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The 4th International Congress on "Green Extraction of Natural Products" (GENP2022)

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Introductory word

Dear colleagues and friends,

On behalf of the Organizing and Scientific Committee, it is a great honor and privilege to welcome you to Poreč (Valamar Diamant) for the 4th International Congress of Green Extraction of Natural Products - GENP2022, which will take place on October 27-28, 2022.

GENP2022 is organized by the Faculty of Food Technology and Biotechnology University of Zagreb with the support of ISEKI-Food Association, EFFoST and the Energy Efficiency and Environmental Protection Fund .

The congress is divided into 4 sections with 6 main topics: Alternative Solvents for Green Extraction, Sustainable and Clean Extraction Technologies, Innovative Design of Extraction Processes, Valorisation of By-Products and Biorefinery, Industrial and Case Studies, and New Tools for Green Extraction Education and Operator Training.

With its themes, this meeting aims to promote constructive dialog and collaboration between experts from universities, research institutes and industry in the fields of cultivation, extraction, processing and recycling applied to the agrofood, nutraceutical, perfume, cosmetic, chemical, fuel and energy sectors.

GENP2022 will also provide an opportunity to present new insights and ideas in the area of new process technologies at the EIT Food Phenolic Workshop, "New Advances in EVOO Production Technologies."

In addition, the 280 participants from 16 countries who contribute posters and oral presentations to this congress will have the opportunity to publish their work in the proceedings of GENP2022 and in a special issue of the journals Applied Food Research (Elsevier) and Antioxidants (MDPI).

The GENP2022 Scientific and Organizing Committee thanks all conference participants for their participation and active contribution. We wish you a pleasant stay in Poreč and much benefit from the multitude of ideas exchanged, contacts made and successful scientific and professional collaborations.

We would also like to take this opportunity to thank our sponsors and exhibitors for recognizing the importance of this congress and supporting it with their valued participation.

Great thanks are also due to the help and valuable contribution of all members of the Scientific and Organizing Committees.

In addition to the scientific program, the city of Poreč on the Istrian peninsula in western Croatia will ensure that our guests and participants can enjoy the beauties of Poreč and its surroundings.

Thank you very much for joining us!

Chair of the conference
Mladen Brnčić

Program

Workshop program

Wednesday, October 26th 2022

14:00 – 18:00	Registration
18:00 – 20:00	EIT Workshop - New Advances in EVOO Production Technologies
20:00	Dinner
21:30	Welcome cocktail party

Conference program

Thursday, October 27th 2022

8:00 – 9:00	Registration
9:00 – 9:30	Opening of the GENP Conference
Session I 9:30	**Alternative solvents for green extraction –** ***HOSTED BY SENSIENT*** Chair: Antonio J. Meléndez-Martínez Damir Ježek Kristina Kljak
9:30 – 10:15 Plenary Lecture	Giancarlo Cravotto The Industrial Green Revolution in Solid/Liquid Extraction and Processing
10:15 – 10:45 Plenary Industrial	Gian Carlo Leocata - Sensient Subcritical Water Extraction: Cleaner Extracts, Sustainably
10:45 – 11:15	Coffee break
11:15 – 11:45 Keynote Lecture	Christian Cravotto Improvement and greener extraction of grape seeds by 2-methyloxolane
11:45 – 12:00	Ombéline Claux Is it possible to perform oilseeds extraction using bio-based 2-methyloxolane instead of hexane?
12:00 – 12:15	Laura Waldschütz Supercritical CO2 - Green Extraction on Industrial Scale
12:15 – 12:30	Manuela Panić Plant-based extracts in natural deep eutectic solvents for industrial application
12:30 – 12:45	Sponsored lecture - Vendor seminar IKA - Božidar Nikić HABITAT - New Generation Reactor

12:45 – 13:00	Sponsored lecture - Vendor seminar RU-VE - Hrvoje Runje Your Lab Partner
13:00 – 14:00	Lunch Break (lunch not included in registration fee)

