proteins, carbohydrates, lipids and other substances. Microalgae are excellent sources of vitamins such as vitamin A, B1, B2, B6, B12, C, E and of minerals such as potassium, iron, magnesium, calcium and iodine (Koyande et al., 2019). Amongst numerous microalgal species, the species that have been highly commercialized are *Spirulina* sp., *Chlorella* sp., *Dunaliella* sp., *Haematococcus* sp., *Botryococcus* sp., *Porphyridium* sp., *Phaeodactylum* sp., *Crypthecodinium* sp., *Chaetoceros* sp., *Nannochloris* sp., *Isochrysis* sp., *Schizochytrium* sp., *Nitzschia* sp., *Skeletonema* sp., and *Tetraselmis* sp. (Sathasivam et al., 2019) (**Table 2**).

Table 2: Biochemical composition of some microalgae (Bernaerts et al., 2019)

Source	Proteins (%)	Carbohydrates (%)	Fats (%)
Chlorella vulgaris	38–53	8–27	5–28
Diacronema vlkianum	24–39	15–31	18–39
Haematococcus pluvialis	10–52	34	15–40
Isochrysis galbana	12–40	13–48	17–36
***Nannochloropsis* sp.**	18–47	7–40	7–48
Odontella aurita	9–28	30–54	13–20
Pavlova Luther	16–43	15–53	6–36
Phaeodactylum tricornutum	13–40	6–35	14–39
Porphyridium cruentum	27–57	12–39	5–13
Scenedesmus sp.	31–56	6–28	8–21
***Schizochytrium* sp.**	10–14	12–24	46–74
Spirulina	43–77	8–22	4–14
***Tetraselmis* sp.**	14–58	12–43	8–33

1.2.1.1 Proteins

Microalgae are a rich source of protein. The studies done on microalgae have not only shown that microalgae produced high amounts of proteins, but also proteins of a high quality and high nutritional values. Evidently, proteins are structurally and metabolically involved in the microalgae composition, whereby they are an integral part of the membrane and the light-harvesting complex. In addition, they form several catalytic enzymes involved in photosynthesis.

As mentioned earlier, the protein content of various microalgal species is of high quality what makes it highly competitive, quantitatively and qualitatively, with the usual protein sources. In terms of quantity-wise, proteins in microalgae species vary, ranging from 42% to over 70% in some cyanobacterial species (Barkia et al., 2019), while higher photoperiods result in higher cellular protein content. In terms of quality-wise, various microalgae species possess most of, or all, the essential amino acids that the human body is unable to synthesize. Additionally, the amino acids in microalgae are well-proportioned and highly comparable to high-quality protein sources, as in egg albumin, soy and lactoglobul (Koyande et al., 2019).

1.2.1.2. Carbohydrates

Carbohydrates, comprising mono-, oligo-, and polysaccharides, have both structural and metabolic functions. Carbohydrates can be either found attached to proteins (glycoproteins) or to lipids (glycolipids), whereas complex polysaccharides are the main constituents of microalgae cell wall. The number of polysaccharides produced by microalgae differ according to the strain. For example, *Tetraselmis suecica* accumulates from 12% to 43% of carbohydrates of its dry weight while the amount is lower for *Phaeodactylum tricornutum* (6% to 35%) (**Table 3**). Even though microalgae are a source of beneficial carbohydrates, their application in the food industry remains limited.

On the other hand, microalgal polysaccharides have been widely used in the cosmetic industry where they have been used to make lotions and creams due to their activity as antioxidants and hygroscopic agents for topical applications. Additionally, polysaccharides present a considerable number of biological properties, such as antiviral, antioxidant, antitumor, anticoagulant, hypoglycemic and immunomodulatory (Chen et al., 2019). For instance, anti-bacterial activities were observed in polysaccharides extracted from several microalgal species such as *Spirulina platensis*, *Anabaena sphaerica*, *Oscillatoria limnetica* and *Chroococcus turgidus* (Swain et al., 2017). Polysaccharides extracted from other species such as *Phaeodactylum tricornutum* and *Chlorella stigmatophora* showed immunomodulatory and anti-inflammatory activities (Guzmán et al., 2003).

1.2.1.3. Lipids

Microalgae comprise a unique profile of lipids which, structurally, can be distributed into two groups; the nonpolar formed by free fatty acids, sterols, acylglycerols, wax, and steryl esters, and the polar lipids compromising glycosylglycerides, phosphoglycerides and sphingolipids (Chen et al., 2018). Basically, the polar lipids are the structural lipids that form the microalgal cells, while the non-polar lipids are mostly responsible for energy storage and have a role in the cell signaling pathways as well as sensing and coping with the environmental changes. The average of these microalgal lipids varies according to the different species as well as growth stage, nutritional and environmental

circumstances (López et al., 2019). Thus the lipid content ranges from 1% to 70% (w/w) while in some species, it can reach 85% of a cell's dry weight (Donot et al., 2013).

A wide group of microalgal species produce lipids such as *Taonia atomaria, Galaxoura cylindriea, Laurencia popillose, Ulva fasciata* and *Dilophys fasciola* but the group of *Dunaliella, Chlorella,* and *Spirulina* species are the most interesting in terms of high concentration of lipids. On the other hand, *Phaeodactylum tricornutum* (marine diatom) is also considered as one of the main producer of EPA (Tripathi & Kumar, 2017).

Besides, microalgae can produce and accumulate high quantities of PUFAs, thus having a great interest over the last years in the nutraceutical industry. Moreover, the lipid content of microalgae has raised significant interest in recent years due to their high content of PUFAs. On the other hand, microalgal lipids, especially PUFAs, have shown to have a role in the prevention of many diseases such as osteoarthritis, diabetes and cardiovascular diseases. Additionally, these PUFAs possess numerous biological activities including antiviral, antimicrobial, anti-tumoral and anti-inflammatory properties (Kendel et al., 2015).

1.2.1.4. Micronutrients

In terms of pigments, three classes of pigments can be produced by microalgae, which are carotenoids, chlorophylls and phycobiliproteins. Microalgae accumulate pigments as secondary metabolites and they are primarily used as a food colorant, particularly the β-carotene and astaxanthin pigments. Moreover, microalgal pigments also possess many biological activities such as antioxidant and anti-inflammatory. These activities have allowed the development of different applications for pigments, so they are also used in cosmetics and cosmeceuticals. The products in which the pigments are used include refreshing and regenerating care products aimed at healing and repairing damage (Silva et al., 2020).

The main algal carotenoids are astaxanthin, fucoxanthin, β-carotene, lutein and zeaxanthin. The antioxidant capacity of astaxanthin, the major carotenoid found in the unicellular green algae

Haematococcus pluvialis, has been reported to be about 10 times greater than β-carotene, lutein, zeaxanthin, canthaxanthin and over 500 times greater than that of α-tocopherol. In addition, researchers have demonstrated the therapeutic effects of astaxanthin against various noncommunicable diseases including atherosclerosis, diabetes, coronary, gastrointestinal, liver, chronic inflammatory and neurodegenerative diseases (Christaki et al., 2013). Besides, a study done on other kind of pigments showed anticancer effects. The study investigated the β-carotene from the microalgae *Dunaliella salina*. Interestingly, β-carotene had an inhibitory effect on neoplasic cells and reduced fibrosarcoma in rats (Villarruel-López et al., 2017). Another activity exerted by microalgal pigments is the antifungal property, which has been found in some microlagal strains such as -carotene, chlorophyll a and chlorophyll b extracted from *Chlorococcum humicola* (Bhagavathy et al., 2011) as well as in phycobiliproteins extracted from *Porphyridium aerugineum* (Najdenski et al., 2013).

1.2.2. Bioactive compounds

Bioactive compounds from microalgae can be obtained directly from primary metabolism, similarly to proteins and fatty acids, or can be synthesized from secondary metabolism such as pigments. Due to their richness in natural bioactive compounds, microalgae have drawn great attention as research targets as well as a promising source for the sustainable production of these valuable bioactive compounds that can be broadly used in different areas such as pharmaceuticals, cosmetics, ingredients, food additives and many more applications. Most importantly, the therapeutic activities whereby microalgae showed antibacterial, antiviral, antifungal, antioxidant, anti-inflammatory, antimalarial and antitumor effects may have a great potential for future commercialization in the near future (Fu et al., 2017). Currently, the fields in which the microalgae biomass have been mostly applied include cosmetics, pharmaceuticals, animal feed, food, human ingestion, wastewater treatment, bioenergy production, CO_2 mitigation and nitrogen-fixing.

1.2.2.1. Bioactive peptides

Bioactive peptides extracted from microalgae draw considerable attention due to their therapeutic applicability in the treatment of numerous diseases. Proteins, peptides, and amino acids are compounds highly beneficial to the human health, these benefits include nutritional benefits, hormones, growth factors and immunomodulators (**Table 3**). Additionally, they can be used in replacing damaged tissues. Furthermore, some species, such as chlorella and spirulina, have been used as nutraceuticals in order to prevent diseases and harm to human tissues due to their rich protein content and amino acid profile (De Morais et al., 2015).

Table 3: health benefits of bioactive compounds extracted from different species of microalgae

Bioactive compounds	Source	Health benefits	References
Peptides	*Navicula incerta*	Cytotoxicity in HepG2/CYP2E1 cells	(Kang et al., 2012)
	Chlorella ellipsiodea	Antioxidant	(Ko et al., 2012)
	Spirulina platensis	Blood pressure reduction	(Carrizzo et al., 2019)
		Antibacterial activity, anticancer	(Sadeghi et al., 2018)
		ACE inhibition	(He et al., 2018)
	Tetraselmis suecica	Antimicrobial activity	(Guzmán et al., 2019)
		Antioxidant and anti-aging activity	(Norzagaray-Valenzuela et al., 2017)
	Dunaliella tertiolecta	Antioxidant, anti-aging activity	(Norzagaray-Valenzuela et al., 2017)
	Nannochloropsis sp.	Antioxidant, anti-aging activity	(Norzagaray-Valenzuela et al., 2017)
Polysaccharides	*Tetraselmis sp*	Antioxidant, antifungal and tyrosinase inhibitory activities	(Amna Kashif et al., 2018)
	Chlorella sp.	Antioxidant activity	(Song et al., 2018)
	Spirulina platensis	Anticancer activity	(Kurd & Samavati, 2015)
	Pavlova viridis	Immunomodulation and antitumor	(Sun et al., 2016)

Table 3: (Cont).

Bioactive compounds	Source	Health benefits	References
Polysaccharides	*Tribonema sp.*	Immunomodulation and anticancer	(Chen et al., 2019)
	Phaeodactylum tricornutum	Anti-inflammatory and immunomodulatory activity	(Guzmán et al., 2003)
Carotenoids	*Phaeodactylum tricornutum*	Anti-obesity	(Koo et al., 2019)
		Antiproliferative and Antioxidant	(Neumann et al., 2019)
	Dunaliella salina	Antioxidant and cytotoxic activity	(Singh et al., 2016)
	Porphyridium aerugineum	Antifungal activity	(Najdenski et al., 2013)
	Chlorella ellipsoidea	Anti-inflammatory	(Soontornchaiboon et al., 2012)
Lipids	*Chlorococcum* sp	Anti-inflammatory and thrombotic activity	(Shiels et al., 2021)
	Nannochloropsis	lower the cholesterol level	(Rao et al., 2020)
	Pavlova lutheri	Anti-inflammatory	(Robertson et al., 2015)

In addition, many studies have shown that peptides derived from microalgal proteins hydrolysate have anti-inflammatory, antihypertensive, antioxidative, and antimicrobial activities that can be also used to promote human health (**Table 3**). For example, one study done on *Tetraselmis suecica* marine microalgae, has proven that antibacterial peptides were efficient against several Gram-negative bacteria as well as against many Gram-positive bacteria (Guzmán et al., 2019).

1.2.2.2. Other secondary metabolites from microalgae

Bioactive compounds of microalgal origin are not only sourced directly from primary metabolism but can also be synthesized from secondary metabolism. Microalgae are mainly a good source of polyphenols, an aromatic class of compounds (Barkia et al., 2019). Polyphenols are typically synthesized to protect cells against pathogens and ultraviolet irradiation, and they possess a wide range of biological activities, including antioxidant properties. Different studies showed that microalgae produce numerous classes of flavonoids, such as flavanones, isoflavones, flavonols and

dihydrochalcones (Sansone & Brunet, 2019). Such phenolic compounds have been found in many microalgae species such as *Arthrospira platensi*s, *Ankistrodesmus sp., Spirogyra sp., Nodularia spumigena and Nostoc sp.* underlining their great variability among different strains. Interestingly, it has been witnessed that the total phenolic content of many microalgal species is comparable to or higher than that in numerous fruits and vegetables (Stojanovic & Silva, 2007). Notably, the greatest amount of phenolic compounds was found in two different microalgal species: *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* (Galasso et al., 2019). One fascinating example of polyphenols of microalgal origin produced by some microalgae is represented by the marennin, a green polyphenol, which is characterized by many interesting bioactivities such as antioxidant, antiviral, antibacterial and inhibitory effects on the growth of several lines of cancerous cells (Galasso et al., 2019). Furthermore, polyphenols isolated from *Spirulina platensis* and *Chlorella pyrenoidosa* had a high anti-allergic activity (Chen et al., 2015) while those isolated from *Nostoc insulare* reported having both antibacterial and antifungal activities (Volk & Furkert, 2006).

On the other hand, sterols and vitamins are also among the secondary metabolites produced by microalgae. These compounds are of high nutritional value and are highly used in the industrial application. The microalgal sterols showed to possess anti-inflammatory and anticancer activities. In addition, they acquire advantageous health effects in some diseases such as in hypocholesterolemia and many neurological diseases like Parkinson disease (Galasso et al., 2019). As for the vitamins, these metabolites are produced by several microalgal species. Generally, microalgal vitamins are vitamins B, C, D and E. They are highly beneficial for human health and are widely used as dietary supplements and antioxidants (Raposo & De Morais, 2015).

1.3 Mycotoxins

Mycotoxins are toxic chemical compounds produced naturally by the secondary metabolism of filamentous fungi. They are contaminants existing in food and feedstuff and can affect the health of humans and animals at critical doses. The diversity of mycotoxins induces diverse toxic effects such

as carcinogenicity, genotoxicity, teratogenicity, dermatogenicity, nephrotoxicity and hepatotoxicity (Hathout & Aly, 2014).

The most abundant sources of dangerous mycotoxins are the fungal genera Penicillium, Aspergillus and Fusarium. Aspergillus species produce Aflatoxins (AFs), Ochratoxin A (OTA) and Patulin (PAT), while Penicillium species produce both OTA and PAT. As for Fusarium species, they produce trichothecenes (HT2, T2, and Nivalenol (NIV), and Deoxynivalenol (DON)), Fumonisins (FB1 and FB2), Zearalenone (ZEA), and emerging mycotoxins (Fusaproliferin (FUS), Moniliformin (MON), Beauvericin (BEA) and Enniatins (ENNs)) (Tolosa et al., 2019).

1.3.1 The occurrence of mycotoxins in fish

Fish in aquaculture farms are frequently fed with a commercial diet based on different plant feedstuffs, such as soybean meals and various cereal grains, all of which may lead to the contamination of the final mixed fish feed with fungi and eventually the production of mycotoxins. For instance, ZEA, OTA and AFB1 were detected in corn and wheat destined for fish feed production (Marijani et al., 2019). In addition, Tolosa et al. (2020), for the first time, identified and documented 40 mycotoxins in farmed fish, as these mycotoxins had been only found in different cereal samples previously (Tolosa et al., 2020).

In terms of fish health, the most harmful mycotoxins are AFB1, which are produced by Fusarium species. Studies showed that the exposure of fish to low doses of aflatoxins over a long period of time may lead to chronic aflatoxicosis and a risk of aflatoxin residue accumulation in the fish tissue. Michelin et al. (2017) reported the accumulation of aflatoxins in lambari (*Astyanax altiparanae*) fish liver and muscle after 90 days of exposure to the detected mycotoxin (Michelin et al., 2017). Likewise, traces of AFB1 were also found accumulated in the muscles of matrinxã fish (*Brycon cephalus*) (Bedoya-Serna et al., 2018). Similarly, El Sayed et al. (2009) reported that high amounts of AFB1 accumulated (4.25±0.85 ppb) in the edible muscles of sea bass suggested a significant risk for transmission of AFB1 to the human food (tolerable daily intake of AFB1 by US Food and Drug

Administration is 5 ppb) (El-Sayed & Hassan, 2009). Another study found that AFB1 residues in the fish liver and pancreas were much higher than that in muscles (Deng et al., 2010). In addition, the content of AFB1 residues in muscle, liver and pancreas of gibel carp and in edible muscle of rainbow trout was evaluated and researchers found higher contents of AFB1 metabolites (aflatoxicol (AFL) and aflatoxin M1 (AFM1)) after dietary exposure (Huang et al., 2011; Nomura et al., 2011).

Then again, the presence of emerging Fusarium mycotoxins (ENs and BEA) was investigated in samples of aquaculture fish and feed for farmed fish, showed the results that all the analyzed feed samples were contaminated with mycotoxins, with 100% coexistence (Tolosa et al., 2014). In addition, the highest incidence of both ENs and BEA was found in muscles and liver of tested aquacultural fish *Dicentrarchus labrax* and *Sparus aurata (*65% of muscle samples positive for EN B and 50% positive for EN B1) (Tolosa et al., 2014). Besides, an acute toxicity and changes in the behavior of the nervous and respiratory system of sea bass associated to OTA mycotoxins were observed, in addition to hemorrhagic patches, fin erosion, rusty spot formation on the belly and dorsal musculature, general congestion of the kidneys and gills, and congestion spots on the periphery of the liver that were determined by an pathological examination (El-Sayed et al., 2009). Thus, the contamination of fish feeds by mycotoxins and the carryover of these toxins into farmed fish and fish-derived products for human consumption remains a serious food safety concern and threatens the ability of aquaculture to supply the global demand of fish.

1.4. Innovative and conventional extraction technologies to recover high-added-value compounds

The conversion of fish side streams and microalgae products into high-added-value components with high economical value can pave the way for the complete valorization of aquaculture discards and side streams, thus increasing the limited resources and deliver the solutions to the related environmental complications. It is fully aligned with the Sustainable Development Goals promoted by the United Nations in the 2030 Agenda. In this line, several conventional extraction techniques such as acid, alkaline, salt and solvent extraction have been traditionally used for the recovery of

nutrients and bioactive compounds from the aquaculture side streams and discards. However, these techniques are time consuming, unsafe, require a large volume of solvents and have low extraction efficiency (Chemat et al., 2020).

The limitations of conventional extraction techniques have drawn the attention into green extraction techniques. In this context, many non-conventional technologies have been reported as safer and more efficient techniques for the recovery of valuable compounds from aquaculture side streams and discards. Ultrasound-assisted extraction (UAE), microwave-assisted extraction, supercritical fluid extraction (SFE) and pulsed electrical fields are among the main and growing green technologies in the recovery and improvement of the recovery efficiency of compounds from aquaculture side streams and discards. Besides, it should be mentioned that these technologies are suitable tools to extract and eliminate undesired compounds, such as mycotoxins, to eventually obtain a safe and healthy product used in the food industries (Gavahian et al., 2020). Below are described the main technologies investigated in the present PhD thesis.

1.4.1. Ultrasound technology

Ultrasound technique is one of those rapidly innovative alternative technologies developed for the use in the process of production of high-quality food products. Commonly, ultrasound is associated with the biomedical field (organs and tumors detection, pre and post-natal handicaps detection, kidney stone removal, physiotherapy, etc.) (Gallo et al., 2018). However, ultrasound has been established in several applications in various other fields as well. In particular, ultrasound has been recently used in the food industry to develop several effective and reliable food processing applications. The most common application of ultrasound consists of cell destruction and extraction of intracellular materials (Carrillo-Lopez et al., 2017). Depending on its intensity, ultrasound is suitable for quality control of vegetables and fruits, detection of honey adulteration, meat tenderization, preservation, inactivation of microorganisms and activation or inactivation of

enzymes in food. Furthermore, ultrasound is used for homogenization, crystallization, freezing, drying as well as filtration (Al Khawli et al., 2020).

1.4.1.1. Ultrasound principle and mechanism of action

Ultrasound is based on the application of mechanical waves at a frequency ranging from 20 kHz to 10 MHz that is above the human's hearing levels and can be classified into two frequency ranges. High frequencies between 100 kHz and 1MHz at intensities lower than $1Wcm^2$ (low intensity ultrasound , while low frequencies range from 20 and 100KHz at high intensities higher than $1Wcm^2$ (high intensity ultrasound) (Chemat et al., 2017). Ultrasound is applicable in three different ways, either directly to the product, or by coupling the product to a device or by immersion in an ultrasonic bath (Ünver, 2016).

The mechanical waves generated by the ultrasound propagate through a solid/liquid media causing compression (high-pressure) and rarefaction (low-pressure) cycles (Barba et al., 2015). This propagation causes the main effect of ultrasound technology "the acoustic cavitation" phenomena, which is the rapid occurrence and formation of the bubbles that grow and collapse when the ultrasound waves propagate across the medium. The oscillation and the collapse of the generated bubbles induce thermal, chemical and mechanical effects throughout the medium. Mechanical effects represent the linear alternating vibrations phenomenon of when ultrasonic waves transfer mechanical energy, manifested by collapse pressure, turbulences, and shear stresses, while the chemical effects appear with the generation of free radicals. In addition, the material will be expanded and compressed because of the opposite pressures in alternate mode, which eventually causes the cell to rupture, all regarding to the ultrasound frequency (Carrillo-Lopez et al., 2017). These effects in the cavitation zone generate a high increase in temperature and pressure (5,000 K and 100 MPa) (Wen et al., 2018). Due to these conditions, especially the high temperature in the center of the cavitation bubble, several chemical reactions are generated. Thus, H^+ and OH^- free radicals are released as the water is hydrolyzed within the wavering bubble (Wen et al., 2018). Besides, during

the treatment two different structures of bubbles are formed. The first kind are the non-linear bubbles, called the stable cavitation bubbles. They are generated when the bubbles are large with equilibrium size during pressure. The second type are the unstable bubbles which are named internal cavitation bubbles and they are generated upon the rapid collapse of the bubbles and their disintegration into small bubbles (Majid et al., 2015). The small bubbles dissolve in the liquid instantly, however as they stretch, the mass-transfer border membrane decreases in thickness whereas the interfacial space becomes thicker than upon the collapse of the bubbles, which means that during the stretching of the bubbles, additional air is transported into the bubble but then it finds its way back out during the implosion phase.

1.4.1.2. Ultrasound instrument

An ultrasonic system basically consists of a sample treatment chamber, a transducer (ultrasound generator), and an electrical power generator that delivers electrical energy to the transducer. The transducers are typically divided into two types: magnetostrictive and piezoelectric. The piezoelectric transducers, which basically are electroacoustic transducers, are highly efficient and durable what makes them the most frequently used (McDonnell & Tiwari, 2017). The most commonly used equipment for the extraction are ultrasonic bath and ultrasonic probe (**Figure 3 A-B**). In bath systems, the ultrasonic waves are applied through the walls of the extraction bath (indirect application), though in the probe systems, the waves are directly applied by submerging the probe in the extraction medium.

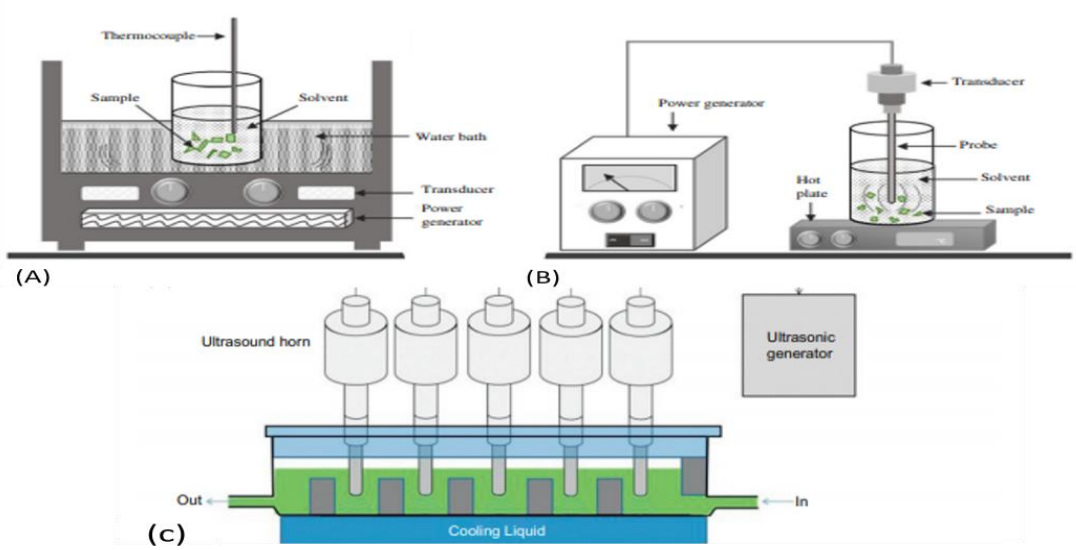


Figure 3: Representation of laboratory-scale ultrasonic systems: (A) Ultrasound bath, (B) Ultrasound probe and (C) continuous ultrasonic probe-based extraction system in industry. Adapted from Barba et al. (2020).

In both systems, ultrasonic energy is transmitted through a transducer, which permits the conversion of the conventional electrical energy (50–60Hz) into low-frequency mechanical ultrasonic waves. Therefore, the choice of ultrasound system depends on the application and the type of the target analytes. For example, the probe systems are usually used for an intensive extraction and recovery of analytes that exist in trace levels. On the other hand, bath systems are chosen for the treatment of large number of samples, since they permit simultaneous extraction of numerous samples. Currently, in an industrial scale, for an exhaustive extraction of compounds, several manufacturers are focusing on designing continuous flow of UAE system as shown in **Figure 3C**.

1.4.1.2.1 Ultrasound bath system

Ultrasonic bath systems are quite inexpensive and most utilized in the purpose of extraction. The system is made of a stainless-steel bath and one or more transducers fixed to the external walls of the bath (**Figure 3A**). Likewise, the baths can be equipped with a heating and cooling system. Most of the commercial ultrasonic bath systems typically operate at a single frequency of around 40–45

KHz. In a typical extraction process, the ultrasonic bath is filled with water and a glass vessel containing the sample and extraction solvent is immersed inside the bath. During the extraction process, it is necessary to consider the position of the vessel as long as the transmission of ultrasounds varies depending on the transducer location. However, it is nearly impossible to keep a homogeneous ultrasound intensity in the bath systems; hence, this kind of extraction is more likely to be non-reproducible. In addition, bath system suffer from lower efficiency due to the water and glass vessel that weaken the applied power (Al Khawli et al., 2020).

1.4.1.2.2. Probe ultrasound system

Probe systems are more efficient and powerful than the bath systems due to the high-intensity ultrasound that is directly delivered through a smaller surface (horn) (**Figure 3B**). Probe systems are usually operated at around 20 kHz, where the probes are made up of titanium which allows the energy to propagate very efficiently. However, in this case the product is at risk of contamination due to the vulnerability of titanium probes to erosion. In order to address this issue, several innovations have been developed such as quartz and Pyrex probes that finally diminished that risk. Moreover, the high-intensity ultrasound is delivered from the probe system that may lead to a rapid increase in the temperature of the medium; thus, increasing the degree of extraction. However, this may also provoke the degradation of the thermolabile target compounds. So in this case, a cooling system should be accompanied with the treatment chamber (McDonnell & Tiwari, 2017).

1.4.2. Ultrasound assisted extraction

A growing interest has been shown regarding the use of UAE as an extraction technology in the food area, especially to recover nutrients and many other beneficial compounds such as bioactive compounds due to the different advantages related to the use of this technology. For example, compared to conventional methods, UAE consumes less energy and reduces the extraction time and operational temperature, which are critical parameters in the extraction of labile compounds. In general, UAE is applied in continuous mode, however, the pulsed mode may provide the best

performance over long extraction times (Pan et al., 2011). In addition, in order to improve the yield of the extraction, UAE can be efficiently utilized in combination with other alternative techniques, such as supercritical CO_2 due to a faster solvation and smaller particle size. Moreover, ultrasound can assist SFE processes producing agitation where the use of mechanical stirrers is not possible (Chemat et al., 2020).

Besides, several parameters have an important influence on the UAE effectiveness and efficiency. It is dependent of several physical parameters that are basically related to the ultrasonic equipment, such as frequency and intensity. Other relevant parameters are related to the medium such as the solvent type, temperature, time extraction, and particle size that can also affect the UAE process (Chemat et al., 2017). Therefore, optimization of all of the above-mentioned parameters is essential in order to improve the extraction yields as well as the selectivity of the valuable substances. In fact, a great number of researchers have focused on the optimization of the UAE process to recover valuable substances.

1.4.2.1. UAE of valuable compounds from aquaculture products

As mentioned above, a lot of research in food science and technology is published in the field of ultrasound application for the extraction of various substances; and recently much attention has been paid to up-scale the UAE and its applications in the industry. Ultrasound in literature showed a great predominance in the extraction of nutrients and other bioactive compounds from marine food matrices. For instance, the UAE was used for the extraction of many valuable compounds, like the protein from marckerel fish (Álvarez et al., 2018), collagen from *Pelodiscus sinensis* calipash tissues (Zou et al., 2017) and oil from shrimp (Bruno et al., 2019), among others.

Other researchers have studied the effects of ultrasound on the recovery of carotenoids from microalgae. In this sense, Jaeschke et al. (2017) found that ultrasound treatment of *Heterochlorella luteoviridis* affected positively to the extraction of carotenoids (Jaeschke et al., 2017). Moreover, Sun et al. used UAE for the extraction of polysaccharides from *Pavlova viridis*, which had a high biological

activity (Sun et al., 2016). In addition, UAE showed an improvement in the extraction yield and a reduction of the extraction time for protein recovery from Spirulina (Vernès et al., 2019). Therefore, UAE can be considered as a useful tool for the recovery of nutrients and bioactive compounds, which are used as food supplements owing to their nutritional properties.

1.4.2.2. UAE of mycotoxins from food

Ultrasound has been widely used to clean and remove contaminants from food matrices such as mycotoxins, heavy metals and pesticides. Many researches have demonstrated the applicability of UAE for the extraction of mycotoxins from various food products. In this sense, Kong et al. (2013) used ultrasound for the extraction of AFs and OTA from nutmeg samples (Kong et al., 2013). Other researches have successfully used ultrasound probe-assisted extraction for the extraction of aflatoxins from different fish species such as gilt-head of sea bass, brown trout, and turbot (Jayasinghe et al., 2020) and ultrasound assisted bath for the extraction of ENs and BEA from *Dicentrarchus labrax* and *Sparus aurata* (Tolosa et al., 2014).

Furthermore, ultrasound was used for the inactivation of the fungi, the mycotoxins producers. The application of ultrasound on two different fungal species *Aspergillus flavus* and *Penicillium digitatum* spores vastly affected the viability of these spores (López-Malo et al., 2005). Also, ultrasound treatment was able to reduce mold fungi content in grains, and subsequently the mycotoxins production (Rudik et al., 2020). Thus, the use of ultrasound in the food industry for fungal inactivation and mycotoxins elimination is definitely feasible.

1.4.3. Supercritical fluid extraction (SFE)

SFE is another green extraction technology that is widely used for the recovery of high-added-value compounds from various matrices in both laboratory and industrial scales. SFE using non-toxic extracting solvent is one the excellent alternatives to the traditional solvent extraction. Mainly, SFE is the separation process of a solute from a solid or liquid matrix using extracting solvents above or near their critical temperature and pressure (Zhou et al., 2021). Carbon dioxide (CO_2) is the most

widely used supercritical fluid due of its intrinsic properties: inert, non-toxic, non-inflammable, low cost, abundant availability, easy recovery from the product and also possessing moderate critical temperature and pressure (T_c = 31.1 °C, P_c = 7.38 MPa) (Herrero et al., 2010). However, due to its non-polar character, it is usually used with organic co-solvents (e.g. ethanol, methanol, acetone), which are also called modifiers since they are used to enhance the solubility of polar substances (da Silva et al., 2016).

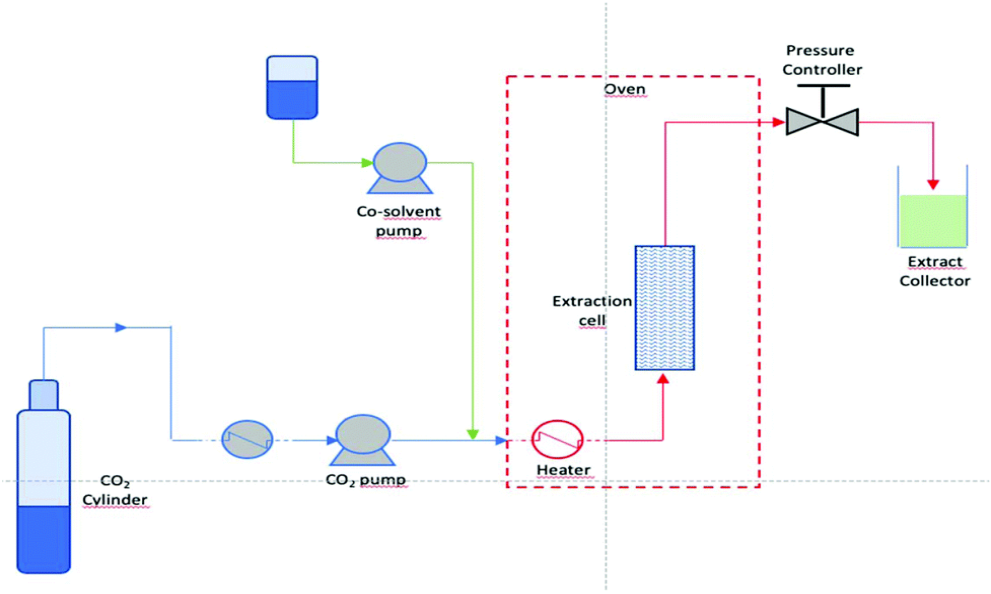


Figure 4: Schematic representation of supercritical CO_2 extraction process (Chemat et al., 2020).

The extraction mechanism of supercritical CO_2 can be simplified by a dispersion of the supercritical fluid into a porous sample matrix immediately followed by a dynamic extraction within the matrix; then the solutes are diffused out of the matrix, and lastly the analytes are collected from the sample during the decompression step. The SFE from solid materials is carried out with autoclaves and installations, it contains four main parts, which are a volumetric pump insuring the correct pumping of the fluid that can be connected to a cooler that transports gaseous components to a liquid phase, a heat exchanger, an extractor where a precise pressure is maintained by a back pressure regulator and a separator (**Figure 4**) (Chemat et al., 2020).

2. OBJECTIVES

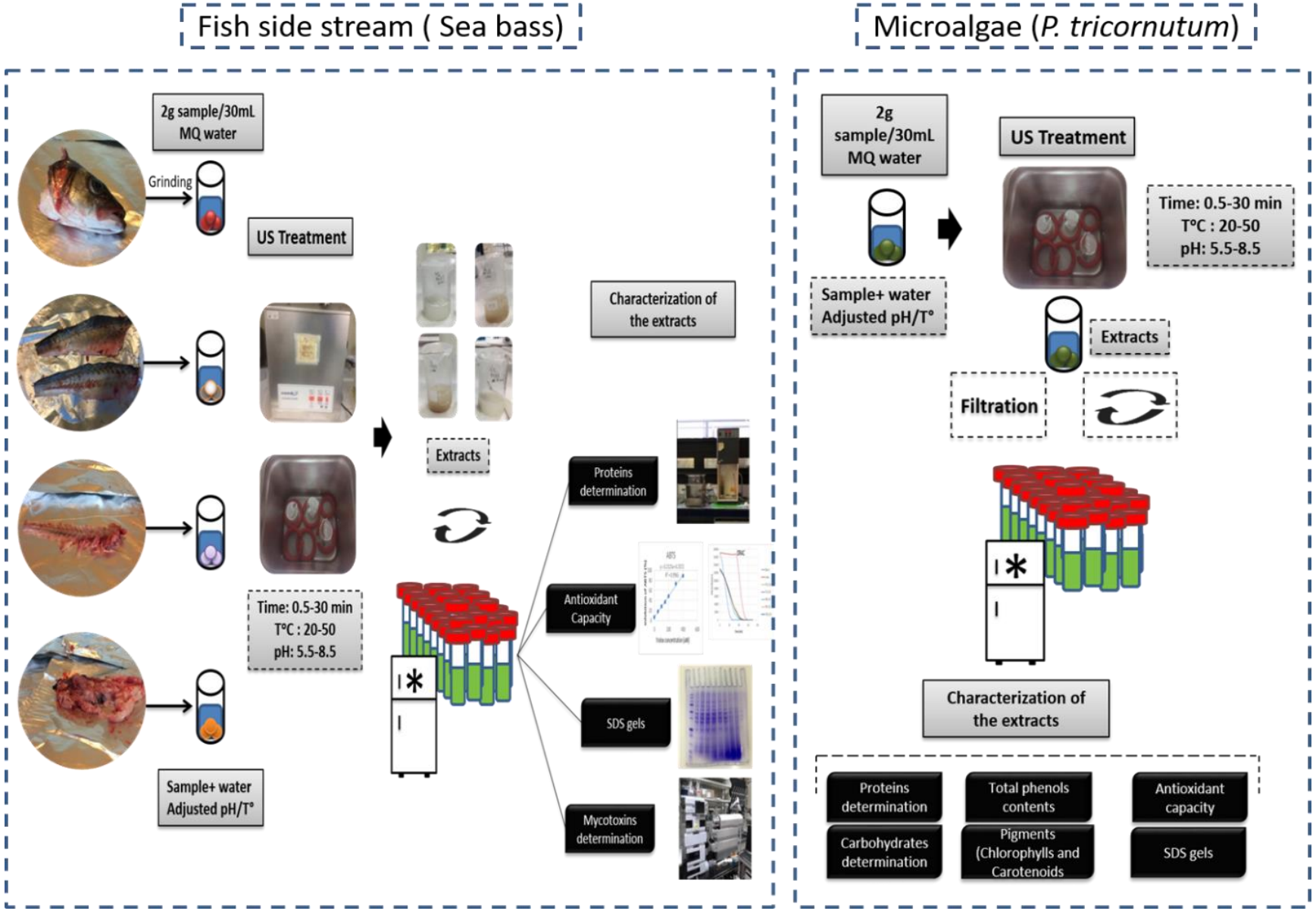
2. Objectives

The **general objective** of this Doctoral Thesis is to evaluate the extraction of proteins and bioactive compounds from fish side streams (i.e., sea bass) and from microalgae (*Phaedoactylum tricornutum*), as well as the mitigation of toxic compounds present in fish side streams. For these purposes, the following **partial objectives** are presented:

- Optimization of ultrasound-assisted extraction (UAE) conditions (extraction time, temperature and pH) using a response surface methodology (RSM), a statistical multifactorial analysis of experimental variables and response.
- Determination of antioxidant capacity of sea bass and *Phaedoactylum tricornutum* extracts
- Determination of carbohydrates and phenolic compounds from *Phaedoactylum tricornutum* extracts.
- Evaluation of the effect of ultrasound treatment on the protein quality through the determination of protein molecular size distribution using SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis).
- Evaluation of the presence of mycotoxins in the fish by product extracts obtained after the treatment. In this regard, spectrophotometric, fluorometric, and LC-MS/MS-QTRAP assays have been carried out.

3. EXPERIMENTAL PLAN

3. Experimental plan



4. RESULTS

4.1. INNOVATIVE GREEN TECHNOLOGIES OF INTENSIFICATION FOR VALORIZATION OF SEAFOOD AND THEIR BY-PRODUCTS

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Innovative Green Technologies of Intensification for Valorization of Seafood and Their by-Products

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Abstract:

The activities linked to the fishing sector generate substantial quantities of by-products, which are often discarded or used as low-value ingredients in animal feed. However, these marine by-products are a prominent potential good source of bioactive compounds, with important functional properties that can be isolated or up-concentrated, giving them an added value in higher end markets, as for instance nutraceuticals and cosmetics. This valorization of fish by-products has been boosted by the increasing awareness of consumers regarding the relationship between diet and health, demanding new fish products with enhanced nutritional and functional properties. To obtain fish by-product-derived biocompounds with good, functional and acceptable organoleptic properties, the selection of appropriate extraction methods for each bioactive ingredient is of the outmost importance. In this regard, over the last years, innovative alternative technologies of intensification, such as ultrasound-assisted extraction (UAE) and supercritical fluid extraction (SFE), have become an alternative to the conventional methods in the isolation of valuable compounds from fish and shellfish by-products. Innovative green technologies present great advantages to traditional methods, preserving and even enhancing the quality and the extraction efficiency, as well as minimizing functional properties' losses of the bioactive compounds extracted from marine by-products. Besides their biological activities, bioactive compounds obtained by innovative alternative technologies can enhance several technological properties of food matrices, enablingtheir use as ingredients in novel foods. This review is focusing on analyzing the principles and the use of UAE and SFE as emerging technologies to valorize seafoods and their by-products.

Keywords: high-added value compounds; seafood by-products; innovative green technologies; functional foods

1. Introduction

Fish is considered to be healthy, and to be among the most nutritious animal-derived foods, due to their content in a high quality of proteins, balanced essential amino acids, high levels of fat-soluble vitamins (A and D) and essential macro- and microminerals (iodine, magnesium, phosphorus and selenium) [1].

Moreover, marine fatty fish contain high levels of long chain highly unsaturated *n-3* fatty acids, which have been associated with reduction of the risk of cardiovascular diseases in humans [2]. Fish nutrient composition, mostly characterized by 15%–30% proteins, 0%–25% lipids and 50%–80% moisture, depends upon fish species, age, gender, health, nutritional status and time of the year. For instance, white fish such as cod and hake are lean species, containing ca. 20% protein, 80% water and rather low lipids levels (0.5%–3%), whereas fatty fish, such as mackerel and salmon, contain 20% protein, 10%–18% lipids, and correspondingly lower water content (62%–70%) [3].

In 2016, fish production worldwide amounted to ca. 171 million tons, 91 million tons deriving from inland and marine fisheries, and 80 million tons from aquaculture, with China being the largest producer [4]. In Europe, Norway and Spain are topping the list of the largest producing countries for capture fisheries (2.03 and 0.91 million of tons, respectively). As a consequence of the activities related to the different fishing sectors, a great amount of fish by-products, not utilized for direct human consumption, are generated every year, and they can represent anything betwen 30% and 85% of the weight of the different catches [5]. The food fish to by-product ratio varies by fishing zone, season, fish size and species [6]. Besides bycatch, fisheries and aquaculture by-products include fish fins, backbones, gills, heads, belly flaps, liver, roe, skin, viscera, among others [7]. Indicatively, heads represent 9 %–12%, viscera 12 %–18%, skin 1 %–3%, bones 9 %–15% and scales ca. 5 % of whole fish weight [8].

Fish by-products can entail significant environmental and food-technical challenges due to their high microbial and endogenous enzyme load, rendering them susceptible to rapid degradation if not

processed properly or stored in appropriate conditions [9,10]. Fish by-products can be classified into two types: One that includes easily degradable products with high enzyme content, such as viscera and blood, and a second one that includes the more stable products (bones, heads and skin) [5]. Timely collection and the treatment of fish by-products is a crucial step in maintaining their quality to be used as raw materials for obtaining high added-value products [5]. Given that fish production, landing and processing locations are spread geographically, it appears that the best management option that would allow the conversion of fish residues into products of greater value is that of processing locally immediately after production [11]. To achieve this, significant investments, for instance, on board fishing vessels, would be required, not easy to justify unless already developed markets for the new end-products are present. By refining seafood by-products, high-added value components for the production of nutraceuticals and bioactive ingredients can be obtained. Processing fish proteins can generate bioactive peptides, amino acids and other bioactive nitrogenous compounds [12], whereas fish oil by-products generated from a fish oil refinery can be utilized as raw materials for the production of the essential long chain, polyunsaturated fatty acids concentrates, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), to be used in food supplements [13].

To succeed in utilizing marine resources in a responsible and good way, it is indispensable to establish efficient and safe methods for the extraction of the target nutrients and bioactives. Downstream processing in the biomass refinery includes, among others, conventional techniques, already widely used for the separation, selective upconcentration and extraction of target compounds, such as in fish meal and fish oil [14] or EPA- and DHA-rich oil production [15]. These methods are efficient, and their main drawback is related to the high energy consumption and potential thermal degradation of target compounds, due to the high processing temperatures. Other extraction methods involving the use of organic solvents would entail risks for human health and the environment, and may also lead to perishable compound degradation, should prolonged extraction periods be involved [16].

In recent years, the concept of green technology, assuming the use of more environmentally-friendly techniques for ingredient processing, has emerged [17]. Innovative alternative extraction technologies, such as supercritical fluid extraction (SFE), ultrasound-assisted extraction (UAE), pulsed electric fields (PEF) or microwave-assisted extraction (MAE), have been identified as green extraction techniques for the separation of high-added value compounds [18,19].

These alternative technologies have several advantages, including rapid extraction, low solvent consumption rates, use of alternative environmentally-friendly solvents, superior compound recovery rates and higher selectivity. This review intends to summarize the potential applications of UAE and SFE, as green technologies, for the extraction of a wide range of bioactive compounds from fish side stream biomasses, and thus achieve the valorization of seafood and their by-products. Moreover, this review also aims to provide detailed information on the potential benefits of applying these innovative technologies for a by-product refinery in both academy and the industry.

2. Valorization of fish by-products

There are multiple possibilities in valorizing marine by-products through processing, as for instance creating more valuable ingredients or extracting specific high-value compounds. Following the European Union (EU) Directive 2008/98/CE, a standard prioritization scheme can be established, visualized by a pyramid in which the obtained product value, as well as the necessary quality of the raw material used, decrease from top to bottom [20]. The main aspect in the model for marine biomass valorization is linked to the application of good practices, and therefore the prevention or reduction of wastes. Millions of tons of captured fish are returned to the sea for failing to comply with regulations regarding legal size, no control over catch rates, or low quality. This forced the European Union to establish a new fisheries' policy that involves actions paving the way towards zero-discards [21]. To meet the goals set by the new policy, novel management measures must be established enabling the valorization of fish side stream biomasses. Maintaining marine catch discards and by-products in the food chain can be practiced either through the commercialization of low-value

fractions, or through the production of ingredients and high-value biomolecules that can be used in the pharmaceutical and nutraceutical industry [22–25], fulfilling the principles of a sustainable circular economy (green approach). This complementary approach allows an efficient use of fish by-products, transforming them into ingredients that can be incorporated into feed, food or other high-value products (**Figure 1**). Use of fish by-products in animal feeds (flours and oils), is the most common option practiced today [26,27]. Finally, waste from the above processes may also have the potential to be used in biofuel production or be exploited in other agronomic and industrial applications, as for instance fertilizers [28,29].

The known healthy compounds and properties associated with fish are also present in their by-products. A great number of bioactive compounds can be obtained from fish by-products [11,13,30–32]: collagen [33], chitin [34], enzymes [35], gelatin [36], glycosaminoglycans [37,38], polyunsaturated fatty acids (PUFA) [39], minerals [40,41], protein and peptides [10,42,43] and vitamins. It should be noted that the long-chain omega-3 fatty acids (LC-PUFAs), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are among the most successful compounds extracted from fish by-products, achieving a high value in the market due to their beneficial health effects [11]. Marine by-product-derived compounds are known to induce positive effects on human health associated with their, e.g., anticancer, antidepressant, anti-diabetic, antihyperglycemic, antihypertensive, anti-inflammatory, antimicrobial, antioxidant, antiproliferative, anti-rheumatoid and immunomodulatory properties [42–44]. Besides their biological activities exploited by pharmaceutical, nutraceutical and cosmeceutical industries [45], marine by-product ingredients can also provide desirable technological properties when included in food products, acting for instance as emulsifying and foaming agents, and facilitating fat binding, solubility and water holding capacity [46,47]. Recent data show that it is possible to modify fish burger technical properties, in terms of hardness, cohesiveness, juiciness and adhesiveness, by the addition of low amounts fish by-product protein powder or fish hydrolysates [48].

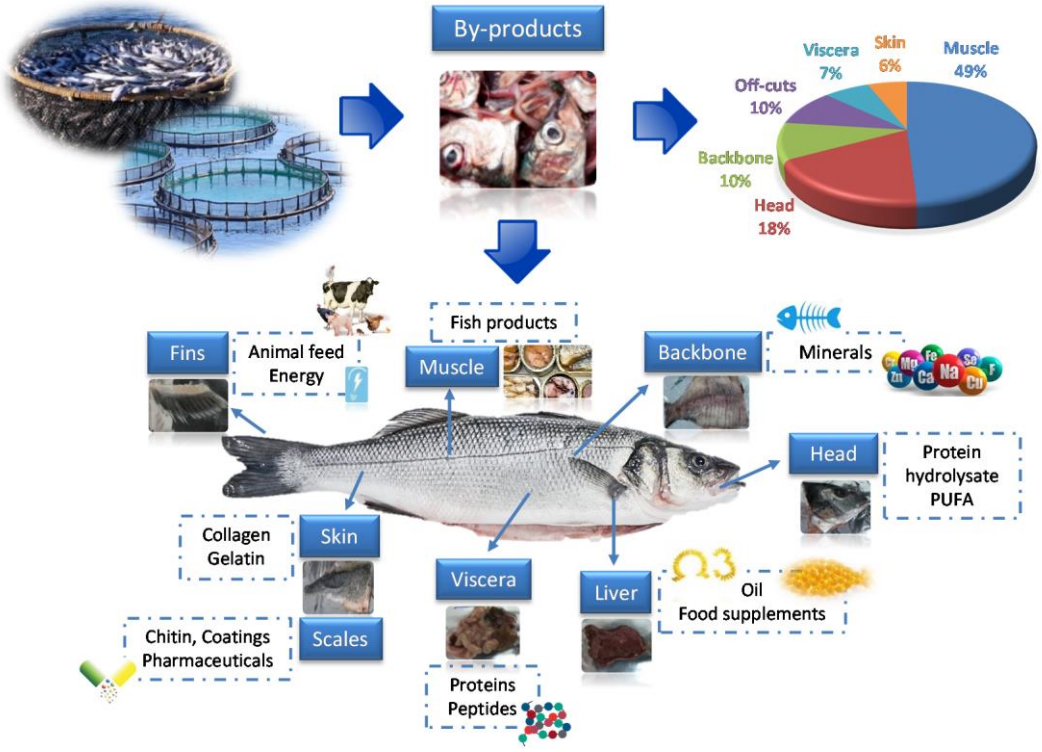

Figure 1: Fish processing by-product generation and end use opportunities

3. Emerging Technologies for the Extraction of Bioactive Compounds from Fishery by-products

Several techniques can be used to extract bioactive compounds, thus valorizing fish by-products. Among the conventional methods that are used for the extraction of fishery by-products. It is possible to highlight enzymatic hydrolysis for the solubilization and up concentration of fish proteins, as reviewed by Aspevik et al. (2017), and among others, lipid extraction by Soxhlet, steam distillation and the use of solvents. Some traditional extraction methods, besides being characterized by low extraction yields, long extraction time, high solvent and high energy consumption and potential health hazards [16], involve extraction conditions (pH, temperature, extraction time, solvent type, concentration, etc.) that can alter the functional properties of potentially valuable compounds. Therefore, there is a need to explore alternative processing technologies that can better preserve target bioactive components [49,50], operating at lower temperatures and avoiding as much as possible the use of solvents. The shortcomings of these conventional methods have stimulated the

interest in emerging green technologies. Several techniques, such as PEF, UAE, MAE, SFE and high pressure can be used to extract bioactive compounds, thus valorizing fish by-products [51,52]. Among these innovative, alternative techniques are ultrasounds-assisted (UAE) and supercritical fluid extraction (SFE), which are the object of the present review.

3.1 Ultrasound-assisted Extraction (UAE)

3.1.1 Fundamentals

The use of ultrasound has increased, and has been applied over the last years with the scope to minimize processing, maximize the quality and ensure the safety of food products. This technique is applied in improving the technological properties of food, such as emulsification ability, solubility and texture, as well as on applications such as preservation, homogenization, viscosity alteration, extraction, drying, crystallization and antifoaming actions and enzymatic activation and inactivation [53]. Nowadays, improvements in ultrasound technology grant the opportunity to extract bioactive compounds with economic advantages, and this is referred to as innovative UAE [53].

Ultrasound works in frequencies above human hearing levels, ranging from 20 kHz to 10 MHz [53], and is classified by the amount of energy generated as sound power (W), sound intensity (W/m^2), or sound power density (W/m^3). The use of ultrasounds can be divided into two types: high intensity and low intensity. Low-intensity ultrasounds with high frequency (100 kHz to 1 MHz), and low-power <1 W/cm^2 are used as non-destructive methods for evaluating the physical and chemical properties in food products [54], whereas high-intensity ultrasounds have low frequency (20 kHz-100 kHz) and high power >1 W/cm^2, and are used to speed up and improve the efficiency of sample preparation, as they can alter the physical or chemical properties of food [54].

UAE is generally recognized as an effective tool used in extraction methods, significantly minimizing the time required to increase both the productivity and the quality of the product. Numerous studies have critically assessed a variety of UAE applications in the industrial extraction of bioactive compounds [53] and found that this extraction technique enhances the yield of extraction,

improving simultaneously their functional properties [55]. UAE efficiency is driven by the creation of acoustic cavitation and mechanical impact in the material matrix (**Figure 2**). Acoustic cavitation when used in plant materials can disrupt cell walls facilitating the solvent penetration into the sample matrix. Ultrasound mechanical impact increases the surface area of contact between the solvent and the extractable compounds, and hence offers greater penetration of solvents into the sample matrix, releasing in this way the bioactive compounds [53,56]. The UAE requires less extraction time and reduced solvent consumption. It can be performed at low temperatures, which can decrease the damages caused by temperature, and reduce the loss of bioactive substances [53]. In contrast, a denaturation of the protein/enzyme can occur when UAE is applied for a long period of time, since it results in high pressures, shear strength and increased temperatures into the medium.

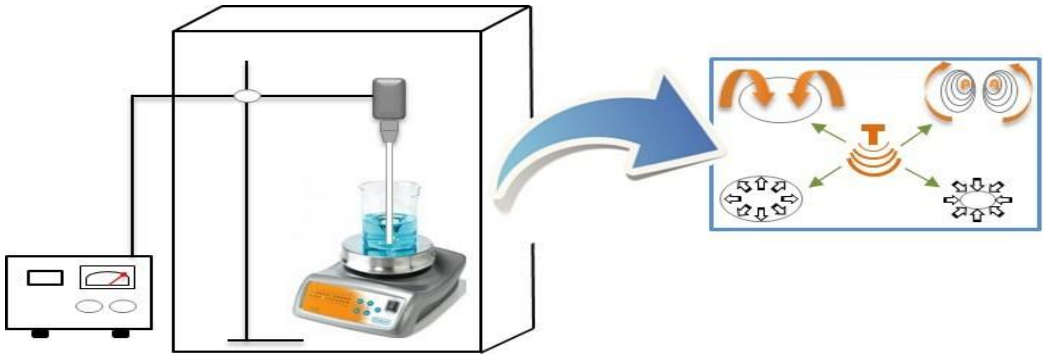

Figure 2: Schematic representation of the ultrasound-assisted extraction (UAE) process and the bubble cavitation phenomenon involved in this extraction technique.

3.1.2 Use of UAE in Fish Industry

The utilization of ultrasound technology in the food industry is not new. Recently, UAE became recognized as an efficient, rapid, clean, reproducible and alternative non-thermal extraction technique as compared to conventional extraction methods [53]. **Table 1** lists the advantages and drawbacks of the employment of UAE in marine products and discards. The application of UAE results in both the disruption of the material cell structures and an increase in the accessibility of the solvent to the internal particle structure, which enhances the intra-particle diffusivity. Hence, with

significant improvements in both the extraction yield and time used, improved efficiency could be achieved when the substrate particle size is reduced [57].

Table 1: Advantages and disadvantages of the application of ultrasound-assisted (UAE) extraction in fish and fish by-products for the extraction of bioactive compounds.

Extraction Technique	Advantages	Drawbacks	Extraction Conditions	Solvent
UAE	Reduction of energy, time and solvent consumption	Can induce lipid oxidation: increasing temperature by cavitation; formation of free radicals by sonolysis; mechanical forces generated by shockwaves and microstreaming	25 kHz 200–2450 W 30-60 min	Ethanol, cyclohexane, other organic solvents
	Safe; does not produce toxic compounds	High power consumption		
	Higher penetration of solvent into cellular material and enhanced release in medium	Difficult to scale up		

In the last decades, researchers have reported that the optimization of several parameters, as for instance ultrasound frequency, propagation cycle (continuous or discontinuous), nominal power of the device, amplitude, type and the geometry of the system (e.g., length and diameter of the probe), improve the efficiency of UAE towards the extraction of target compounds [58]. Currently, UAE is widely used for the recovery of several valuable compounds from seafood by-products (**Table 2**) [54].

For instance, several studies reveal that UAE can be used successfully for collagen extraction from fish by-products (skin and scales), reducing processing time and increasing yield [59,60]. In the processing skin of Japanese sea bass (*Lateolabrax japonicus*) for the extraction of collagen using UAE, it was shown that the extraction yield differed according to the amount of acid added, the treatment time and the amplitude of the ultrasonic waves [60]. More in detail, when the treatment time was increased for a long period (24h), unknown components were obtained, most probably deriving from

a breakdown of collagen, and conducting further optimization trials determined the most effective conditions for the extraction of pure collagen using USE (80% amplitude with 0.1 M acetic acid for 3 h of treatment).

Another important peptide for its emulsifying, foaming and gelling properties, is gelatin [61]. Gelatine is a polypeptide, which results from the denaturation of insoluble collagen, shown to have valuable functional properties, such as emulsifying, foaming, gelling, fat binding and water holding capacity [62]. Although the most widely used gelatins are of mammalian origin, the appearance of bovine spongiform encephalopathy (BSE, or mad cow) disease and religious restrictions regarding the consumption of porcine and bovine products, places marine collagen in a favorable position, rendering it as the most important alternative source. Several studies report the potential of using fish by-products, especially skins and bones, as novel sources of marine gelatin [32]. Limiting factors for the large-scale development of the fish gelatin industry are its inferior rheological properties, the lack of sufficient available raw materials and the variable quality of marine gelatin. In addition, other intrinsic quality factors related to odor, color, bloom strength and the viscosity of fish gelatin also limit the use of this gelatin [62].

In a study using the scales of bighead carp (*Hypophthalmichthys nobilis*), UAE (200 w, 60 ◦C, different extraction times from 1 h to 5 h) allowed an increase in extraction yields (30.94–46.67%) and the quality of the gelatin obtained as compared to using a water bath [63]. The authors reported that the extraction yields obtained with an ultrasound bath at 60 ◦C (46.67%) was also higher than those obtained with the water bath (36.39%) [63]. Furthermore, fish scales gelatins extracted with UAE are shown to have higher gelling and melting points, gel strength, apparent viscosity and emulsifying properties, compared to those obtained with a water bath extraction [59]. In another study, gelatin extracted by UAE was shown to have higher thermal stability compared with gelatin extracted by a conventional extraction.

However, the application of a higher ultrasound intensity (over 200 W) and a more extended extraction time (above 5 h) can lead to the decrease in gel strength and melting points of gelatin, which may cause protein degradation due to acoustic cavitation [63].

Table 2: Bioactive compounds obtained from fish and shellfish by-products by UAE.

By-Product	Source	Bioactive Compound and Product	Extraction Conditions	Main Effects	Ref.
Head	*Labeo rohita*	Oil	UAE: 20 kHz, 40% amplitude, for 5, 10 and 15 min. Enzymatic hydrolysis: Protamex ratio of 1:100 (w/w), 2 h, 150 rpm, 55 ◦C.	Pretreatments with UAE improved the extraction yield of oil, showing higher oil recoveries (67.48% vs. 58.74 % for SFE and untreated samples, respectively).	[64]
Scales	Bighead carp *(Hypophthalmichthys nobilis)*	Gelatin	Temperature: 60, 70 and 80 ◦C Extraction time: 1 h	Improved technological properties: highest storage modulus (5000 Pa), gelation point (22.94 ◦C), and melting point (29.54 ◦C).	[59]
	Bighead carp *(Hypophthalmichthys nobilis)*	Gelatin	Temperature: 60 ◦C Extraction time: 1, 3 and 5 h	Extraction yield: 46.67% for ultrasound bath versus 36.39% for water bath.	[63]
Shells	Prawns *(Macrobrachium rosenbergi*i)	Chitin	Extraction time: 0, 1, and 4 h 0.25M NaOH at solid to liquid ratio of 1:40 (w/v) Power: 41 W/cm	Decrease of the crystallinity indices and extraction yield of chitin as the time of sonication increased.	[65]
Skin	Japanese sea bass *(Lateolabrax japonicus)*	Collagen	UAE: 20 kHz, 80% amplitude, 0.1 M acetic acid, 3 h	UAE did not alter the major components of collagen (α1, α2 and β chains).	[60]
Whole fish	Mackerel	Proteins	ISP: Isoelectric solubilization precipitation.	UAE: 40 kHz, 60% amplitude, 0.1 M NaOH, 10 min. Significant increase of protein recovery, recovering more than 95% of total protein from mackerel by-products.	[66]

Chitin, a polysaccharide presents in the exoskeleton of crustaceans (shells) and the endoskeleton (pen) of cephalopods [67], is another compound that can be extracted with UAE. The influence of

sonication time (0, 1 and 4 h) on yield, purity and crystallinity was evaluated during the extraction of chitin from North Atlantic shrimp (NAS) shells (*Pandalus borealis*). The investigation showed that the crystallinity indices and the extraction yield of chitin decreased as the sonication time increased (from 8.28% to 5.02% after 4 h of sonication treatments). Meanwhile, the extraction yield increased from 7.45% to 44.01% after 4 h of sonication treatment (**Table 2**) [65]. The combination of UAE with other technologies has also been studied in processing different fish by-products in order to improve the extraction efficiency and the quality of extracted bioactive compounds. In summary, pre-treatment with emerging technologies has the potential to increase the quality of the extracted compounds and thus their beneficial properties, as by using these techniques it is possible to nearly maintain their composition and structure intact. Combining different novel technologies, such as UAE with enzymes, has also been demonstrated to improve extraction yields, facilitating an increase in collisions between enzyme and substrate [68]. Ultrasound-assisted enzymatic extraction is considered as a promising method for the improvement of the extraction yield of oil from marine matrices. Bruno et al. [64] evaluated the effects of pretreatments with UAE before enzymatic extraction on the extraction yield, fatty acid profile, oxidative stability and rheological properties of oil extracted from *Labeo rohita* heads (**Table 2**). The results showed higher oil recoveries, higher PUFA contents and higher oxidative stability in the samples subjected to a pretreatment with UAE before enzymatic hydrolysis. Besides, lower apparent viscosity and sensitivity to temperature changes were observed in the oil extracted using both UAE and enzymes as compared to enzymes alone [64].

In addition, Álvarez et al. [66] investigated the influence of UAE in the protein extraction yield from mackerel by-products by isoelectric solubilization precipitation (ISP). ISP is an emerging technology that uses pH changes to promote protein extraction. Several parameters influence the yield of extraction using this technology, such as the raw material quality as well as the extraction conditions (pH, temperature and extraction time). It was reported that by applying 60% of amplitude

for 10 min in 0.1 M NaOH solution it was possible to recover ≈94% of total raw material protein in a single extraction step. It was also shown that lower amplitudes (20%) of ultrasonic bath increases the yield of the extraction when compared to traditional ISP. Furthermore, applying UAE to alkaline extraction allowed the recovery of more than 95% of total protein from mackerel by-products [66]. Therefore, the use of UAE in combination with ISP for protein extraction from fish by-products can give higher yields, using lower extraction times and less solvent [69].

3.2 Supercritical Fluid Extraction (SFE)

3.2.1 Fundamentals

SFE is an alternative extraction method that has attracted a growing attention in food industries in the last decade. It is considered a green technology due to the utilization of non-toxic organic solvents, which results in more sustainable processing and reduced energy use and environmental pollution (**Table 1**) [70]. In SFE, solvents are used at above or near their critical temperature and pressure to separate solutes from a liquid or solid matrix under pressurized conditions. Under these conditions the solvents have intermediary properties between gases and liquids, which facilitates the extraction of the target compounds (**Figure 3**). Carbone dioxide (CO2) is the most widely used SFE solvent in food applications, since it is generally recognized as safe (GRAS) [71]. CO2 is not only cheap and easily available at high purity, but also lacks toxicity and flammability. It has a moderate critical temperature and pressure (31.1 ◦C and 7.4 MPa), and can be readily removed by a simple pressure reduction [72]. Furthermore, its higher diffusion coefficient and lower viscosity allow the rapid penetration through the pores of heterogeneous matrices, like gas, helping to dissolve the solute like a liquid. The efficiency of the SFE process is mostly affected by pressure, extraction temperature, extraction time, CO2 density, CO2 flow rate and co-solvent concentration [73]. The SFE selectivity is achieved by adjusting temperature and pressure, resulting in alterations of the density. This selectivity can also be adjusted by the use of a co-solvent, either to increase or decrease the polarity

of CO2. The most frequently used co-solvent is ethanol, because it meets the green technology requirements.

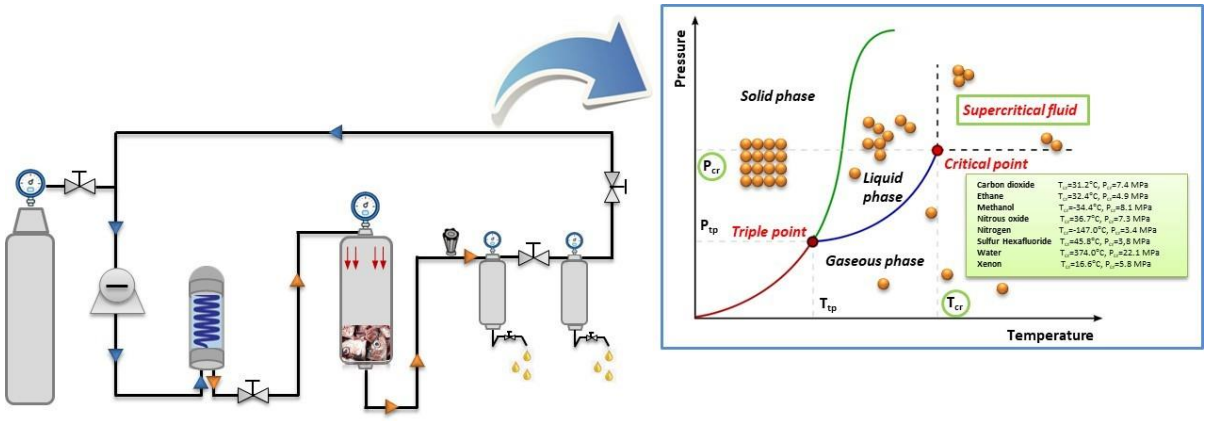

Figure 3: Schematic representation of supercritical fluid extraction (SFE) and the mechanism involved in this extraction technique.

3.2.2 Application of SFE in By-Products from Fish Industry

SFE has been used widely in several areas of food technology for food safety, food drying and sterilization, and food oil removal applications. This extraction technique is already being applied in the extraction of valuable compounds from natural materials, such as plant and marine sources. Several natural compounds, such as vitamins, flavors, natural pigments and essential oils, are extracted with SFE, thus avoiding the use of organic solvents and high temperatures [74]. So far, most of the studies that have evaluated the potential of SFE to extract biomolecules from fish by-products have focused upon lipid-soluble and antioxidant compounds [73,75]. **Table 3** collects the advantages and drawbacks of the employment of SFE in processing marine products and discards.

Nowadays, the large demand on fish oil by consumers linked to the large amount of fish by-products generated every year that are discarded has increased the interest regarding the extraction of edible fish oil from fish by-products (**Table 4**) using SFE. During SFE, the extraction parameters used (extraction time, flow-rate of CO2, pressure and temperature) play a key role on the extraction yield and the lipid composition of the functional products obtained. SFE has been applied to extract

an oil fraction from fish meal. Fish meal is one of the primary products obtained from fish processing [76].

Table 3: Advantages and disadvantages of the application of supercritical fluid (SFE) extraction in fish and fish by-products for the extraction of bioactive compounds.

Extraction Technique	Advantages	Drawbacks	Extraction Conditions	Solvent
SFE	Green extraction Technique. No need for organic solvent, and therefore the extract is very pure. Lipids can be used immediately	Very expensive and complex equipment operating at elevated pressures	25–40 MPa 40–80 ◦C CO2 flow > 2 mL/min 45 min-6 h	Co-solvent: Ethanol
	Maintain the quality of the final product. Low operating temperatures (40–80 ◦C)	No polar substances are extracted		
	Free of heavy metals and inorganic salts	High power consumption		
	Very effective because of its low viscosity and high diffusivity. Fast and high yield			

Its composition stands out for its higher protein content and balanced amino acid profile, characterized by good digestibility. Fish meals can be used to obtain fish protein concentrates intended for human consumption, as well as low-fat protein hydrolysates, thus achieving consumer demands for healthier fish products [77]. SFE allowed us to reduce the fat content of the produced fish meal without affecting protein quality. Extraction conditions of pressure (10–40 MPa), temperature (25–80 ºC), and CO_2 flow-rates of 9.5 g/min resulted in a product with a 90% reduction of fat and a lighter color, as with this method pigments such as astaxanthin were also extracted.

Moreover, SFE-extracted oils have also been shown to have higher radical scavenging activity and longer oxidative stability [84]. Using a gas saturated solution process, employing similar extraction conditions as that of SFE, in mackerel muscle, resulted in a more stable and less oxidized oil. However, the yields were low, obtaining oil concentrations of 4.00 g/20 g of mackerel muscle [83].

Longtail tuna (*Thunnus tonggol*) heads have also been used to obtain PUFA using SFE [80,81]. Tuna oil, besides omega-3 PUFA, also contains substantial levels of saturated fatty acids (SFA) and

undesirable impurities which were extracted by simultaneous fractionation using SFE with ethanol as a co-solvent. In this process, fish oil was extracted and simultaneously collected into six fractions based on molecular weight. The short chain SFA fraction was extracted early, while the latter fractions were dominated by long-chain fatty acids, especially monounsaturated fatty acids (MUFA) and PUFA, particularly rich in DHA among other omega-3 and omega-6 fatty acids, resulting in a refined product with added value for health. The conditions that yielded optimal results in terms of obtaining a PUFA-rich fraction with a high quality and storage stability were 65 ºC, 40 MPa, with a CO2 flow of 3.0 mL/min during 120 min. The results of this study demonstrate that, in applying SFE, the utilization of ethanol as the co-solvent allows us to achieve an upconcentration of PUFA (omega-3 and omega-6) in an effective way, and that using SFE for the extraction of fish oil from fish by-products can play an important role in obtaining economic and nutritional benefits, reducing environmental risks [84] (**Table 4**).

Sahena et al. compared different techniques for oil extraction from Indian mackerel (Rastrelliger kanagurta) skin [86]. Oil from this by-product fraction was extracted by SFE at different pressures (20–35 MPa) and temperatures (45–75 ºC), and was compared to Soxhlet extraction [70,86]. The authors observed that their oil extraction yield increased with pressure and temperature, being 53.2% for SFE co-solvent, 52.8% for soaking pressure and 24.7% for the continuous technique at 35 MPa and 75 ºC. The Soxhlet method achieved the highest extraction yield (53.6%) compared to that obtained with SFE. Other studies have demonstrated that the pressure swing and soaking techniques are among the most effective ones in extracting oil from fish skin [70,86].

Létisse et al. [44] also evaluated the influence of SFE conditions (pressure, temperature and CO2 rate) on the up concentration of EPA and DHA in oil from sardine heads and tails. The obtained results confirmed that conditions of 30 MPa, 75 ºC, 2.5 mL CO_2/min and 45 min of extraction time allowed the obtaining of yields of 10.36%, and contents of EPA and DHA of 10.95% and 13.01%, respectively. Rubio-Rodríguez et al. [91] found that the application of lower pressure and temperature (25 MPa,

40 ºC), higher CO_2 flow (10 kg CO_2/h) and an up flow direction through the offcuts from two hake species (Merluccius capensis–Merluccius paradoxus) during 3 h resulted in extracting more than 96% of the total oil contained in the raw material. High contents of EPA and DHA (about 6% and 14%, respectively, of the total fatty acids) were obtained in the extracted oil [91]. Furthermore, the application of the aforementioned conditions of temperature and pressure on the off-cuts of orange roughy (*Hoplostethus atlanticus*) and Atlantic salmon (*Salmo salar*), as well as on liver from jumbo squid (*Dosidicus gigas*) resulted in fish oils with reduced PUFA oxidation and less impurities [85]. The application of SFE in tuna livers also allowed to result in oil both rich in n-3 PUFA and vitamins [82].

Fish by-products, such as caviar and viscera, are also an important source of bioactive compounds, especially of monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids [79]. In the case of viscera, the application of conditions of 400 bar, 60 ºC and a CO_2 flow rate of 0.194 kg/h resulted in high yields (above 50 g/100g), which are similar to those obtained with petroleum ether, and the production of omega-enriched fish oils (DHA and EPA). Lisichkov et al. [90] studied the influence of operating parameters (pressure: 200, 300, 350 and 400 bar; temperatures: 40, 50 and 60 ºC; CO_2 flow rate: 0.194, 0.277 and 0.354 kg/h; and extraction time: 30, 60, 120 and 180 min) on the SFE extraction of PUFA from the viscera of common carp. For this purpose, authors used the 3D respons surface methodology (RSM) and found that an equilibrium state was achieved after 180 min, where the curve of the extraction yield and the extraction time reached a plateau.

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Table 4: Bioactive compounds obtained from fish and fish by-products by SFE.

By-product	Source	Bioactive compound	SC-CO_2 conditions	Outcomes	Ref.
Canned by-product	Tuna	Oils	Temperature ≥ 40 °C Pressure ≥ 25 MPa CO_2 flow ≥ 10 kg/h Extraction time: 3 h	Extracted oils showed better conditions, quality (type of compounds and indicators of lipid oxidation) and yield.	[78]
Caviar, fillet and viscera	Carp (*Cyprinus carpio* L.)	Oil	Temperature: 40, 50 and 60 °C Pressure: 200, 300, 350 and 400 bar CO_2 flow: 0.194 kg/h Extraction time: 180 min	Omega-enriched fish oils (DHA and EPA). High yields, above 50 g/100g in viscera, which are similar to those obtained with petroleum ether.	[79]
Fish meal	n.a. [1]	Oil	Temperature: 25-80 °C Pressure: 10-40 MPa CO_2 flow with ethanol: 9.5 g/min	High reductions of fat (90%). Extract with a lighter colour due to astaxanthin extraction.	[77]
Head	*Thunnus tonggol*	Fatty acid	Temperature: 65 °C Pressure: 40 MPa CO_2 flow with ethanol: 3 mL/min Extraction time: 2 h	SC-CO_2 (co-solvent) is a good technique to extract omega3/6 after fractionations of oil.	[80]
		PUFA	Temperature: 65 °C Pressure: 40 MPa CO_2 flow with ethanol: 2.4 mL/min Ethanol flow: 0.6 mL/min Extraction time: 120 min	Good quality of extracted PUFA-rich fraction, even 60 days after storage.	[81]
Heads and tails	Sardine	DHA and EPA	Temperature: 75 °C Pressure: 300 bar CO_2 flow: 2.5 mL/min Extraction time: 45 min	Increase of the extraction yields: DHA (59%), EPA (28%).	[44]

Table 4: (cont.)

By-product	Source	Bioactive compound	SC-CO_2 conditions	Outcomes	Ref.
Liver	Tuna	Fatty acids	Step of freeze-drying (12h) Temperature: 40 °C Pressure: 35 MPa Continuous CO_2 flow: 3mL/min (at 20°C) Extraction time: 4h	High quality and excellent yield obtained 98.45%.	[82]
Muscle	Mackerel	Vitamins	Temperature: 45 °C Pressure: 15-25 MPa CO_2 flow: 27 g/min Extraction time: 2 h	High extraction of vitamins A, D2, D3 and α-tocopherol.	[83]
Muscle, bone and skin	Salmon	Oil (PUFA)	Temperature: 45 °C Pressure: 250 bar CO2 Flow: 27g/min Extraction time: 3 h	Premium quality oil of physical, biochemical and biological properties. Yield 76.12 %–86.99%.	[84]
Muscle	Mackerel	Oil (EPA and DHA)	Temperature: 45 ◦C Pressure: 15–25 MPa CO2 flow: 27 g/min Extraction time: 2 h	The extracted oil presented significant contents of PUFAs (EPA, DHA). Higher stability compared with n-hexane extracted oil.	[83]
Off-cuts	Hake (*Merluccius capensis–Merluccius paradoxus*) Orange roughy (*Hoplostethus atlanticus*)	Oil	Temperature: 313 K Pressure: 25 MPa CO_2 flow: 880 kg/m^3	PUFA extraction. Reduction of fish oil oxidation. Reduction of certain impurities. Co-extraction of some endogenous volatile compounds.	[85

Table 4: (cont.)

By-product	Source	Bioactive compound	SC-CO_2 conditions	Outcomes	Ref.
Off-cuts	Salmon (*Salmo salar*)	Oil	Temperature: 313 K Pressure: 25 MPa CO_2 flow: 880 kg/m^3	PUFA extraction. Reduction of fish oil oxidation. Reduction of certain impurities. Co-extraction of some endogenous volatile compounds.	[85
Liver	Jumbo squid (*Dosidicus gigas*)				
Skin	Mackerel (*Rastrelliger kanagurta*)	Oil	Temperature: 45–75 °C Pressure: 20–35 MPa		[86]
			Continuous system: Pressurized for 5 min, CO_2 flow 2ml/min	Yield very close to those obtained with Soxhlet technique.	
			Co-solvent technique: CO_2 and ethanol (80-20% at 2 ml/ min) for 6 h	PUFA constituents of co-solvent, soaking and pressure swing techniques were similar to the Soxhlet method.	
			Soaking technique: Samples were soaked with pure CO2 for 10h then extracted for 6 h	The largest recoveries of PUFA, especially the ω-3 family, were achieved from the soaking and pressure swing techniques at 35 MPa and 75ºC.	
			Pressure swing: Samples were pressurized, with pure Co2 for 2 h then extracted for 3 h		

Table 4. Bioactive compounds obtained from fish and fish by-products by SFE.

By-product	Source	Bioactive compound	SC-CO_2 conditions		Outcomes	Ref.
Viscera	Squid (*Todarodes pacificus*)	Enzymes	Temperature: 35-45 °C Pressure: 15-25 MPa CO_2 flow: 22 g/min Extraction time: 2.5 h		Thermal stability of enzymes was slightly higher than *n*-hexane treated squid viscera. Denaturation of proteins did not occur.	[87] (Uddin et al., 2009) (Uddin et al., 2009) (Uddin et al., 2009) (Uddin et al., 2009)
		Amino acids	SFE: Temperature: 35-45 °C Pressure: 15-25 MPa CO_2 flow: 22 g/min Extraction time: 2.5 h	SWH: Temperature: 180-280 °C Pressure: 0.101-6.41 MPa Extraction time: 5 min	Positive effects of use SFE as pretreatment method. Amino acids were 1.5 times higher than those obtained in non deoiled samples.	[88]

		Lecithin	Temperature: 35-45 °C Pressure: 15-25 MPa CO_2 flow: 22 g/min Extraction time: 2.5 h	Extraction yield was higher at highest temperature and pressure (0.34 g/g squid viscera at 45 ◦C and 25 MPa). Lecithin isolated had in its composition polyunsaturated fatty acids (EPA and DHA) with a high oxidative stability.	[89]
	Common carp (*Cyprinus carpio* L.)	PUFA	Temperature: 40, 50 and 60 °C Pressure: 200, 300, 350 and 400 bar CO_2 mass flow: 0.194, 0.277 and 0.354 kg/h Extraction time: 30, 60, 120 and 180 min	Adequate for the isolation of bioactive components. Positive impact on the total yield and extraction time.	[90]

The higher extraction yield was achieved at 180 min of extraction time, 60 ºC of temperature, 400 bar of pressure and with a 0.354 kg/h CO_2 flow rate. A positive impact of the increase of pressure and CO_2 flow rate was observed on the extraction time and the total extraction yield, whereas the operating temperature had a complex influence, depending on the values of the operating pressure at isobaric conditions (**Table 4**) [90]. The yield and quality of oil extraction using different conventional versus emerging technologies were also evaluated by Fang et al. [82], who concluded that the best results were obtained using SFE and SC-dimethyl ether (SDEE), as these methods prevented the oxidation of lipids and reduced the damage of PUFA and vitamins, as compared with conventional methods (wet reduction and enzymatic extraction). Moreover, only a minor difference between the resulting material levels in volatile compounds and vitamins was observed in both SFE and SDEE, which was related to the used solvents' solubility [82]. The disadvantages of SFE are related to high energy consumptions due to the application of high pressures and the need for material preparation by freeze-drying [82]. The limitation of SDEE is its lower critical point density and the related environment hazards [92]. Likewise, Taati et al. [78] found that SFE gives high extraction yields preventing oil oxidation, especially in oils with a high level of triacylglycerol (TAG) and PUFA, and attributed this result to the vacuum conditions and absence of free atmospheric oxygen during processing.

Finally, following extraction, the residues of fish by-products can also be used as a source of other valuable ingredients, such as amino acids, facilitated by the defattening amounts of the raw material which allows the extraction of other biomolecules [88]. Accordingly, Uddin et al. [88] evaluated the combined effect between SFE and sub-critical water hydrolysis (SWH) in order to obtain valuable materials from squid viscera. SWH is a technique considered as a non-conventional extraction method (green technology) that uses water in a sub-critical state as the solvent (from 100 ºC to 374 ºC at 0.10 MPa and 22 MPa, respectively). This enables the extraction of bioactive compounds of an ionic, polar and non-polar nature. This method has been used in several studies for the extraction of

peptides and amino acids from animal by-products by hydrolyzing and breaking down the protein [93]. The results obtained in deoiled squid viscera confirmed that the use of SFE before SWH had positive effects on the recovery of amino acids, since the contents obtained in pretreated samples were 1.5 times higher than those obtained from raw squid viscera (51% vs. 76%, respectively).

The viscera of squid (*Todarodes pacificus*) was also processed to obtain other bioactive compounds such as enzymes and lecithin [87,89]. In the first case, n-hexane treatment of squid viscera resulted in the highest extraction yield; however, the thermal stability of digestive enzymes (protease, lipase and amylase) were slightly greater in SFE-treated samples [87]. High oxidative stability was also found in squid viscera lecithin subjected to a defattening step using SFE, despite its significant content in LC-PUFAs (EPA and DHA) [89].

3.2.3 Application of SFE in by-Products from Processing Shellfish

Shellfish are marine organisms rich in several bioactive components with potential health benefits, which makes them interesting as functional food ingredients [12]. SFE has also been used to extract PUFAs from shrimp by-products (**Table 5**). Northern shrimp (*Pandalus borealis* Kreyer) processing by-products, such as heads, shell and tail could be used as a natural source for the development of beneficial health products (omega-3 PUFA) [94]. Depending on the extraction conditions used, different extraction yields and qualities can be obtained. The use of low pressure conditions (15 MPa and 50 ºC) with flow rates of 3–5 L/min during 90 min showed high selectivity for DHA and EPA, while moderate pressures (35 MPa and 40 ºC) showed increase extraction efficiency but lower yields than those obtained with organic solvents (137 mg oil/g vs. 206 mg oil/100 g and 178 mg oil/g, for SFE, acetone and n-hexane, respectively). In contrast, the obtained extract by SFE contained higher total free fatty acids (795 mg/g), and similar levels of EPA (7.8%) and DHA (8.0%) to conventional solvent extraction (Soxhlet using acetone and n-hexane as solvents), but with lower extraction times (90 min vs. 8 h, for SFE and Soxhlet extraction, respectively).

Table 5: Bioactive compounds obtained from shellfish by-products by supercritical fluid extraction (SFE).

By-product	Source	Bioactive compound	SC-CO_2 conditions	Outcomes	Ref.
Head, shells and tails	Brazilian redspotted shrimp (*Farfantepenaeus paulensis*)	Lipids and carotenoids	Temperature: 50 °C Pressure: 30 MPa CO_2 flow: $4.2 \cdot 10^{-5}$ kg/s Extraction time: 20 min Solvent for compounds recovery: *n*-hexane	Increase extraction yield: Astaxanthin (36%)	[95]
			Temperature: 50 °C Pressure: 30 MPa CO_2 flow with ethanol: $8.3 \cdot 10^{-5}$ kg/s Ethanol flow: $4.4 \cdot 10^{-6}$ kg/s Extraction time: 200 min Solvent for compounds recovery: *n*-hexane	Increase extraction yield: Astaxanthin (57.9%)	
			Temperature: 43 °C Pressure: 370 bar CO_2 flow: 1.5 L/min Extraction time: 200 min Solvent for compounds recovery: *n*-hexane	Increase extraction yield: Astaxanthin (39%)	[96]
	Northern shrimp (*Pandalus borealis* Kreyer)	PUFA	Temperature: 40 °C Pressure: 35 MPa CO_2 flow: 3-5 L/min Extraction time: 90 min	Lower yields (137 mg oil/g) than those obtained in organic solvent extraction. Higher contents of total fatty acid content (795 mg/g), DHA (8%), EPA (7.8%).	[94]
Liver	Rock lobsters (*Jasus edwardsii*)	PUFA and vitamins	Temperature: 50 °C Pressure: 35 MPa Continuous CO_2 flow: 0.434 kg/h Extraction time: 4h	Enrichment in PUFAs (DHA, EPA) *vs*. Soxhlet extraction. Reduction in the amounts of toxic heavy metals.	[97]
Shell	Crawfish	Pigments	Temperature: 50-70 °C Pressure: 13.8-31.0 MPa CO_2 flow: 1.0-1.5 L/min Co-solvent: 10% ethanol	Increase extraction yield: Astaxanthin (197.6 mg/kg)	[98]

Recently, PUFA-rich lipids, in particular DHA and EPA, have been recovered with high yields (94% relative to the yield of Soxhlet extraction) from Rock lobster livers by SFE extraction [97]. Besides the use of this technique to obtain essential fatty acids for human consumption from this discard material, it also allowed us to reduce the presence of heavy metals in a product usually characterized by high contamination levels of arsenic and cadmium. This is due to the ability of SFE to carry out selective extraction of low-polar lipid compounds, retaining polar impurities such as some organic derivatives with heavy metals [85]. Another important compound that can be obtained from shellfish by-products is astaxanthin. As commented previously, astaxanthin is a pigment present in marine foods [99], such as fish (salmon and trout) and shellfish (shrimp and lobster). SFE is a selective and precise method that allows the extraction of astaxanthin from crustacean samples [95,98,100,101], achieving yields of total carotenoid extraction up to 98%, vs. 84% obtained with conventional extraction methods [100]. Depending on the extraction conditions, it is possible to achieve astaxanthin yields of about 40% [101]. Redspotted shrimp (*Farfantepenaeus paulensis*) heads, shells and tails are another source of astaxanthin, but the yields obtained by SFE in the published study by Sánchez-Camargo et al. (2011) were low [95]. The use of ethanol as co-solvent in different ratios improved the extraction of astaxanthin, as it allowed one to extract more than non-polar compounds [102], increasing the recoveries significantly (65.2% vs. 36%) [103]. Crawfish shell is also a source of astaxanthin. The application of similar SFE conditions (50 ^{o}C, 22.4 MPa, 1.0-1.5 L/min of CO_2 flow rate, 10% of ethanol) to previously reviewed studies resulted in a significant increase of the extraction yield (197.6 mg/kg) [98].