Session II 14:00	**Alternative solvents for green extraction** **Sustainable and clean extraction technologies** Chair: Giancarlo Cravotto Marija Badanjak Sabolović Ivana Radojčić Redovniković
14:00 – 14:45 Plenary Lecture	Axel Schmidt Green Manufacturing Technology for Natural Products
14:45 – 15:15 Keynote Industrial	Leon Skaliotis - Flavourtech Unique Technologies for the Production of Natural Aromas and Concentrates
15:15 – 15:30	Siti Ramli Optimization of the oil extracted from Sardinella lemuru waste with supercritical fluid extraction (SC-CO2) using response surface methodology (RSM)
15:30 – 15:45	Lauriane Bruna Supercritical fluids extraction of bioactive molecules from Apple Pomace
15:45 – 16:15	Coffee break
16:15 – 16:30	Angeles Moron-Ortiz Preliminary studies for the optimization of ultrasound-assisted extraction of microalgal carotenoids
16:30 – 16:45	Marilena Muraglia Combined experimental designs for screening and optimizing the ultrasound-assisted extraction of polyphenols from ripe carob pods (Ceratonia siliqua L.)
16:45 – 17:00	Sónia Santos Green extraction methodologies for the sustainable recovery of linear diterpenes from Bifurcaria bifurcata
17:00 – 17:15	Alberto Alessandro Casazza Cascade recovery of biocompounds from Chlorella vulgaris
17:15 – 17:30	Emilie Isidore Optimization of the Supercritical Extraction of Rosmarinic Acid from Clary Sage and the Antioxidant Activity of the Extracts
17:30 – 18:30	Poster Session 1

Friday, October 28th 2022

Session III 9:00	**Innovative extraction process design** **New tools for green extraction education and operator training** Chair: Giorgio Grillo Antonela Ninčević Grassino Sven Karlović
9:00 – 9:45 Plenary Lecture	Francisco J. Barba Nutrient and Bioactive Compounds´ Recovery from Mushrooms Assisted by Pulsed Electric Fields, pressurized liquid extraction and SC-CO2
9:45 – 10:15 Keynote Lecture	Želimir Kurtanjek Causal Artificial Intelligence Molecular Model of CO2-H2O Extraction Coefficient
10:15 – 10:30	Nenko Nenov Pressurized hot water extraction vs. Energy consumption - are they contradicting?
10:30 – 10:45	Anita Šalić Deep eutectic solvents aqueous two-phase system based protein extraction in a microextractor
10:45 – 11:00	Sponsored lecture - Vendor seminar MILESTONE - Luca Bertoli High-purity extracts from natural products through a fast and solvent-free approach
11:00 – 11:30	Coffee break
11:30 – 11:45	Ana Jurinjak Tušek CFD modelling of the continuously operated microextraction of proteins - a shortcut to new microextractor designs
11:45 – 12:00	Morag Davidson Optimization of an ultrasound-enzymatic assisted extraction for the simultaneous recovery of polyphenols and oil from raspberry pomace using a Definitive Screening Design
12:00 – 12:15	Larissa Knierim; Axel Schmidt Digital twins with process analytical technology under quality by design regulations towards autonomous operation of natural products manufacturing
12:15 – 12:30	Margherita Pettinato Solid-liquid multivariable extraction (SoLVE) of lycopene from tomato waste
12:30 – 13:30	Lunch Break (lunch not included in registration fee)

Session IV 13:30	**- Valorisation of by products and biorefinery** **- Industrial and case study applications** Chair: Sandra Voća Jana Šic Žlabur Suzana Rimac Brnčić
13:30 – 14:15 Plenary Lecture	Antonio J. Meléndez-Martínez Carotenoids and Apocarotenoids: Actions in Nature, Importance for Food Security and Applications
14:15 – 14:30	Pablo Méndez-Albiñana Obtaining pectin of Premium quality from industrial orange juice by-products
14:30 – 14:45	Michał Ochnik The antiviral activity of the blend of double-standardized extracts of black chokeberry and elderberry against human influenza A virus and betacoronavirus-1
14:45 – 15:00	Mia Dujmović Ultrasound as a sustainable technology for the isolation of polyphenols from coffee grounds
15:00 – 15:30	Coffee break
15:30 – 15:45	Matsia Sevasti Biostimulant extraction and analytical characterization of marine organism residual products
15:45 – 16:00	Lavinia-Florina Călinoiu Integrated sustainable pre-treatments approach for cereal bran valorization
16:00 – 16:15	Marilena Muraglia Unripe Apulian carob: a future perspective in nutraceutical and food supplements fields
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CAROTENOIDS AND APOCAROTENOIDS: ACTIONS IN NATURE, IMPORTANCE FOR FOOD SECURITY AND APPLICATIONS

Antonio Jesús Meléndez Martínez [1]