4. Conclusions

There is a great increase in interest for the extraction of bioactive compounds from fish and shellfish by-products due to their nutritional value and potential health benefits. The valorization strategy of seafood by-products based on the development of novel products can lead to the more environmentally sustainable use of marine resources and higher economic benefits for the sector. It

is thus critical to define appropriate extraction technologies that allow minimizing processing, maximizing quality and yield and ensuring product safety (non-toxic organic solvents) meeting thus the objectives for sustainable development in achieving food safety and food security for the increasing global human population. UAE and SFE are two emerging technologies that allow enhancing the extraction of thermolabile bioactive compounds, maintaining their quality and oxidative stability. Combining UAE and SFE with other extraction methods (ISP, SWH or enzymatic methods) can further increase extraction yields and reduce the presence of undesirable compounds (heavy metals). Finally, the use of UAE and SFE as a pretreatment to other methods offers the possibility of extracting even more valuable compounds from fish by-product matrices.

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4.2 AQUACULTURE AND ITS BY-PRODUCTSAS A SOURCE OF NUTRIENTS AND BIOACTIVE COMPOUNDS

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Aquaculture and its by-products as a source of nutrients and bioactive compounds

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Abstract:

Underutilized marine resources (e.g., algae, fish, and shellfish processing by-products), as sustainable alternatives to livestock protein and interesting sources of bioactive compounds, have attracted the attention of the researchers. Aquatic products processing industries are growing globally and producing huge amounts of by-products that often discarded as waste. However, recent studies pointed out that marine waste contains several valuable components including high-quality proteins, lipids, miner- als, vitamins, enzymes, and bioactive compounds that can be used against cancer and some cardiovascular disorders. Besides, previously conducted studies on algae have shown the presence of some unique biologically active compounds and valuable proteins. Hence, this chapter points outrecent advances in this area of research and discusses the importance of aquaculture and fish processing by-products as alternative sources of proteins and bioactive compounds.

1. Introduction

The current food supply system based on intensive agriculture has not only limited the number of plants and animal species in our dietbut also contributed to the depletion of natural resources (Nadathur,Wanasundara, & Scanlin, 2016). About 37% of cultivated land worldwideis used for feed production to generate animal protein. Besides, the pro- duction of vegetable proteins requires less water, land, nitrogen, and fos- sil fuel than those of animal-derived protein. Therefore, sustainable strate-gies (e.g., obtaining of proteins from alternative sources and the creation of new high-value products from underutilized waste streams) have been proposed in order to reduce environmental footprint (Henchion, Hayes, Mullen, Fenelon, & Tiwari, 2017). There are several options of highly nu-tritious protein sources still not mass exploited (e.g., indigenous pulses and root crops, ancient grains, insects, lower organisms, marine algae or by-products from different industries) (Bleakley & Hayes, 2017; Hayes, 2018; Kim et al., 2019; Nadathur et al., 2016)

In addition to environmental concerns, consumers are increasingly interested in safe, nutritious, and healthy food products. Moreover, the excessive intake of proteins from terrestrial animals, linked to saturated fattyacids and cholesterol, is considered as a risk factor of certain chronic dis-eases development. On the one hand, the consumption of aquatic prod- ucts is believed to have several healthy effects, and consequently, the production of different edible marine species through aquaculture tech- niques for human consumption has risen over the last years (FAO, 2018a), leading to high volumes generation of by-products from fish processing. On the other hand, healthy bioactive compounds from natural sources, including those of marine origin, have gained great importance in recent years. Marine environment is constituted by a great amount and biodiversity of plants, animals and microorganisms adapted to so varied environmental conditions that the substances they produce forsurvival exhibit a broad panel of interesting biological activities (de Veraet al., 2018; Herrero, Mendiola, Plaza, & Ibañez, 2012; Ibañez, Herrero, Mendiola, & Castro-Puyana, 2012). In this sense, several types of sec- ondary metabolites and a large mixture of biogenesis metabolites have been

isolated from marine organisms, as well as many biological activities (antimicrobial, antitumor, antidiabetic, anticoagulant, antioxidant, anti-in-flammatory, antiviral, antimalarial, antitubercular, anti-aging, antifouling, and antiprotozoal) with industrial and therapeutic potential have also been described (Alves et al., 2018).

It is estimated that by 2050 the world population will exceed 9 billion people (Tian, Bryksa, & Yada, 2016). This expected increase in the global population will be associated with a high demand for food products with high nutritional quality that can be produced in an environmen-tally friendly way. Therefore, achieving a healthy diet through sustainable foods will be one of the challenges for researchers and food producers in the next years. In this context, special attention is given to protein and bioactive compounds from both underutilized marine species and fish processing by-products. In this regard, this chapter describes the valuable compounds that might be obtained from algae and seafood processing by-products, highlighting their biological activities and to a lesser ex-tent their potential applications.

2. Fish by-products

The amount of fish produced worldwide reaches around 171 million tons, of which 80 million tons are from aquaculture (Marc Antonyak,Lukey, & Cerione, 2018). In 2016, a great diversity of species was raised in aquaculture, among them, common carp (*Cyprinus carpio*) (8%), Nile tilapia (*Oreochromis niloticus*) (8%), bighead carp (*Hypophthalmichthys nobilis*) (7%), Catla (*Catla catla)* (6%), Atlantic salmon (*Salmo salar*) (4%), and rainbow trout (*Oncorhynchus mykiss*) (2%) were the major species produced (Marc Antonyak et al., 2018).

In 2015, fish accounted for about 17% of animal protein consumed bythe global population (Marc Antonyak et al., 2018). Moreover, FAO reported that the fish consumption raised an average rate of about 1.5% peryear, i.e., from 9.0 kg in 1961 to 20.2 kg in 2015 (in per capita terms) (Marc Antonyak et al., 2018). Moreover, seafood is a valuable source of bioactive compounds such as peptides, amino acids, omega-3 long-chain polyunsaturated fatty acids (PUFAs), vitamins (e.g., vitamins A and D) and

minerals such as calcium, potassium, and zinc (Kundam, Acham, & Girgih, 2019; Marc Antonyak et al., 2018). The fish composition consists of 15–30% proteins, 0–25% fat, and 50–80% moisture depending on the species, age, gender, health, and harvesting season (Caldeira et al., 2018). For example, white fish, such as cod and hake, contains around 20% protein, 80% water, 0.5–3% oil, minerals, vitamins, carbohydrates, and other compounds. On the other hand, oily fish, such as mackerel and salmon, contain 20% protein, 10–18% oil, and 62–70% water (Kundamet al., 2019). Among 20% and 80% of fish is considered as waste by the fish processing industry, depending on several parameters such as the fishtype and the processing specifications (Caldeira et al., 2018).

This waste usually includes head, viscera, skin, bones, and scales withranges of 9–12%, 12–18%, 1–3%, 9–15% and 5% of the whole fish weight, respectively (Villamil, Váquiro, & Solanilla, 2017). It should be noted that recent studies showed that such waste can be considered as a valuable by-product source of value-added compounds. Consequently, considerable attention has been paid in the nutrients and bioactive com- pounds present in fish by-products. These materials are considered as sustainable sources for pharmaceutical, nutraceutical and cosmeceutical in- dustries (Kundam et al., 2019; Marc Antonyak et al., 2018).

2.1 Nutrients and bioactive compounds from fish by-products

Fish bioactive compounds are substances present in fish by-products with biological activity. These constituents are beneficial to human health(Kundam et al., 2019). These health benefits are accomplished through multiple biological activities, including antioxidant activity, hormones mediation, immune system enhancement and facilitation of substancestransition through the digestive tract, butyric acid production in the colon(it favors acidification, which improves intestinal health), and absorptionand/or dilution of substances in the gut (Kundam et al., 2019).

Thus, fish by-products are an effective source of bioactive compounds that may be used as nutritional supplements and provide medical and health benefits. **Figure 1** shows the main by-products from fish processing and some compounds obtained from them. Many studies have been

con- ducted to extract bioactive compounds from different by-products. Some of these studies are listed in **Table 1**.

2.1.1 Proteins

Fish proteins are rich sources of essential (e.g., leucine and lysine) and non-essential amino acids (e.g., aspartic and glutamic acids) (Shahidi & Ambigaipalan, 2018). Protein-rich by-products include backbone, skin, head, viscera, and blood that may be used to produce collagen/gelatin and proteoglycan, bioactive peptides, protein hydrolysates, among others.

Up to 10–20% of total fish protein can be present in the fish by-products (Zamora-Sillero, Gharsallaoui, & Prentice, 2018). Both these essential amino acids and the bioactive peptides obtained from fish by-prod- ucts have great potential as beneficial compounds for improving health (Hamed, Özogul, Özogul, & Regenstein, 2015).

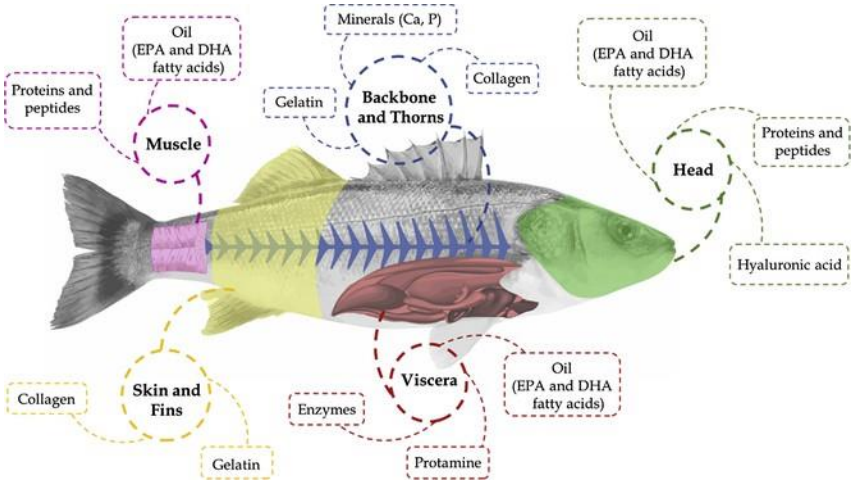

Figure 1: Fish by-products and main compounds obtained from them. *Adapted from Marti-Quijal et al. (2020).*

2.1.1.1 Collagen and gelatin

Collagen is a fibrous and structural protein present in the extracellular space of fish and contributes to the physiological function of tissues in bones, tendons, skin, head, cartilage, and muscle (Raman & Gopakumar, 2018). It is the most abundant single protein present in fish, representing 25% of the total protein (Caldeira et al., 2018). Collagen has a wide range of applications in the health-related

sectors, specifically in cosmetics, pharmaceutical industry and medical care (including plastic surgery, orthopedics, ophthalmology, and dentistry) (Silva et al., 2014). In fact, there are many types of collagen however the most common form in fish by-products is Collagen type I and it is found in the connective tissues, skin, muscles, bone (Caldeira et al., 2018) and cornea (Raman & Gopakumar, 2018). Actually, collagen has been obtained from the skin of different fish types (Chi et al., 2014). Furthermore, fish collagen, following their extraction, may be further enzymatically hydrolyzed to release physiologically active peptides. Especially, some collagen derived peptides could exhibit interesting antioxidant activity (Chi et al., 2014), antimicrobial activity against different strains of bacteria (Ennaas, Hammami, Beaulieu, & Fliss, 2015), potent antihypertensive activity through ACE inhibitory properties (Alemán, Gómez-Guillén, & Montero, 2013).

Gelatin is a proteinaceous macromolecule obtained by thermal denaturation of collagen with a kinetic irreversible process. It shares some of thecollagen's properties because of their similar composition. Thus, it can beused to improve the consistency, elasticity, and stability of foods, as well as, to produce edible and biodegradable films that increase the shelf life offood products (Caldeira et al., 2018). Moreover, it was also reported that fish gelatin had higher antioxidant activity those of synthetic ones (Ishak & Sarbon, 2018). Several studies extracted gelatin from fish by-products whereas fish skin was the main source of gelatin (Irwandi et al., 2009). Itwas extracted from the skin of seabass (*Lates calcarifer*) (Sae-leaw et al.,2016) and pacific cod (*G. macrocephalus*) (Ngo et al., 2016). Also, gelatinwas extracted from the bones (*Black tilapia*) (Zakaria, Hidayah, & Bakar, 2015) and scales bighead carp (*Hypophthalmichthys nobilis*) (Huang et al., 2017) and the head of mackerel (*Scomber scombrus)* (Khiari, Rico, & Martin-Diana, 2011).

Table 1: Valuable compounds with biological activities that are extracted from the by-products of various marine species

Side streams	Source	Valuable compounds	Biological activities	References
Skin	Tilapia (*Oreochromis niloticus*)	Gelatin hydrolysate/ active peptides	ACE inhibitory simulated gastrointestinal digestion	Thuanthong, De Gobba, Sirinupong, Youravong, and Otte (2017)
	Chum salmon (*Oncorhynchus keta*)	Collagen peptide/ bioactive peptides	Antioxidant	Pei et al. (2010)
	Atlantic salmon (*Salmo salar* L.)	Collagen hydrolysates	Antihypertensive	Gu, Li, Liu, Yi, and Cai(2011
	Salmo (*Oncorhynchus keta*)	Oligopeptides	Antidiabetic	Zhu, Peng, Liu, Zhang, and Li (2010)
	Seabass (*Lates calcarifer*)	Gelatin hydrolysate/ bioactive peptides	Antioxidant, immunomodulatory, antiproliferative	(Sae-leaw et al., 2016)
		Gelatin hydrolysate/ bioactive peptides	Antioxidant	Mirzapour-Kouhdasht, Sabzipour, Taghizadeh, and Moosavi-Nasab (2019)
	Pacific cod (*Gadus macrocephalus*)	Gelatin hydrolysate/ bioactive peptides	ACE inhibitory	Ngo, Vo, Ryu, and Kim (2016)
	Bluefin leatherjacket (*Navodon septentrionalis*)	Bioactive peptides	Antioxidant	
	Tilapia (*Oreochromis niloticus*)	Three bioactive peptides	Antidiabetic	Wang et al. (2015)

Table 1: (cont).

Side streams	Source	Valuable compounds	Biological activities	References
Scales	Tilapia (*Oreochromis niloticus*)	Gelatin hydrolysate/ bioactive peptide	ACE inhibitory	Zhang, Tu, Shen, and Dai (2019)
Head	Bluefin leatherjacket (*Navodon septentrionalis*)	Protein hydrolysate/ bioactive peptides	Antioxidant	Chi, Wang, Wang, et al. (2015)
	Bluefin tuna (*Thunnus thynnus*)	Protein hydrolysate	Antioxidant	Bougatef et al. (2012)
	Tilapia (*Oreochromis niloticus*)	Bioactive peptides	Antimicrobial	(Robert et al., 2015)
	Sardinelle (*Sardinella aurita*)	Four bioactive peptides	Antioxidant	Bougatef et al. (2010)
	Salmon (*Oncorhynchusketa*)	ω-3 PUFAs, EPA, and DHA	Nitric oxide (NO) inhibitory, tumor necrosis factor alpha (TNFα) inhibitory, and anti-inflammatory	Ahmad, Rudd, Kotiw, Liu,and Benkendorff (2019)
Bone	Indian mackerel *Rastrelliger kanagurta*	Protein hydrolysate/ bioactive peptides	Antioxidant	(Sheriff et al., (2014)
	Alaska Pollack (*Theragra chalcogramma*)	Bioactive peptides	Ca-binding	(Jung et al., 2006)
	Tuna (*Thunnus alalonga*)	Bioactive peptides	Antioxidant	(Je et al., 2007)
	Hoki (*Johnius belengerii*)	Calcium peptide	Ca-binding	Kim and Jung (2007)
		Bioactive peptide	Antioxidant	Kim, Je, and Kim (2007
liver	Atlantic cod (*Gadus Morhua*L.)	ω-3 PUFAs, EPA, and DHA	Antibacterial	Ilievska, Loftsson, Hjalmarsdottir, and Asgrimsdottir (2016)

Table 1: (cont).

Side streams	Source	Valuable compounds	Biological activities	References
Viscera	Rain bow trout (*Oncorhynchusmykiss*)	Protein hydrolysates/ bioactive peptides	Antibacterial	Wald, Schwarz, Rehbein, Bußmann, and Beermann (2016)
	Black Pomfret, *Parastromateus niger*	Protein hydrolysates/ bioactive peptides	Antioxidant	Jai Ganesh, Nazeer, and Sampath Kumar (2011)
	Black scabbardfish (*Aphanopus carbo*)	Protein hydrolysates/ bioactive peptides	Antioxidant	Batista, Ramos, Coutinho, Bandarra, and Nunes (2010)
	Sardinella (*Sardinella aurita*)	Protein hydrolysates/ bioactive peptides	Antioxidant	Souissi, Bougatef, Triki- Ellouz, and Nasri (2007
	Smooth hound (*Mustelus mustelus*)	Protein hydrolysates/ bioactive peptides	Antioxidant, anti-ACE, antibacterial activities	(Abdelhedi et al., 2016)
	Sardine (*Sardinopssagax*)	Omega-3 PUFAs, EPA, and DHA	Nitric oxide (NO) inhibitory, tumor necrosis factor alpha (TNFα)	Ahmad et al. (2019)
	Red snapper (*Lutjanus campechanus*) Seer fish (*Scomberomorus commerson*) Great barracuda (*Sphyraena barracuda*)	Protolithic enzyme(protease	Protolithic activity	Sabtecha, Jayapriya, and Tamilselvi (2014

ACE, angiotensin-converting enzyme; PUFA, polyunsaturated fatty acids; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

2.1.1.2 Bioactive peptides

Fish bioactive peptides mainly consist of 2–20 amino acids, which are available in all parts of fish or incorporated in fish protein. However, thesepeptides are inactive within the native proteins and are activated after being released by digestion in vivo (proteolysis) or by enzymatic hydrolysis in vitro

which is the best method to obtain protein hydrolysate or bioactive properties (Zamora-Sillero et al., 2018).

Moreover, active peptides extracted from fish by-products display multiple biological activities based on amino acid composition and sequence. They also play a great role in pharmaceutical and medical ap- plications which result in the promotion of human health and may be helpful in the prevention and treatment of several chronic diseases. Thus, the obtained peptides can act as antioxidants, antidiabetic, immunomodulatory, antiproliferation and antimicrobial agents among others (Kim & Wijesekara, 2010).

Different fish species by-products are rich in bioactive peptides. Several studies have shown that head, viscera, skin, and backbone are good sources of protein hydrolysates. Seven antioxidant peptides were puri- fied from the combined head and viscera of sardinella (*Sardinella au- rita*); these peptides demonstrated high antioxidant activity, measured with DPPH radical scavenging assay (Bougatef et al., 2010). Tilapia by-product (head, frames, and viscera) hydrolysate had a high peptide content and a well-balanced amino acid profile; Robert et al. (2015) characterized the peptide fraction that yielded 1374 unique peptides and high- lighted the high peptide diversity of the hydrolysate. Also, bioactive peptides, isolated from the head (Bougatef et al., 2012) and bone (Je et al., 2007) of tuna, have shown good antioxidant activities.

In addition to the antioxidant peptides that can be naturally present, peptides from protein hydrolysates have been reported to have bioactivity. In this context, bioactive peptides isolated from Atlantic salmon (*Salmo salar*) skin, bone and muscle extraction of gelatin hydrolysate have sev- eral biological activities such as antioxidant, ACE inhibitory and antidiabetic activity through DPP-IV inhibition (Neves et al., 2017).

Moreover, peptides hydrolysates were extracted by other authors fromskin gelatin of seabass (*Lates calcarifer*) (Sae-leaw et al., 2016) and unicorn leatherjacket (Karnjanapratum, O'Callaghan, Benjakul, & O'Brien, 2016) having both immunomodulatory and antiproliferative activties.

Moreover, Gu et al. (2011) isolated 11 peptides from salmon skin collagen after enzymatic hydrolysis, which showed an important ACE inhibitory activity and might be functional as useful foods and antihy- pertensive agents. In another study, Alaska Pollock collagen skin was used to generate iron-chelating peptides after being treated by commercial enzymes and one tripeptide contained amino acid sequence with highiron-chelating activity was detected (Guo et al., 2013).

2.1.2 Lipids

Fish oils containing omega-3 PUFAs and providing a myriad of health benefits have been produced from fish by-products (Soldo et al., 2019). They diminish the likelihood of vascular disease, cancer, diabetes and depression (Ivanovs & Blumberga, 2017). They also affect the immune system and ensure a proper neural development (Ivanovs & Blumberga, 2017). One of the main sources of omega-3 PUFAs (DHA and EPA) is fatty fish such as herring, sardine, salmon, and mackerel (Hamed et al., 2015; Kundam et al., 2019). The quantity and composition of these oils are highly dependent on the species, season and location of catching sites(Hamed et al., 2015).

In the whole, fish fatty acids (FA) are found in the subcutaneous tis- sue, viscera, muscle tissue, liver, mesenteric tissue, and head. Consideringfish by-products, FA can be obtained mainly in the fish, skin, gut, head and bone from different fish species. For example, extraction of PUFAs was obtained from the bones of cod, blue whiting, salmon, trout, herring,mackerel and horse mackerel (Toppe, Albrektsen, Hope, & Aksnes, 2007)and from the viscera of tilapia *(Oreochromis niloticus*) (Shirahigue et al.,2016) and common carp (*Cyprinus carpio* L.) (Lisichkov, Kuvendziev, Zeković, & Marinkovski, 2014). Also, PUFA (omega-3) was extracted from mackerel skin (Sahena et al., 2010).

In one study, lipids from Australian sardine (*Sardinops sagax*) viscera and salmon (*Salmo salar*) head were extracted and large amounts of omega-3 PUFAs, EPA, and DHA were found (Ahmad et al., 2019). In addition, it is well known that fish oils are a rich source of vitamins (A and D). Vitamin A is concentrated mostly in fish liver oils. Halibut, sardine, and cod contain vitamin A and D in their liver (Kundam et al., 2019), while herring, mackerel, trout and salmon have vitamin D in their tissues, also

yellow tuna contain vitamin D in its bone (Talib & Zailani, 2017). These vitamins are commonly included in dietary supplements for several applications, such as bone health or antioxidant formulations (Harris, Morrow, Titgemeier, & Goldberg, 2017).

2.1.3. Minerals

Fishbones generate a huge amount of minerals. Inorganic minerals con- stitute approximately 60% of fish bones. Thus, fish bones are an important source of hydroxyapatite, calcium, phosphate, zinc, selenium, and iron (Bruno, Ekorong, Karkal, Cathrine, & Kudre, 2019). Minerals were isolated from various fish species. Seabass (*Lates calcarifer*) bone was a source of calcium and phosphor (Pal et al., 2017). Also, calcium, phosphor, magnesium, and strontium were isolated from the scale of *Catla catla* fish (Paul, Pal, Roy, & Bodhak, 2017). These minerals are important compounds in nutraceutical formulations destinated to improve health, mainly bone health but also cardiovascular or immunological diseases (Webb, 2015).

3. Shellfish by-products

After industrial processing, 75% of shellfish weight ends up as by-products (Hamed, Özogul, & Regenstein, 2016). These by-products are currently disposed of by incineration or returning them to the environment, which might lead to health and environmental concerns. So, it isa real challenge both industrially and ecologically the processing of this waste (Yadav et al., 2019). From these by-products, compounds with dif-ferent biological properties of great interest can be obtained; as chitin/ chitosan and its derivatives, carotenoids, glycosaminoglycans (GAG) or bioactive peptides, among others (**Figure 2**). Therefore, a good approach forthe utilization of this waste would reduce its environmental impact while revaluing.

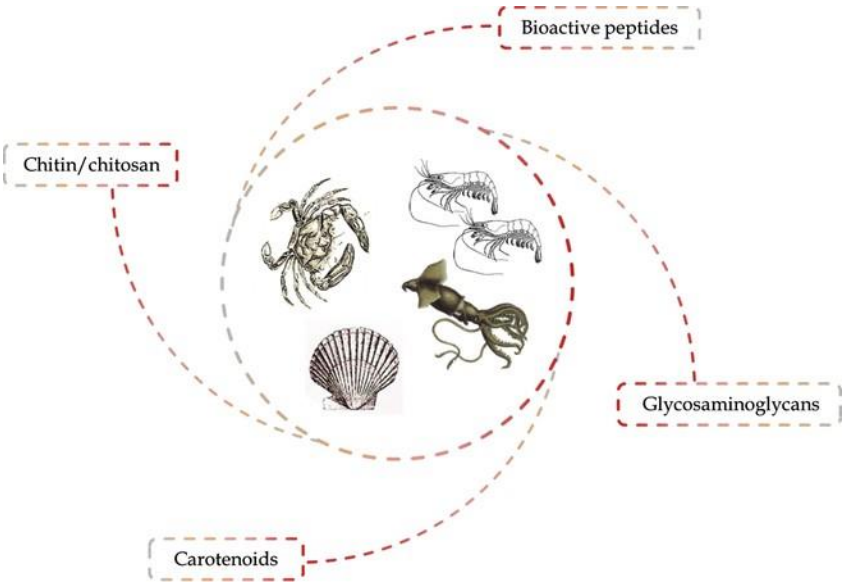

Figure 2: Shellfish and main bioactive compounds obtained from them.

3.1 Chitin/chitosan

Chitin is a biopolymer that can be obtained from shellfish waste, mainly from the exoskeletons of crustaceans. The shell of crustaceans is com- posed of 13–42% chitin, in addition to having a content of 30–50%mineral salts and 30–40% protein (Vo & Kim, 2014). It is the secondmost abundant polysaccharide in the world, after cellulose (Hamed etal., 2016). Both chitin and derivatives have great importance in biomedi cine as they are biocompatible and non-potential toxic compounds. They are also renewable and biodegradable so their environmental impact is low.

Since chitin is a water-insoluble compound, its transformation into chitosan is often chosen. To obtain chitosan, a chemical or enzymatic process of deacetylation is followed. Chitosan is the name given to chitinwhen an acetyl group is removed, and different degrees of deacetyla-tion can be achieved (Menon & Lele, 2015a, 2015b). When 50% of the acetylated form is exceeded with respect to the non-deacetylated form thecompound becomes soluble in an acid solution (Shavandi, Hou, Carne, McConnell, & Bekhit, 2019). To be suitable for use, at least 70% deacety-lation of chitosan is required (Menon & Lele, 2015a, 2015b).

Both chitin and chitosan have applications in the biomedical field. Among other uses, they are intended for the production of drugs, asit can be used to control their bioavailability (Nivethaa, Martin, Frank-Kamenetskaya, & Kalkura, 2020). It also has great applications in tissue engineering and wound healing (as a biomaterial) (De Masi et al., 2019; Jangid, Hada, & Rathore, 2019).

However, it is not only used as a carrier or excipient, but also forits own bioactivity. Among the most outstanding biological activities of chitin and its derivatives, it also has antitumor, antimicrobial, antioxidant dant, anticoagulant and even antifungal activity. The antitumor activity of these compounds has been demonstrated both in vivo and in vitro, mainly as consequence of a direct action of chitosan on tumor cells (Simonaitiene, Brink, Sipailiene, & Leskauskaite, 2015), by increasing the production of natural killer (Chatterjee, Chatterjee, & Guha, 2014; Lopez-Moya et al., 2015), or by inhibiting the angiogenesis of tumor cellsand suppressing the tumor (Li et al., 2019). Its antimicrobial activity has also been demonstrated, and the mechanism of action depends entirely on the molecular weight of the polysaccharide. If the polysaccharide has a high molecular weight, it can bind to the bacterial cell wall and interfere with the ion exchange of the cell (Rahaiee, Shojaosadati, Hashemi, Moini, & Razavi, 2015; Salis et al., 2015). In contrast, polysaccharides with a small molecular weight penetrate the bacteria and interfere with the processes of DNA transcription and mRNA synthesis (Lindborg etal., 2015). It has been proven that the antimicrobial activity of chitosan ismore intense in Gram-negative bacteria (Zeng et al., 2014).

Another remarkable biological activity of chitosan is its anticoagulant capacity. It has been reported that it has a slightly lower anticoagulant capacity than heparin, suggesting an alternative use (Arasukumar, Prabakaran, Gunalan, & Moovendhan, 2019; Yang et al., 2013).

On the other hand, chitosan displayed antioxidant properties. The antioxidant capacity depends on both its molecular weight and the degreeof acetylation, since they also depend on the molecular weight, being more active a lower molecular weight and a higher degree of deacetylation(Anraku et al., 2018). The mechanism by which this is explained could berelated to the chemical structure of the

chitosan molecule, more specifically to the amino group present in C2 and the hydroxyl group present in C6 (Park, Koppula, & Kim, 2010). Due to its antioxidant capacity, chi- tosan has been proven effective in diseases in which oxidative stress hasa great implication, such as metabolic syndrome or chronic renal failure (Anraku et al., 2018).

Finally, it has also been shown that chitin/chitosan has an antifungal activity that varies according to the fungus and plants that it contami nates depending on molecular weight and degree of acetylation (Verlee, Mincke, & Stevens, 2017). This could have great potential in the field of agriculture.

3.2 Carotenoids:

Carotenoids are lipophilic compounds responsible for yellow and red colors in nature, both in plants and animals (Wade, Gabaudan, & Glencross,2017). They can be divided into two groups: in the first one, the compounds are only composed of C and H atoms (e.g., carotene and xanthophylls), while in the second group the compounds have at least one func- tional group with O atoms (e.g., astaxanthin and lutein) (Shavandi et al., 2019). Specifically, the carotenoid responsible for the pink pigmentation of crustaceans is astaxanthin, so this carotenoid can be recovered from its by-products (Zhao et al., 2019).

For its extraction, the process consists of a deproteinization and demineralization of the sample followed by carotenoid extraction through the ap- plication of organic solvents (Shavandi et al., 2019). As for the biological activity of astaxanthin, its high antioxidant potential can be highlighted which is 500 times higher than that of vitamin E (Mao, Guo, Sun, & Xue, 2017), which makes it the largest natural antioxidant in the world. This high antioxidant power is due to the high presenceof double bonds in its structure (Zhao et al., 2019). Thanks to its high antioxidant capacity, astaxanthin has different biological activities, such as antitumor activity, anti-inflammatory activity, prevention of cardiovascular diseases and atherosclerosis, liver protection and protection of the ner-vous system against diseases with a high component of oxidative stress (Amengual, 2019; Atalay, Kuku, & Tuna,

2019; Dutta, Mahalanobish, Saha, Ghosh, & Sil, 2019; Fakhri, Abbaszadeh, Dargahi, & Jorjani, 2018;Ni et al., 2015; Prameela et al., 2017).

3.3 Glycosaminoglycans (GAGs)

The GAGs are polysaccharides composed of repetitions of disaccharides linked by an oxygen atom. These disaccharides are generally formedby a unit of uronic acid and a unit of an amino sugar (Valcarcel, Novoa-Carballal, Pérez-Martín, Reis, & Vázquez, 2017). Within the GAG we find chondroitin sulfate, dermatan sulfate or heparin sulfate among others. These GAGs are part of the connective tissue, forming the extracellular matrix together with collagen and other structural molecules (Menon & Lele, 2015a, 2015b).

These molecules have various biological activities. One of the most important and also best known is anticoagulant activity. In this sense, several studies have obtained heparin from shellfish by-products, specifically from shrimp heads. However, its effectiveness as an anticoag-ulant is less than heparin from mammals (Brito et al., 2014; Chavante et al., 2014).

On the other hand, its anti-inflammatory activity is also remarkable. This property is also related to heparins. In fact, it has been seen that he- parin obtained from shrimp has anti-inflammatory activity, reducing the activity of metalloproteinase 9, an enzyme involved in the inflammatory response (Brito et al., 2008).

Another widespread use of GAGs is their use in the field of regenerative medicine. The GAGs can bind to proteins and form proteoglycans. These proteoglycans can capture growth factors, which have great rele- vance in the process of differentiation and cellular function (Place, Evans,& Stevens, 2009). Therefore, this makes GAGs especially suitable for tissue regeneration. It was reported that hyaluronic acid and chondroitin sulfate are among the most important GAGs used in regenerative medicine (Salbach et al., 2012).

In addition to these very relevant applications, their use in other diseases, such as cancer, has been explored. In this sense, some studies haveshown in vitro how sulfated GAGs, mainly dermatan

sulfate and heparansulfate, obtained from Norway lobster, have antiproliferative activity in human colon tumor cells (Sayari et al., 2016). This capacity can be explained by the high presence of sulfur in its composition.

Finally, they also have antiviral properties. Glycosaminoglycans ob- tained from squid have demonstrated their antiviral activity against viruses such as herpes simplex virus, T-cell leukemia virus or dengue (Valcarcel et al., 2017). It has also been seen that heparin sulfate groups can inhibit the human immunodeficiency virus (HIV) by electrostatic interaction with basic amino acids (Chen & Huang, 2018).

3.4 Bioactive peptides

Several articles have described different biological activities of peptides obtained from the by-products of shellfish. Bioactive peptides are amino acid sequences that are inactive when they are included in a protein but active when are released. They contain between 2 and 20 amino acids, and their bioactivity is based on the amino acid sequence and its length (Lorenzo et al., 2018). Specifically, peptides with antihypertensives (inhibiting angiotensin-converting enzyme), antioxidants, and antimicro bials have been obtained from shellfish by-products (Menon & Lele, 2015a, 2015b).

The antioxidant activity has been related to the presence of amino acids such as histidine, tyrosine, methionine, and cysteine in the peptide sequence. It is also related to other hydrophobic amino acids suchas hydroxyproline, leucine, alanine, proline, glycine, valine and repetitive glycine-proline sequences (Neves, Harnedy, & FitzGerald, 2016). Suárez-Jiménez et al. (2019) have obtained peptides with antioxidant activity from hydrolysates of squid by-products. In this study, we observe that the peptides with the highest activity are those with a smaller size. In addition, these authors obtained peptides with antiproliferative activityand related the mechanism of action with the ability of these peptides to act directly on tumor cells and their cytotoxic effect.

On the other hand, antihypertensive activity is related to very short peptide sequences (less than nine amino acids), in whose sequence are theamino acids glycine, tyrosine, valine, phenylalanine,

isoleucine, arginine or asparagine (Amado, González, Murado, & Vázquez, 2016; Neves et al., 2016). In this sense, Apostolidis, Karayannakidis, and Lee (2016) obtained peptides with antihypertensive activity from hydrolysates of squid by-products. This observation indicates that the most active peptides are those with a lower molecular weight.

The ability of some peptides to suppress appetite has also been de-scribed, and this is due to the structural similarity of these molecules withgastrin or cholecystokinin (Neves et al., 2016). Cudennec, Ravallec-Plé,Courois, and Fouchereau-Peron (2008) obtained peptides that stimulate cholecystokinin release in vitro from brown shrimp protein hydrolysates.

Peptides with antimicrobial activity are related to the presence of positively charged residues (Menon & Lele, 2015a, 2015b). Antimicrobial peptides obtained from shrimps and prawns have in their structure ahigh presence of proline, arginine and glycine residues (Hayes & Flower,2013). Jiang et al. (2018) and Jiang, Liu, Yang, and Hu (2018) obtained antibacterial peptides from crab shells and squid by-products. In addition,several authors have obtained antimicrobial peptides from shrimp, scallop, abalone, and oyster (Harnedy & Fitzgerald, 2013; Hayes & Flower,2013).

4 Marine algae (Macro and Micro)

Marine algae are a diverse group of photosynthetic organisms fromaquatic environments. They are usually classified as macro- and microalgae. Macroalgae or seaweeds are multicellular organisms that can be divided into brown algae (Phaeophyta), red algae (Rhodophyta) and green algae (Chlorophyta), while microalgae are unicellular organisms constituted by prokaryotic green-blue algae (cyanobacteria) and eukaryotic microalgae (microalgae) (Martínez-Francés & Escudero-Oñate, 2018). The chemical composition of marine algae depends on the species, habitat, and environmental conditions**. Figure 3** shows some compounds obtained from macro- and microalgae with nutritional value. From a nutritional point of view, edible seaweeds are rich in minerals and vitamins, be-ing recognized as an ideal food source of iodine as well as one of the few vegetable sources of vitamin B_{12} (Chandini, Ponesakki, Suresh, & Bhaskar, 2008). For centuries, different species of

seaweeds such as *Ulva* (Chlorophyta), *Porphyra* (Rhodophyta), *Undaria*, *Laminaria*, *Himanthalia* and *Saccharina* (Phaeophyceae) have been harvested for human consumption, especially in coastal areas of the Asiatic continent.In the same way, the microalgae known as *Spirulina* have been consumed in Central America and Africa regions (Pereira & Carvalho, 2014). Later, the interest was in applying edible algae as food ingredients to improve the quality of different food products.

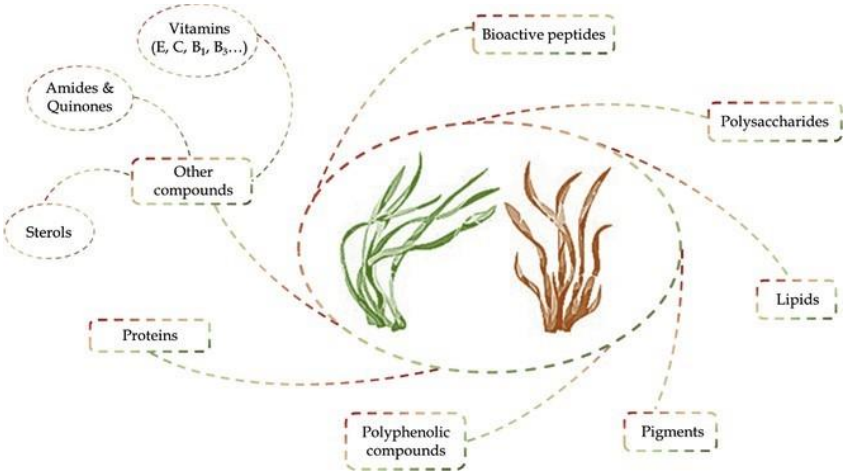

Figure 3: Main proteins and bioactive compounds obtained from macro- and microalgae.

For example, sea spaghetti (*Himanthalia elongata*), nori (*Porphyra umbilicalis*) and wakame (*Undaria pinnatifida*) increased the content of sulfur amino acids and minerals in meat products, while *Ulva lactuca* and *Laminaria algae* were added to bread processing (Ścieszka & Klewicka, 2018). In contrast, European countries focused the interest on the gelling properties of algal polysaccharides and therefore, carrageenan, agar and alginates have beenused as additive agents in the food industry (Pereira & Carvalho, 2014). In addition to the nutritional and industrial characteristics, the most recent scientific knowledge about their composition and biological activitieshave placed marine algae as a promising source of proteins and bioactivecompounds. As a result, the high demand for both macro- and microalgae worldwide, sustainably led their production through aquaculture techniques.

Current aquaculture is the main source of edible aquatic plants, accounting for 96% of production in 2016. The volume of global farmed algae has increased by about 55% in the last two decades. Of

the total mil- lion tons of algae from aquaculture in 2016, seaweeds represented 30 million tons while only 89,000 tons were recorded for microalgae (although this last value is understated because of unavailable data from impor- tant producers and farmed algae for scientific purposes are not included) (FAO, 2018a). There are more than 200 commercialized macroalgae, butonly 10 species are intensively cultivated: *Saccharina japonica*, *Undaria pinnatifida* and *Sargassum fusiforme* for brown seaweed; *Porphyra* spp., *Eucheuma* spp., *Kappaphycus alvarezii* and *Gracilaria* spp. for red seaweed; and *Enteromorpha clathrata*, *Monostroma nitidum*, and *Caulerpa* spp. for green seaweed (FAO, 2018b). Regarding farmed microalgae, themain cultivated species are *Spirulina* spp., *Chlorella* spp., *Haematococcus pluvialis*, and *Nannochloropsis* spp. (FAO, 2018a).

4.1. Nutritional value

4.1.1. Proteins

From the nutritional point of view, algae are an interesting alternative source of proteins. Protein content in algae varies depending on thespecies, habitat and seasonal period. Microalgae present higher protein concentration than macroalgae. In general, protein fraction (dry weight) of green and red seaweed ranged from 10% to 47% while the protein percentage of brown seaweed is less than 15% (except for wakame (*Undariapinnatifida*) whose protein level is 11–24%) (Fleurence, 1999; Herrero et al., 2012). Microalgae can contain more than 60% protein. The cyanobac-teria *Spirulina platensis* present a protein composition of 43–63% so it is considered a food supplement (Villarruel-López, Ascencio, & Nunõ, 2017). In addition, the essential amino acid profile of algae meets the requirements of the Food and Agriculture Organization of the United Nations (FAO). In this sense, the essential amino acid content of themost common microalgae and cyanobacteria has been reviewed by Barba,Grimi, and Vorobiev (2014). However, in vitro bioaccessibility studies suggest that unprocessed seaweed proteins have reduced digestibility compared to that of other protein sources (Villarruel-López et al., 2017).

On the other hand, other non-nutritional protein compounds such as enzymes produced by algae and peptides derived from their proteinshave also been considered as bioactive compounds. For example, researchers revealed that various types of enzymes, such as mannuronan C5 epimerase, can be produced by algae (Parte, Sirisha, & D'Souza, 2017). Many of the algal producing enzymes are known to be important for the food and pharmaceutical industries (Inoue et al., 2016; Levy-Ontman, Fisher, Shotland, Tekoah, & Malis Arad, 2015). For instance, enzymesof *Closterium*, *Cylindrotheca*, and *Chaetoceros muelleri* are reported to be effective in diethyl phthalate degradation (Gao & Chi, 2015). Besides,glutathione peroxidase, ascorbate peroxidase, and catalase can be pro- duced by algal (Babu et al., 2014; Moenne, González, & Sáez, 2016). Also, alginate can be produced from the brown algae wherein a symbiotic association between bacteria and seaweeds results in the production of alginate lyases (Ertesvåg, 2015). Moreover, a variety of bioactive pep-tides have been produced by enzymatic hydrolysis of the proteins of algal (Beaulieu, 2019). In this context, an investigation showed that the health state of bread can be enhanced by the incorporation of an algae renin in- hibitory dulse protein hydrolysates (Fitzgerald et al., 2014). Such studies indicate that bioactive peptides obtained from algae can be consideredvaluable ingredients that can be used for food production.

4.1.2. Polysaccharides

Marine algae contain mucopolysaccharides, and storage and cellwall-structured polysaccharides. Some seaweed species contain polysaccharides in a range from 4% to 76% (dry weight), with the highestlevels found in species such as *Ascophyllum*, *Palmaria*, *Porphyra* and *Ulva* (Usman, Khalid, Usman, Hussain, & Wang, 2017). Both, the cell wall structure and storage polysaccharides, are species-specific. Green algae contain sulfuric acid polysaccharides, sulfated galactans, and xy- lans. Also, the brown algae presents alginic acid, fucoidan, laminarin, and sargassan. Besides, the red algae contain agars, carrageenans, xylans, floridean starch, water-soluble sulfated galactan, as well as the mu- copolysaccharide porphyrin (Chandini et al., 2008; Kraan, 2012). As polysaccharides do not

participate in the nutritional value of algae, they are considered as a source of dietary fiber resistant to enzymatic hydrolysis of the intestinal microflora of the human digestive tract.

The dietary fibers included in marine algae are divided into insoluble (cellulose, mannans and xylene) and water-soluble (agars, alginic acid, furonan, laminaran, and porphyrin) dietary fibers (Kraan, 2012). The diverse chemical composition of dietary fiber polysaccharides has been considered responsible for their possible biological activities. In this sense, sulfated polysaccharides have shown many health benefits as anticoagulant, antioxidant, antiproliferative, antitumoral, anti-inflammatory, antiviral, and cholesterol lowering agents (Mišurcová, Orsavová, & Ambrožová, 2015).

Fucoidan, in particular, has been shown to exhibit antiviral and anti-in-flammatory properties as well as anti-metastatic effects in metastasized invasive human lung cancer cells (Khalid, Abbas, Saeed, Bader-Ul-Ain, & Ansar Rasul Suleria, 2018). In addition, a recent review concluded that fucoidan, laminarin sulfate, and carrageenan directly slowed the progres- sion of the atherosclerotic lesion while alginate, ulvan (sea lettuce), and agar reducing the accompanying risk factors (Patil et al., 2018).

4.1.3. Lipids

The lipid content from marine algae differs between macro- and microalgae. While seaweeds usually present a low percentage of lipids (1–3%of the dry weight), many microalgae contain 20–50% of lipids (dry bio-mass) and even values ranging from 1% to 70% have also been re ported (Barkia, Saari, & Manning, 2019). However, the lipid profile of both types of algae has raised considerable interest in recent years due to the high content of PUFAs. Typical PUFAs from seaweeds are α-linolenic (18:3n-3), octadecatetraenoic (18:4n-3), arachidonic (20:4n-6), and eicos-apentaenoic (20:5n-3) acids (Kendel et al., 2015). On the other hand, the complete lipid profile (saturated, monounsaturated and PUFAs) from the most common used microalgae and cyanobacteria have been shown by researchers (Barba et al., 2014). In addition to playing an

important role in the prevention of cardiovascular diseases, osteoarthritis, and diabetes, these PUFAs possess antimicrobial, antiviral, anti-inflammatory and anti-tumoral properties (Kendel et al., 2015). PUFAs and glycolipids obtainedfrom *U. armoricana* and *S. chordalis* have been shown to have promising antitumor activities (Kendel et al., 2015). As current unbalanced di- ets do not provide sufficient amounts of omega-3 PUFAs to satisfy human physiological requirements, marine algae are one the new alternative sources for helping to support healthy diets for people (Tocher, Betancor,Sprague, Olsen, & Napier, 2019).

4.1.4. Vitamins

In general, marine algae contain both water and fat soluble vitamins. Apart from the considerable vitamin functions for the body, vitamin E (α-tocopherol), vitamin C (ascorbic acid), and partially vitamin B1 and niacin have been considered responsible for the algal antioxidant activity (Škrovánková, 2011).

4.2. Bioactive and antioxidant compounds

Seaweed pigments are chlorophylls and carotenoids such as carotenes (β-carotene) and xanthophylls (fucoxanthin, violaxanthin, antheraxanthin,zeaxanthin, lutein, neoxanthin, among others) (Aryee, Agyei, & Akanbi, 2018). The most studied algae for natural carotenoids include brown sea-weed (*Laminaria* spp. and *Undaria pinnatifida*), red seaweed (*Corallina elungata* and *Jania rubens*), and green microalgae (*Dunaliella salina*, *Chlorella* spp., *Haematococcus pluvialis*, and *Spirulina* spp.) (Christaki, Bonos, Giannenasa, & Florou-Paneria, 2013). Main algal carotenoids are astaxanthin, fucoxanthin, β-carotene, lutein and zeaxanthin. The antioxidant capacity of astaxanthin, the major carotenoid found in the unicellular green algae *Haematococcus pluvialis*, has been reported to be about 10 times greater than β-carotene, lutein, zeaxanthin, canthaxanthin and over 500 greater than that of α-tocopherol. In addition, the in vitro and in vivo studies have shown the effectiveness of astaxanthin against coronary, chronic inflammatory, diabetes, gastrointestinal, liver and neu-

rodegenerative diseases as well as against atherosclerosis, ischaemic brain development and metabolic syndrome (Christaki et al., 2013).

Moreover, the β-carotene produced from the halophilic microalgae *Dunaliella salina* inhibited neoplastic cells and reduced fibrosarcoma in Wistar rats (Villarruel-López et al., 2017). Pigments from macroalgae have also shown health benefits. Fucoxanthin, the most important bioac- tive carotenoid in the chloroplasts of brown seaweeds such as *Ascophyllum nodosum* and *Laminaria* spp. has been reported for showing antiproliferative effects on prostate and human colon cancer cells, efficacy in thetreatment of obesity and type 2 diabetes as well as anti-inflammatory andantioxidant properties (Christaki et al., 2013; Herrero et al., 2012). In addition, complex compounds constituted by protein-bound pigments that exhibit bioactivity have also been found. For example, phycobiliproteins,only present in red algae (phycoerythrin) and blue-green algae (phycocyanin), are characterized by containing the phycobilin pigment in their structure and this pigment has been related to hepatoprotective, anti-inflammatory and antioxidant properties of phycobiliproteins (Herrero et al., 2012).

Polyphenols are plant secondary metabolites whose structures vary from simple molecules to highly polymerized compounds. As aquatic plants, macro- and microalgae are the main marine sources of polyphenolic compounds. Green and red algae contain bromophenols, phenolic acids, and flavonoids while only in brown algae have been found phlorotannins (Gómez-Guzmán, Rodríguez-Nogales, Algieri, & Gálvez, 2018).

In general, these phytochemicals have been considered bioactive compounds with potential health benefits in numerous human diseases due totheir antioxidant activity as well as enzyme inhibitory effect and antimicrobial, antiviral, anticancer, antidiabetic, antiallergic and anti-inflammatory activities (Gómez-Guzmán et al., 2018).

Phlorotanin, in particular, has been associated with anti-HIV, anticancer, bactericidal, radioprotective, antiallergic, and other health beneficial biological activities shown by *Ecklonia cava*,

Ecklonia stolonifera, *Ecklonia kurome*, *Eisenia bicyclis*, *Ishige okamurae*, *Sargassum thun- bergii*, *Hizikia fusiformis*, *Undaria pinnatifida*, and *Laminaria japonica* (Freile-Pelegrín & Robledo, 2013; Khalid et al., 2018). Regarding microalgae, there is limited information about specific phenolic compoundsin microalgae and the activity they provide. Jerez-Martel et al. (2017) identified and quantified the six widely distributed phenols in nature (gallic acid, (+) catechin, (−) epicatechin, syringic acid, protocatechuic acid, and chlorogenic acid) in crude extracts from several cyanobacteria and microalgae. They determined their antioxidant activity and observed a direct relation between the phenolic compounds and the activity tested for some strains (particularly, *Euglena cantabrica).*

Sterols are another interesting group of compounds extracted from marine algae. Not only sterols but also some of their derivatives have shown anticholesterol, anti-inflammatory and anticancer properties (Ibañez et al.,2012; Michalak & Chojnacka, 2015). On the other hand, as in bacteria and plants, glycolipids such as mono- and digalactosyl diacylglycerol as well as sulfoquinovosylacylglycerol are present in marine algae and they couldhave an important role in inflammatory diseases (Talero et al., 2015). Finally, several secondary metabolites (quinone-based natural products, small amides, and hierridin B) produced by different cyanobacteria have exhibited cytotoxicity toward HT-29 colon cancer cells (Olsen, Toppe, &Karunasagar, 2014; Talero et al., 2015).

5. Conclusion

Aquaculture provides a new source of high-quality food for the growing population. Besides, marine by-products offer many beneficial capabilities that make them valuable materials for the food and phar- maceutical industry. The nutritional value and bioactivity of these compounds and their derivates enlarge the scope of their applications. Researchers pointed out the potential value-added products that can be produced from aquatic waste. These include nutrients (i.e., high-quality oils and proteins, polysaccharides, etc.) and bioactive compounds such as bioactive peptides, polyphenols, among others. These findings might be later appreciated by the industry, resulting in developing commercial valorization techniques for processing the aquatic processing waste.

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4.3 RECENT ADVANCES IN THE APPLICATION OF INNOVATIVE FOOD PROCESSING TECHNOLOGIES FOR MYCOTOXINS AND PESTICIDE REDUCTION IN FOODS

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Recent advances in the application of innovative food processing technologies for mycotoxins and pesticide reduction in foods.

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Abstract

Background: Agricultural products are a vital component of the human diet. However, these products can be contaminated by health-threatening pesticides and mycotoxins due to improper farming and storage practices. Besides, pesticide pollution can be also regarded as an environmental pollution and pesticide reduction is among the Sustainable Development Goals (SDGs). While these hazardous chemicals are stable during several traditional food processing, innovative food processing technologies, including high-pressure processing (HPP), pulsed electric fields (PEF), cold plasma (CP), supercritical carbon dioxide (SC-CO_2), and ultrasound (USN) processing, have been found to have good potential for mycotoxin and pesticide reduction. However, the extent that each of these technologies can degrade pesticides and mycotoxins, as well as the mechanisms involved, is not well-discussed in the literature.

Scope and approach: The present study aims to provide a narrative review of recent findings in pesticide and mycotoxin removal through HPP, PEF, CP, SC-CO_2, and USN processing. In this regard, the data published in the literature were retrieved and the efficiency of these emerging technologies in pesticide and mycotoxin removal was evaluated.

Key findings and conclusion: Innovative technologies can prevent mycotoxin formation and can cause mycotoxins and pesticide reduction in foods. Besides, different innovative processing technologies have different efficiency in removing pesticides and mycotoxins and pesticide pollution, depending on processing parameters, the type of pesticide/mycotoxin, and the food matrix. Therefore, some reports showed promising results (e.g. 100% removal of deoxynivalenol and zearalenone toxins by HPP) but some others showed only a limited amount of target hazardous material can be removed by emerging technologies (e.g. maximum degradation of dimethoate was 35% after PEF treatment).

Keywords: Innovative food processing technologies; Pesticide pollution; Pesticide Reduction; Mycotoxin; Cold plasma; Pulsed electric fields; Ultrasound; Supercritical carbon dioxide.

1. Introduction

Nowadays, there is a trend among food consumers for healthy and convenient foods that are free from agrochemical and toxins. Regardless of the progress in organic farming, unfortunately, recent studies pointed out that several foods in many regions of the world are contaminated by pesticides and mycotoxins (Campagnollo et al., 2016; Mousavi Khaneghah et al., 2019; Nabizadeh et al., 2018). The main reasons for the high rate of contamination are raw materials such as polluted fruit, vegetables, and cereals (Bhat, 2008; Gomiero, 2018; González, Marquès, Nadal, & Domingo, 2019). Many of these products are will be polluted before being received by the food factories either in the farm (e.g. by misapplication of pesticides) or during inappropriate transportation and storage (e.g. by the growth of mycotoxin forming fungi) (Danezis, Anagnostopoulos, Liapis, & Koup-paris, 2016; Narenderan, Meyyanathan, & Babu, 2020; Stoev, 2013). It should be mentioned that pesticide reduction is among the Sustainable Development Goals (SDGs) to ensure sustainable consumption and production patterns. Therefore, the food processing industry is looking for technologies that can remove such hazardous chemicals from the food materials. Due to such a demand, researchers around the world explored the applicability of innovative processing technologies for reducing the level of pesticides and mycotoxins in food products (Bhil-wadikar, Pounraj, Manivannan, Rastogi, & Negi, 2019; Gonçalves, Coppa, de Neeff, Corassin, & Oliveira, 2019; Ioi, Zhou, Tsao, & Marcone, 2017; Pankaj, Shi, & Keener, 2018). Considering the needs of the industry and scientific society for an updated list of the innovative technologies that can remove both pesticides and mycotoxins along with their potential benefits and limitations, the present review was carried out. Therefore, the research aims to provide recent advances in pesticide and mycotoxin removal by the applications of innovative food processing technologies are discussed (**Figure. 1 and 2**).

2. High pressure processing

Traditionally, high-pressure processing (HPP) has been used for food preservation purposes. This technology is mainly based on the 3-D concept regarding the application of pressure, temperature

and time to the food matrix, thus inducing microbial inactivation and structural modifications in some specific food components (Barba, Terefe, Buckow, Knorr, & Orlien, 2015; Barba, Koubaa, do Prado-Silva, Orlien, & Sant'Ana, 2017; Kultur, Misra, Barba, Koubaa, & Alpas, 2017). For that purpose, it has been documented as a useful tool for removing pesticides and mycotoxins. Although some studies documented significant decreases in the concentration of pesticide after HPP of foods, some researchers hypothesized that such observations are because of the physical transportation of toxins, not their chemical degradation. They claimed that HPP may simply result in the transfer of pesticides from the outer layer to the inner layers of the product. Moreover, there is no report proving that HPP generates toxic intermediates from pesticides and mycotoxins. This can also support the above-mentioned hypothesis. Despite being a controversial technique for removing pesticides, it is generally believed that HPP is a good technique for reducing the concentration of mycotoxins.

a. Pesticide removal by HPP

In one of the early studies, researchers studied the effects of HPP (0.1–400 MPa/5–25 °C/30 min) on the concentration of a common pesticide in vegetables (i.e. tomatoes and Brussels sprouts), that is, chlorpyrifos (Iizuka, Maeda, & Shimizu, 2013; Iizuka & Shimizu, 2014, 2014). They documented that the optimal process conditions for pesticide removal in this specific case were the pressure of 75 MPa and temperature of 5 °C, which allowed the removal of three-quarters of the pesticide in the sample. However, recently, there are not many published papers that recommend pesticide removal by HPP (**Table 1**). Hence, HPP can be suggested mainly for other applications such as non-thermal preservation.

b. Mycotoxin removal by HPP

According to the literature, HPP is an effective tool that can inactivate the spore fungi and delay their growth (**Table 2**). As an example when HPP (600 MPa) was applied combined to an ultrasound treatment (24 kHz/0.33 W/mL) at 75 °C for 30 min on strawberry pureé, it effectively inactivated the ascospores of Byssochlamys nivea, which is a thermal resistant mycotoxins-producing mold

(Evelyn & Silva, 2015). Similarly, researchers found that the application of an HPP (600 MPa) + ultrasound (24 kHz/0.33 W/mL) at 75 °C on apple juice samples significantly inactivated ascospores of Neosartorya fischeri, which is a thermal resistant mycotoxins-producing mold (Evelyn, Kim, & Silva, 2016). Likewise, Evelyn and Silva (2017) explained that HPP (600 MPa) + ultrasound (24 kHz/0.33 W/mL) at 75 °C can inactivate spores of B. nivea and N. fischeri in strawberry pureé and apple juice, respectively. The authors observed 2.7 log and 2 log reductions in the population of B. nivea and N. fischeri spores, respectively. These observations suggested that the type of mold spore and food sample can affect the efficiency of HPP to inactive mycotoxin forming fungi. Moreover, it can be also mentioned that the combination of HPP with ultrasound can enhance its decontamination effects and this is why several studies, such as the above-mentioned papers, explored the combination of HPP + ultrasound.

In 2018, a model to predict the reduction of *F. graminarum* as a function of process pressure, time, and temperature was proposed by Kalagatur et al. (2018). The authors reported that HPP (380 MPa/60 ºC/0.5h) prevented the germination of *F. graminarum* spores when they were suspended in peptone water. An interesting result, that is, 100% reduction in CFU, was reported by these researchers when they applied 500 MPa/45 ºC/20 min to maize. The potential of HPP treatment as a tool to reduce mycotoxin contents in food has been also studied by some researchers.

For instance, Avsaroglu, Bozoglu, Alpas, Largeteau, and Demazeau (2015) explored the possibility of degrading 5, 50 and 100 µg/L PAT in apple juices by HPP and pulsed HPP process. HPP (300–500 MPa) process was applied for 5 min at temperatures of 30–50 ºC for pulsed-HPP, the researchers used the following plans: 2 pulses x 150 s and 6 pulses x 50 s. According to the authors, HPP process, pulsed-HPP at 6 pulses x 50 s, and pulsed-HPP at 2 pulses x 150 s reduced PAT by 0–51%, 0–62%, and 0–45%, respectively.

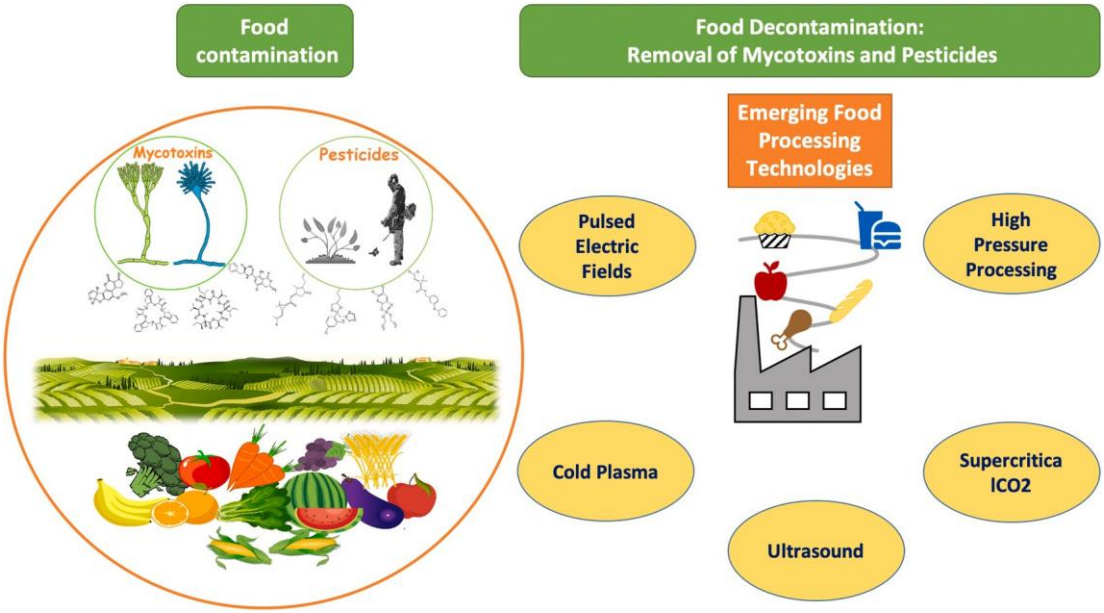

Figure 1: Removal of mycotoxins and pesticides from food using different technologies.

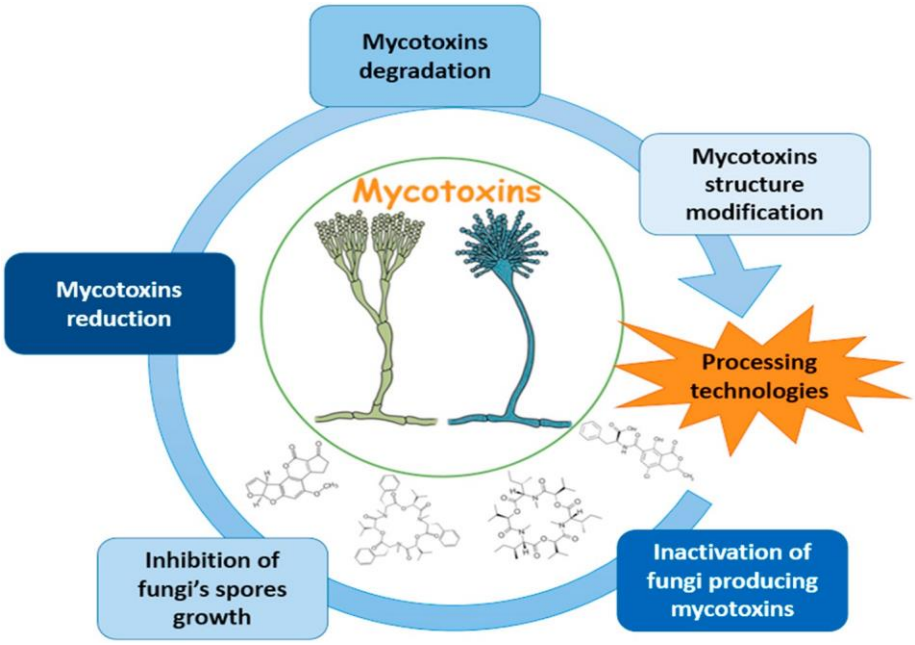

Figure 2: Mechanism of action of processing technologies on mycotoxins.

Table 1: Effect of high pressure processing (HPP) on pesticide removal from food products.

Matrix	Compound	HPP treatment	Main findings	References
Brussels sprouts	Chlorpyrifos	(0.1–400 MPa/5 or 25 ºC/30 min)	≈89% removal under 200 MPa/5 ºC/30 min+ ethanol (10% v/v)	(Iizuka & Shimizu, 2014a)
Cherry tomatoes	Chlorpyrifos	(0.1–400 MPa/5 or 25 ºC/30 min)	≈75% removal under 75 MPa/5 ºC/30 min	(Iizuka et al. 2013)
		(0.1–400 MPa/5 or 25 ºC/30 min) various HPP time periods (0, 0.5, 1, 6, and 12 h)	Increased HPP time decreased the levels of chlorpyrifos. Ethanol helped removing pesticides.	(Iizuka & Shimizu, 2014b)

Hao, Zhou, Koutchma, Wu, and Warriner (2016) investigated the degradation of 200 µg/L PAT in various fruit juices composition by HPP. Then, these juices were subjected to different HPP treatments (400–600 MPa/11 ºC/0–300 s). The authors reported that the most effective treatment was HPP at 600 MPa for 300 s, which reduced the PAT from 200 to 60 µg/L in juice sample.

In another study, Tokus¸oğlu, Alpas, and Bozoğlu (2010) treated olive samples previously spiked with citrinin (CIT) at concentrations of 1, 1.25, 2.5, 10, 25 and 100 µg/kg under HPP (250 MPa/5 min) and observed reduction average percentages of CIT ≈100, 98, 55, 37, 9 and 1.3%, respectively. They also found a reduction (≈90%) of mold flora contaminant.

Huang et al. (2014) applied HPP (600–800 MPa) observing the inhibition of mycotoxigenic fungi *A. flavus* growth in crushed peanuts, and consequently the reduction of AFs accumulation after 30 days of storage period. The values of AFs reported by these authors after treatment were 0.26 µg/g and 0.22 µg/g, respectively. These values were much lower than that of the control sample (9.08 µg/g). In addition, DON and ZEA reduction (almost 100%) was observed in maize samples treated by 550 MPa HPP at 45 ºC for 20 min (Kalagatur et al., 2018).

3. Pulsed electric fields

The previously published reports have explained the feasibility of using pulsed electric fields (PEF) for food treatment, including extraction of high-added value compounds, microbial inactivation, improvement of osmotic dehydration, drying and freezing processes (Barba, Terefe, Buckow, Knorr,

& Orlien, 2015; Gabric et al., 2018; Koubaa et al., 2016; Misra et al., 2018, 2017; Putnik et al., 2018). Specifically, new research revealed the possibility of reducing mycotoxins and pesticides in food by PEF treatments. Besides, it seems that such decontamination processes did not deteriorate the quality parameters of the product. Although it seems that decontamination by PEF wont's deteriorate the quality parameters of food as compared with the traditional processes, such hypothesis needs to be verified at severe process conditions such as very high voltages (e.g. >30 kV/cm). Therefore, optimization processes (e.g. using Response surface methodology (Al-Hilphy et al., 2020), Taguchi-gray analysis (Chung et al., 2020) can be recommended for designing such a process.

a. Mycotoxin removal by PEF

It is generally believed that PEF can degrade mycotoxins (**Table 3**). For example, research showed that *Aspergillus flavus* generated aflatoxins G1 and B1 can be removed by applying PEF (Eisa, Ali, El-Habbaa, Abdel-Reheem, & Abou-El-Ella, 2003). These researchers reported up to 83% reduction in the population of *A. flavus* after one day of PEF processing. They explained that protein and carbohydrates contents of the samples treated by PEF were very similar to those of the control samples (Eisa et al., 2003). Later on, Subramanian, Shanmugam, Ranganathan, Kumar, and Reddy (2017) evaluated the effects of PEF processing in combination with thermal treatment for removing aflatoxin from potato dextrose agar. According to the authors, such combinations can enhance the performance of the PEF process.

One year later, Vijayalakshmi, Nadanasabhapathi, Kumar, and Sunny Kumar (2018) evaluated the effects of a PEF treatment (pulse width of 10–26 μs) on the concentrations of aflatoxin in potato dextrose agar (the pH of 4–10). They reported that sample pH was among the main parameters affecting the efficiency of PEF. These authors also pointed out the value of determination of optimal process conditions for effective PEF-induced removal of mycotoxin (Vijayalakshmi et al., 2018).

b. Pesticide removal by PEF

An overview on the application of PEF for reducing the pesticide concentration in food commodities is presented in **Table 4**. Many years ago, Chen et al. (2009) discovered that 8–20 kV/cm PEF processing with pulse number of 6–26 of apple juice can degrade chlorpyrifos and methamidophos. They also proved that the decontamination effects of PEF differ according to the type of pesticides when they observed that chlorpyrifos was much more sensitive to PEF compared to methamidophos. These researchers also explained that increasing the electrical field can enhance the decontamination effects of PEF due to the rotation and vibration of polar molecules. Three years later, PEF was employed to degrade diazinon and dimethoate of apple juice (Zhang et al., 2012). This study showed a huge degradation of these chemicals after PEF. It also revealed that increasing process time and the strength of electric field can enhance the effectiveness of PEF. According to the authors, the maximum diazinon degradation (48%) and dimethoate degradation (35%) were observed when 20 kV/cm was applied.

Table 2: Effect of high pressure (HPP) on toxin formation in food products.

Matrix	Targeted compound/fungi	HPP treatment	Main findings	References
Apple juice	*Neosartorya fischeri* ascospores	HPP 600 MPa+ ultrasound processing (24 kHz, 0.33 W/mL)+75 ºC	HPP at 75 °C resulted in 3.3 log reduction after 10 min, vs no inactivation after applying either US treatment at 75 °C or thermal treatment (75 °C) alone	Evelyn et al. (2016)
Apple, celery, cucumber, kale, lemon mixture juice parsley, Romaine, spinach, and	Patulin	(400-600 MPa, 0-300 s, 11 ºC)	Up to 60 µg/L decrease after 600 MPa for 300 s.	Hao et al. (2016)
Maize	*Fusarium graminarum* Deoxynivalenol and Zearalenone	(380 MPa/60 °C/30 min) and (500 MPa/45 °C/20 min)	Spore germination inactivation of *F. graminarum* after HPP (380 MPa/60 °C/30 min) in peptone water. Complete reduction in CFU was observed after applying HPP (500 MPa/45 °C/20 min). Complete reduction of DON and ZEA in maize after HPP treatment (550 MPa/45 °C/20 min).	Kalagatur et al. (2018)
Strawberry puree	*Byssochlamys nivea* ascospores	HPP 600 MPa+ ultrasound processing (24 kHz, 0.33 W/mL)+75º C	HPP at 75 °C resulted in 1.4 log reduction after 10 min, vs no inactivation after applying either US treatment at 75 °C or thermal treatment (75 °C) alone	Evelyn & Silva, 2015)
Table olives	*Penicillium* spp. Citrinin	(250 MPa/5 min/35 °C).	≈90% reduction of mold flora. Citrinin reduction from 64 to 100%.	(Tokus¸oğlu et al., 2010)

Later on, the effects of PEF on vinclozolin, pyrimethanil, procymidone, and cyprodinil (four different types of fungicides) were evaluated in wine samples (Delsart et al., 2015). This study showed that PEF can effectively degrade all the studied fungicides. These authors also explained that

the effects of the electrical field strength and applied energy were more profound when compared to that of process duration.

4. Cold plasma

Cold plasma attracted notable attention as an innovative decontamination method (Gavahian, Sheu, Tsai, & Chu, 2020; Gavahian, Chu, & Jo, 2019; Gavahian, Peng, & Chu, 2019). The increasing research introduced plasma as an efficient means for the degradation of mycotoxins (e.g. enniatins, deoxynivalenol, aflatoxins, fumonisin, and zearalenone), and inactivation of dangerous molds that are capable to produce mycotoxin (e.g. Aspergillus, Alternaria, Fusarium, and Penicillium). Such studies verified the feasibility of using non-thermal plasma for decontamination of several food products such as dairy products and cereals which have bad reputations in terms of being contaminated by mycotoxins (Gavahian & Cullen, 2020). Besides, according to the published works, cold plasma can degrade several types of pesticides including paraoxon, parathion, omethoate, malathion, dichlorvos, azoxystrobin, fludioxonil, cyprodinil, cypermethrin, and chlorpyrifos. Such decontaminations happen usually because of plasma-generated reactive species such as reactive oxygen species that can attack the chemical bonds of food pesticide molecules (Gavahian & Khaneghah, 2020).

a. Mycotoxin removal by cold plasma

The role of plasma in mycotoxin elimination can be viewed in two parts. First, cold plasma can inactivate the mycotoxin producing fungi and prevent the generation of mycotoxin in foods. Besides, reactive species that are generated by cold plasma can attack the chemical bounds of mycotoxin molecules, resulting in their degradation or conversion to other products.

Regarding the inactivation of mycotoxin-producing microorganisms, a group of researchers employed an atmospheric pressure cold plasma at a frequency of 25 kHz and a power of 700 W for inactivation of *A. parasiticus* and *A. flavus* spores that were inoculated on hazelnut surface. According

to the authors, 5 min of treatment reduced the *A. parasiticus* and *A. flavus* by 4.5 log and 4.2 log, respectively (Dasan, Mutlu, & Boyaci, 2016).

Table 3: Some mycotoxins in food as affected by pulsed electric fields.

Sample	**Type of toxins**	**PEF conditions**	**Major observation**	**Source**
Model solution	Ricin	(30 kV/cm, 10-300 ns per pulse)	PEF reduced the toxicity of ricin and altered the secondary structure of this compound	Wei et al. (2016)
Potato dextrose agar	Aflatoxin	PEF: Pulse frequency (50 Hz) Burst: 10 Energy: 1kJ Time: 10 s. (They also used thermal process for 10–23.4 min at 110–119 ºC)	Combination of PEF and thermal treatment was found to be an effective approach to degrade aflatoxin	Subramanian et al. (2017)
Model system	Aflatoxins	Voltage percentage: 20–65%, Puls:10–26 µs pH: 4–10	Increased both voltage and pulse width improved the degradation	Vijayalakshmi et al., (2018)

Table 4: Some examples of pesticides from food samples as affected by pulsed electric.

Matrix	Compound	PEF treatment	Main findings	References
Juice of apple	Methamidophos Chlorpyrifos,	40 °C, 8-20 KV/cm 6-26 pulses 60–260 µs	Both pesticides were degraded significantly Chlorprifos was degraded easier than methamidophos. Increasing electric field and time improved the degreadation	Chen et al. (2009)
Juice of apple	Dimethoate, Diazinon	15-23.5 °C 8-20 kV/cm 60-260 µs	Both pesticides were degraded significantly Treatment time and electric field strength had significant effects	(Y. Zhang et al., 2012)
Wine	Cyprodinil, Vinclozolin, Procymidone, Pyrimethanil	5-20 kV/cm 0.5-2 ms 10-160 kJ/L	All the studied pesticides were degraded significantly Increasing PEF strength and energies improved the degradation process.	Delsart et al. (2015)

Similarly, another research team studied the effects of 0–0.5 h of 40–60 W plasma processes on A. flavus and A. parasiticus that were inoculated on the surface of groundnuts. These researchers documented that 24 min of plasma processing at a power of 60 W inactivated A. flavus and A. parasiticus by 99% and 98%, respectively (Devi, Thirumdas, Sarangapani, Deshmukh, & Annapure, 2017). Later on, it was discovered that cold plasma can massively alter the structure of A. flavus hyphae (Šimončicová,et al., 2018).

Regarding mycotoxins degradation, a very first study in the last decade revealed that a 1000 W microwave-argon plasma device can effectively degrade AFB1, DON, and NIV which are among the common mycotoxins in food materials. According to these researchers, only 5 s of the plasma process could eliminate almost all the mycotoxin molecules (Park et al., 2007).

Ten years later, another research team investigated the possibility of removal of the mycotoxins produced by *Fusarium, Aspergillus* and Alternaria. These mycotoxins include T2, DON, ENNs, ZEN, and FB1 (generated by *Fusarium*), sterigmatocystin (generated by *Aspergillus*), and AAL toxin (generated by *Alternaria alternata*). This research team explained that plasma process can affect the mycotoxin structure and the decontamination effects vary depending on the type of mycotoxin. For

example, a faster degradation was observed for FB1 and AAL compared to that of sterigmatocystin. This research team also elaborated on the fact that the effects of cold plasma on pure mycotoxin sample will be different from that of mycotoxin inoculated in a food product such as rice. Indeed, their research confirmed that food matrix may slowdown the plasma-induced degradation of mycotoxins (tenBosch et al., 2017). Another study conducted by Devi et al. (2017) showed that plasma processing of peanut can effectively eliminate aflatoxins B1, B2, G1, and G2. They also explained that the longer plasma processing times and higher powers increased the detoxifying effects of plasma. Their results also confirmed that plasma decontamination effects depend on the type of mycotoxin, that is, various types of mycotoxins have different degrees of resistance against plasma processing. For instance, 15 min of 40 W plasma processing reduced the aflatoxin B1 by 74% while the same processing conditions reduced aflatoxin G1 by 99% (Devi et al., 2017). In another study, Ren et al. (2017) studied the effects of peanut composition (e.g. moisture content and α-tocopherol), on the degradation of AFB1 by 100 s of a 170 V plasma treatment. When they increased the moisture content of the sample by 6%, the degradation rate of AFB1 increased from 62 to 98%. On the other hand, the detoxification effects of plasma decreased when the peanut sample was fortified with α-tocopherol (Ren et al., 2017). In another study in the same year, Shi, Ileleji, Stroshine, Keener, and Jensen (2017) explored the effects plasma treatment time (1–30 min), type of working gas (a selected gas mixture vs. atmospheric air), and relative humidity (5–80%) on aflatoxin degradation in corn samples. They found that using high relative humidity, longer processing times, and using the selected gas mixture (65% O2, 30% CO_2, and 5% N2) can enhance the aflatoxin degradation rate. For example, increasing the processing time from 1 to 10 min, altered the degradation rate of aflatoxin from 62 to 82% when the relative humidity was 40% (Shi et al., 2017).

b. Pesticide removal by cold plasma

The possibility of degradation of various types of pesticides, including parathion, fludioxonil, dichlorvos, paraoxon, azoxystrobin, omethoate, cyprodinil, and pyriproxyfen, has been documented

in the literature (Gavahian & Khaneghah, 2020). In a very first investigation, Kim, Kim, and Kang (2007) confirmed the feasibility of paraoxon and parathion degradation by an atmospheric pressure cold plasma and explained that reactive species that are generated by the plasma system degraded the pesticides through the oxidation process (Kim et al., 2007). Indeed, the effects of plasma-induced reactive species and plasma-induced oxidation should be considered when a sample, such as a pesticide-contaminated food, is treated by cold plasma (Gavahian, Chu, Mousavi Khaneghah, Barba, & Misra, 2018). More recently, a gliding arc discharge plasma was employed by a research team to remove cypermethrin and chlorpyrifos pesticides from the surface of mango (Phan et al., 2018). According to the authors, 5 min of plasma processing degraded 63% of cypermethrin and 74% of chlorpyrifos while enhancing the carotenoid content of the mango. Taking into account the findings of this research team, plasma treatment not only removed the pesticide but also improved the product quality. In another study, Zhou et al. (2018) successfully, i.e., the decontamination rate of 99.6%, eliminated organophosphorus from wolfberry samples by a plasma discharge system (Zhou et al., 2018). They also reported that this process did not affect product quality parameters. Hence, optimization of plasma processing conditions can enhance the decontamination effects of this emerging processing condition (Gavahian et al., 2018; Gavahian, Peng, & Chu, 2019; Gavahian et al., 2020).

5. Supercritical carbon dioxide (SC-CO_2)

According to several published studies in the available literature, SC-CO_2 has been utilized in the field of food processing to isolate not only natural components, but also to eliminate unnatural organic chemical contaminants such as mycotoxins and pesticides. For instance, several studies were carried out to extract and eliminate mycotoxins and pesticides from different types of food. Some of the main findings are listed below.

a. Mycotoxins removal by SC-CO_2

Regarding the potential strategies to reduce the level of mycotoxins in food assisted by SC-CO_2, there are 3 main potential mechanisms of action: first, extraction of mycotoxins to reduce the content in the final food (De Boevre et al., 2018); second, modify the mycotoxin structure; and third, to inactivate the microorganisms producing mycotoxins (Park & Kim, 2013). Concerning the inactivation of microorganisms, SC-CO_2 has been proved to be able to inactivate mycotoxin-forming from food. Researchers studied the inactivation of mycotoxin-forming fungi such as *Penicillium oxalicum* spores (*P. oxalicum*) assisted by SC-CO_2 and using ethanol as a cosolvent. The authors achieved the complete inactivation of *P. oxalicum* spores of 10^7 CFU/mL after applying 10 MPa and 40 ºC, within 45 min (Park & Kim, 2013). Similarly, Park, Lee, Kim, Choi, and Kim (2012) investigated the effects of SC-CO_2 using water on *P. oxalicum* spores inoculated on wheat. Results showed that the inactivation yield of *P. oxalicum* spores increased significantly when the process time, temperature, and the water found in the sample was increased. Particularly, the amount of water was the most important factor for inactivation of spores in wheat grains. The optimal conditions were a temperature of 44 ºC, 233 µL of water, and a treatment time of 11 min which produced a 6.41 log CFU reduction of spores. Thus, with the SC-CO_2 treatment and the utilization of water as co-solvent, high inactivation yields of fungal spores were obtained. However, the use of this method may affect the germination of wheat grains so further studies need to be done (Park et al., 2012). Regarding mycotoxins, Kang, Lee, Kim, Yun, and Chun (2012) analyzed wheat flour samples treated by SC-CO_2. In this regard, the authors treated five kg of flour for 3 h by 4.2 L/h of ethanol and 60 L/h of carbon dioxide at a temperature of 40 ºC. The authors confirmed the absence of aflatoxins in the treated samples compared with non-treated samples in which detected aflatoxins were about 0.6 ppb. This study implies that SC-CO_2 treatment is an efficient tool to eliminate aflatoxins from wheat flour (Kang et al., 2012). In another study conducted by Zougagh, Téllez, Sánchez, Chicharro, & Ríos (2008), the SC-CO_2 method was carried out for the isolation and removal of macrocyclic lactone mycotoxins, such

as ZEN, from maize flour. Several experimental conditions such as CO_2, time of extraction, temperature and flow rate, were optimized. The results showed that the use of SC-CO_2 combined with methanol as a co-solvent allowed 100% recovery for all mycotoxins.

b. Pesticides removal by SC-CO_2

Over the last decades, SC-CO_2 has been applied in food pollutants extraction and analysis, mainly pesticide residues (**Table 5**). For example, Saito-Shida, Nemoto, and Matsuda (2014) reported a method for the extraction of pesticides using SC-CO_2, where the authors extracted 117 pesticides from tomato and cucumber. In another study, Cutillas, Galera, Rajski, and Fernandez-Alba (2018) assessed the application of SC-CO_2 for the extraction of pesticides from food samples. Moreover, Tao et al. (2018) suggested that this approach may be scaled up for the examination of the stereoselective degradation and conversion of fenbuconazole and its chiral metabolites in plant matrices, which decreases the risk of these compounds on both human health and the environment. Recently, Sartori, Higino, Bastos, and Mendes (2017) evaluated the effects of SC-CO_2 (20–50 MPa, 40–80 ºC) on the extraction of 27 types of pesticides that contaminated banana flour. The results showed that the pesticides yield enhanced when the pressure increased. It was reported that the optimal conditions when 50 MPa and 60 ºC were applied. Rissato, Galhiane, Knoll, and Apon (2004) explored the extraction of pyrethroid, organohalogenated, organonitrogenated, and organophosphorus from honey at temperatures of 40–90 ºC and the pressure 20–60 MPa. They reported that the efficiencies ranged between 75 and 94% and the optimal result was obtained when the temperature and pressure were 90 ºC and 40 MPa, respectively. The result of this study demonstrated that the use of SC-CO_2 is fast, accurate and specific for the multi-residue pesticide analyses in honey samples.

6. Ultrasound

Other promising innovative and green processing technology is ultrasound (USN). This technique offers some advantages, it is simple, relatively cheap and energy-saving (Chemat et al., 2020; Majid, Nayik, & Nanda, 2015). It is used in food processing, preservation and extraction processes

maintaining food quality aspects such as texture, color, and nutritional components (Koubaa et al., 2016; Pinela & Ferreira, 2017; Rosello-Soto et al., 2015; Zinoviadou et al., 2015). It has been widely studied to inactivate and remove pathogenic microorganisms from food alone or in combination with other techniques or antimicrobials (Barba et al., 2017; Chen, 2017; Misra et al., 2017). The ultrasonic degradation of pesticides in wastewater has received special attention in the last years (Azam et al., 2020; Yuting et al., 2013). In foods and water solutions, USN shows promising applications in cleaning and decreasing chemical contaminants such as pesticides or mycotoxins. The main limitations of USN treatment to be incorporated in the industry may be solved with the combination with other treatments or compounds. To improve the efficiency of ultrasound in the removal of food contaminants (e.g. pesticides and Mycotoxin), the mechanisms involved in this innovation processing technology should be considered. Sonication mainly works based on cavitation, i.e., the result of generation, growth, and implosion of gas bubbles which will collapse on the surface of the food sample and discharge high pressure and temperature. This results in the creation of shock waves and micro-fractures (Gavahian, Chen, et al., 2018). Besides, the mechanical and chemical effects of ultrasound should be considered. A combination of the above-mentioned mechanisms may result in the release and degradation of food contaminants. Hence, optimization of process parameters (e.g. power, frequency, duration, temperature, sample pH, etc.) to enhance these effects may improve the efficiency for the sonication process. It should be noted that the quality parameters of the food should be considered in such an optimization process.

Table 5: Effect of supercritical carbon dioxide (SC-CO_2) on the recovery of pesticides from food products.

Matrix	SC-CO2 conditions	Main finding	References
Tomato	P = 16.4Mpa T = 40 ºC Time 30min	Recoveries of 117 pesticides Higher recoveries of polar pesticides.	Saito-Shida Cucumber et al. (2014)
Onion	Density: 0.7–1.0 g mL^{-1} T: 40–70 ºC Volume of CO_2: 10–40 mL	Optimum extraction condition: Volume of 29 mL, density of 0.90 g mL^{-1}, and temperature of 53 ◦C. Increasing the density of enhanced the extraction recovery of 2,4′ dichlorodiphenyldichloroethane and endrin.	Tolcha, Gemechu, AlHamimi, Megersa, and Turner (2020)
Rice wild rice wheat	P = 20.4 MPa T = 50 ºC Methanol as a cosolvent	High recovery for all pesticides from all matrices with an average higher than 70%.	Valverde, Aguilera, Rodríguez, and Brotons (2009)

a. Mycotoxin removal by ultrasound

Evelyn et al. (2016) studied the effectiveness of ultrasound (USN) (24 kHz, 0.33 W/mL) and HPP processing (600 MPa) up to 40 min in combination with a temperature of 75 ºC, compared with the thermal process alone for inactivate ascospores of *N. fischeri* (mycotoxigenic mold) in apple juice and observed that HPP at 75 ºC process was the most effective, resulting in 3.3 log reductions after 10 min. No inactivation was observed after applying thermosonication (75 ºC, 10 min) and thermal processing (75 ºC). However, thermosonication during 25 min resulted in higher inactivation (0.5 log) comparing to thermal processing. Similar results were reported by these authors in *B. nivea* spores of strawberry puree, observing that HPP at 600 MPa during 10 min at 75 ºC was better treatment than thermosonication and thermal process. However, after 15 min of treatment, thermosonication (75 ºC), produced a comparable inactivation than HPP (Evelyn et al., 2016). Despite this, the industry requires shorter times for better productivity that make ultrasounds unfeasible for commercial applications.

More recently, Evelyn and Silva (2017) investigated the efficacy of HPP (600 MPa) and USN treatments (24 kHz, 0.33 W/mL) at 75 ºC for the inactivation of 4–12 week old spores of *B. nivea* and *N. fischeri* in samples of strawberry puree and apple juice, respectively. The results obtained by these authors revealed that the resistance of mold spores depends on species and age, increasing the spore resistance with the age. Furthermore, HPP treatment was more effective than USN treatment, like it was observed in previous studies. USN treatment (0.33 W/mL) at 75 ºC was not appropriate for *N. fischeri* and *B. nivea* spores inactivation, since more than 60 min was necessary to only achieve 1–1.5 log reduction of 12-week old spores, so it is necessary to investigate higher USN intensity treatments.