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ABSTRACT

Carotenoids are widespread isoprenoids that participate in many actions in Nature. They can be converted into a myriad of derivatives enzymatically or non-enzymatically, which extends the spectrum of actions carotenoids are involved in. Carotenoids are therefore polivalent natural compounds and not just natural pigments or (some of them) provitamins A. Thus, carotenoids and their cleavage derivatives (usually termed "apocarotenoids") elicit expanding interest in photosynthesis, plant science, agriculture, food science and technology, nutrition, health, pharma or cosmetics. Currently, efforts need to be directed towards the production of sustainable and health-promoting carotenoid-rich products and their extraction from natural products is very important in this context. The evaluation of different treatments (ultrasounds, microwaves, pressurized liquid extraction, among others) and emerging green solvents (ethyl acetate, 2-methyl oxolane, natural deep-eutectic solvents, etc.) is undoubtedly a timely research topic.

Keywords*: carotenoids, carrots, microalgae, microwave, pressurized liquid extraction, ultrasound.*

1. INTRODUCTION: CAROTENOIDS AND DERIVATIVES IN NATURE

Carotenoids are biosynthesized by all photosynthetic organisms (plants, algae, cyanobacteria) and by some non-photosynthetic bacteria and fungi. In general, animals cannot synthesize them *de novo*, although recently it has been shown that some arthropods can biosynthesize them thanks to the presence in their genomes of carotenogenic genes [1]. Carotenoids are widespread and have been described in ~ 700 organisms belonging to the three domains of life, that is Archaea, Bacteria and Eukaryotes [2]. Indeed, carotenoids can be found in organisms adapted to the most disparate environmental conditions, from the bottom of the oceans to glaciers, thermal ponds, hypersaline waters or in in radioactive locations [3]. Their interest in food science and technology and nutrition relied for many years in their colorant properties and in the fact that some could be converted into retinoids exhibiting vitamin A activity. However, a large body of evidence generated in the last 3 decades indicates that carotenoids could be involved in health-promoting biological actions contributing to the reduction of the risk of developing diverse diseases (cancer, cardiovascular disease, skin, bone or eye conditions, metabolic disorders, etc.). They are also eliciting interest in relation to their beneficial actions in relation to cognition and child development [4]. The chemical sstructures of the major carotenoids found in humans are shown in Figure 1. Interestingly, carotenoids can be cleaved into a myriad of compounds that also intervene in a wide variety of actions. The cleavage derivatives are commonly referred to as apocarotenoids and can be obtained enzymatically or not. In this sense, carotenoid cleavage enzymes, specifically oxygenases, have also been described in organisms belonging to the three domains of life. Some of these apocarotenoids and their importance have been long known. Examples are the retinoids exhibiting vitamin A

activity in humans (retinal, retinol, retinoic acid), the phytohormone abscisic acid or the scents β-ionone or β-cyclicitral (Figure 2).