Lopez-Malo, Palou, Jiménez-Fernandez, Alzamora, and Guerrero (2005) evaluated the effects of USN (frequency: 20 KHz; amplitude: 0, 60, 90,120 μm); temperature: 52.5,55,57.5 and 60 ºC); water activity (0.99 or 0.95) with or without 500 ppm of vanillin or potassium sorbate) on *Aspergillus flavus* and *Penicillium digitatum* inactivation, observing that the non-viability of USN treatment in the food industry may be solved with the combination with antimicrobials or heat.

Better results were obtained by Rudik, Morgunova, and Krasnikova (2020) who proposed USN treatment at low frequencies 24–26 kHz and no more than 1 W/cm^2 intensity as a useful strategy to reduce mold fungi content in grains, and subsequently mycotoxins production (Rudik et al., 2020). Concerning the application of USN to reduce mycotoxins levels, Liu, Li, Bai, & Bian (2019) studied the potential of USN technology at a frequency of 20 KHz in AFB1, DON, ZEA and OTA removal from maize and aqueous solution considering the influence of different factors such as the initial concentrations, the power intensity, the duration of the treatment and the duty cycle. These authors reported high degradation rates of 96.5, 60.8, 95.9 and 91.6% for AFB1, DON, ZEA and OTA, respectively at a duty cycle of 25%. Mycotoxins reduction was significantly affected at intensities of 2.2–11 W/cm^3 and treatment times ranging from 10 to 50 min (Liu et al., 2019). In another study, the same research group explored the effect of USN exposure of AFB1 in aqueous solution prepared at a concentration of 10 mg/L and treated under the frequency of 20 kHz during processing times of 30, 40, 60, or 80

min and power intensity of 6.6 W/cm^3 observing an AFB1 degradation percentage of 85.1% after 80 min (Liu et al., 2019). Furthermore, these authors also identified eight AFB1 degradation products after the treatment.

Slight lower reductions were obtained by Mortazavia, Sania, and Mohsenib (2015) after treating standard solutions of AFs at a concentration of 17.7 ppb under USN irradiation at the frequency of 20 kHz, intensities of 20, 60 and 100% and time periods of 10, 20 and 30 min, observing reduction about 41% for AFs, after the constant frequency of 20 kHz with 60% intensities during 10 min.

b. Pesticide removal by ultrasound

Several studies have evaluated the application of USN to decontaminate pesticides from wastewater, which may be a problem in the agricultural industry. For instance, Wang and Liu (2014) studied the decontamination of alachlor herbicide (initial concentration of 50 mg/L) in wastewater by an USN (20 kHz and output power of 100 W)/Fe^{2+}/H_2O_2 process in continuous dosing mode. Data obtained by these authors revealed that the maximum alachlor degradation (near 100%) was obtained at pH 3, 20 mg/L of Fe^{2+}, 2 mg/min of H_2O_2, 20 ºC and duration of 60 min with 46.8% total organic carbon removal. These authors also observed that lower pH values enhanced the alachlor degradation and mineralization. Adequate dosages of Fe^{2+} and H_2O_2 in combination with US can save operational cost and produce better results than US process alone (Wang & Liu, 2014). In a subsequent study, the same research group (Wang & Shih, 2016) studied the effect of USN facilitated by Fenton's and Fenton-like reagents on diazinon insecticide at 50 mg/L and observed that USN in combination with Fenton's and Fenton-like reagents degraded effectively diazinon and reduced its toxicity. The optimal experimental conditions were: Fe^{2+} 20 mg/L, H_2O_2 150 mg/L, 25 ºC and pH 3. After processing during 60 min a 98% of diazinon reduction was reached with a mineralization efficiency of 30% (Wang & Shih, 2016). Farooq, Shaukat, Khan, and Farooq (2008) also observed that the combination of USN with H_2O_2 produced better results than USN alone in the decomposition of the methidathion

pesticide. The decomposition rate observed in methidathion at 200 mg/L by these authors was about 88% at an amplitude of 120 μm, pH of 3 and treatment time of 90 min in combination with H_2O_2.

Recently, Schieppati et al. (2019) studied USN-assisted photocatalytic degradation of isoproturon herbicide at a concentration of 20 ppm in water and observed that with an ultrasonic power of 50W cm^2, the degradation ≈100% was obtained after 1 h of treatment. Furthermore, the authors also observed that USN coupled with photocatalysis lead to lower molecular weight by-products compared to photocatalytic treatments alone (Schieppati et al., 2019). Suri and Kamrajapuram (2003) studied the effect of H_2O_2 and/or silica in combination with USN treatment for the destruction of 2-chlorophenol (2-CP). The effect of different parameters such as silica dosage (1, 5, 10, and 20 g/L), peroxide dosage (50, 75, and 100 mg/L) and pH (3,7, or 11) was also examined, observing that lower pH was more effective to reduce the levels of 2-CP. Peroxide (100 mg/L) and silica (5 g/L) enhanced the 2-CP destruction (approximately 84%) in 60 min, corresponding to a factor of 2 as compared with USN treatment alone (Suri & Kamrajapuram, 2003). In all these studies, the combination of USN with other chemicals or oxidation processes was suggested as an efficient strategy to remove pesticides from wastewater.

Regarding the effect of USN removing pesticides from food matrices, Lozowicka, Jankowska, Hrynko, and Kaczynski (2016) studied the removal of 16 pesticide residues from strawberries employing USN cleaning with a frequency of 40 kHz, power 2×240W peak/period and period times of 1, 2 and 5 min. These authors obtained that USN cleaning reduced efficiently the contents of all analyzed pesticides with reduction rates between 45.1% and 91.2% after 5 min of treatment. The shockwaves formed during USN cleaning contributed to a more efficient pesticide reduction than that obtained only using cleaning (Lozowicka et al., 2016). Buakham, Songsermpong, and Eamchotchawalit (2012) also evaluated the potential of USN cleaning (60 kHz and 140 W) to remove carbamate group (carbosulfan) from coriander, kale, yard long bean, and red chili vegetables. The

authors found that USN cleaning was a more efficient technique than cleaning by soaking in water to remove pesticides from those matrices.

In another study, Cengiz, Bas¸lar, Basançelebi, and Kiliçli (2017) evaluated the effects of electrical current at low intensities (200–1400 mA) and two kinds of USN treatments including an ultrasonic bath (UB) at 40 kHz and ultrasonic probe (UP) at 24 kHz. Better reductions for captan (94.24%), thiamethoxam (69.80%) and metalaxyl (95.06%) were obtained using the following combinations of ultrasound + electrical current: 1400 mA + 40 kHz, 800 mA + 24 kHz and 1400 mA + 24 kHz, respectively (Cengiz et al., 2017). Recently, Zhu et al. (2019) studied the effect of USN treatment to remove chlorothalonil, pyrazophos, and carbendazim residues form pakchoi at a concentration of 10 mg/L, obtaining reduction rates in the range of 13.87–74.86%, 21.74–45.68%, and 5.12–24.63%, for chlorothalonil, pyrazophos and carbendazim, respectively. The authors also observed that frequency of 28 kHz and powder of 0.45 W/cm^2 were the most efficient USN treatment to remove these pesticides. In addition, they also found that USN was more efficient in pesticide removal than traditional water soaking (Y. Zhu et al., 2019).

In juices, Zhang, Xiao, et al. (2010) evaluated the potential of USN irradiation as a promising process to remove organophosphorus pesticides such as malathion and chlorpyrifos spiked at 2 and 3 mg/L. The maximum degradations rates observed by the authors were 41.7% for malathion and 82.0% for chlorpyrifos after USN treatment with 25 kHz and 500 W during 120 min. In another study, the same research group observed similar results for diazinon in apple juice (Zhang, Zhang, et al., 2010). After USN treatment during 30 min, a degradation percentage of 51.3% was obtained with an initial concentration of diazinon (7.82 μmol/L). These authors also suggested that the initial concentration and USN power can influence the degradation percentage achieved.

7. Possibility of combined methods

An innovation approach will be using a combination of innovative processing technologies together (e.g. ohmic heating and ultrasound) (Gavahian et al., 2017). Also, a combination of emerging

technologies (e.g. ohmic heating and fermentation process) can be interesting (Gavahian & Tiwari, 2020). The possibility of such combinations for removing pesticides and mycotoxins can be considered in future studies to enhance the decontamination efficiency.

8. Conclusions

Innovative processing technologies, including high-pressure technology, pulsed electric fields, cold plasma, supercritical carbon dioxide, and ultrasound, are found to be effective processing technologies for degradation of pesticides and mycotoxins provided that they use after an optimization study for degrading a specific compound (e.g. a specific type of pesticide) which is located in a specific food matrix. For example, optimization of pulsed electric field process in terms of applied voltage and pulsation can enhance the degradation rate. Besides, combination of conventional technologies and innovative processing technologies (e.g. thermal processing and pulsed electric field) has been shown to be an effective approach for degradation of hazardous compounds from food materials. Also, closer look into the mechanisms of degradation and the degradation products suggest that further studies on the safety of degradation byproducts are needed. Therefore, researchers in this area should strive to prepare a scalable platform for the industry which can efficiently remove various types of pesticides and mycotoxins from various types of agricultural products. Considering the growing interest in this area and previously reported promising data, such progress may happen soon.

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4.4 MIND THE GAP IN THE KNOWLEDGE OF THE POTENTIAL FOOD APPLICATIONS OF ULTRASOUND BASED ON ITS MECHANISM OF ACTION

Chapter 1 in book: Design and Optimization of Innovative Food Processing Techniques Assisted by Ultrasound (2020)

Mind the gap in the knowledge of the potential food applications of ultrasound based on its mechanism of action

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1. Introduction

Over the last years, the demand for minimally processed food in food industries, reduced processing times, and improved shelf life of food products has led to significant modifications in the processing methods as some processing techniques applied under critical conditions decrease nutrient level and bioavailability by generating physical and chemical changes, thus minimizing the acceptability of organoleptic properties. Thereby, instead of such conventional techniques, nonconventional processing methods in the food industry have been developed in order to retain sensory characteristics, nutrient, and non-nutrient compounds (bioactive) (Chemat & Khan, 2011; Putnik et al., 2017; Putnik et al., 2018). Ultrasonic method is one of those rapidly innovative alternative techniques developed for use in the process of production of high-quality food products (Chemat & Khan, 2011; Misra et al., 2017). Ultrasound technique uses mechanical waves with either high or low frequencies (Misra et al., 2018; Soria & Villamiel, 2010). High-frequency with a low-power ultrasound (LPU), as an analytical technique, is used to obtain information on the physicochemical properties of food such as acidity, firmness, sugar content, and ripeness, etc. (Arvanitoyannis, Kotsanopoulos, & Savva, 2017). On the other hand, low frequency with a high-power ultrasound (HPU)is used to change the physical and chemical properties of food by inducing pressure, shear, and temperature difference in the medium through which they propagate and is capable of producing cavitationin order to extract bioactive compounds (Agregán et al., 2018;Ciğeroğlu et al., 2018; Roselló-Soto et al., 2015; Zhu et al., 2017), inactivate microorganisms (Zinoviadou et al., 2015), accelerate and inactivate enzymes in foods (Arvanitoyannis et al., 2017). Ultrasound technology finds its application in a wide range of food industry such as in quality control of fresh vegetables and fruits, cheese and milk processing, wheat products, emulsified food fat products, and frozen food (Majid, Nayik, & Nanda, 2015). Furthermore, ultrasound is suitable for the detection of the adulteration of honey and protein analysis, tenderization of meat, decreasing the adhesiveness of dry-cured ham, homogenization,

crystallization, freezing, drying as well as filtration, foam production, and reduction (Carrillo-Lopez, Alarcon-Rojo, Luna- Rodríguez, & Reyes-Villagrana, 2017; Perez-Santaescola´stica et al., 2018).

Although ultrasound has been widely studied for food processing, its range area increases and the parameters that affect the ultrasound efficiency remain to be determined for various systems. This chapter will mainly review the mechanism of action and the application of ultrasound in food technologies.

2. Principle and mechanism of action of ultrasound

Ultrasound technology is based on the application of mechanical waves at a frequency above the human's hearing levels ranging from 20 kHz to 10MHz (Gallo, Ferrara, & Naviglio, 2018) and can be classified into two frequency ranges: LPU, or synonymous low intensity ultrasound (high frequencies between 100 kHz and 1MHz at intensities lower than $1Wcm^2$), while HPU, or synonymous high-intensity ultrasound, uses intensities higher than $1Wcm^2$ at low frequencies between 20 and 100 kHz (Gallo et al., 2018). Ultrasound can be applied using three different methods: (1) directly to the product; (2) coupling the product to a device; (3) immersion in an ultrasonic bath (Soria & Villamiel, 2010).

First, ultrasound generates mechanical waves requiring a material medium to propagate, causes acoustic cavitation; which is the occurrence of the bubbles generation, growing, and implosion (Majid et al., 2015). The ultrasound waves propagate through a liquid medium inducing a cavitation of bubbles, which oscillate and collapse causing thermal, mechanical and chemical effects (Misra et al., 2018; Soria & Villamiel, 2010). Chemical effects include the creation of free radicals, while mechanical effects consist of collapse pressure, turbulences, and shear stresses. In addition, positive and negative pressures in an alternative mode cause expansion or compression of the material, thus the cell rupture, depending on the frequency of ultrasound (Carrillo-Lopez et al., 2017). The effects in the cavitation zone generate a high increase in temperature and in pressure (5000K and 100MPa) (**Figure 1**) (Wen et al., 2018). The consequence of the high-temperature conditions within the core

of the cavitation bubble is the generation of a variety of chemical reactions. Therefore, ultrasound leads to the hydrolysis of water inside the wavering bubbles producing H^+ and OH^- free radicals (Wen et al., 2018). Additionally, during ultrasound treatment, the generated bubbles are grouped into two types based on their structure: stable and internal cavitation bubbles. Stable cavitation bubbles (nonlinear) are formed when the bubbles are large with equilibrium size during pressure while the internal cavitation bubbles (unstable) are formed when bubbles are rapidly collapsing and disintegrating into small bubbles (Majid et al., 2015). The small bubbles dissolve in the liquid immediately but while they stretch, the mass transfer boundary membrane becomes thinner while the interfacial space becomes bigger than during the bubbles collapse, which means that during the stretching of the bubbles, more air is transferred into the bubble and later on, during the implosion phase, the air leaks out.

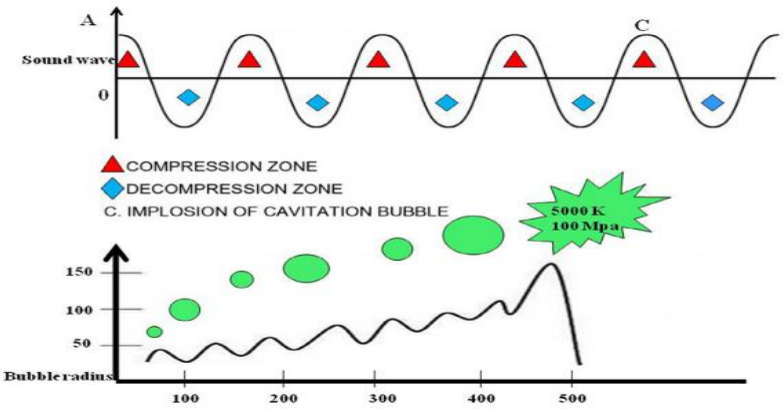

Figure 1: Cavitation mechanical effect of ultrasound technology (Wen et al., 2018).

3. High-power ultrasounds in food technology

HPU can change the physical and chemical properties of food, in sterilization, extraction, food preservation, emulsification, filtration, drying, stripping, tempering, bleaching, and other aspects of more applications (Dolatowski, Stadnik, & Stasiak, 2007). Energy, strength, pressure, velocity, and temperature are the main parameters affecting high-energy ultrasound. HPU can be described by the following modes: $P_a = P_{amax} * \sin(2\pi ft)$. P_a refers to acoustic pressure, which depends on the time, frequency, and maximum pressure range of the wave (sinusoidal wave) (Patist & Bates, 2008). P_{amax}

is related to the input power or strength of the sensor: $I= P_{amax}/2\rho v$. ρ is the density of the medium and v is the velocity of sound in the medium.

The thermal, mechanical, and chemical effects of HPU are attributed to the rapid formation and rupture of cavitation bubbles in the liquid, resulting in strong shear stress. These bubbles burst in the positive pressure cycle, producing highly turbulent flow conditions and extremely high pressures and temperatures. The thermal effect refers to the effect of the conversion of the energy of a large amplitude sound wave into heat, resulting in local temperature rise and instantaneous strong pressure, which can produce strong sonochemical effects (Sillanpaa, Shrestha, & Pham, 2011). In addition, the cavitation effect is the phenomenon of compression, expansion, and high-frequency mechanical oscillation generated by the ultrasonic wave. It will crush and strain the liquid medium in local space, resulting in cavitation, which has a mechanical bond breaking effect on macromolecules (Wu & Nyborg, 2008). Mechanical effect is the phenomenon of linear alternating vibration when ultrasonic waves transfer mechanical energy, which is mainly manifested in the aspects of vibration velocity, origin displacement, sound pressure, and so on. Higher energy is transferred to the medium with more intense acoustic vibrations, enhancing mechanical effects, and promoting heat and mass transfer, these three functions complement each other so that ultrasonic technology plays an important role in industrial applications.

3.1. HPU applications

There are many benefits of the application of high energy ultrasonic in the food industry, including increased physical mixing, improved mass and heat transfer, reduced processing time, lower processing temperature, selective extraction, increased yield, increased efficiency, and so on. HPU is simple to operate, which can achieve continuous, repeated processing. The application of ultrasonic technology in food processing has achieved good results.

3.1.1. Extraction

One of the main applications of high-energy ultrasound in the food industry is to use the mechanical shear action generated by its cavitation effect to change the physical properties of food, such as the destruction of food surface structure, so as to accelerate the solvent extraction speed to improve the extraction efficiency in the food industry (**Figure 2**) (Rostagno, Palma, & Barroso, 2003). Substances commonly extracted from food include bioactive ingredients of plant and animal origin, such as fats, proteins, polysaccharides, and some bioactive substances (Jiang et al., 2018; Li et al., 2018). A more detailed description of the potential application of ultrasound to extract highly added value compounds from food products will be discussed in Chapter 5.

3.1.2. Preservation

During the ultrasonic preservation process, instant high-temperature and high-pressure changes occur in the food system. The combined effect of the two effects causes microbial proteins and physiologically active substances to mutate, resulting in the loss of vitality and death of the microorganisms, achieving the purpose of food sterilization (Li et al., 2016). In the process of ultrasonic application to food, the sound wave produces a longitudinal wave when it contacts the medium, followed by the cavitation phenomenon and the formation of bubbles. The ultrasonic energy is not enough to maintain the gas phase of bubbles, so it will produce rapid compression, leading to intense collisions between molecules, resulting in shock wave, high temperature, and high pressure (Piyasena, Mohareb, & McKellar, 2003; Zinoviadou et al., 2015). In addition, the effectiveness of ultrasound for food preservation is related to the type of microorganisms, and related studies have focused on Listeria, Salmonella, Escherichia coli, Staphylococcus aureus, Bacillus subtilis, and other microorganisms. Conventional ultrasound itself has a certain bactericidal effect, but when ultrasound is combined with pressure and heat treatment, that is, thermal ultrasound (heat + ultrasound), pressure ultrasound (pressure+ultrasound), and pressure ultrasound (heat and pressure + ultrasound), which can further increase the preservation rate. Although ultrasonic food preservation

technology has been widely used in the food industry, there has been a lot of research on ultrasonic over the last recent years. For a more detailed description, see Chapter 3.

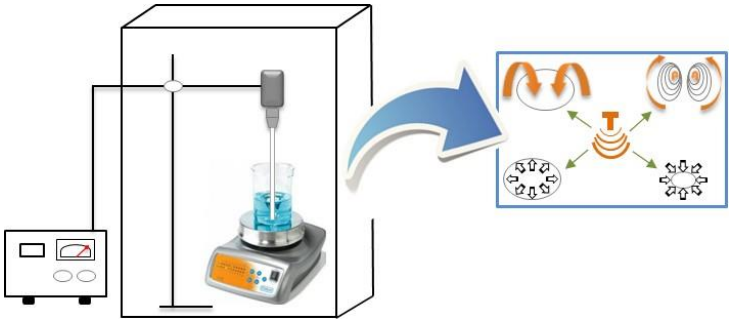

Figure 2: Ultrasound-assisted extraction process and the bubble cavitation phenomenon involved in this extraction technique.

3.1.3. Improvement of food properties

Emulsification

Emulsifying food using ultrasound is an early and relatively mature technology. Foods using phacoemulsification technology worldwide include ketchup, mayonnaise, margarine, baby food, chocolate, salad oil, etc. A homogeneous dispersion or emulsion is usually produced by means of a surfactant (emulsifier). If the mixing process does not occur spontaneously (microemulsions cannot be formed), the emulsification process needs to input energy through mechanical stirring or sonication to promote the formation of small droplets (Gavahian, Chen, Mousavi Khaneghah, Barba, & Yang, 2018). The emulsification of food using ultrasound technology can improve the quality and production efficiency of corresponding products (Awad, Moharram, Shaltout, Asker, & Youssef, 2012; Pinton et al., 2019). Compared with conventional emulsification methods such as propellers and homogenizers, phacoemulsification has the following advantages: the average droplet size of the emulsion formed is small; the emulsion concentration is higher and more stable; it can form two water-in-oil and oil-in-water emulsion systems; the production process requires less power and saves energy (Dolatowski et al., 2007). Jonathan's research shows that soy protein isolate treated with ultrasound has improved both in emulsifying ability and emulsion stability (O'Sullivan, Park, &

Beevers, 2016). The possible reason is that the molecular structure of soybean protein is loose after being modified by ultrasound, which exposes some nonpolar groups inside, which improves the emulsifying properties of soybean protein.

Crystallization

Ultrasound has a good effect on the formation of seed crystals, the growth of crystals, and the prevention of crusting of crystals under freezing conditions, ensuring continuous and effective heat transfer in the above process. In the food industry, foods that have been stored at low temperatures are prone to quality problems after thawing, which often affects consumers' perception of food and has a negative impact on food sales, especially some meat products and fruits. This problem is mainly related to the principle of water freezing in food. The small ice crystals in the cells inside the food will continue to grow during freezing. When these small ice crystals grow up, they will destroy the cell wall inside the food, thereby destroying the cell structure (Li & Sun, 2002). Ultrasound, as a technique is used to easily control the nucleation of ice crystals, which can improve the repeatability of the freezing process. The application of ultrasonic technology and the freezing process can effectively promote the formation of crystal nuclei in supercooled solutions. Under the action of ultrasound, the temperature of the material decreases faster, and the seed formation is more rapid, resulting in a shorter "swelling time." In addition, there are a large number of seed crystals, and the final size of the ice crystals and the degree of cell damage are greatly reduced (Li & Sun, 2002).

In the process of food freezing, the ice crystals formed inside the food cells are significantly reduced and the distribution is more uniform after ultrasonic treatment, which makes the quality of the product not deteriorate after thawing. Islam et al. used ultrasonic-assisted infusion to freeze mushrooms, shortening the ice crystal nucleation time and total freezing time during mushroom soaking and freezing, and increasing the hardness of mushrooms and reducing water loss (Islam, Zhang, Adhikari, Xinfeng, & Xu, 2014). In addition to current applications, the high energy ultrasound can also cooperate with other new freezing technologies, such as ultra-high-pressure freezing

technology and biological frozen protein technology, to broaden the range of ultrasonic action parameters and reduce its technical and material requirements (see Chapter 11 for a more detailed description).

Filtration and drying

Different types of membranes are commonly used for filtration in food processing. The disadvantage of these membrane filters is that they are often blocked and need to be cleaned or replaced frequently. Ultrasound can avoid the disadvantages of membranes when applied to filtration in the food industry. First, ultrasonic treatment can make fine particles agglomerate, which will increase their filtration speed; second, ultrasonication can generate enough vibration energy to suspend some particles in the liquid, which leaves more channels for dissolved components and can also be effective in preventing clogging (Kyllonen, Pirkonen, & Nystrom, 2005).

The use of ultrasound in the production of fruit extracts and beverages can improve the efficiency of apple juice filtration to produce apple juice. Ordinary vacuum filtration can reduce the moisture from 85% to 50% at the beginning, and ultrasonic technology can reduce the moisture to 38%, which can improve product quality and has potential commercial value (Mason, Paniwnyk, & Lorimer, 1996). Compared with conventional methods, ultrasonic drying can be carried out at a lower temperature, thereby reducing the oxidation or degradation of the product, and it will not blow materials around or spoil materials like high-speed air drying (see Chapter 4 for a more detailed description).

4. Low-power ultrasound in food technology

LPU mainly refers to the ultrasonic wave with a frequency greater than 100 kHz and energy less than 1w/cm^2, it was used in the food industry as early as the 1940s. This kind of sound wave has low intensity and is less destructive. It can not only track the process of food processing but also can provide the physical and chemical properties of food, used in food analysis and detection, and has the advantage of fast preparation and low degree of damage (Turanta, Ba, & Birol, 2015). Different from

the low frequency ultrasound, the highfrequency ultrasound has a stronger chemical effect than physical effect and produces more OH and H_2O_2, which changes the physical and chemical properties of molecules. The detection of meat products by LPU has been widely reported. In one study, Lyng, Allen, and McKenna (1997) showed that meat could not be tenderized at LPU. Got et al. (1999) used high-frequency ultrasound to treat beef. The results showed that although high-frequency ultrasound could induce prerigor phase ultrastructure, it could not change the tenderization of meat. Other studies have shown that ultrasound is also important for thawing meat. For example, Miles, Morley, and Rendell (1999) found that ultrasound can reduce the thawing time of meat. In addition, LPU has also been applied to dairy products. In this sense, Juliano et al. (2014) explored the effects of different frequencies, temperatures, time, and energy on lipid oxidation in different types of milk. The results showed that the lipid oxidation in milk was optimized by controlling ultrasound frequency, time, temperature, and other factors. McClements (1995) determined the degree of homogenization of milk by detecting ultrasonic attenuation, so as to predict the degree of emulsification, indicating that the chemical properties of food could serve as the basis for ultrasonic analysis. Besides, LPU has been used in the study of wheat quality. Ali Salimi, Sijo Joseph, and Ames (2018) measured the properties of wheat flour and compared the effects of ultrasonic parameters on it. The results showed that ultrasonic parameters had a significant correlation with protein and ash content of wheat. Thus, because ultrasonic signals are sensitive to proteins, starch, and microstructure, wheat can be screened by low-intensity ultrasound. Daugelaite et al. (2017) used 11MHz ultrasound to explore the influence of glucose oxidase and mechanical properties of frozen noodles, so as to evaluate the rheological properties of dough. Furthermore, Park and Han (2016) used high-frequency ultrasound to treat brown rice, the results showed that the rice tasted better and gelatinized more easily after ultrasonic treatment, possibly because ultrasound degraded starch particles and made them more likely to bind to water.

Ultrasonic technology has also been used to detect the structure of fruits and vegetables. At the same time, high-frequency ultrasound produces active free radicals that can react with food ingredients. For example, Stojanovic and Silva (2007) took blueberry as the experimental material and found that high-frequency ultrasound would damage the anthocyanins in blueberry and their antioxidant properties. Also, Zhang et al. (2017) pretreated with LPU, and the results showed that LPU preconditioning could increase the chlorophyll content and reduce the browning degree of carotenoids. In other study, after 986MHz ultrasound treatment, the total phenolic content of red raspberry sauce increased, and the total antioxidant activity also changed (Golmohamadi, Moller, Powers, & Nindo, 2013). On the other hand, using ultrasonic technology in the processing of frozen vegetables can significantly shorten the frozen nucleation time of radish, broccoli, etc., and increase the nucleation temperature (Xin, Zhang, & Adhikari, 2014). High frequency ultrasound is also used in other food industries. Camara and Laux (2010) took advantage of high-frequency ultrasound to determine the moisture of honey by ultrasonic reflectometry with a frequency of 10MHz. Gunther et al. found that ultrasound can also degrade L-ascorbic acid (Portenlanger & Heusinger, 1992). In addition, because LPU is not enough to rupture cell walls, it also has a positive effect on the preservation of food nutrients (Vallespir, Crescenzo, Rodríguez, Marra, & Simal, 2019). LPU has a great influence on the physical and chemical properties of different types of food and the degradation of substances. In addition, the free radical effect of high-frequency ultrasound on the degradation of macromolecules, as well as the removal of food residues can be further explored.

5. Advantages and limitations of ultrasound

The application of ultrasound offers various advantages in the food industry as well as some limitations that could appear due to the exposition of food to ultrasound waves. **Table 1** outlines some of the advantages and disadvantages of ultrasound technology utilization.

Table 1: Advantages and drawbacks of ultrasound in food industry.

	Advantages / drawbacks	Ref.
Advantages	Safe, doesn't generate toxic compounds, eco-friendly	Bruno, Ekorong, Karkal, Cathrine, and Kudre (2019)
	Easy technology, low cost and efficient power output	Zhao, de Alba, Sun, and Tiwari (2019)
	Feasible as ultrasonic assisted extraction of bioactive compounds on the industrial scale.	Wen et al. (2018)
	Provide extract yield well compared with conventional techniques	Álvarez, Lelu, Lynch, and Tiwari (2018)
	Enhance preservation in food, aid in thermal treatments, improved mass transfer, and alteration of food texture and analysis	Awad et al. (2012
Drawbacks	The mechanical effects such as shear stress developed by eddies from the shock waves may cause the inactivation of the released products	Majid et al. (2015)
	Free radical could be responsible for changes in food compounds that leads to the degradation of the product, thus a deficiency in the product quality.	Awad et al. (2012
	Possible oxidation of fats, inactivation of valuable enzymes and denaturation of proteins	Jambrak et al. (2009)

6. *Conclusions*

Due to the characteristic mechanism of ultrasound technology, it can be considered as an interesting tool for different food applications. Ultrasound has high efficiency, is safe, and environmentally friendly. This technology can be effectively used for extraction of high added value compounds from food and byproducts, as well as to preserve foods, at the same time protecting the nutritional and bioactive ingredients of food. It can, therefore, influence the quality of various food systems, improving their productivity and performance. Thus, the attention of the food industry has turned into ultrasound technology and towards its promising potential in the processing and preservation

of foods such as fruits, vegetables, cereals, and meat, etc. However, although a huge variety of applications and potential uses have been proposed, wide-ranging research is still required to further progress the industrial applicability of this technology and fully examine the effects of ultrasounds on the characteristics of foods.

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4.5 ULTRASOUND EXTRACTION MEDIATED RECOVERY OF NUTRIENTS AND ANTIOXIDANT BIOACTIVE COMPOUNDS FROM *PHAEODACTYLUM TRICORNUTUM* MICROALGAE

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Ultrasound extraction mediated recovery of nutrients and antioxidant bioactive compounds from *phaeodactylum tricornutum* microalgae

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Abstract:

In recent years, a growing interest has been shown in the use of microalgae due to their interesting nutritional and bioactive profiles. Green innovative processing technologies such as ultrasound-assisted extraction (UAE) avoid the use of toxic solvents and high temperatures, being a sustainable alternative in comparison with traditional extraction methods. The present study aims to evaluate the recovery of high added-value compounds from assisted by ultrasound. To optimize the UAE of proteins, carbohydrates, pigments and antioxidant compounds, a response surface methodology was used. Carbohydrate extraction was positively affected by the temperature. However, for the extraction of carotenoids, the most influential factor was the extraction time. The total polyphenols were only significantly affected by the extraction time. Finally, the antioxidant capacity, measured by 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), was strongly modulated by the extraction time, while for the oxygen radical antioxidant capacity (ORAC) assay, the most important parameter was the temperature, followed by the extraction time. The optimal conditions for the maximum extraction of nutrients, bioactive compounds and antioxidant capacity were 30 min, 50 ºC and a pH of 8.5. Finally, it has been seen that with these conditions, the extraction of fucoxanthin is allowed, although no differences were found between an ultrasound-assisted extraction and a shaking extraction (control).

Keywords: microalgae; ultrasounds; bioactive compounds; nutrients; extraction; optimization; Phaeodactylum tricornutum

1. Introduction

Over the last two decades, there has been growing interest in the use of microalgae as food to partially replace conventional food products (e.g., meat) or to be used as a source of high added-value compounds [1]. Marine microalgae consist of prokaryotic or eukaryotic photosynthetic microorganisms that are able to grow rapidly due to their unicellular or simple multicellular structures [2]. The main reason for the increased microalgae exploitation is due to their interesting nutritional and bioactive profiles (e.g., high protein content, healthy lipid profile and micronutrient (vitamins and minerals) composition) [3]. Moreover, microalgae is considered a sustainable biomass and can be also used to remove heavy metals from marine waters and industrial waste [4,5]. However, the nutritional and bioactive profiles of microalgae differ according to the target species. For that reason, there is a need to explore and evaluate each microalgae species separately. *Phaedoactylum. tricornutum* consists of a unicellular marine diatom with a high growth rate under optimal conditions. It is considered an important source of n-3 polyunsaturated fatty acids (PUFAs), particularly eicosapentaenoic acid (EPA), being an interesting alternative in the industry for EPA production [6]. Moreover, *P. tricornutum* is a promising source of other interesting bioactive components such as fucoxanthin, a primary marine carotenoid [7]. In order to recover the different high added-value compounds from microalgae, different approaches have been taken into account. For example, traditionally, the use of liquid–liquid or solid–liquid extraction using organic solvents and high temperatures has been used. However, due to recent concerns regarding the use of toxic solvents and non-sustainable approaches, there is a growing search for green and efficient methods to avoid them [8–10].

Nowadays, innovative alternative technologies, such as supercritical fluid extraction (SFE), pressurized liquid extraction (PLE), ultrasound-assisted extraction (UAE), pulsed electric field-assisted extraction (PEF) and microwave-assisted extraction (MAE), are being studied to extract interesting compounds from microalgae [11–15]. These techniques produce a low environmental

impact, since no organic solvents are used and there is less volume consumption. Moreover, they operate under low extraction temperatures and short extraction times, being a sustainable alternative in comparison with traditional extraction methods [16].

Among the different techniques, ultrasound has been used by different authors to extract high added-value compounds from microalgae species (e.g., *Nannochloropsis* spp., Spirulina spp. and Chlorella spp.). Parniakov et al. [17] obtained an efficient recovery of phenolic compounds and chlorophylls from the microalgae *Nannochloropsis* spp. after ultrasound (USN) pretreatment. Adam et al. [18] reported the effective extraction of lipids from *Nannochloropsis oculata* under USN treatment. On the other hand, Vernès et al. [11] and Hildebrand et al. [19] proposed USN technology as an effective tool for the rapid extraction of proteins from *Spirulina* and *Chlorella vulgaris* microalgae, respectively. In general, all these authors obtained better results employing ultrasound than those obtained with conventional extraction. However, there is a lack of information regarding the effects of this technology on the recovery of nutrients and bioactive compounds from *Phaedoactylum tricornutum*. Ultrasound research is focused on various applications, such as food preservation, the stimulation of fermentation and enzyme reactions and the modification of food constituents or product structures, as well as the improvement of mass and heat transfer during the drying or extraction processes. This technology implies the application of ultrasound waves with a range of frequencies between 20 kHz and 100 MHz, thus leading to a constant growth of gas bubbles in the medium, resulting in the collapse and cavitation of the bubbles. This phenomenon causes the breakdown of liquid–solid interfaces, with the consequent release of bioactive compounds from the food matrix [20]. In recent years, this technology has also shown promising applications in cleaning and decreasing chemical contaminants in food, such as mycotoxins or pesticides [21].

Taking into account the extraction yield and profile differences according to each microalgae species, in a previous review, Barba et al. [8] established the need to evaluate each microalgae species separately. Therefore, the present study aims to evaluate the recovery of high added-value

compounds from *Phaedoactylum tricornutum* using an optimization strategy. Moreover, the influence of ultrasound on the protein molecular size distribution will be evaluated using SDS-PAGE electrophoresis.

2. Materials and Methods

2.1 Chemicals and Reagents

Ethanol (99.8%) and glacial acetic acid were obtained from Panreac (Castellar del Vallés, Barcelona, Spain). Sodium carbonate (Na_2CO_3), dimethyl sulfoxide (DMSO) and metanol (99.9%) were acquired from VWR (Saint-Prix, France). The 2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), Folin–Ciocalteu reagent, gallic acid, D-glucose, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), phenol, 2,2′-azobis-(2- amidinopropane) dihydrochloride (AAPH) and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (Steinheim, Baden-Württemberg, Germany). The Tris-HCl, sodium dodecyl sulfate (SDS) and Trizma® base and glycerol were obtained from SigmaAldrich (St. Louis, Missouri, USA). A quantity of 5–250 kDa of molecular weight pattern Precision Plus Protein™, 8–16% Mini-PROTEAN® TGX™ Precast gels and Coomassie brilliant blue R-250 were purchased from BioRad (Hercules, CA, USA). Dithiothreitol (DTT) and acetonitrile were obtained from VWR (Leuven, Belgium). Methanol and sulfuric acid (96%) were purchased from Merck (Whitehouse Station, NJ, USA). Tris(hydroxymethyl) aminomethane, potassium phosphate monobasic (Na_2HPO_4), potassium phosphate dibasic (K_2HPO_4) and sodium phosphate dibasic (Na_2HPO_4) were purchased from Merck (Darmstadt, Germany). Sodium fluorescein was obtained from Fluka Chemie AG (Bunds, Switzerland). Deionized water (resistivity >18 MΩ cm−1) was prepared in the laboratory using a Milli-Q SP reagent water system (Millipore Corporation, Bedford, MA, USA).

2.2 Samples

Phaedoactylum tricornutum microalgae were produced in four 800 L GemTube (LGEM, Rotterdam, The Netherlands) photobioreactors at the National Algae pilot plant in Mongstad (NAM), Norway,

located in a greenhouse exposed to natural light and additionally equipped with artificial illumination (EAX 170W LED lights, Evolys AS, Oslo, Norway). The photobioreactors operated in dual mode under the conditions of a pH of 7.8, on demand CO_2 addition and culture temperatures in the range of 15–35 °C. The microalgae were grown in a modified WUR (Wageningen University & Research) medium, which was based on natural seawater (Fensfjorden, Mongstad, salinity of 31 ppt) enriched with a nutrient stock solution. After harvesting, the biomass was dewatered by employing a spiral plate centrifuge (Evodos 25, Evodos b.v., Raamsdonksveer, The Netherlands), resulting in a paste of approximately 22% dry weight which was vacuum packed and stored until at −20 °C.

2.3 Ultrasound-Assisted Extraction (UAE) Technology

A Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA) was employed to carried out the experiments. The instrument operated under a frequency of 20 KHz, power of 100 W and treatment time of 0.5, 15 or 30 min. The bath temperature was set at 20, 30 or 50 °C and was checked during all the extractions. For the extraction experiments, two grams of microalgae were weighed and placed with 30 mL of distilled water in a 100 mL beaker. Prior to the extractions, the water pH was adjusted to 5.5, 7 or 8.5. The resulting extracts were placed in 15 mL tubes and preserved at −20 °C until further determinaions.

2.4 Total Protein Content and Profile by SDS-PAGE Electrophoresis

The protein content (mg of bovine serum albumin equivalent/g of dry sample) was obtained by a bicinchoninic acid (BCA) microtiter plate assay, in accordance with the work of Smith et al. [22] and adapted by Parniakov et al. [23]. For this, 0.1 mL of the sample extract was mixed with 2 mL of the solution of a BCA protein assay kit (Pierce Biotechnology, Inc., Waltham, MA, USA). Then, the samples were kept at room temperature (20 °C) for 2 h. Finally, the absorbance of each sample was read at 562 nm, employing a VICTOR3 1420 multilabel plate counter (PerkinElmer, Turku, Finland). Bovine serum albumin (Thermo scientific, Waltham, MA, USA) was used for calibration.

The different sizes of proteins contained in the extracts were evaluated after the UAE treatment by SDS-PAGE electrophoresis, according to the method previously described in [24]. Briefly, the proteins were precipitated by adding acetone to the sample (1:4 ratio, v/v for the acetone sample), and then they were centrifuged at 11,000×rpm at 4 °C for 10 min. The pellet was resuspended in deionized water. The resulting suspension was mixed with the same volume of sample buffer (62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 50 mM dithiothreitol and 0.01% bromophenol blue) and heated for 5 min at 95 °C. Then, 10 µL of this mixture was loaded on the prepared gel (8–16% Mini-PROTEAN® TGX™ Precast gels, Bio-Rad). Electrophoresis was carried out in a Mini-PROTEAN® tetra cell (Bio-Rad) at 120 V for the first 30 min and then at 80 V until the front line was arriving at the end of the gel. Precision Plus Protein™ in the amount of 5–250 kDa was used to estimate the molecular weight. The running buffer used during electrophoresis was prepared with SDS (0.1%), glycine (192 mM) and Trizma® base (25 mM) in deionized water. Once the electrophoresis process was finished, the gel was stained with 0.125 % Coomassie brilliant blue R-250 for 30 min. After this, it was destained using methanol:acetic acid:water (deionized) at a ratio of 2:1:7 (v/v/v). Finally, the picture of the gel was analyzed using ImageJ® (National Institutes of Health, Bethesda, MD, USA).

2.5 Carbohydrate Determination

The total carbohydrate content was measured, employing the phenol–sulfuric acid method [25]. First, a 5% phenol solution was prepared. Then, 500 µL of it was placed with 500 µL of a sample extract or standard in a tube and they were mixed. The tubes were kept at room temperature for 15 min, allowing a phenol reaction with glucose. After the incubation time, 60 µL of this mixture was transferred to a 96-well plate, 150 µL of 96% sulfuric acid was added to each well containing the calibration solution or the sample, and they were mixed well by upward pipetting. Then, the 96-well plate was incubated at room temperature for 5 min. A D-glucose calibration curve was performed in the range of 25– 500 µg/L. Finally, the absorbance of each sample was read at 490 nm by a VICTOR3 1420 multilabel plate counter (Perkin-Elmer, Turku, Finland), and the concentration of

carbohydrates was calculated based on a D-glucose calibration curve. Analyses were performed in triplicate.

2.6 Antioxidant Capacity and Compounds

The ABTS assay was performed according to the methodology proposed by Parniakov et al. [23]. An ABTS radical cation ($ABTS^{+\cdot}$) was produced by reacting 25 mL of ABTS (7 mM) with 440 µL of potassium persulphate ($K_2S_2O_8$) (140 mM). The mixture was kept in darkness at room temperature for 12–16 h. Then, the ABTS radical cation was diluted with ethanol at a ratio of 1:100 (v/v) to obtain an absorbance of 0.700 (±0.020) at a wavelength of 734 nm. The Trolox standard curve at different concentrations (0, 50, 100, 150, 200, 250 and 300 µM) was prepared, using ethanol as a solvent. The absorbance of 2 mL of the ABTS*+ working solution was the initial point of reaction (A0). Then, 0.1 mL of diluted sample extracts or Trolox standards were added, and the absorbance was determined after 3 min (Af). All absorbances were read at a wavelength of 734 nm in a PerkinElmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Rodgau-Jügesheim, Germany). The percentage of inhibition was calculated with the following equation: % Inhibition = (1 − (Af/A0)) * 100 (1)

The antioxidant activity was calculated using a Trolox standard curve and expressed as µM rolox equivalents (TEs). Experiments were performed in triplicate. The oxygen radical antioxidant capacity (ORAC) method was evaluated, following the methodology proposed by De la Fuente et al. [26] with some modifications. Sodium fluorescein and an AAPH working solution were prepared at a concentration of 0.015 mg/mL and 120 mg/mL, using a 75 mM phosphate buffer (pH 7). In a 96-well microplate, 50 µL of the sample extract was mixed with 50 µL of fluorescein, and the mixture was preincubated at 37 °C for 10 min. Then, 25 µL of the AAPH solution were added, and the plates were immediately placed in the VICTOR^3 1420 multilabel plate counter reader (PerkinElmer, Turku, Finland), and the fluorescence was recorded every minute for 60 min under an excitation wavelength of 485 nm and an emission wavelength of 528 nm. The phosphate buffer was used as a blank, and Trolox (100 µM) was used as the antioxidant standard. Each extract was analyzed in five replicates,

and the differences in areas under the curve (AUCs) of the fluorescein decay between the blank and the samples were used to calculate the antioxidant activity. The results were expressed as μM Trolox equivalents (TEs).

For the total phenolic compound (TPC) determination, the Folin–Ciocalteu method was employed according to Parniakov et al. [12]. Briefly, a 50% v/v Folin-Ciocalteu reagent, 2% Na_2CO_3 and the gallic acid diluted standards were prepared. First, 100 μL of the sample extract or standard were mixed with 3 mL of Na_2CO_3. Then, 100 μL of a Folin Ciocalteu reagent solution was added to the mixture, and the samples were incubated at room temperature for 1 h. Finally, the absorbance of the samples was read at a wavelength of 750 nm using a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Rodgau-Jügesheim, Germany). The gallic acid calibration curve was used for quantification of the total phenols. Analyses were performed in triplicate.

The total carotenoid and chlorophyll A contents were obtained spectrophotometrically in a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Rodgau Jügesheim, Germany). This method consisted of the determination of the carotenoid and chlorophyll contents based on their maximum absorbances: chlorophyll A $\lambda \approx 664.1$ nm, chlorophyll B $\lambda \approx 648.6$ nm and total carotenoids $\lambda \approx 470$ nm. The sample extracts were diluted with distilled water, and the absorbance (A) was read at wavelengths of 470, 648.6 and 664.1 nm, respectively. Finally, the chlorophyll A, chlorophyll B and carotenoid contents were obtained according to the following equations: C chlorophyll A (μg/mL) = $13.36\ A_{664.1} - 5.19A_{648.6}$ (2) C chlorophyll B (μg/mL) = $27.43\ A_{648.6} - 8.12A_{664.1}$ (3) C total carotenoids (μg/mL) = $(1000A_{470} - 2.13\ C_a - 97.64\ C_b)/209$ (4).

2.7 Experimental Design and Statistical Analyses

The ultrasound-assisted extraction conditions were optimized using a response surface method, with a Box–Behnken experimental design with two central points. The studied parameters were as follows: extraction time 0.5–30 min, temperature 20–50 °C and pH 5.5–8.5, with 3 levels each one (minimum, central and maximum) leading to 15 different combinations of these variables, with

repetition of the central conditions to check the stability and reproducibility of the results. **Table 1** shows the randomized design of the 16 experiments.

In order to obtain significant differences ($p < 0.05$) between the results, an analysis of variance (ANOVA) was performed, followed by the least significant differences (LSD) test in order to indicate the samples with significant differences. For comparison of the 23 kDa band in the SDS-PAGE gel, a Student's paired t-test was performed. For this purpose, GraphPad Prism 8.0.2® (GraphPad Software, San Diego, CA, USA) was used, and values of $p < 0.05$ were considered significant. The response surface methodology design and the rest of statistical analyses were performed using Statgraphics Centurion XV® software (Statpoint Technologies, Inc., The Plains, VA, USA).

Table 1: Conditions of time of extraction (min), temperature (°C) and pH for the 16 experiments included in the response surface optimization.

Run #	Time of Extraction	Temperature (°C)	pH
1	15	20	7
2	30	20	8.5
3	30	20	5.5
4	0.5	20	8.5
5	0.5	20	5.5
6	15	35	7
7	15	35	7
8	15	35	8.5
9	15	35	5.5
10	30	35	7
11	0.5	35	7
12	15	50	7
13	30	50	8.5
14	30	50	5.5
15	0.5	50	8.5
16	0.5	50	5.5

3. Results

3.1 Impact of Extraction Time, Temperature and pH on the Selective Extraction of Nutrients and Antioxidants

The ultrasound-assisted extraction (UAE) was optimized using a response surface methodology Box–Behnken design with two central points. The optimization was carried out to obtain the maximum values of all the studied responses: proteins, carbohydrates, chlorophyll A, total carotenoids, total phenolic compounds, TEAC (Trolox Equivalent Antioxidant Capacity) and ORAC (Oxygen Radical Absorbance Capacity). For the optimization, the following parameters were taken into account: extraction time 0.5–30 min, temperature 20–50 ºC and pH 5.5–8.5.

3.1.1 Nutrients (Proteins and Carbohydrates)

The protein and carbohydrate values for each extraction condition are shown in **Table 2**. As can be seen in the table, the protein values ranged from 4.14 to 6.10 g/100 g of dry matter, being the optimal conditions for the maximal protein recovery (24.4 min, 20 °C and pH 8.5) obtained under this condition: 5.96 g of proteins/100 g dry matter. On the other hand, the values for the carbohydrates ranged from 1.39 g/100 g dry matter to 2.52 g/100 g dry matter. In this case, the optimal conditions for the highest carbohydrate extraction were 30 min, 50 °C and a pH of 8.5, obtaining 2.53 g of carbohydrates/100 g dry matter.

The influence of the extraction time, temperature and pH for the proteins and carbohydrates is shown in Figures 1 and 2, respectively. Regarding the protein, as can be seen in **Figure 1**, although it seems that both the temperature and time affected protein extraction, the ANOVA analysis revealed that these changes were not significant (p = 0.0553 for temperature and 0.1690 for the time of extraction). The pH did not show any significant influence (p = 0.6355). On the contrary, regarding the carbohydrate extraction, the temperature clearly influenced the extraction (p = 0.0040), while the pH and time of extraction did not have a significant influence (p = 0.2954 and 0.6061, respectively). Regarding the temperature, as can be seen in **Figure 2**, an important increase in

carbohydrate extraction was found, obtaining the maximum yield at temperatures between 45–50 °C. This fact can be explained due to the modification of the integrity of the cell wall after the increase in temperature, then the solvent solution contact with the intracellular compounds was facilitated, thus improving their extraction [27]

In a similar way, other authors did not observe any significant influence of these parameters for protein extraction from microalgae. In this sense, Lupatini et al. [28] did not find a significant effect from the sonication time, temperature or pH when extracting proteins from Spirulina platensis. Moreover, the same authors reported a significant and positive influence of the temperature for carbohydrate extraction. On the other hand, Sánchez-Zurano et al. [29] optimized the UAE of protein extraction from Spirulina microalgae, using a response surface method with a central composite design. They found that the extraction time (10–120 min) and pH (9–11) had a significant effect on protein solubilization. Finally, Hildebrand et al. [19] also reported that ultrasound treatment increased the protein extraction from Chlorella vulgaris, especially at a basic pH (in a NaOH medium).

Table 2: Proteins and carbohydrates (g/100 g dry matter) obtained after ultrasound-assisted extraction at different times, temperatures and pH levels

Run	Time of Extraction (min)	Temperature (°C)	pH	Proteins (g/100 g Dry Matter)	Carbohydrates (g/100 g Dry Matter)
1	15	20	7	5.19	1.48
2	30	20	8.5	6.10	1.56
3	30	20	5.5	5.37	1.79
4	0.5	20	8.5	4.74	1.39
5	0.5	20	5.5	5.47	2.16
6	15	35	7	5.03	2.13
7	15	35	7	4.80	2.01
8	15	35	8.5	5.14	2.33
9	15	35	5.5	5.40	2.03
10	30	35	7	4.28	1.90
11	0.5	35	7	4.71	1.91
12	15	50	7	5.34	2.10
13	30	50	8.5	5.23	2.52
14	30	50	5.5	4.69	2.03
15	0.5	50	8.5	4.14	1.84
16	0.5	50	5.5	4.95	2.22

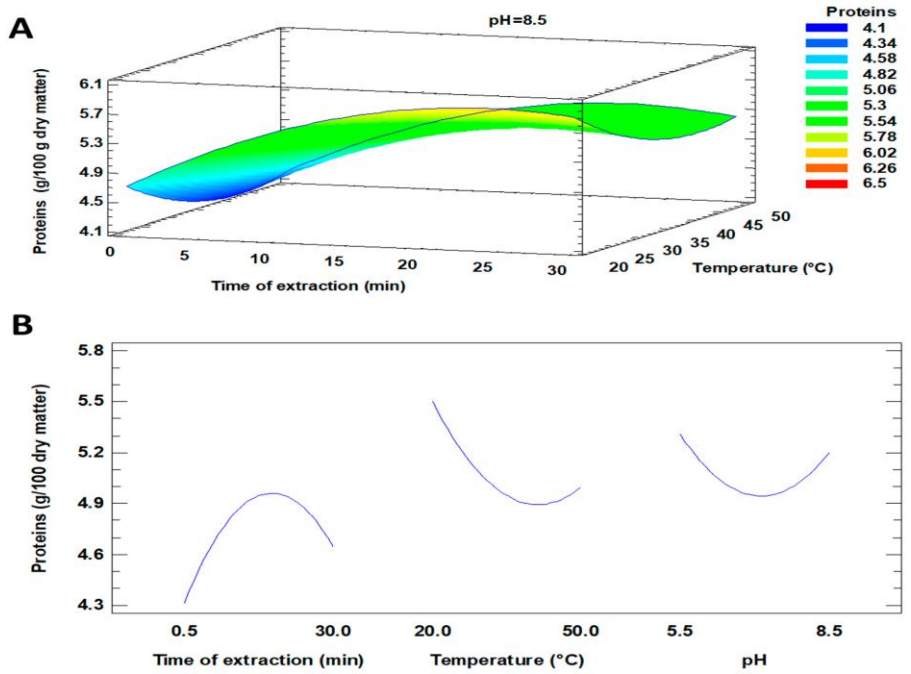

Figure 1: Influence of the different extraction conditions (**A**) and main effects chart for these conditions (**B**) on the protein recovery yield (g/100 g dry matter). The least relevant factor (highest *p*- value) has been set at its optimal value.

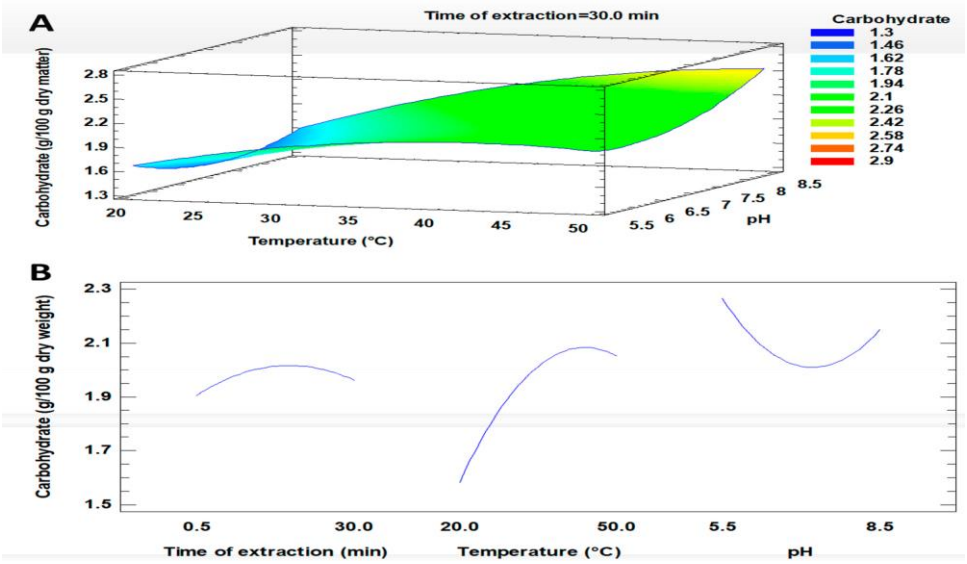

Figure 2: Influence of the different extraction conditions (**A**) and main effects chart for these conditions (**B**) on the carbohydrate recovery yield (g/100 g dry matter). The least relevant factor (highest *p*-value) has been set at its optimal value.

3.1.2 Antioxidant Capacity and Compound

Regarding pigment extraction, the chlorophyll A values ranged from 17.99 to 37.95 mg/100 g dry matter, and the carotenoid values were quite smaller, ranging from 0 to 4.93 mg/100 g dry matter (**Table 3**). The optimal conditions for the highest extraction of chlorophyll A were 0.5 min, 20 °C and a pH of 5.5, obtaining a theoretical value of 36.28 mg/100 g dry matter. The optimal conditions for carotenoid recovery were 30 min, 20 °C and a pH of 8.5, reaching a theoretical value of 4.87 mg/100 g dry matter. As can be observed in the table, the theoretical optimal values were really close to those obtained experimentally with the same conditions (36.28 vs. 35.56 for chlorophyll A and 4.87 vs. 4.93 mg/100 g dry matter for the carotenoids). On the other hand, the total phenolic compounds (TPCs) ranged from 316.76 to 761.55 mg GAE/100 g dry matter (**Table 3**). The optimal conditions for their extraction were 16.07 min, 20.05 °C and a pH of 5.5, obtaining a value of 854.70 mg GAE/100 g dry matter.

Table 3: Chlorophyll A, total carotenoids (mg/100 g dry matter) and total phenolic compounds (TPC) (mg GAE/100 g dry matter) obtained after ultrasound-assisted extraction at different times, temperatures and pH levels. GAE: Gallic Acid Equivalent.

Run #	**Time of Extraction (min)**	**Tª (°C)**	**pH**	Chlorophyll A (mg/100 g Dry Matter)	Carotenoids (mg/100 g Dry Matter)	TPC (mg GAE/100 g Dry Matter)
1	15	20	7	27.44	1.91	731.00
2	30	20	8.5	37.95	4.93	761.55
3	30	20	5.5	22.53	2.76	659.63
4	0.5	20	8.5	21.57	1.91	474.73
5	0.5	20	5.5	35.56	0.00	645.69
6	15	35	7	26.75	1.67	689.54
7	15	35	7	22.47	1.95	680.89
8	15	35	8.5	20.90	2.11	707.85
9	15	35	5.5	33.45	3.22	672.36
10	30	35	7	21.22	2.16	461.25
11	0.5	35	7	28.34	0.59	316.76
12	15	50	7	22.94	1.55	736.82
13	30	50	8.5	35.07	2.64	599.00
14	30	50	5.5	28.35	0.49	598.96
15	0.5	50	8.5	17.99	1.38	514.40
16	0.5	50	5.5	24.16	1.48	719.68

For chlorophyll A, none of the parameters studied significantly influenced its extraction, obtaining p values much higher than 0.05. In this sense, Parniakov et al. [17] did not find differences in the chlorophyll extraction in aqueous media for Nannochloropsis spp. when increasing the time of the ultrasound treatment. Although they were not significant, it seemed that the extraction time had a positive influence on the chlorophyll A extraction, while the temperature increase had a negative effect on it (**Figure 3**).

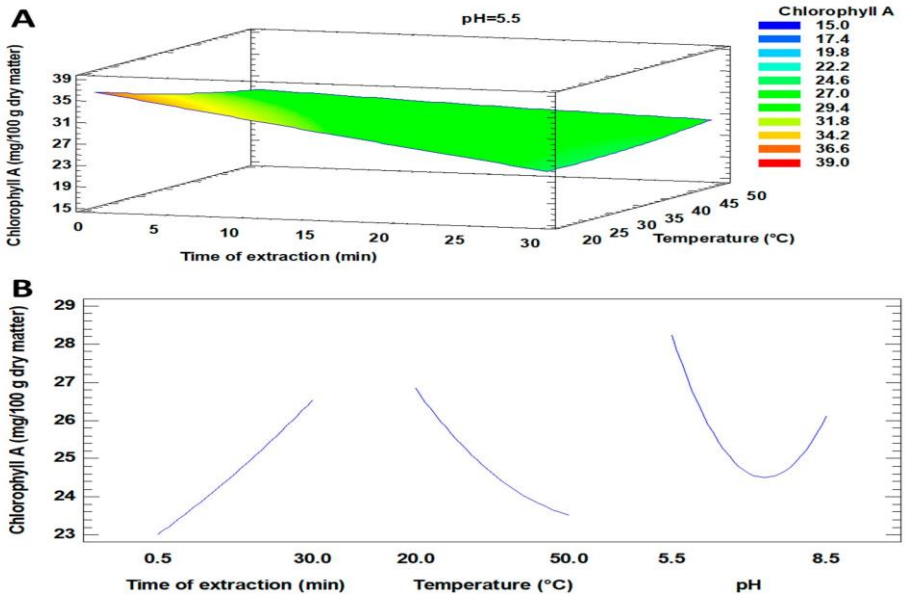

Figure 3: Influence of the different extraction conditions (A) and main effects chart for these conditions (B) on chlorophyll A's recovery yield (mg/100 g dry matter). The least relevant factor (highest *p*-value) has been set at its optimal value

For the carotenoids, the extraction time had a strong influence (p = 0.0192), although the temperature and pH did not have a significant effect (p = 0.1493 and 0.0815, respectively). The extraction time clearly improved carotenoid extraction, while the temperature had a slight impact, decreasing carotenoid recovery. Additionally, at pH = 7, the carotenoid extraction was at its minimum, while the maximum value was obtained at a pH level of 8.5 (**Figure 4**). Gilbert-López et al. [30], who optimized the microwave and pressurized liquid extraction of carotenoids from P.

tricornutum, also described a reduction in carotenoid extraction when the temperature increased, probably due to compound degradation.

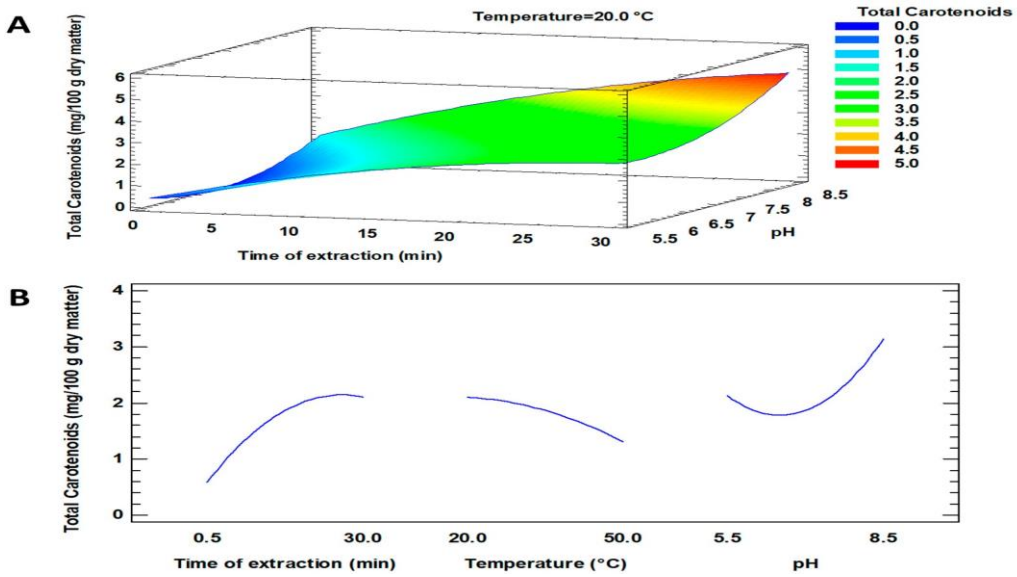

Figure 4: Influence of the different extraction conditions (**A**) and main effects chart for these conditions (**B**) on the total carotenoid recovery yield (mg/100 g dry matter). The least relevant factor (highest *p*-value) has been set at its optimal value.

Regarding the TPC, the most influential parameter in their extraction was the time (p = 0.0496). The efficiency of the extraction increased with the passage of time (up to 16 min) when the TPC value was at its maximum. After 16 min, this value decreased (**Figure 5**). This is in close agreement with the results reported by Parniakov et al. [17] for *Nannochloropsis* spp., who found that the optimal extraction of the total phenolic compounds assisted by ultrasound (W= 400 W) was achieved after 15 min. Martínez-Sanz et al. [31] also found a huge increase in the TPC extraction from *Nannochloropsis gaditana*, *Scenedesmus almeriensis* and *Spirulina platensis* after ultrasound treatment for 5 min. The temperature and pH did not significantly influence the polyphenol extraction (p = 0.5568 and 0.2021, respectively). Other authors, such as Yucetepe et al. [32], also reported that neither the temperature nor the pH had a significant influence on the UAE of TPC from *Spirulina platensis*.

Finally, regarding the total antioxidant activity, the ABTS values ranged from 563.82 to 760.11 μM TE, while the ORAC values where higher, ranging from 1416.81 to 2340.01 μM TE. The optimal conditions for the maximum antioxidant capacity measured by the ABTS method were 28.36 min, 20 °C and pH = 5.5, obtaining a theoretical value of 758.28 μM TE. On the other hand, for the ORAC assay, the best conditions were 30 min, 47.65 °C and pH = 8.5. The theoretical antioxidant activity measured by the ORAC assay applying the optimal conditions was 2338.54 μM TE, really close to the experimental values obtained with the extraction for 30 min at 50 °C and pH = 8.5 (2340.01 μM TE) (**Table 4**). These values of the antioxidant capacity are in the same range as those described in the literature for *P. tricornutum* [30,33].

As can be seen in **Figure 6**, the antioxidant activity measured by the ABTS assay was strongly influenced by the extraction time ($p = 0.0044$), but the temperature ($p = 0.1386$) and pH ($p = 0.9547$) did not show a great impact. The increase of the extraction time led to a clear improved antioxidant capacity, from 698.57 μM at 0.5 min to 760.11 μM at 30 min (at the optimal conditions for the temperature and pH, 20 °C and 5.5, respectively). This can be explained by the increase in the antioxidant compounds´ extraction with the passage of time. Akyıl et al. [34] also found a significant influence of the extraction time on the antioxidant activity, measured by DPPH, when they optimized the bioactive compound extraction from *P. tricornutum*. However, Kokkali et al. [15] found a minimal decrease in antioxidant activity, measured by ABTS in *P. tricornutum* aqueous extracts, comparing 4 h and 24 h of extraction. The differences with our study can be explained regarding the huge time interval. For instance, in the present study, a time range was established from 0.5 min up to 30 min, while in the study conducted by Kokkali et al. [15], the time range was from 4 h to 24 h.

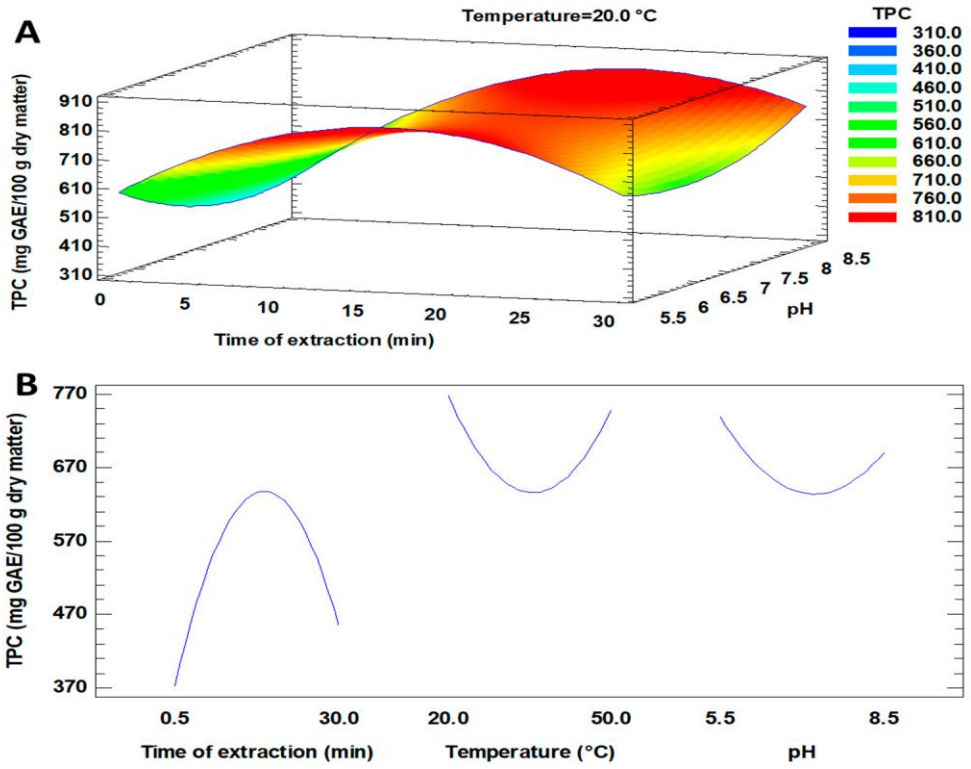

Figure 5: Influence of the different extraction conditions (**A**) and main effects chart for these conditions (**B**) on the total phenolic compounds (TPC) recovery yield (mg/100 g dry matter). The least relevant factor (highest *p*-value) has been set at its optimal value.

Table 4: Antioxidant activity (µM TE) measured by the ABTS and oxygen radical antioxidant capacity (ORAC) methods, obtained after ultrasound-assisted extraction at different times, temperatures and pH levels

Run#	Time of Extraction (min)	Temperature (°C)	pH	ABTS (µM TE)	ORAC (µM TE)
1	15	20	7	658.89	1416.81
2	30	20	8.5	701.41	1842.10
3	30	20	5.5	760.11	1681.80
4	0.5	20	8.5	696.02	1766.48
5	0.5	20	5.5	698.57	1693.02
6	15	35	7	673.39	1972.92
7	15	35	7	690.40	1863.11
8	15	35	8.5	715.77	2048.95
9	15	35	5.5	700.56	1924.95
10	30	35	7	726.47	1973.97
11	0.5	35	7	563.82	1541.58
12	15	50	7	670.33	1910.16
13	30	50	8.5	721.49	2340.01
14	30	50	5.5	718.51	1892.14
15	0.5	50	8.5	638.03	1812.60
16	0.5	50	5.5	600.73	1805.78

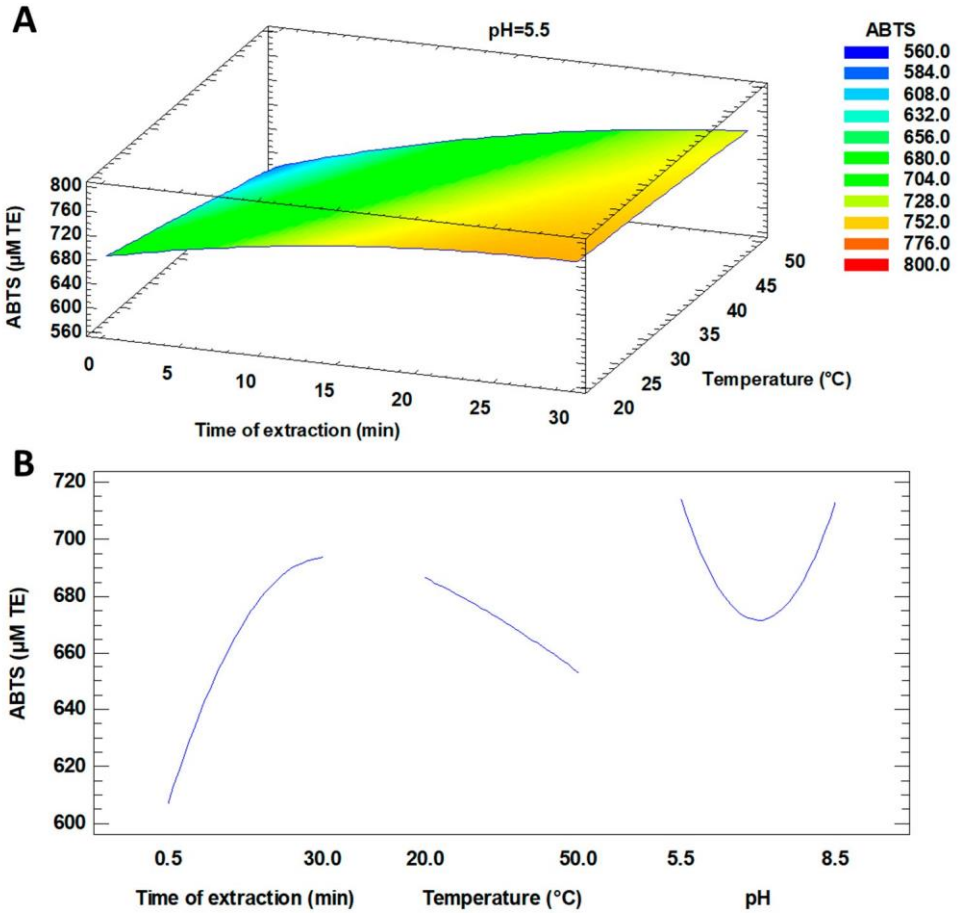

Figure 6: Influence of the different extraction conditions (**A**) and main effects chart for these conditions (**B**) on the total antioxidant activity (µM TE), measured by the ABTS method. The least relevant factor (highest *p*-value) has been set at its optimal value.

On the other hand, the extraction time also significantly influenced (p = 0.0364) the antioxidant activity, measured by the ORAC method. Moreover, the temperature also affected the ORAC value even stronger (p = 0.0167) than the extraction time (**Figure 7**). Concerning the extraction time, it can be seen that at 20 °C and pH = 8.5, the antioxidant activity was 1766.48 µM TE at 0.5 min. However, when the time increased up to 30 min, the antioxidant activity was augmented (1842.10 µM TE). Furthermore, increasing the temperature also resulted in an improvement of the antioxidant activity. In our study, at pH = 8.5 and after 30 min of extraction (optimal conditions), the antioxidant activity measured by the ORAC assay was enhanced from 1842.10 µM TE at 20 °C up to 2340.01 µM TE at 50 °C. As can be seen, the ORAC values increased with the increasing temperature and extraction time,

despite the fact that all the possible antioxidant compounds studied in the present work decreased under these conditions. This may be due to the presence of other compounds not identified in this study that may have had an impact on the antioxidant capacity measured by the ORAC method. It has been seen that the ORAC assay had a higher affinity for lipophilic compounds. Therefore, it could be that with these conditions, the amount of antioxidant lipid compounds extracted increased, contributing to the enhanced antioxidant capacity values measured by the ORAC assay [33].

German-Baez et al. [35] found lower values for the ABTS and ORAC assays when measuring the antioxidant capacity of *P. tricornutum* residual biomass (67.93 and 106.22 μM TE/g dry weight, respectively). However, this difference can be explained by their use of a microalgae by-product from biofuel production instead of full microalgae. Then, the presence of the antioxidant compounds would be lower than in the original microalgae.

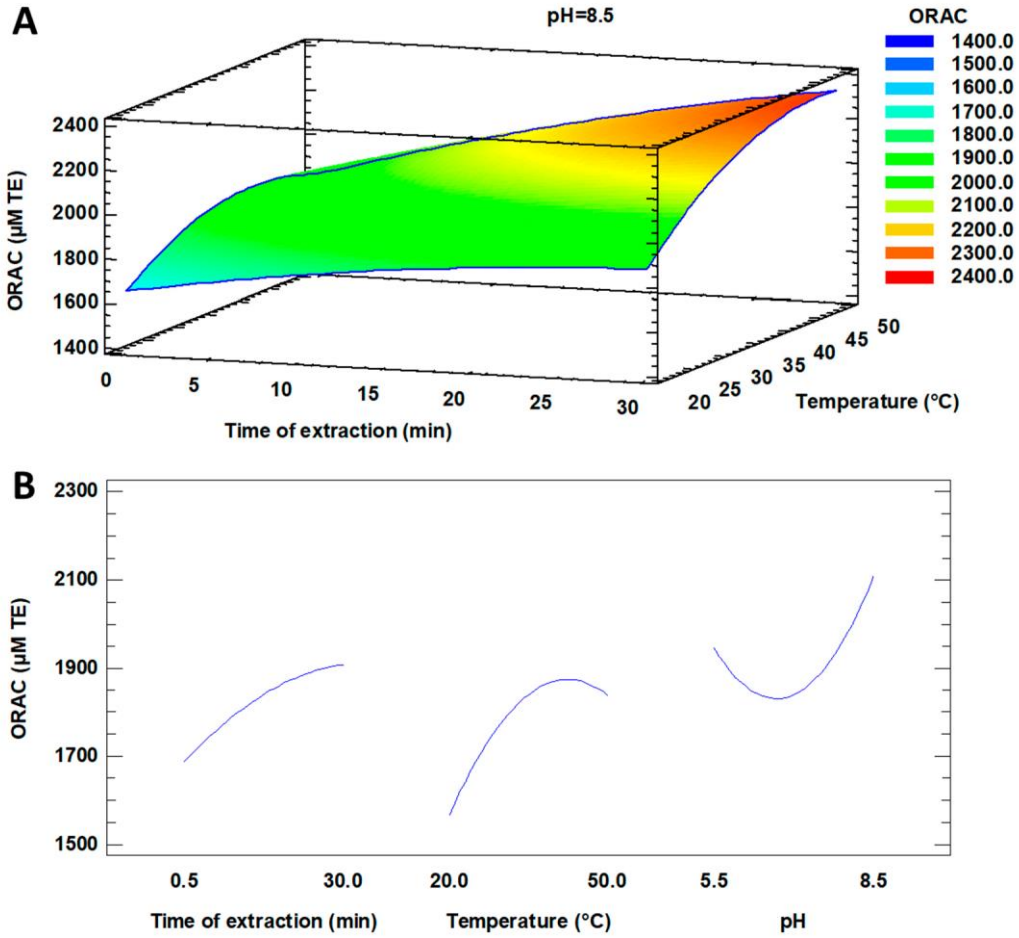

Figure 7: Influence of the different extraction conditions (A) and main effects chart for these conditions (B) on the total antioxidant activity (µM TE), measured by the ORAC method. The least relevant factor (highest *p*-value) has been set at its optimal value.

3.1.3 Optimization

Once the optimal conditions for each response studied were obtained, the method was optimized to achieve the maximum yield in all of them. After multiple optimizations, the overall optimal conditions obtained were 30 min of extraction at 50 °C and a pH level of 8.5 (**Table 5**). As can be seen in **Figure 8**, the desirability obtained at the optimal conditions was 0.72. This low result can be due to the different behavior of the studied responses. For instance, in this study, pigment extraction decreased when the temperature increased, while the antioxidant activity had the opposite behavior. This could explain why the temperature did not influence the overall response.

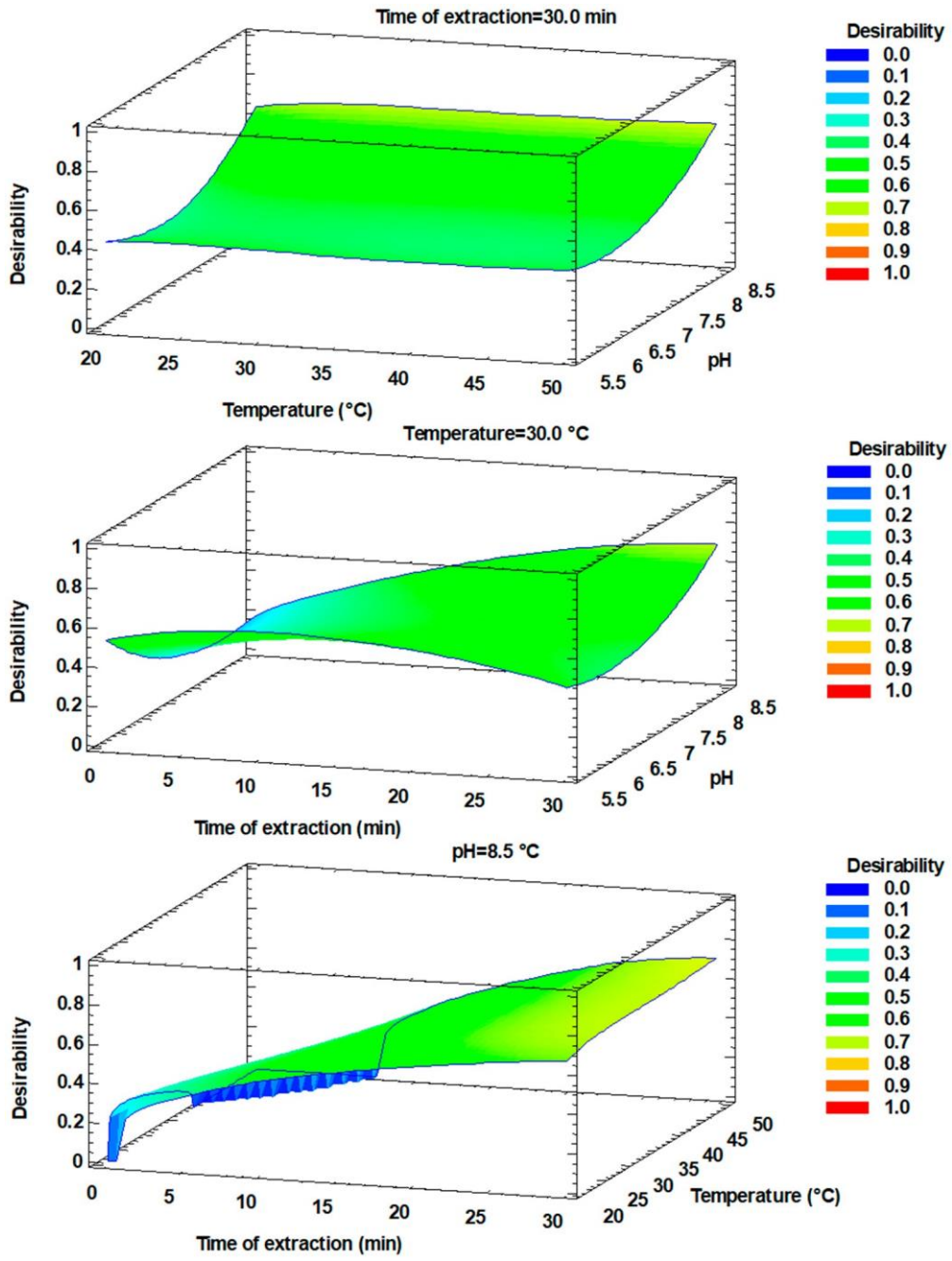

Figure 8: Influence of the different extraction conditions on the optimization desirability for the higher yield of all the studied responses. The fixed factor in each graph has been set at its optimal value.

Table 5: Time of extraction, temperature and pH ranges and optimal values obtained after the optimization of the response surface model.

Parameter	Min	Max	Optimal
Time of extraction	0.5	30.0	30.0
Temperature (°C)	20.0	50.0	50.0
pH	5.5	8.5	8.5

It would be necessary to further investigate whether longer extraction times could improve the recovery of these compounds and the total antioxidant capacity. However, it must be taken into account that prolonged ultrasound treatment could end up degrading the extracted compounds. On the other hand, it is not advisable to increase the temperature further because it can degrade bioactive compounds, such as pigments [17]. Finally, a broader range of pH should be also studied.

3.2 Influence of the Extraction Method on the Protein Profile and Molecular Weight Distribution

After obtaining the optimal conditions for the UAE of the nutrients and bioactive compounds with antioxidant capacity, the protein extraction was compared with a control sample. To obtain the control sample, an extraction was carried out with the same optimal parameters (30 min, 50 °C and pH = 8.5) by shaking and without ultrasound treatment. In **Figure 9A**, the protein profiles of both the optimal and control samples are shown. As can be seen in the figure, there is a strongly marked band above 23 kDa in all of them. According to previous studies, this band fit with fucoxanthin, which has a molecular weight of 17–23 kDa from the fucoxanthin–chlorophyll complex [36–39]. The quantification of these bands, based on the BSA (Bovine Serum Albumin) standard of 60 μg/mL, is shown in **Figure 9B**. As can be appreciated, there were no significant differences between the control samples and the optimal ones. Then, it can be concluded that both treatments had a similar fucoxanthin extraction efficiency. Moreover, both treatments were also similar concerning the protein profile, due to there being only one marked band in both treatments.

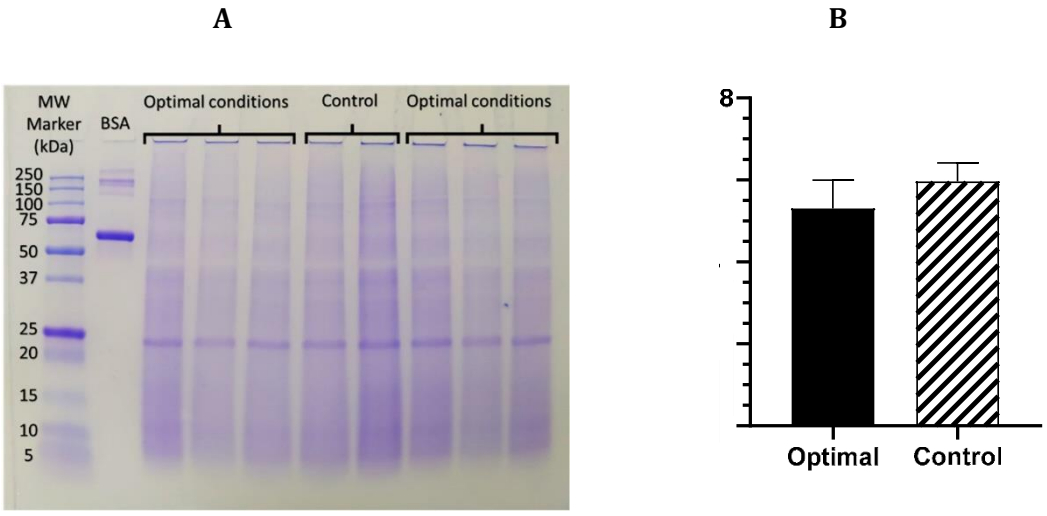

Figure 9: (**A**) Protein profile and molecular weight distribution of the *P. tricornutum* extracts, comparing the ultrasound-assisted extraction (UAE) optimal conditions vs. the control conditions (shaking). The optimal conditions with UAE had six replicates, while the control conditions for extraction (shaking) had two replicates. (**B**) Relative quantification of the band at 23 kDa (fucoxanthin) based on a BSA sample at 60 µg/mL.

4. Conclusion

The optimization of the extraction of nutrients, pigments and polyphenols, in addition to the antioxidant activity, using the response surface methodology gave the optimal extraction conditions of a time of 30 min, a temperature of 50 °C and a pH of 8.5. The influence of the parameters studied (extraction time, temperature and pH) differed according to the target compounds, showing different behaviors depending on the nutrients and antioxidant high added-value components. Therefore, it can be concluded that the microalgae *P. tricornutum* is a good source of nutrients, chlorophyll and phenolic compounds. However, the limitations of the present work are related to the use of relatively short extraction times, as well as a narrow pH range. It should be also mentioned that UAE conditions were applied only at a frequency of 20 kHz and a power of 100 W, so it would be interesting to investigate other treatments and UAE modalities (sonotrode) in future studies. More studies are needed in order to improve the efficiency of the extraction of high added-value compounds from P.

tricornutum, reducing costs, increasing the yield and evaluating the potential scalability of the UAE process.

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4.6 SEA BASS SIDE STREAMS VALORIZATION ASSISTED BY ULTRASOUND. LC-MS/MS-IT DETERMINATION OF MYCOTOXINS AND EVALUATION OF PROTEIN YIELD, MOLECULAR SIZE DISTRIBUTION AND ANTIOXIDANT RECOVERY

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Sea bass side streams valorization assisted by ultrasound. LC-MS/MS-IT determination of mycotoxins and evaluation of protein yield, molecular size distribution and antioxidant recovery

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Abstract:

Sea bass side streams obtained from the fish industry can be a good source of nutrients such as high-quality protein, lipids, and antioxidants. In this context, it is interesting to develop innovative approaches to extract the added-value compounds from fish side streams. In this study, a strategy to obtain valuable compounds and to minimize the presence of toxins from fish side streams assisted by ultrasound technology is presented. For this purpose, ultrasound-assisted extraction (UAE) conditions have been optimized based on a response surface methodology (RSM) with the dependent variables: time (0.5–30 min), pH (5.5–8.5), and temperature (20–50 °C). After the treatment, protein extraction and antioxidant activity were evaluated in the extracts obtained from sea bass side streams using some spectrophotometric and fluorometric methods. Furthermore, mycotoxin presence was evaluated by LC-MS/MS-QTRAP. The results obtained revealed a high recovery percentage of proteins and antioxidant activity in the UAE extracts, especially those obtained from viscera, when the time and temperature increased to 30 min and 50 °C. Furthermore, none of the analyzed mycotoxins were detected in the sea bass side streams extracts under the studied variables. The experimental values obtained were close to the expected values, confirming the validity of the model employed to establish the optimal UAE conditions.