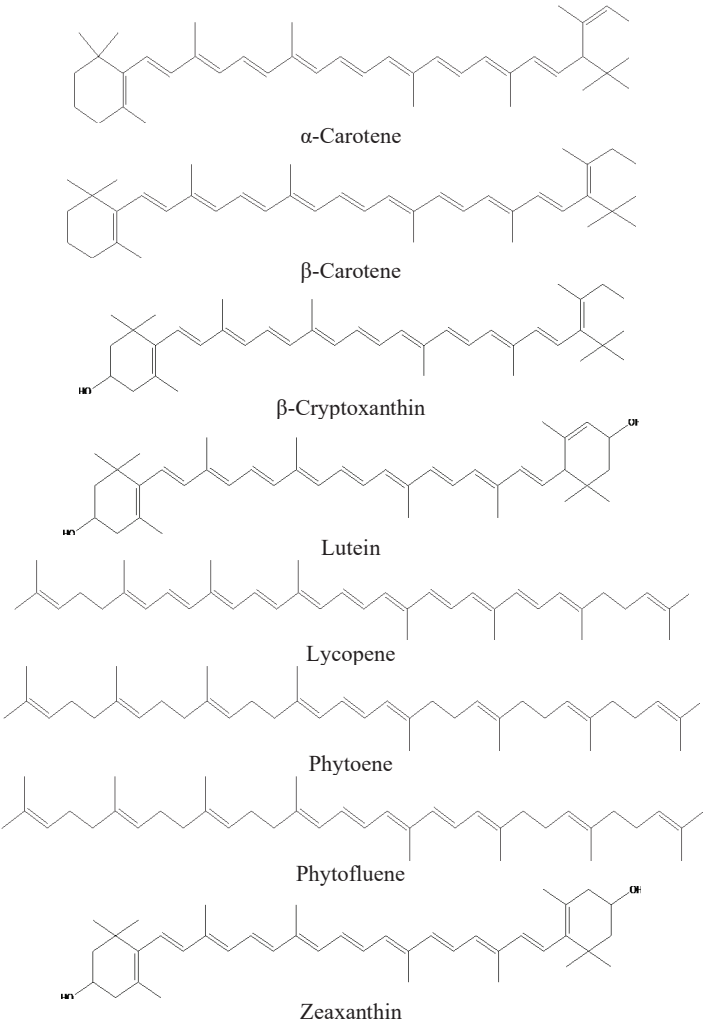

Figure 1. Chemical structures of the main carotenoids found in humans

2. ACTIONS IN NATURE

Carotenoids play key roles in photosynthesis and are also important from an ecological point of view as their color convey important information for the communication within and among species. They are also found in the eyes of many animals where they can improve vision.

Besides they are located in membranes, being important for the modulation of their properties, which is essential for the survival of microbes in harsh conditions. They can also act as antioxidants, although depending on the conditions they can exhibit prooxidant properties. On the other hand, there is evidence that they can be beneficial for the reproduction and fertility of some animals [5]–[7]. Concerning apocarotenoids, they can intervene in key processes with vitamin (for instance vitamin A) or hormone (for instance abscisic acid or strigolactones) modes of action. They can also act as aromas, antimicrobials, pheromones or allelopathic compounds [8]–[11].

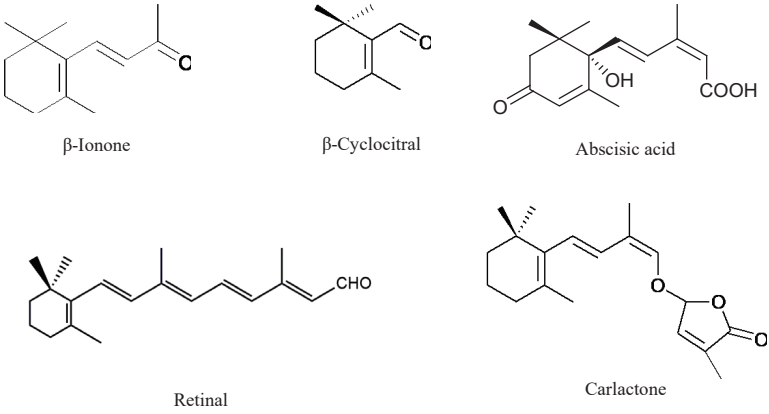

Figure 2. Chemical structures of some carotenoid cleavage derivatives ("apocarotenoids")

3. IMPORTANCE FOR FOOD SECURITY

Carotenoids are essential for photosynthesis, the primary engine of food production as photosynthetic organisms are or provide food for both aquatic and terrestrial animals. Besides, carotenoid-derived colours and aromas are key for both pollination and seed dispersal, essential processes for plant propagation and therefore for food production. Abscisic acid and strigolactones are hormones that regulate important processes for plant development, resilience and propagation such as symbiosis in the rhizosphere, resistance to abiotic stresses or even seed germination. On the other hand evidence is accumulating that carotenoid-derived signals can be important at different levels for plants, not to mention the already commented evidence that carotenoids can be beneficial for the reproduction of some animals [5]–[11]. Altogether, it is indisputable that, given their versatility, carotenoids and their derivatives are essential in food production at different levels.

4. APPLICATIONS

Carotenoids have been long used as food and feed additives and as components of food supplements for both health promotion and cosmetic purposes. Some such as β-carotene and canthaxanthin have been used therapeutically to treat the skin condition erythropoietic protoporphyria. Retinoids are also used to treat skin conditions [12]–[15]. Apart from these, other applications can be envisaged, such as agrochemicals with different actions, textile dyes or even as molecules for artificial photosynthesis.