Keywords: sea bass side streams; ultrasound technology; antioxidant capacity; proteins; mycotoxins; LC-MS/MS-QTRAP; response surface methodology

1. Introduction

According to the Food and Agriculture Organization (FAO), total fish production reached up to 171 million tonnes in 2016 [1]. It has been estimated that ≈20–80% of fish weight are side streams (i.e., head, skin, bones, viscera, scales, and tails), which have been traditionally considered as a waste with low added-value, thus representing a potential negative environmental impact [2]. However, they are a great source of nutrients such as high quality protein, fat, and antioxidants, which can protect the human body from free radicals, thus delaying the development of many noncommunicable diseases [3]. For instance, some previous studies have evaluated the use of fish side streams from sardine [4], tuna [5,6], salmon [7], mackerel [8], seabass [9,10], among others, as a source of protein hydrolysates and antioxidant peptides using conventional recovery strategies. However, there is a lack of information regarding the use of innovative approaches to recover proteins from sea bass side streams, and about their impact on protein molecular size distribution and the antioxidant yield.

Ultrasound-assisted extraction (UAE) is a nonconventional technology that has emerged over the last few decades. UAE utilizes acoustic cavitation that promotes molecular movement of solvent and sample, showing some advantages such as efficiency, reduced extraction time, low solvent consumption, and high level of automation. UAE has been reported as an interesting tool for the extraction of protein from the whole fish [11]. It has also been shown as a useful strategy to extract collagen and gelatin from different fish side streams (i.e., skin and scales) [12]. In this line, UAE has been used with different methods, including the green, environmentally friendly solvents, such as the deep eutectic solvents (DES) and their natural equivalents, the natural deep eutectic solvents (NADES) to improve the efficiency of the extraction process, and the tailored recovery of target compounds [13].

Moreover, UAE combined with other techniques can be an efficient tool for mycotoxin extraction from fish [14]. Mycotoxins are toxic chemical compounds resulting from the secondary metabolism of fungi, which can occur on different substrates under certain environmental conditions. They are

natural micropollutants present in food and can affect consumers and animals health at subtoxic doses, due to their simultaneous presence in food and their continued ingestion throughout life. Mycotoxins are related with adverse effects such as hepatotoxicity, nephrotoxicity, estrogenicity, immunotoxicity, mutagenicity, teratogenicity, carcinogenicity, and diabetic action [15]. The toxigenic fungal species most frequently found in food belong to the genera Aspergillus, Fusarium, and Penicillium. Aflatoxins (AFs) are produced by Aspergillus species, and Ochratoxin A (OTA) and Patulin (PAT) by both Aspergillus and Penicillium. Fusarium species produce trichothecenes (HT2, T2, Deoxynivalenol (DON), and Nivalenol (NIV)), Zearalenone (ZEA), Fumonisins (FB1 and FB2) and emerging mycotoxins (Fusaproliferin (FUS), Moniliformin (MON), Beauvericin (BEA) and Enniatins (ENNs)) [16].

Maximum concentrations have been established for some mycotoxins in different raw materials and processed foods based on their toxicity and consumption habits [17], however in fish products maximum levels have not been legislated yet.

Mycotoxin carryover from feed to edible fish tissue has been previously reported in bibliography. Huang et al. [18] and Nomura et al. [19] reported AFB1 contents in muscle and hepatopancreas of gibel carp and in edible muscle of rainbow trout. Moreover, they also found higher contents of AFB1 metabolites (aflatoxicol (AFL) and aflatoxin M1 (AFM1)) after dietary exposure. On the other hand, ENNs were reported in fish species and FUS-X and ENN B in gula substitute samples [20,21].

Due to the low mycotoxin contents in food and the complexity of food matrices, there is a need for sensitive and specific analytical methods in order to determine mycotoxins. Furthermore, an appropriate sample preparation and an exhaustive preconcentration method are also required to efficiently extract the mycotoxins from tested samples prior to their analysis [22]. In this line, the use of UAE has shown promising results for this purpose [23]. For instance, Jayasinghe et al. [14] successfully applied UAE in the extraction of aflatoxins trace amounts from fish. Taking into account that aquaculture fish is frequently exposed to feed-borne mycotoxins and that several studies have

estimated the presence of mycotoxins residues in fish organs and tissues [24], it is necessary to verify if mycotoxins are present in the extracts obtained after UAE extraction [25].

In this work, a strategy to obtain valuable compounds and minimize the presence of mycotoxins from sea bass side streams is presented. For this purpose, UAE conditions were optimized using a response surface methodology (RSM), a statistical multifactorial analysis of experimental variables and response for protein and antioxidant recovery. Moreover, the effect of ultrasound treatment on the protein quality was evaluated through the determination of protein molecular size distribution using SDS–PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis). Furthermore, mycotoxins presence has been evaluated in the extracts obtained after the treatment. For that purpose, some spectrophotometric, fluorometric, and LC-MS/MS-QTRAP assays have been carried out.

2. Materials and Methods

2.1 Chemicals and Reagents

Glacial acetic acid and ethanol (99.8%) were obtained from Panreac (Castellar del Vallés, Barcelona, Spain). High-performance liquid chromatography (HPLC) grade acetonitrile (ACN), methanol (MeOH), and chloroform (CHCl3) (99%) were purchased from Merck (Darmstadt, Germany). Ethyl acetate (EtOAc) (HPLC-grade, >99.5%) was obtained from Alfa Aesar (Karlsruhe, Germany). Sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), and dimethyl sulfoxide (DMSO) were acquired from VWR (Saint-Prix, France). Sulfuric acid (96%) and hydrochloric acid (HCl) were obtained from Merck (Whitehouse Station, NJ, USA). Deionized water (resistivity >18 MΩ cm−1) was prepared in the laboratory using a Milli-Q SP Reagent Water System (Millipore Corporation, Bedford, MA, USA). ABTS (2,2′-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic acid), Trolox (6-hydroxy2,5,7,8-tetramethylchroman-2-carboxylic acid), AAPH (2,2′-azobis-(2-amidinopropane) dihydrochloride), and potassium persulfate ($K_2S_2O_8$) were purchased from Sigma–Aldrich (Steinheim, Baden-Württemberg, Germany). Tris(hydroxymethyl) aminomethane, potassium phosphate monobasic

(K_2HPO_4), potassium phosphate dibasic (K2HPO4), and sodium phosphate dibasic (Na2HPO4) were purchased from Merck (Darmstadt, Germany). Sodium fluorescein was obtained from Fluka Chemie AG (Bunds, Switzerland); 8–16% Mini-PROTEAN® TGX™ Precast gels, molecular weight marker Precision Plus Protein™ 5–250 kDa, and Coomassie brilliant blue R-250 were purchased to BioRad (Hercules, CA, USA). Dithiothreitol (DTT) was obtained from VWR (Leuven, Belgium).

Mycotoxins standards of AFB1 (≥98% purity), AFB2 (≥98%), AFG1 (≥98%), AFG2 (≥98%), ZEA (≥99%), OTA (≥98%), BEA (≥97%), ENA (≥95%), ENA1 (≥95%), ENB (≥95%), and ENB1 (≥95%) were supplied by Sigma (St. Louis, MO, USA). Individual stock solutions were prepared at 100 mg/L in methanol. All solutions were stored in the dark at −20 °C until LC-MS/MS-IT analysis.

2.2 Samples

Sea bass fresh fish samples were collected from a local supermarket and transported on ice. Side streams (heads, skin, bones, and viscera) were manually obtained from the sea bass fish samples (see **Figure 1**). Each side stream was homogenized using a grinder and then packaged and stored at −20 °C until analysis.

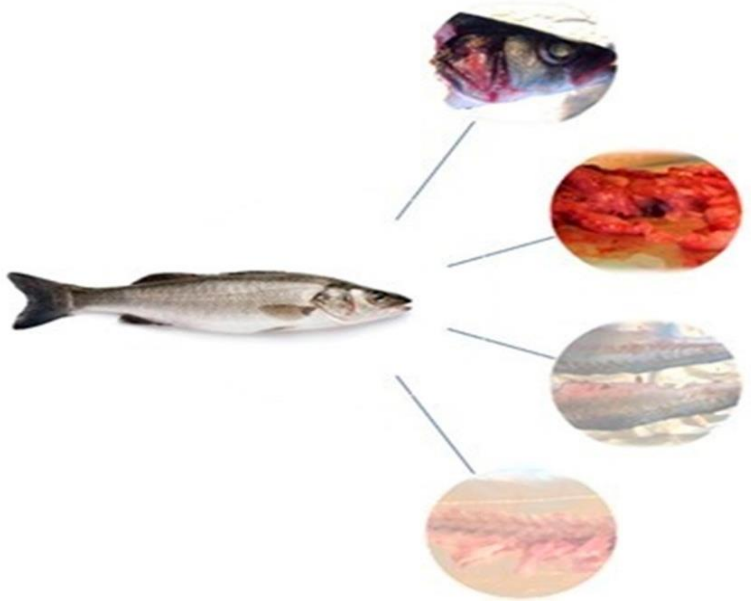

Figure 1: Sea bass side streams (head, viscera, skin, and bones)

2.3 Ultrasound-Assisted Extraction

The ultrasound-assisted extractions were carried out using a Branson 5200 ultrasonic bath (Branson Ultrasonic Corp., CT, USA) under 20 KHz frequency and power of 100 W. For the extraction, two grams of each fish side stream sample were placed in a 100 mL beaker containing 30 mL of distilled water. Temperature and pH were adjusted in the parameters set. The beaker was then sealed with paraffin and placed in the ultrasonic bath. The extracts were placed in 15 mL tubes and preserved at −20 °C for further tests.

2.4 Determination of Total Protein and Molecular Size Distribution using SDS-PAGE Electrophoresis

The total protein content of the extracts obtained was determined using the Kjeldahl assay (AOAC) with some modifications [26]. Briefly, 2 g of sample, 3 g of potassium sulfate and 4–5 drops of copper sulfate were digested with 10 mL of sulfuric acid. Then, the digested sample was distillated with sodium hydroxide (40%) and distilled ammonia was collected in an Erlenmeyer flask with boric acid (4%). Finally, it was valorated with hydrochloric acid 0.1 N. Total protein content was calculated by multiplying by the conversion factor of 6.25.

SDS-PAGE electrophoresis was performed based on the method previously described by Marti-Quijal et al. [27]. After the precipitation of proteins with acetone (in a relation 1:4 (v/v) for sample:acetone) and subsequent centrifugation, the pellet was resuspended in distilled water. This suspension was mixed with the same volume of sample buffer and denaturalized at 95 °C for 5 min. Then, 10 µL were loaded on an 8–16% Mini PROTEAN® TGX™ Precast gel and the electrophoresis was run at 120 V for the first 30 min and then at 80 V. In order to estimate the molecular weight, Precision Plus Protein™ 5–250 kDa was used. When electrophoresis finished, the gel was stained using 0.125% Coomassie brilliant blue R–250 and afterwards it was destained using a mixture of methanol (20%) and acetic acid (10%). For the analysis of the gel, the ImageJ software® was used. Sample buffer was prepared by mixing 62.5 mM Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, 50 mM

dithiothreitol, and 0.01% bromophenol blue. Running buffer was prepared by mixing glycine (192 mM), Trizma® base (25 mM), and SDS (0.1%).

2.5 Determination of Total Antioxidant Capacity

The ABTS assay was performed following the method described by Marti-Quijal et al. [27]. ABTS radical cation was generated by reacting 25 mL of ABTS (7 mM) with 440 µL of potassium persulfate (140 mM). The mixture was incubated in dark conditions for 12–16 h at room temperature. Prior to assay, ABTS radical cation was diluted with ethanol 1:100 to obtain an absorbance of 0.70 (±0.02) at 734 nm. The standard curve of prepared Trolox (5 mM) was constructed at different concentrations (0, 50, 100, 150, 200, 250, 300 µM) employing ethanol. The assay was performed with 2 mL of ABTS+ working solution as the initial point of reaction (A0). Then, 0.1 mL of diluted sample extracts or Trolox standards were added and the absorbance was determined as (Af). The initial absorbance (A0) and the final absorbance (Af) (after 3 min) were read using spectrophotometry at 734 nm in a Perkin-Elmer UV/Vis Lambda 2 spectrophotometer (Perkin-Elmer, Rodgau-Jügesheim, Germany). The percentage of inhibition was calculated as: % inhibition = (1 − (Af/A0)) × 100 (1).

The antioxidant activity was determined using the Trolox standard curve and expressed as µM trolox equivalents (TE). Oxygen radical absorbance capacity (ORAC) was determined according to the method previously detailed by De la Fuente et al. [28], with some modifications. The reaction was carried out in 75 mM phosphate buffer (pH 7), for a final reaction volume of 125 µL. Fifty microliters of sample, loaded onto a 96-well microplate, were mixed with 50 µL of fluorescein, and the mixture was preincubated at 37 °C for 10 min. Then, 25 µL of AAPH solution was added rapidly, using micropipette multimode. The plates were immediately placed in the reader Multilabel Plate Counter VICTOR3 1420 (Perkin-Elmer, Turku, Finland) and the fluorescence recorded every minute for 60 min with an excitation wavelength of 485 nm and emission wavelength of 528 nm. The phosphate buffer (as blank) and the Trolox (as standard) were used in this assay. Each extract was analyzed in

five replicates, and the differences in areas under the fluorescein decay curve (AUC) between the blank and the sample were used to calculate the antioxidant activity.

2.6. Determination of Mycotoxins

Selective methods are required for quantitative mycotoxins extraction from the original food matrix. The mycotoxins extraction from the sample is a critical step and some important parameters can be optimized, such as the nature of the extraction solvent, temperature, time, and purification steps. For multiple mycotoxin analysis, good recoveries are obtained with different solvents such as acetonitrile (AcN), or a mixture of AcN/methanol (MeOH), usually using acidic conditions. There is not to be expected an important extraction of mycotoxins with only water, due to their low solubility in this solvent. For instance, in this work, our purpose for using water was to extract the high-added-value compounds (protein and antioxidants) from sea bass side streams, but not the mycotoxins.

In a previous work carried out in our laboratory, UAE resulted to be a good procedure for mycotoxins extraction, being an effective tool for emerging mycotoxins extraction after applying ultrasound (20 kHz, 100 W, 30 min, 30 °C) using AcN as an extraction solvent, obtaining mycotoxin recoveries ranging from 78 to 91% [21]. In the present work, water was tested as a solvent to extract mycotoxins from the sea bass side streams, in the same conditions of time and temperature detailed above. For this, recovery experiments were performed for 11 mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, ENNA, ENNA1, ENNB, ENNB1, and BEA) comparing absolute peak areas of each analyte in a viscera blank sample spiked before the extraction and absolute peak areas of each analyte spiked after the procedure. However, in this case, the recovery percentages obtained after UAE treatment were lower than 25%, showing the low affinity of water to extract mycotoxins from the sea bass side streams. After UAE extraction, dispersive liquid–liquid microextraction (DLLME) was used to preconcentrate and purify mycotoxins in the sea bass side streams extracts before the determination.

2.6.1. Dispersive Liquid–Liquid Microextraction Method (DLLME)

Mycotoxins were extracted from fish side streams aqueous extracts obtained after UAE treatment by employing the DLLME procedure according to Pallarés et al. [29]. The method was readjusted to the sample volume available, 1 mL in this case. For this, 1 mL of the extract was placed with 0.2 g of NaCl in a 15 mL conical tube and shaken for 1 min. Next, 523 µL of the combination of dispersant and extractant solvents AcN/EtOAc prepared in the proportion (9.50 mL/6.20 mL) were added. After shaking for 1 min, a cloudy solution of the three components was formed. The mixture was centrifuged for 5 min at 4000× rpm to allow the separation of the phases; the organic phase separated at the top of the tube was recovered and placed in another tube. Then, in a second step, 523 µL of the dispersant and extractant solvents mixture MeOH/CHCL3 (prepared with 9.50 mL/6.20 mL, respectively) were added to the remaining residue. Next, the mixture was shaken and centrifuged. After centrifugation, the organic phase, located in this case at the bottom of the tube, was separated and placed with the organic phase separated before. Finally, both recovered organic phases were evaporated together to near dryness under a nitrogen stream using a Turvovap LV Evaporator (Zymark, Hoptikinton, MA, USA). The dried residue obtained was reconstituted with 500 µL of 20 mM ammonium formate (MeOH/AcN) (50/50 v/v) and filtered through a 13 mm/0.22 µm nylon filter prior to the determination by LC-MS/MS-IT.

2.6.2. LC-MS/MS-IT Identification and Determination of Mycotoxins

Mycotoxins determination a carried out using an Agilent 1200 chromatograph (Agilent Technologies, Palo Alto, CA, USA) equipped with 3200 QTRAP® (Applied Biosystems, AB Sciex, Foster City, CA, USA) with Turbo Ion Spray (ESI) electrospray ionization. The QTRAP analyzer combines a fully functional triple quadrupole and a linear ion trap mass spectrometer. A Gemini-NX column C18 (Phenomenex, 150 mm × 4.6 mm, 5 particle size) preceded by a guard column was employed. The injection volume was fixed at 20 µL, the flow rate at 0.25 mL/min, and the oven temperature was 40 °C. Mobile phases consisted of 5 mM ammonium formate and 0.1% formic acid water (mobile phase A) and 5 mM

ammonium formate and 0.1% formic acid methanol (mobile phase B). The chromatographic gradient started with a proportion of 0% for mobile phase B, increasing to 100% in 10 min, then decreased to 80% in 5 min, and finally decreased to 70% in 2 min. Then, in 6 min, the column was cleaned and readjusted to the initial conditions and equilibrated for 7 min. Turbo Ion Spray operated in a positive ionization mode (ESI+). Nitrogen served as nebulizer and collision gas. To perform the analysis, the following parameters were set: ion spray voltage at 5500 V; curtain gas, 20 arbitrary units; GS1 and GS2, 50 and 50 psi, respectively; probe temperature (TEM) at 450 °C. The spectrometric parameters (collision energy, cell exit potential, and declustering potential) and the fragments monitored (quantification and confirmation ions) are shown in **Table 1**.

Table 1: Spectrometric parameters of liquid chromatography ion trap tandem mass spectrometry (LC-MS/MS-IT).

Mycotoxin	Retention Time (min)	DPa	Precursor Ion	Quantification Ion Q			Confirmation Ion q		
				CE b	Product Ion	CXP	CE	Product Ion	CXP
AFB1	9.13	46	313.1	39	284.9	4	41	241.0	4
AFB2	9.03	81	315.1	33	286.9	6	39	259.0	6
AFG1	8.86	76	329.0	39	243.1	6	29	311.1	6
AFG2	9.37	61	331.1	27	313.1	6	39	245.1	4
ZEA	10.40	26	319.0	15	301.0	10	19	282.9	4
OTA	10.27	55	404.3	97	102.1	6	27	239.0	6
ENNA	12.62	76	699.4	35	210.1	14	59	228.2	16
ENNA1	12.22	66	685.4	37	210.2	8	59	214.2	10
ENNB	11.60	51	657.3	39	196.1	8	59	214.0	10
ENNB1	11.89	66	671.2	61	214.1	10	57	228.1	12
BEA	12.00	116	801.2	27	784.1	10	39	244.1	6

a DP: declustering potential (volts). b CE: collision energy (volts). c CXP: cell exit potential (volts).

2.6.3. Method Validation

The DLLME method was characterized for the analysis of AFs, OTA, ZEA, ENNs, and BEA in sea bass side streams according to the Commission Decision [30] (**Table 2**). The analytical parameters

determined for method validation were recoveries, repeatability (intraday precision), reproducibility (interday precision), matrix effects, linearity, limit of detection (LOD), and limit of quantification (LOQ). For recoveries at level of 10×LOQ, the intraday and interday precision were between 68 and 120%. Matrix effects revealed that there was no significant signal suppression/enhancement (SSE) for the analyzed mycotoxins with SSE values ranging from 65 to 105%. The LODs and LOQs were obtained using the criterion for both transitions predetermined per each analyzed mycotoxin of S/N ≥ 3 for calculating LOD and S/N ≥ 10 for LOQ. LODs values ranged from 0.05 to 5 μg/l and LOQs from 0.2 to 17 μg/l. Regarding the linearity and regression coefficients obtained, all were higher than 0.990.

Table 2: Analytical parameters for method validation.

Mycotoxin	Recovery c ± RSD d (%)		SSE (%) b	LOD a	LOQ a
	Intraday Precision	Interday Precision			
AFB1	78 ± 6	68 ± 8	75	0.7	2.3
AFB2	96 ± 7	114 ± 9	104	2.4	8.0
AFG1	90 ± 5	120 ± 10	93	0.7	2.3
AFG2	106 ± 8	73 ± 12	86	0.5	1.7
ZEA	80 ± 6	77 ± 7	65	0.2	0.7
OTA	115 ± 9	120 ± 10	72	5	17
ENA	100 ± 7	95 ± 8	85	0.4	1.3
ENA1	99 ± 2	100 ± 6	89	0.2	0.7
ENB	115 ± 5	105 ± 7	105	0.05	0.2
ENB1	98 ± 7	93 ± 8	75	0.1	0.3
BEA	94 ± 8	89 ± 11	99	0.4	1.3

[a] LOD and LOQ are limits of detection and quantification. [b] SSE: signal suppression/enhancement. [c] Recoveries: analysis performed at concentrations of 10 × LOQ. [d] RSD: relative standard deviation.

2.7. Response Surface Methodology Design and Statistical Analysis

The UAE conditions were optimized using the response surface methodology: Box– Behnken design with two central points. Treatment time (X_1: 0.5–30 min), pH (X_2: 5.5–8.5), and temperature (X_3: 20–50 °C) parameters were optimized. The responses studied were total protein content and antioxidant capacity (ORAC and ABTS assays). Fifteen different experiments were established by using the minimum, central, and maximum value for each parameter. Moreover, the central point was

duplicated in order to check the variability and reproducibility. The different combinations are shown in **Table 3**.

In order to obtain the significant differences ($p < 0.05$) between the results, an analysis of variance (ANOVA) followed by least significant differences (LSD) test was performed. All the statistical analysis were performed using Statgraphics Centurion XVI® (Statpoint Technologies, Inc., The Plains, VA, USA). A $p < 0.05$ was considered significative.

Table 3: Dependent variable conditions for the ultrasound-assisted extraction studied.

Run #	Time of Extraction	Temperature (°C)	pH
1	15	20	7
2	30	20	8.5
3	30	20	5.5
4	0.5	20	8.5
5	0.5	20	5.5
6	15	35	7
7	15	35	7
8	15	35	8.5
9	15	35	5.5
10	30	35	7
11	0.5	35	7
12	15	50	7
13	30	50	8.5
14	30	50	5.5
15	0.5	50	8.5
16	0.5	50	5.5

3. Results and Discussion

3.1 Protein Extraction

In order to determine the percentage of recovered proteins from the different sea bass side streams after applying UAE extraction, the Kjeldahl method was used. The results are shown in **Table 4**. It was found that the highest percentage of proteins recovered from head extracts (39.89%), which was observed after 15.25 min of extraction at 35 °C and 5.5 pH, while 31.68% of proteins were recovered from skin extracts after 30 min of extraction at 35 °C and pH 7. Additionally, the bone extracts yielded

75.07% of proteins after 30 min of UAE at 50 °C and pH of 8.5. Lastly, 30 min of ultrasound at 50 °C and a pH 5.5, allowed the extraction of 99.37% of proteins from the viscera extracts.

Similar protein recoveries were obtained by Tian et al. [31]. These authors observed protein yields that reached 62.60% when they evaluated protein recovery from tilapia fillets assisted by UAE combined with alkaline conditions. Moreover, higher protein yields were obtained by Álvarez et al. [11] under UAE + alkaline conditions, with a recovery ≈95% of total protein from mackerel byproducts. In our study, a similar percentage of protein recovery was observed in viscera extracts (99.37%). In general, protein recovery reported in the literature by other authors varies in a range between 42% and 90%. Moreover, data available in the literature revealed that alkaline solubilization usually results in higher protein recoveries than acidic conditions [32]. In our work, proteins recovery optimal pH differed according to the side stream studied.

Table 4: Percentage of protein recovered from sea bass side streams extracted using UAE at different extraction times (min), temperature (°C), and pH.

Run #	Time of Extraction (min)	Temperature (°C)	pH	Protein recovery %			
				Head	Skin	Bon	Viscera
1	15	20	7	17.4	25.07	75.07	93.21
2	30	20	8.5	12.5	19.15	45.36	70.10
3	30	20	5.5	15.7	13.64	23.63	80.48
4	0.5	20	8.5	12.8	31.68	44.26	93.66
5	0.5	20	5.5	24.1	14.11	56.50	85.35
6	15	35	7	17.8	12.54	31.87	85.93
7	15	35	7	14.9	12.49	63.91	82.26
8	15	35	8.5	33.4	18.95	37.94	90.03
9	15	35	5.5	17.4	20.26	33.52	86.42
10	30	35	7	21.8	19.23	54.75	92.50
11	0.5	35	7	31.1	17.12	35.09	84.81
12	15	50	7	20.3	17.19	36.04	93.01
13	30	50	8.5	25.3	12.41	42.56	81.38
14	30	50	5.5	28.1	24.95	38.66	99.37
15	0.5	50	8.5	39.8	24.78	38.28	84.73
16	0.5	50	5.5	31.1	18.79	36.68	77.22

Figure 2A,C,E,G represents the estimated response surface by plotting the protein recoveries from sea bass head, skin, bone, and viscera versus the extraction time, temperature, and a fixed pH,

for each side stream, while **Figure 2**B,D,F,H represents the influence of the studied parameters on the protein recovery. As can be observed in **Figure 2**A,B, under the tested treatment conditions, the protein recovery from head extracts increased with the elapse of extraction time from 0.5 to 15.25 min and increased temperature (from 20 to 35 °C), respectively. However, when both extraction time and temperature increased up to 30 min and 50 °C, the protein recovery reached a plateau and slowly decreased. Nevertheless, the effects of these parameters are not statistically significant ($p > 0.05$). On the other hand, the pH significantly ($p < 0.05$) affected the recovery of proteins, where a lower pH lead to a higher recovery ($p = 0.0091$). According to RSM, the optimal conditions for the recovery of proteins (40.65%) from head extracts are 15 min of UAE at 35 °C and 5.5 pH.

As shown in **Figure 2**C,D, increasing extraction times from 15 to 30 min with simultaneous increase of temperature up to 35 °C, progressively increased the recovery of proteins from skin. However, none of the studied parameters had a statistically significant impact ($p > 0.05$) on the recovery of proteins from skin extracts. The optimal conditions generated by RSM were extraction time 30 min, temperature 37 °C, pH 5.5 with a 28.13% protein recovery.

For the bone and viscera (**Figure 2**E–H), the percentage of protein recovery significantly increased as the extraction time increased ($p \leq 0.01$). Higher pHs had a positive effect on the recovery of protein from bone ($p = 0.0125$). On the other hand, higher temperatures strongly affected the recovery from the viscera extracts ($p = 0.0072$). Consequently, under the optimal conditions of UAE (30 min, 50 °C, and 8.5 pH), 70.25% of proteins were recovered from bone extracts. Likewise, 96.07% of proteins from viscera extracts were recovered under optimal UAE (30 min, 50 °C, pH 5.5).

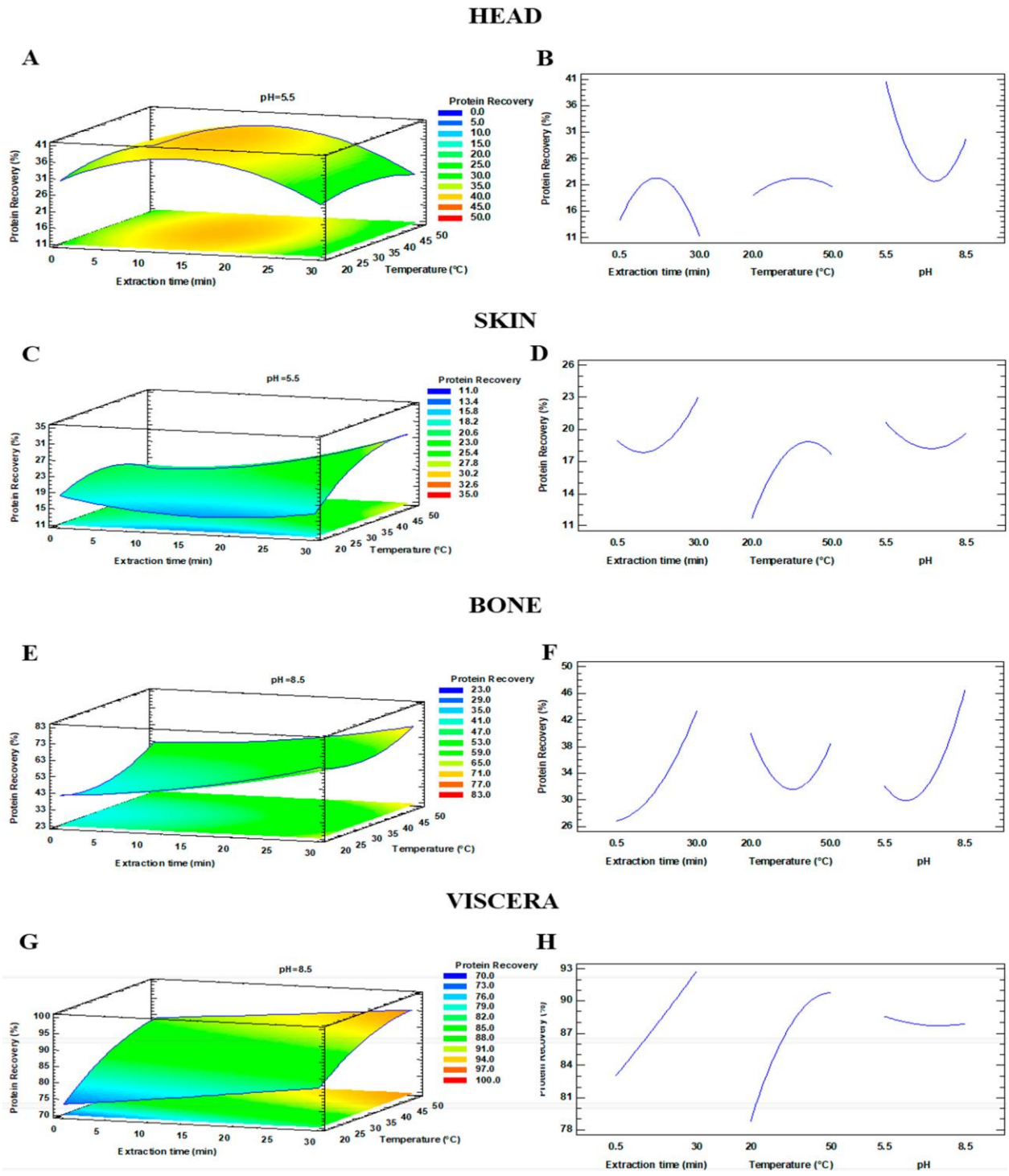

Figure 2: Plots shown in (A,C,E,G) indicate the response surface plot for the percentage of recovered protein as a function of the extraction time (min) and temperature (°C) at fixed pH. The plots in (B,D,F,H) show the influence of the different parameters (extraction time, temperature, and pH) on the recovery of protein (%) from sea bass side streams.

3.2 Determination of Antioxidant Capacity

The ABTS values for each extraction are shown in **Table 5**. The antioxidant activity from the head extracts ranged from 9.37 to 129.38 μM TE, obtaining the highest values after 30 min of UAE extraction at 20 °C and pH 8.5. The highest activity observed from the skin extracts was found after UAE at 30 min, 20 °C, and pH 5.5, whereas for the bone extracts, the values ranged from 28.94 to 276.23 μM TE, achieving the maximum value at 30 min, 20 °C, and pH 5.5. Lastly, the uppermost activity (516.02 μM TE) from the viscera extracts was obtained after applying UAE 30 min, at 50 °C, and pH 8.5.

Table 5: Antioxidant capacity values obtained by ABTS assay (μM TE) from sea bass side streams extracts using UAE at different extraction times (min), temperature (°C), and pH

Run #	Time of Extraction (min)	Temperature (°C)	pH	Antioxidant Capacity (ABTS, μM TE)			
				Head	Skin	Bon	Viscera
1	15	20	7	126.	74.84	161.9	516.02
2	30	20	8.5	11.0	31.62	34.46	213.51
3	30	20	5.5	9.91	43.59	28.94	137.87
4	0.5	20	8.5	43.9	125.8	134.3	450.35
5	0.5	20	5.5	98.2	285.9	276.2	432.54
6	15	35	7	21.7	164.8	173.2	492.30
7	15	35	7	129.	207.6	291.9	427.19
8	15	35	8.5	36.5	90.85	210.8	487.78
9	15	35	5.5	37.8	124.6	160.6	439.34
10	30	35	7	9.76	42.04	57.83	186.63
11	0.5	35	7	9.37	48.75	45.61	253.96
12	15	50	7	19.8	154.8	164.3	496.85
13	30	50	8.5	93.6	214.0	349.6	487.57
14	30	50	5.5	74.1	124.7	217.8	347.36
15	0.5	50	8.5	29.6	156.2	197.1	442.86
16	0.5	50	5.5	28.9	13.46	42.25	171.50

Figure 3A,B shows the main effects observed for the antioxidant capacity of the extracts obtained from head at different temperatures and extraction times at a constant pH of 8.5. It is clearly observed how increased extraction times significantly increased the antioxidant capacity of the extracts (p = 0.0006). Besides, neither the pH nor the temperature affected antioxidant capacity (p = 0.2855 and p

= 0.1469, respectively). Regarding the skin (**Figure 3**C,D), all the studied parameters affected the antioxidant capacity of the extracts with different degrees, obtaining p values of 0.0001, 0.0034, and 0.0045 for extraction time, temperature, and pH, respectively. As shown in **Figure 3**E–H for both bone and viscera, a significant increase in the antioxidant activity was observed with augmented extraction times ($p < 0.001$). On the other hand, as in the case of head, no significant effect was observed regarding the temperature and pH. Accordingly, the optimal conditions for the antioxidant activity of the extracts obtained from the studied side streams measured with ABTS assay are shown in **Table 6**.

Table 6: Optimal conditions for ABTS optimal values.

Side stream	Extraction time (min)	Temperature (°C)	pH	Antioxidant Capacity (ABTS, µM TE)
Head	30	20	8.5	128.13
Skin	30	20	5.5	278.37
Bone	23	20	5.5	318.65
Viscera	21	50	8.5	535.70

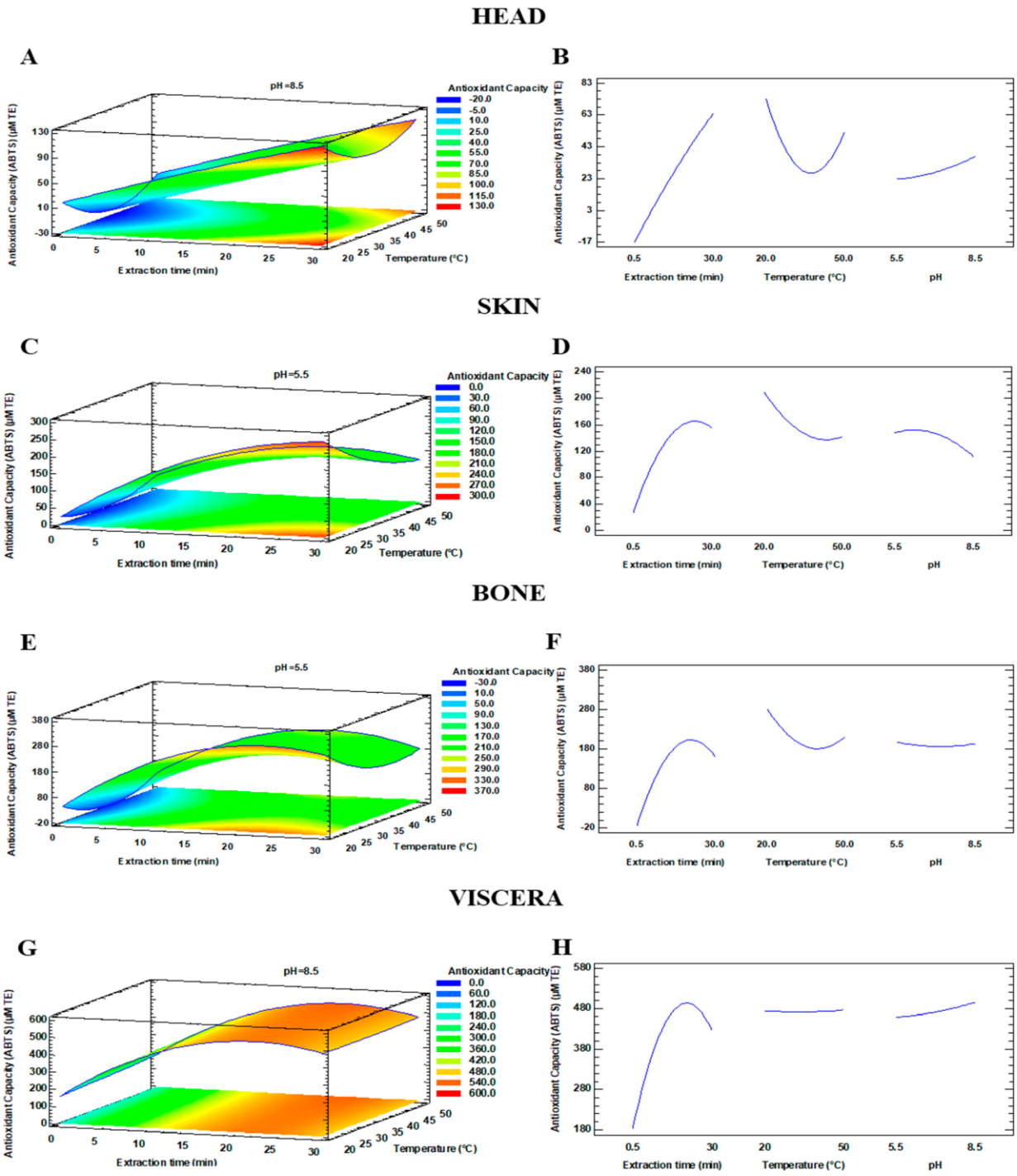

Figure 3: Plots shown in (A,C,E,G) indicate the response surface plot for the percentage of antioxidant capacity as a function of the extraction time (min) and temperature (°C) at a fixed pH. The plots in (B,D,F,H) show the influence of the different parameters (extraction time, temperature, and pH) on the antioxidant capacity determined as µM trolox equivalent using ABTS assay.

The effects of the extraction conditions on the antioxidant activity determined by ORAC assay are shown in **Table 7**. As can be expected, the highest ORAC values were found after 30 min of UAE in the four studied side streams, 20 °C for skin and bone, and 50 °C for head and viscera, obtaining the maximum ORAC values at pH = 5.5 for head and skin and pH = 8.5 for bone and viscera.

Table 7: Antioxidant capacity values obtained by oxygen radical absorbance capacity (ORAC) assay (μM TE) from fish side streams extracts at different UAE (ultrasoultrasound-assisted extraction) times (min), temperatures (C), and pH.

Run #	Time of Extraction (min)	Temperature (°C)	pH	Antioxidant Capacity (ORAC, μM TE)			
				Head	Skin	Bon	Viscera
1	15	20	7	350.	287.7	241.4	5794.64
2	30	20	8.5	173.	139.7	299.9	2124.46
3	30	20	5.5	123.	140.1	218.4	2813.98
4	0.5	20	8.5	215.	302.9	263.6	4684.92
5	0.5	20	5.5	248.	401.4	617.3	2611.02
6	15	35	7	262.	339.2	264.3	4042.80
7	15	35	7	316.	248.4	698.9	2410.56
8	15	35	8.5	209.	303.5	265.1	3991.56
9	15	35	5.5	259.	264.7	366.7	5206.57
10	30	35	7	325.	168.5	223.9	3538.50
11	0.5	35	7	158.	156.9	167.6	3914.67
12	15	50	7	234.	303.8	228.4	4394.47
13	30	50	8.5	247.	226.7	581.2	3493.03
14	30	50	5.5	399.	331.8	173.9	5355.38
15	0.5	50	8.5	145.	289.5	334.2	3648.70
16	0.5	50	5.5	155.	239.8	208.3	2082.74

Three-dimensional response surface plots and the graphs of influence of the studied parameters are presented in **Figure 4**. As shown in **Figure 4**A,B, the extraction time is the only parameter that significantly increased the antioxidant activity (p = 0.157) for head. Similar trends were also observed for skin, where only the extraction time had a significant positive impact on the antioxidant activity (p = 0.0012). On the other hand, concerning the bone and viscera side streams, the pH did not have any significant impact (p > 0.05). For the bone, the antioxidant activity was enhanced as extraction time increased and temperature decreased (p = 0.008 and p = 0.0016, respectively). As for the viscera, the antioxidant activity was strongly affected by the temperature. The increase of

temperature and extraction time resulted in higher antioxidant activity (p = 0.0000 and p = 0.0008, respectively). The optimal conditions for ORAC assay and their theoretical response are shown in **Table 8**.

Table 8: Optimal conditions for ORAC optimal values.

Side stream	Extraction time (min)	Temperature (°C)	pH	Antioxidant Capacity (ORAC, µM TE)
Head	30	50	8.5	369
Skin	28	25	5.5	389
Bone	20	20	7.8	679
Viscera	30	50	7.0	5996

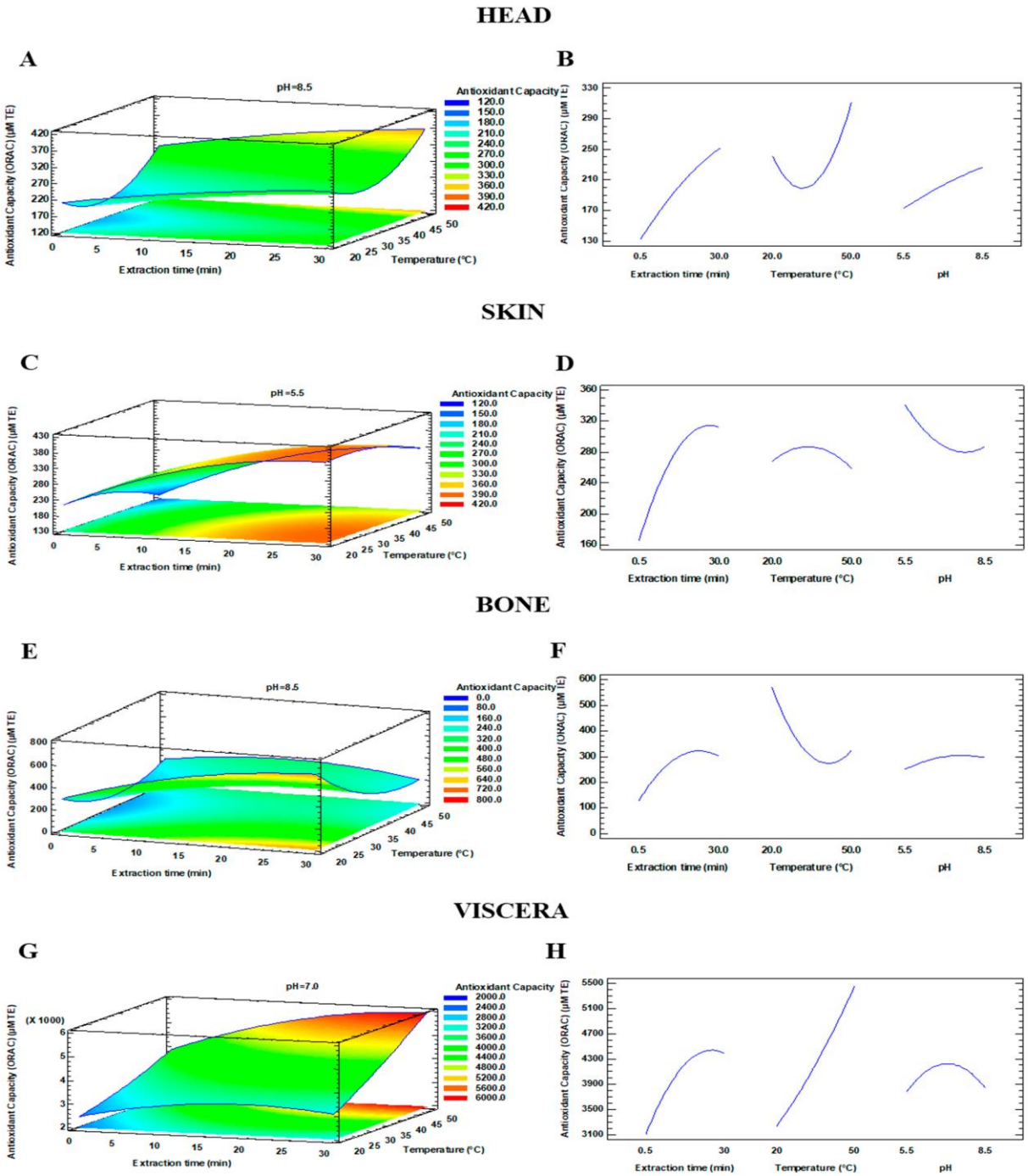

Figure 4: Plots shown in (A,C,E,G) indicate the response surface plots for the percentage of antioxidant capacity as a function of the extraction time (min) and temperature (°C) at a constant pH. The plots in (B,D,F,H) represent the plot of the influence of the different studied parameters on the antioxidant capacity values determined as µM TE (Trolox equivalent) using the ORAC assay.

The results obtained showed that sea bass side streams extracts are a great source of compounds with antioxidant potential. Regarding the information in the available literature about the

antioxidant capacity of fish side streams, Franco et al. [33] studied the application of aqueous and hydroethanolic mixtures assisted by pulsed electric fields (PEF) to recover antioxidants of sea bream and sea bass residues (gills, bones, and head). These authors found the highest antioxidant values after PEF-assisted extraction in aqueous media. They also observed that among the different side streams studied, gill extracts showed the highest antioxidant capacity, obtaining DPPH values in sea bass gills ranging from 105.93 to 313.87 μg Trolox/g sample. In the present study, viscera was the side stream with the highest antioxidant capacity, with values of ABTS and ORAC up to 516.02 μM TE and 5794.64 μM TE, respectively. Moreover, our results are in close agreement to those obtained by Franco et al. [33], who reported an antioxidant activity after using aqueous media, thus suggesting that substances with higher polarity can have more antioxidant capacity.

In other study, Nasyiruddin et al. [34] investigated the effect of low-frequency ultrasound treatment at different times (6–14 min) on the properties of silver carp myofibrillar protein and observed a significant effect on antioxidant activity (DPPH inhibition from 16.07 to 36.51% and ABTS inhibition from 14.17 to 22.58%), obtaining the highest antioxidant activity after the UAE treatment at 12 min.

On the other hand, other treatments such as mechanical separation resulted in lower antioxidant capacity (<50 μg Trolox/g sample) in sea bass, gilthead sea bream, and rainbow trout samples [35]. For instance, ultrasound could improve the extraction of antioxidant compounds by two mechanisms: i) the release of antioxidant compounds from inside of cells and ii) the induction of proteolysis, producing antioxidant peptides [33,36]. The higher antioxidant activity observed in the present study for sea bass viscera compared with the other side streams (head, bone, and skin) could be due to its high content of peptides with low molecular weight. In this sense, the antioxidant activity of peptides increases as their molecular weight decreases [37].

3.3 Optimization and Verification of Predictive Responses

Based on the interaction of the three critical parameters (extraction time, temperature, and pH), the UAE process was optimized in order to obtain the highest yield of protein recovery and antioxidant activity (ABTS and ORAC values). The optimal UAE conditions obtained are presented in **Table 9**. Furthermore, in order to confirm the accuracy and the reliability of the optimal conditions and to validate the adequacy of the model, additional experiments were carried out under the optimal conditions. The predicted and the experimental values for the different responses are shown in **Table 9**. As it can be seen, the experimental values were close to the expected values, confirming the validity of the model. Thus, this model has high accuracy in predicting the experimental optimal conditions, and it can be greatly applicable and operable.

Table 9: Optimal conditions, predicted values, and experimental responses of protein recovery and antioxidant activities (ABTS and ORAC) for different fish side streams.

	Optimal conditions for US			Protein Recovery (%)		ABTS (mM TE)		ORAC (uM TE)	
	Time (min)	**Temperature (°C)**	**PH**	**Predicted values**	**Experimental values**	**Predicted values**	**Experimental values**	**Predicted values**	**Experimental values**
Head	**25**	**20**	**5.5**	**32.19**	**31.7±0.1**	**90.91**	**142.6±25**	**260.60**	**327.71±12.15**
Skin	**30**	**32**	**5.5**	**24.63**	**33.7±0.7**	**189.73**	**240.9±26**	**384.48**	**359.08±13.01**
Bone	**30**	**20**	**8.5**	**66**	**54.2±0.0**	**292.92**	**139.5±22**	**673.43**	**584.68±67.09**
Viscera	**26**	**50**	**8.5**	**94.52**	**94.6±1.0**	**516.02**	**412.3±32**	**5705.61**	**5475.65±357.5**

3.4 Comparison of Optimal Extraction Conditions with the Lowest UAE Treatment

In addition, the optimal results obtained in this study were compared to those obtained with the lowest extraction time (0.5 min) of UAE at the optimal temperature and pH of each side stream (**Figure 5**). As can be seen in the table, the percentage of protein recovered was very similar for head and viscera side streams, compared to the optimal condition for time of extraction with the lowest one (0.5 min). However, a higher protein recovery was obtained for skin and bone, reaching 33.7 and 54.2%, respectively, under the optimal condition. The antioxidant activity obtained (measured with

ABTS and ORAC values) was higher for all side streams under the optimal condition. Moreover, in general, better results were observed by increasing the treatment time.

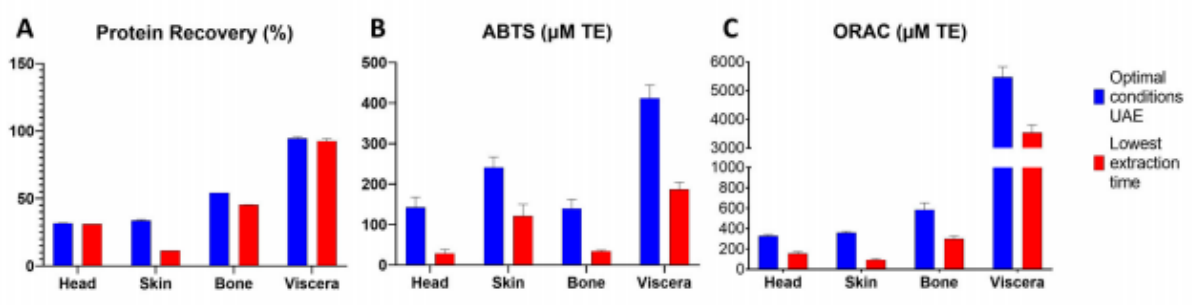

Figure 5: Comparison of the optimal condition with the lowest treatment of UAE (0.5 min): (A) protein recovery (%), (B) ABTS values (μM TE), and (C) ORAC values (μM TE).

3.5 Comparison of Optimal Conditions with Conventional Extraction

Moreover, the results obtained after applying the optimal conditions were also compared to those obtained after using a conventional treatment (stirring from 0 to 180 min) in head side streams extracts (as a model matrix). As can be observed in **Figure 6**, the protein recovery was very similar after employing both treatments, around 32%. However, higher values of ABTS and ORAC were reached under UAE optimal conditions, with levels ranging from 149.64 to 377.54 μM TE and from 319.29 to 974.52 μM TE, respectively. In this sense, UAE treatment could improve the extraction of antioxidant compounds.

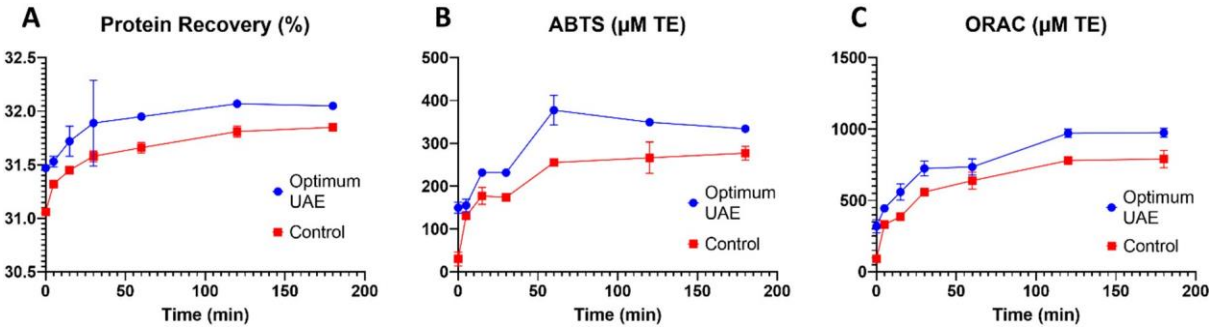

Figure 6: Optimal condition of UAE vs. conventional extraction (Control) for sea bass head: (A) protein recovery (%), (B) ABTS values (μM TE), and (C) ORAC values (μM TE).

3.6 SDS-PAGE Electrophoresis

The results obtained after performing the electrophoresis assays revealed a higher abundance of proteins in the extract obtained under the optimal UAE conditions (30 min) compared to lowest UAE (0.5 min), except for skin side streams, which presented a higher abundance in the lowest treatment (**Figure 7**).

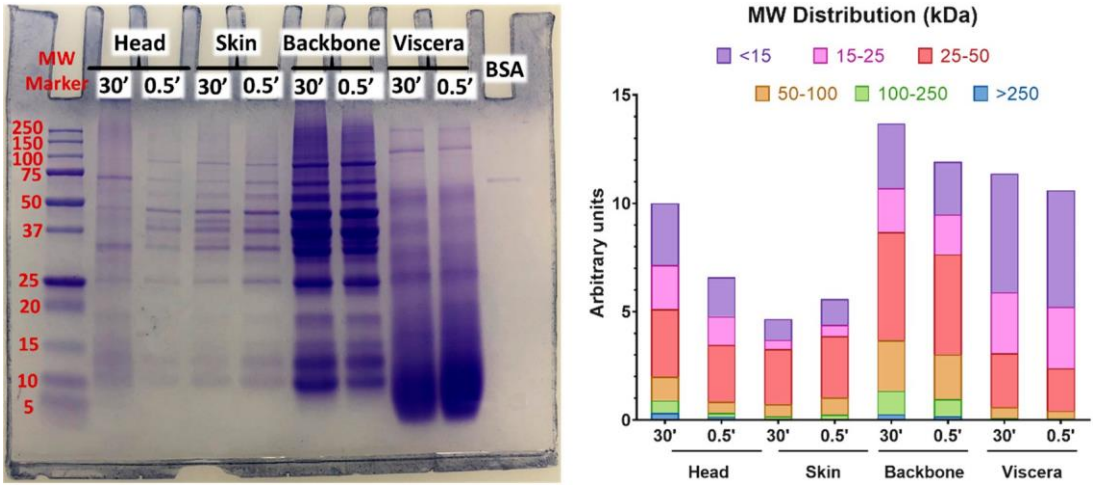

Figure 7: SDS-PAGE analysis of extracts obtained by UAE from sea bass byproducts (head, skin, bone, and viscera) (MW, molecular weight).

Moreover, different protein profiles were observed between the different side streams (head, skin, backbone, and viscera). The higher protein concentrations were detected in backbone and viscera extracts. In general, for all side streams the main part of proteins extracted had a low molecular weight, ranging from <15 to 50 kDa, with most presenting in the molecular weight band of 25–50 kDa. However, the size of the main part of proteins was <15 kDa in viscera. This fact can be attributed to a higher protein hydrolysis in this specific side stream. It should be highlighted that in head and backbone ex- tracts, proteins of high molecular weight (100–250 kDa) were also identified.

Similar results were also reported by Álvarez et al. [11]. These authors analyzed the protein size from mackerel side streams extracts obtained after ultrasound-alkaline-assisted extraction. They observed a low content of large proteins (100–500 kDa) and a high content of proteins ranging from 10 to 40 kDa. This fact could suggest that some hydrolytic process of large proteins is taking place

during UAE. In this line, Kim et al. [38] also reported changes in the collagen fiber structure and its breakdown after ultrasound treatment. As it is known, proteins or hydrolysates of low molecular weight are more digestible [39].

3.7. Mycotoxin Presence in Sea Bass Side Streams

The analyzed mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, ENNA, ENNA1, ENNB, ENNB1, and BEA) were detected below the LODs in sea bass side stream (head, skin, bones, and viscera) extracts obtained after applying UAE conditions under the studied variables. This confirmed that the use of aqueous media combined with UAE did not facilitate the recovery of mycotoxins from the sea bass side streams extracts evaluated in this study.

Contrary to our results, Deng et al. [40] observed the presence of AFB1, T-2, and OTA at levels of 0.58–0.89, 0.55–1.34, and 0.36–1.51 μg/kg, respectively, in dried seafood after ultrasound treatment for 60 min at 20 °C. However, these authors employed an acetoni- trile/water mixture (85/15, v/v) as an extraction solvent. It is important to point out the importance of the solvent employed in mycotoxins recovery.

4. Conclusions

UAE technology is presented here as a good strategy to obtain high-added-value compounds and to avoid the presence of mycotoxins from sea bass side streams extracts. The study for the optimization of the UAE treatment based on the interaction of time, temperature, and pH parameters by response surface methodology proved that this technology was suitable to obtain a high yield of proteins and antioxidants from all sea bass side streams studied. Concretely, the highest protein recovery and the highest antioxidant capacity (ABTS and ORAC) values were observed in viscera extracts. In general, increased values were obtained with the elapse of extraction time. On the other hand, no mycotoxins were detected in the extracts obtained after the UAE treatments. Compared to conventional treatment, better results were obtained for head side streams under UAE technology, observing higher values for ABTS and ORAC, up to 377.54 μM TE

and to 974.52 μM TE, respectively. Finally, it was seen that ultrasound treatment could reduce the molecular weight of the extracted proteins, making these proteins more digestible. These results highlight that fish side streams and innovative extraction tools such as UAE are a good combination. It should be evaluated as a potential tool to obtain high-added-value compounds, with potential applications in the food and pharmaceutical industry, and valorizing fish side streams.

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5. SUMMARY OF THE RESULTS AND DISCUSSION

5. Summary of results and general discussion

5.1 Impact of ultrasound extraction time, temperature and pH on the selective extraction of proteins, antioxidants capacity and the molecular weight of extracted protein from fish by-products

After the application of different ultrasound-assisted extraction conditions (time/temperature/pH) on sea bass side streams, a different range of protein recovery was found varying from 12.51–39.89% (heads), 12.41–31.64% (skin), 23.63–75.07% (bone) and 70.10–99.37% (viscera). Moreover, the highest percentage of recovered proteins was observed after the application of different conditions of 15.25 min/35 °C/5.5 (39.89%), 30 min/35 °C/7 (31.68%), 30min/50 °C/8.5 (75.07%) and 30 min/50 °C/5.5 (99.37%) from head, skin, bone and viscera extracts, respectively. In this line, in a study conducted in 2015, the authors used the UAE technique to isolate proteins from tilapia (*Oreochromis niloticus*) fillets, obtaining that the application of UAE achieved a high yield of protein recovery, reaching up to 62.60% in alkaline conditions (Tian et al., 2015).

Besides, the behavior of each single variable (extraction time, temperature, and pH) on recovered protein differed according to each sea bass side stream extract. For example, protein recovery was clearly improved by increasing the extraction time for the skin, bone and viscera samples. On the other hand, increasing temperature enhanced the amount of proteins obtained only for viscera samples. Moreover, it should be noted that the pH value mainly influenced protein extraction from head and bone, while it was not a determining factor for skin and viscera. In this sense, higher protein yields were obtained under UAE and alkaline conditions, with a proximate protein recovery of 95% of total protein from mackerel byproducts (Álvarez et al., 2018). Similarly, the higher percentage of protein recovery in our study was detected in viscera extracts (99.37%). Actually, protein recovery reported in previous studies varied in a range between 42% and 90%. Moreover, the data available in the literature revealed that alkaline solubilization usually results in higher protein recoveries than that obtained in acidic conditions (Gehring et al., 2011). In general, our study showed that longer

extraction time, alkaline pH and high temperature may positively affect the yield of protein depending on the sea bass side stream studied.

Regarding the total antioxidant capacity (TEAC and ORAC assays), sea bass side streams reveled a high antioxidant activity. A wide variety of antioxidant activity values were observed among the different fish by product extracts. The values ranged from 9.37 to 516.02 μM TE (TEAC) and from 123.73 to 5794.64 μM TE (ORAC). Moreover, the highest antioxidant capacity (ABTS) was observed after the application of different conditions of 30 min/20 °C/8.5 (129.38 μM TE), 30 min/20 °C/5.5 (285.69 μM TE), 30 min/50 °C/8.5 (276.23 μM TE) and 30 min/50 °C/8.5 (516.02 μM TE) for head, skin, bone and viscera extracts, respectively. While for the ORAC assay, the highest values were found after 30 min of UAE in the four studied side streams (head (399.12 μM TE), skin (401.45 μM TE), bone (698.96 μM TE) and viscera (5794.64 μM TE)), 20 °C for skin and bone and 50 °C for head and viscera, obtaining the maximum ORAC values at pH = 5.5 for head and skin and pH = 8.5 for bone and viscera. This variation in the obtained values is mainly due to the various applied conditions, in addition to the response of each side stream to the variables used in the optimization (extraction time, temperature and pH). A treatment of UAE of 30 min demonstrates to result in the higher antioxidant capacity for all the tested by-products, although the effects of the temperature and pH on the antioxidant capacity differ between the side streams. It should be mentioned that the highest antioxidant activity (ABTS and ORAC) was observed for sea bass viscera, compared with the other side streams (head, bone, and skin). Since the antioxidant activity of peptides increases as their molecular weight decrease (Lin et al., 2019), this outcome could be due to the richness of viscera with low molecular weight peptides.

Concerning the data available in the literature about the antioxidant capacity of fish side streams, de la Fuente et al. (2021) studied the antioxidant capacity of extracts from sea bass side streams (muscles, head, skin, viscera and tailfins) by pressurized liquid extraction. The highest values of antioxidant capacity were found in muscle samples whereas the highest antioxidant capacity in head

extracts was found to be 986 μM TE (TEAC) and 1949 μM TE (ORAC). Regarding the remaining sea bass side streams (viscera, skin, and tails), the antioxidant capacity values were approximately below 600 μM for TEAC and 1500 μM for ORAC tests (de la Fuente, Pallar, et al., 2021). In our study, the antioxidant capacity values from head, skin and bone extracts were below 349.63 and 617.38 μM TE for TEAC and ORAC tests, respectively, while for viscera, the highest antioxidant capacity values were obtained for ORAC assay (5794.64 μM TE). In addition, the application of different mixtures (aqueous and hydroethanolic) assisted by pulsed electric fields (PEF) on the seabass and sea bream (gills, bones and head) side streams showed that the highest antioxidant values after PEF-assisted extraction were obtained in an aqueous media. Among the different side streams studied, gill extracts demonstrated the highest antioxidant capacity, with DPPH values ranging from 105.93 to 313.87 μg Trolox/g sample (Franco et al., 2020). In our study, we chose water as the extraction media, as it is the green solvent and is the cheapest solvent. Moreover, sea bass viscera was the side stream with the highest antioxidant capacity among the different extracts, with values of ABTS and ORAC up to 516.02 μM TE and 5794.64 μM TE, respectively. Furthermore, our results are in close agreement to those obtained by Franco et al., who reported an antioxidant activity after using aqueous media, thus suggesting that substances with higher polarity can have more antioxidant capacity (Franco et al., 2020).

In a different study, the influence of low-frequency ultrasound treatment on the activities of silver carp myofibrillar protein, at diverse times (6–14 min), was investigated. The results showed that the DPPH inhibition increased from 16.07 to 36.51% and similarly, that of ABTS reached 22.58% after an initial 14.17%. Thus, the study demonstrated a noteworthy effect on antioxidant activity, where the highest antioxidant activity was obtained after 12 min of UAE treatment (Liuhartana Nasyiruddin et al., 2019).

5.1.1. UAE optimization and verification of the applicability of RSM

The influence of extraction time, temperature and pH on the development of protein extracts with antioxidant capacity of sea bass side streans obtained by UAE was studied using a response surface methodology. The optimization of the obtained UAE conditions using the RSM showed that an optimal condition (extraction time/temperature/pH) leads to an optimal recovery of protein (%) and antioxidant activities (µMTE) (protein %/ABTS/ORAC µMTE). Therefore, the predicted optimal conditions and responses for the head (25 min/50 °C/5.5) and (32.19%/90.91/327.71 µMTE), for the skin (30 min/32 °C/5.5) and (24.63%/189.73/384.48 µMTE), for the bone (30 min/20 °C/8.5) and (66%/292.92/673.43) and for the viscera (26 min/50 °C/8.5) and (94.52%/516.02/5705.61 µMTE) were confirmed experimentally. The predicted and experimental values were comparable. Hence, this model is proved to be highly applicable since it showed high accuracy in the prediction of the experimental optimal values.

In addition, the comparison of the obtained optimal results of all the studied side streams with the lowest extraction time (0.5 min) of UAE, showed that the highest antioxidant activities were observed with the optimal extraction time (25-30 min), which confirms that increasing the treatment time may generate a frequent number of components (Kim et al., 2013) which, in turn, can lead to the high antioxidant activity. Moreover, under the optimal conditions, a higher protein recovery was obtained from skin and bone, reaching 33.7% and 54.2%, respectively. However, the percentage of recovered proteins was very similar to head and viscera side streams, compared to the optimal condition for time of extraction with the lowest one (0.5 min). This similarity, can be explained by the proteins already released via the bubble cavitation from ultrasound, hence additional extraction time has not improve the proteins recovery (Hadiyanto & Adetya, 2018).

Furthermore, in order to justify the latest data, head side streams were used as a model. A conventional treatment (stirring from 0 to 180 min) was applied to the side streams and the values were compared with those treated with UAE under optimal temperature and pH. The protein

recovery (≈32%) was very similar after employing both treatments. However, the ABTS and ORAC resulted in higher values under the optimal conditions, with levels ranging from 149.64 to 377.54 μM TE and from 319.29 to 974.52 μM TE, respectively, thus indicating that the UAE had a significant positive influence on the antioxidant activity. In contrast, extraction yields were not affected by increasing the extraction times. This is probably caused by the early release of molecules through the bubble cavitation from ultrasound irradiation (Hadiyanto & Adetya, 2018).

Lastly, the protein electrophoresis was performed in order to study the influence of UAE on the molecular weight of extracted protein and the amounts of proteins obtained after UAE treatment with the optimal condition for 30 min, in comparison with the lowest treatment time of 0.5 min. Bone and viscera extracts showed the highest protein concentrations. Generally, the main parts of extracted proteins ranged from 15 to 50 kDa, of which mostly ranged from 25 to 50 kDa. Except for the viscera extracts which showed abundance of molecules <15KDa, which may be explained with higher protein hydrolysis occurring to this side stream. In contrast, the protein profiles showed proteins of higher molecular weight (100–250 kDa) from head and backbone extracts. Comparable results were observed in other studies in which ultrasound-alkaline-assisted extraction was applied to mackerel side streams and later on examined the protein size of the extracts (Álvarez et al., 2018). The results showed that most of the proteins ranged from 10 to 40 kDa, whether less proteins were in the range of 100–500 kDa. These results, along with the results that we have obtained in our research, could possibly indicate that large proteins are being hydrolyzed during the UAE.

5.1.2. Influence of UAE on mycotoxins extraction

As we know fish feeding contain mycotoxins and other contaminants that can be transmitted to fish. For example, some mycotoxins were identified in wheat and corn meant for fish feed production (Marijani et al., 2019). Furthermore, after 90 days of exposure to aflatoxins in lambari (*Astyanax altiparanae*), the studied aflatoxin was detected in fish liver and muscle (Michelin et al., 2017). Moreover, UAE was used for the extraction of mycotoxins from fish. In this sense, ultrasound was

successfully applied for the extraction of aflatoxins from as gilt-head of sea bass, brown trout, and turbot (Jayasinghe et al., 2020) and of ENs and BEA from *Dicentrarchus labrax* and *Sparus aurata* (Tolosa et al., 2014). In our study, the examined mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, ENNA, ENNA1, ENNB, ENNB1, and BEA) were detected underneath the LODs in sea bass side stream extracts (head, skin, bones, and viscera), which were obtained after applying UAE conditions under the studied variables. These results prove that using aqueous media combined with UAE difficulted the recovery of mycotoxins from the sea bass side streams extracts. On the contrary, in another study, Deng et al. (2020) 40 applied ultrasound treatment for 60 min at 20 ℃ on dried seafood and used acetonitrile/water mixture (85/15, v/v) as an extraction solvent. In their research, the authors detected the mycotoxins AFB1, T-2, and OTA at levels of 0.58–0.89, 0.55–1.34, and 0.36–1.51 μg/kg, respectively (Y. Deng et al., 2020). These results emphasize the significance of the solvent employed in mycotoxins recovery. Moreover, de la Fuente et al. (2021) tested the presence of 223 mycotoxins in the extracts of sea bass by-products after Pressurized Liquid Extraction. These authors found that the mycotoxin deoxynivalenol was observed only in viscera side stream (de la Fuente, Pallar, et al., 2021). In addition, in another study, the same authors investigated the possible occurrence of mycotoxins in muscle, head, viscera, skin, and tailfin of gilthead sea bream after the same treatment and they found a total absence of mycotoxins in all studied sea bream side streams (de la Fuente, Pallarés, et al., 2021).

5.2 Impact of ultrasound assisted extraction time, temperature and pH on the selective extraction of nutrients, pigments and antioxidants capacity and the molecular weight of extracted protein from microalgae (P. tricornutum)

After UAE treatment the proteins and carbohydrates values ranged from 4.14 to 6.10 g/100 g of dry matter (DM) and 1.39 g to 2.52 g per 100 g DM, respectively. Moreover, the optimal conditions to recover 5.96 g of proteins/100g DM and 2.53 g of carbohydrates/100 g DM were 24.4 min/20 °C/pH 8.5 and 30 min/50 °C/pH of 8.5, respectively. On the other hand, the extraction time and temperature

affected the extraction of proteins but statistically the changes in both parameters were not significant. Moreover, it was also observed that the pH changes were not significant. Regarding the carbohydrate extraction, only the temperature showed a strong influence and an important increase in the extraction yield was observed between 45 and 50 °C. This can be attributed to the alteration in the cell wall integrity thus facilitating the interference of the solvent with the intracellular molecules which can assist the extraction of these molecules (Roselló-Soto et al., 2019). These results are in reasonable agreement with those obtained by Luize et al, (2017) who evaluated the extraction of proteins and carbohydrates from *S. platensis* biomass applying ultrasound and mechanical agitation, under alkaline conditions. The authors observed that none of the sonication time, temperature and pH had a significant influence on the extraction of proteins from *Spirulina platensis,* while only the temperature significantly and positively affected the extraction of carbohydrates (Luize et al., 2017).

Furthermore, some articles reported the optimization of UAE for protein and nutrients high recovery. In this sense, Hadiyanto et al, (2018) optimized the process for extracting proteins and lipids from dry *Spirulina sp.* biomass using ultrasonic osmotic shock. The authors optimized the following parameters: osmotic NaCl concentration (10–30%), solvent/biomass ratio (5–15 v/ w) and extraction times (20–50 min) using RSM. The use of ultrasound showed an increase in the lipid yields to 6.65% with the optimal parameters (11.9% NaCl, 12:1 v/w and 22min), and in the protein yields to reach 43.96% with 15.12% NaCl, 10:1 v/w and 30 min (Hadiyanto & Adetya, 2018). In addition, UAE was also optimized for the protein extraction from *Arthrospira platensis* microalgae, using a RSM with a central composite design. They found that the extraction time (10–120 min) and pH (9–11) had a significant effect on protein solubilization (Sánchez-Zurano et al., 2020). Additionally, it was reported that ultrasound treatment increased the protein extraction from *Chlorella vulgaris*, especially at a basic pH (NaOH medium), which was in line with our obtained optimal pH (8.5) (Hildebrand et al., 2020).

Besides, proteins and carbohydrates can be extracted from microalgae, and the UAE has been widely used for the recovery of microalgal pigments. The optimization of the UAE parameters is critical to increase the recovery of pigments. In this regard, the recovery of chlorophyll A in *P. tricornutum* varies under the changes of the extraction conditions (17.99 - 37.95 mg/100 g DM). The highest theoretical value (36.28 mg/100 g DM) was observed at 0.5 min/20 °C/pH of 5.5. In another study, where the authors evaluated the effects of the extraction time of UAE on the chlorophyll extracted in aqueous media from *Nannochloropsis spp*, they showed that increasing the extraction time, did not affect chlorophyll extraction (Parniakov et al., 2015). While, even that the three other studied parameters did not show any significant effect on the chlorophyll extraction, but it appears that increasing the extraction time had a positive effect on the chlorophyll yield which in return is negatively affected with the increase of temperature. Similarly, Amin et al. (2018) who optimized the extraction time and temperature of UAE of chlorophylls extracted from *Chlorella sp*. UAE, found that the maximum recovery of total chlorophylls was (17.15 µg/ml) and it was achieved at 30 ºC and 120 min. This study also showed that the increase in the extraction time elevated the yield, while increased temperatures decreased it (Amin et al., 2018). In fact, it is well known that microalgae pigments are highly susceptible to thermal degradation which results in decreasing the chlorophylls yields at elevated temperature (Poojary et al., 2016).

Likewise, the temperature had a slight impact on carotenoids recovery, which induced a decrease of the yield. While the extraction time had a strong positive effect on the extraction of carotenoids and the pH did not show any significant effect, the maximum value was obtained at a pH level of 8.5. Moreover, the recovery of carotenoids was quite fewer, ranging from 0 to 4.93 mg/100 g DM with an optimal recovery of 4.87 mg/100 g DM at optimal conditions (30 min/20 °C/pH 8.5). Likewise, the optimization of the microwave and pressurized liquid extraction of carotenoids from *P. tricornutum*, demonstrated that a reduction in carotenoid extraction was observed when the temperature increased (Gilbert-lópez et al., 2017).

In addition, UAE coupled with a microextraction technique was applied for extracting a considerable amount of carotenoids with antioxidant activities (lutein) from marine microalgae *Chlorella salina*. The authors optimized the frequency of the ultrasound for the extraction of lutein, in addition to the extraction time and temperature. The results showed that the maximum yield of extraction was achieved after 30 min of extraction with 35-kHz frequency (Gayathri et al., 2018). In our case, for *P. tricornutum* the frequency of ultrasound used was 20 KHz and the maximum yield of carotenoids was established after 30 minutes of extraction.

Besides, a recent study showed that *P. tricornutum* had the highest amount of carotenoids (especially all-E-fucoxanthin) and phenolic contents, as well as antioxidant activities (65.5%) compared to *Nannochloris* sp, *Tetraselmis suicica*, and *Nannochloropsis gaditana,* with respective antioxidant activity of 56.8%, 54.9%, and 51.1% (Haoujar et al., 2019)

On the other hand, the UAE of phenolic compounds from microalgae was optimized in some studies. For instance, Parniakov et al. (2015) investigated the application of UAE for the extraction of total phenols from the microalgae *Nannochloropsis spp.* They found that the optimal extraction of the total phenolic compounds assisted by ultrasound (W= 400 W) was achieved after 15 min (Parniakov et al., 2015). Likewise, the UAE was more efficient as the extraction time increased to reach 16 min and the TPC reached its maximum value of 761.55 mg GAE/100 g DM. Additionally, neither the pH nor the temperature had a significant effect on the TPC extraction by the UAE, which is in agreement with the study of Yucetepe et al. (2018) who evaluated the effect of UAE conditions on TPC from *Spirulina platensis* (Yucetepe et al., 2018). Besides, in our study the optimal conditions for the extraction of phenolic compounds were 16.07 min, 20.05 °C and 5.5 pH. These conditions yielded a value of 854.70 mg GAE/100 g DM, which is similar to the values obtained in another study (800 mg GAE/100 g DM), where *P. tricornutum* was pre-treated with pulsed electric fields+DMSO 50% in water (Kokkali et al., 2020).

As we know, antioxidants play a main role in protecting the tissues from free radicals, thus protecting the living organism against infections and degenerative diseases. The antioxidant activity of the extracts indicates the presence of compounds that can interact with free radicals and act via donation of an electron (Tirado et al., 2017). Furthermore, the exploration of natural antioxidant composition and antioxidant capacity of novel microalgae biomass is gaining an ever increasing importance. In this regard, different studies on the evaluation of the antioxidant activity of specific species of microalgae such as Phaeodactylum species (Banskota, 2019; Gato et al., 2001).

In addition, UAE has been proved as a promising technology for the extraction of antioxidant compounds. In this sense, the total antioxidant activity of *P. tricornutum* extracts varies with the variation in the UAE conditions. The highest value of antioxidant capacity was 2340.01 µM TE, obtained by ORAC assay and the lowest one was 563.82 µM TE obtained by the ABTS assay. Besides, the optimal conditions for the highest antioxidant capacity measured by the ABTS method (758.28 µM TE) were 28.36 min, 20 °C and pH = 5.5. On the other hand, for the ORAC assay, theoretically, 2338.54 µM TE were obtained with the optimal conditions (30 min, 47.65 °C and pH 8.5) which is very close to the experimentally obtained value (2340.01 µM TE), at the same conditions. These values of the antioxidant capacity are in the same range as those described in the literature for *P. tricornutum* (Gilbert-lópez et al., 2017).

Moreover, the effect of the UAE time positively affected the antioxidant activity measured by ABTS. At an optimal temperature and pH, there was an increase in the antioxidant capacity by increasing the extraction time from 0.5 min to 30 min. This can be explained by the increase of the extraction of the antioxidant compounds as the time passes. In a recent study conducted in 2020, the authors optimized the extraction of bioactive compounds from *P. tricornutum* and they found that the extraction time had a significant effect on the antioxidant capacity investigated by DPPH (Akyıl et al., 2020). Similarly, the temperature and pH did not have a great impact on the antioxidant capacity (p = 0.1386 and p = 0.9547, respectively).

On the other hand, the extraction time affected positively the antioxidant capacity measured by the ORAC assay, it was found that at 0.3 min of UAE (20 °C and pH = 8.5) the antioxidant activity from *P. tricornutum* extracts was 1766.48 µM TE. However, when the time increased up to 30 min, the antioxidant activity also increased (1842.10 µM TE). Furthermore, at pH = 8.5 and after 30 min of extraction (optimal conditions), the antioxidant activity was enhanced from 1842.10 µM TE at 20 °C up to 2340.01 µM TE at 50 °C. Hence, ORAC values increased as the time and temperature increased, whereas with the same parameters, the investigated antioxidant compounds showed a decrease. This could imply that other compounds, which were not detected during our work, had an effect on the antioxidant activity measured by ORAC. Since the ORAC assay has a greater affinity for lipophilic compounds, these results may suggest that the extraction conditions enhanced the extraction of lipid compounds, which in return enhanced the antioxidant activity (Banskota, 2019). On the contrary, another study showed decreased antioxidant values by ORAC (106.22 µM TE/g dry weight) and ABTS (67.93 µM TE/g) of *P. tricornutum* residual biomass. In this particular study, the researchers did not use the whole microalgae, but instead they used a microalgae by-product from biofuel production which could explain the decreased antioxidant values that were obtained.

As a final point, after the application of ultrasound optimal condition (30 min, 50 °C and pH = 8.5) and application of control condition (30 min stirring without US, 50 °C and pH = 8.5) on *P. tricornutum* biomass, the protein profile showed a strongly marked band above 23 kDa in all extracts. This band fits with fucoxanthin, which has a molecular weight of 17–23 kDa from the fucoxanthin–chlorophyll complex (Gelzinis et al., 2015; Stack et al., 2018). The quantification of these bands was based on the BSA (Bovine Serum Albumin) standard of 60 µg/mL. There were no significant differences between the control samples and the optimal ones. Then, it can be concluded that both treatments had a similar fucoxanthin extraction efficiency. Moreover, both treatments were also similar concerning the protein profiles, due to the occurrence of only one marked band in both treatments.

6. CONCLUSIONS

6. Conclusions

From the results obtained in the present PhD thesis it can be concluded that:

1. Sea bass side streams (head, skin, bone and viscera) are a valuable source of nutrients and antioxidant compounds. As for the microalgae *P. tricornutum*, it can be considered as a remarkable source of nutrients and antioxidant compounds such as chlorophyll and phenolic compounds
2. Alternative technologies such as UAE and SFE have been proved to be promising tools to recover nutrients and bioactive compounds from different matrices as well as efficient tools to eliminate contaminants from food such as mycotoxins and pesticides. In our studies UAE confirmed to be a good strategy to obtain valuable compounds and to avoid the existence of mycotoxins from all sea bass side streams extracts.
3. The optimization of the UAE parameters showed that the highest values of protein recovery and antioxidant capacity in sea bass extracts were observed in viscera extracts. In general, longer extraction time, alkaline pH and high temperatures may positively affect the yield of protein, differing according to the target sea bass side stream.
4. A treatment of UAE during 30 min demonstrates to yield the highest antioxidant capacity for all the tested by-products, while the effects of the temperature and pH on the antioxidant capacity differ according to the side streams. Higher antioxidant activities (ABTS and ORAC) were observed in the sea bass viscera compared with the other side streams (head, bone, and skin) which is likely attributed to its high content of peptides with lower molecular weight. In addition, when comparing optimal UAE conditions with conventional treatment, improved results were obtained for head side streams under UAE technology, observing higher values for ABTS and ORAC, up to 377.54 μM TE and to 974.52 μM TE, respectively.
5. The technique of UAE could reduce the molecular weight of the extracted proteins, making these proteins more digestible. These results highlight that fish side streams and innovative

extraction tools such as UAE are a good combination. It should be evaluated as a potential tool to obtain high-added-value compounds, with potential applications in the food and pharmaceutical industries, and valorizing fish side streams.

6. The optimization of the UAE of nutrients, pigments and polyphenols in addition to the antioxidant activity from *P. tricornutum*, using the RSM gave the optimal extraction conditions at a time of 30 min, a temperature of 50 °C and a pH of 8.5, thus promoting the extraction of fucoxanthin.
7. The influence of the studied parameters differed according to the target compounds for *P. tricornutum*, showing different behaviors depending on the nutrients and antioxidant high-added-value components. The extraction time showed a positive influence on the carotenoids extraction as well as on the polyphenols extraction. However, the temperature was the prominent factor for the extraction of carbohydrates. The temperature showed a positive influence on the carbohydrate extraction. However, for the extraction of carotenoids, the most influential factor was the extraction time. The total polyphenols were only significantly affected by the extraction time.

These findings pose future challenges as there is a need to conduct additional studies using alternative methods for the extraction of compounds from marine by-products, which should mainly focus on the evaluation of the extraction parameters as a whole. In this sense, it would be of interest to investigate other extraction treatments such as power/frequency and other UAE modalities in future studies. On the other hand, it is necessary to focus on choosing the appropriate extraction method, extraction costs, applicability and sustainability concepts. In this regard, other innovative extraction technologies, such as supercritical fluid extraction, could be useful to extract bioactive compounds. Likewise, it would be necessary to evaluate the existence of other bioactive compounds and nutrients of interest that could be present in marine side streams. Besides, studying other biological activities such as the cytoprotective effects are of particular significance to the

pharmaceutical, cosmetic and food industries. As a final point, it is of great significance to study the effects of the combination of different technologies on the extraction of high added value components.

6. Conclusiones

De los resultados obtenidos en la presente tesis doctoral se puede concluir que:

1. Los subproductos de la lubina (cabeza, piel, espinas y vísceras) son una valiosa fuente de nutrientes y compuestos antioxidantes. En cuanto a la microalga *P. tricornutum*, se puede considerar como una fuente notable de nutrientes y compuestos antioxidantes como la clorofila y los compuestos fenólicos.
2. Se ha demostrado que las tecnologías alternativas como UAE y SFE son herramientas prometedoras para recuperar nutrientes y compuestos bioactivos de diferentes matrices, así como herramientas eficientes para eliminar contaminantes de los alimentos como micotoxinas y pesticidas. En nuestros estudios, se confirmó que UAE es una buena estrategia para obtener compuestos valiosos y evitar la presencia de micotoxinas en todos los extractos de subproductos de lubina.
3. La optimización de los parámetros de UAE mostró que los valores más altos de recuperación de proteínas y capacidad antioxidante en extractos de lubina se observaron en extractos de vísceras. En general, un mayor tiempo de extracción, pH alcalino y altas temperaturas pueden afectar positivamente al rendimiento de proteína, difiriendo según el subproducto de lubina objeto.
4. El tratamiento de UAE durante 30 min permite obtener la mayor capacidad antioxidante para todos los subproductos ensayados, mientras que los efectos de la temperatura y el pH sobre la capacidad antioxidante difieren según los subrpoductos. Se observaron mayores actividades antioxidantes (ABTS y ORAC) en las vísceras de la lubina en comparación con los otros supbroductos (cabeza, espinas y piel), lo que probablemente se atribuya a su alto contenido en péptidos con menor peso molecular. Además, al comparar las condiciones óptimas de los UAE con el tratamiento convencional, se obtuvieron mejores resultados para

los subproductos de cabeza bajo la tecnología de los UAE, observándose valores más altos para ABTS y ORAC, hasta 377,54 μM TE y 974,52 μM TE, respectivamente.

5. La técnica de UAE podría reducir el peso molecular de las proteínas extraídas, haciendo estas proteínas más digeribles. Estos resultados destacan que los subproductos de pescado y las herramientas de extracción innovadoras como los UAE son una buena combinación. Debe ser evaluada como una herramienta potencial para la obtención de compuestos de alto valor añadido, con aplicaciones potenciales en la industria alimentaria y farmacéutica, y valorización de los subproductos pesqueros.
6. La optimización de los UAE para recuperar nutrientes, pigmentos y polifenoles además de la actividad antioxidante de *P. tricornutum*, utilizando la RSM dio las condiciones óptimas de extracción en un tiempo de 30 min, una temperatura de 50 ° C y un pH de 8.5, promoviendo la extracción de fucoxantina.
7. La influencia de los parámetros estudiados difirió según los compuestos diana para *P. tricornutum*, mostrando diferentes comportamientos en función de los nutrientes y componentes antioxidantes de alto valor añadido. El tiempo de extracción mostró una influencia positiva tanto en la extracción de carotenoides como en la extracción de polifenoles. Sin embargo, la temperatura fue el factor destacado para la extracción de carbohidratos. La temperatura mostró una influencia positiva en la extracción de carbohidratos. Sin embargo, para la extracción de carotenoides, el factor más influyente fue el tiempo de extracción. Los polifenoles totales solo se vieron afectados significativamente por el tiempo de extracción.

Estos hallazgos plantean futuros desafíos, ya que es necesario realizar estudios adicionales utilizando métodos alternativos para la extracción de compuestos de subproductos de origen marino, que deben centrarse principalmente en la evaluación de los parámetros de extracción en su conjunto. En este sentido, sería de interés investigar otros tratamientos de extracción como potencia /

frecuencia y otras modalidades de UAE en futuros estudios. Por otro lado, es necesario centrarse en escoger el método de extracción adecuado, los costes de extracción y los conceptos de aplicabilidad y sostenibilidad. En este sentido, otras tecnologías de extracción innovadoras, como la extracción con fluidos supercríticos, podrían ser útiles para extraer compuestos bioactivos. Asimismo, sería necesario evaluar la existencia de otros compuestos bioactivos y nutrientes de interés que pudieran estar presentes en los subproductos de origen marino. Además, el estudio de otras actividades biológicas como los efectos citoprotectores son de especial importancia para las industrias farmacéutica, cosmética y alimentaria. Como conclusión, es de gran relevancia estudiar los efectos de la combinación de diferentes tecnologías en la extracción de componentes de alto valor añadido

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