Within this context of carotenoid versatility, the main research line of our group is the study of carotenoids and derivatives and their applications in agro-food, health and cosmetics. Recently, we are focusing in the application if diverse treatments in carotenoid-rich products for the obtaining of extracts or the improvement of their quality. Specifically, in our current projects ultrasound and microwaving are being used as well as pressurized liquid extraction (PLE) or pulsed electric fields, the latter two in collaboration with other teams. These methodologies can contribute to sustainability by reducing the time of extractions and the usage of solvents while increasing the extraction of target compounds compared to more traditional treatments. In a very recent study, remarkably increases in the potential bioavailability of the colourless carotenoids phytoene and phytofluene, lutein and the provitamin A carotenoids α- and β-carotene (Figure 1) have been observed in microwave-cooked carrots (manuscript under revision). On the other hand, the effect of milling and sonication in the extraction of carotenoids from phytoene-accumulating and wild-type microalgae (*Chlorella, Dunaliella*) is being assessed. Our preliminary results indicate that the extraction yield of emerging green solvents such as ethyl lactate and 2-methyl oxolane is comparable if not higher than that observed in some food-grade solvents (please, see the specific proceeding). Furthermore, the usefulness of PLE for the obtaining of carotenoid-rich extracts from *Arthrospira platensis*, *Phaeodactylum tricornutum* and *Chlorella sp.* has been recently demonstrated [16].

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POTENTIAL APPLICATIONS OF AVOCADO BYPRODUCTS IN VARIOUS INDUSTRIES

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ABSTRACT

The avocado (*Persea americana*) is a fruit widely consumed throughout the world and is an important dietary source of valuable essential nutrients and phytochemicals. Due to the high consumption and industrial processing of avocado, large amounts of residues, peels and seeds are generated, which represent a major environmental problem due to the large production volumes and the inadequate and insufficient use of avocado residues. Currently, most of this waste is discarded and underutilized. The pulp of the avocado and its waste (peel, seeds, pulp after oil extraction) contain bioactive compounds such as ascorbic acid, vitamin E, phenolic compounds and carotenoids, high levels of proteins, carbohydrates (starch, fiber), monounsaturated fatty acids and minerals (especially potassium). Extracts from all parts of the avocado fruit exhibit antioxidant, anti-inflammatory, antimicrobial, anticancer, antidiabetic, and anti-atherogenic properties. Due to their numerous health benefits, the consumption and production of avocados have increased greatly in recent years. Therefore, the pulp and waste of avocado are a potential source of bioactive compounds suitable for use in foods or dietary supplements. In addition, avocado seed waste is the main source of biomaterials such as starch and can therefore be used as an alternative starch source in the textile industry. Avocado seeds are also rich in antioxidants, natural dyes, biodiesel, and a phenolic compound that can be used for various applications in the pharmaceutical and cosmetic industries. The processing of avocados produces large quantities of avocado peels, which contain large amounts of valuable compounds. Avocado peels are a remarkable source of biomolecules, including phenolic and flavonoid compounds, and can be used as a food preservative, antioxidant, or functional ingredient in novel food formulations. The use of avocado byproducts as a source of natural compounds is a way to extract value-added ingredients while having a significant impact on avocado industrial applications and the environment.

Keywords: avocado, byproducts, bioactive compounds, peel, seed

1. INTRODUCTION

The avocado (*Persea americana* Mill.) is a subtropical/tropical fruit native to Mexico and Central America, where it has been a staple food for at least 9000 years [1]. It belongs to the Lauraceae family and the Persea genus, of which more than 150 species are known. Global production in 2019. was 7.31 million tons, primarily in Mexico (31,5%), followed by the Dominican Republic (9,1%), Peru (7,3%) and Colombia (7,3%) [2,3].

The avocado fruit consists of pulp, peel and seed (Figure 1). Recent studies have shown that the avocado fruit has high nutritional quality. The avocado is rich in soluble and insoluble fiber, as well as minerals and vitamins. Provitamin A, vitamin E and vitamin C particularly stand out as they act as antioxidants and neutralize oxidative stress, which helps the body maintain homeostasis. Avocado is also a very good source of vitamin B6, which can reduce the risk of ischemic stroke due to its role in the conversion of homocysteine to cysteine [4,5].

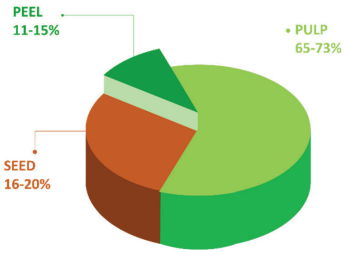

Figure 1. An average avocado composition [4]

Both, direct consumption and industrial processing of avocados, produce large amounts of residues, peels, and seeds, accounting for 11% and 16% of the total weight of the fruit, respectively [6]. Due to the large production volumes and the inappropriate and insufficient use of avocado residues, this is a major environmental problem. Currently, most of this waste is discarded and underutilized [7]. A number of important industrial products can be obtained from avocado waste, such as animal feed, oil, microbiological culture media, adsorbents/bioremediants, biocatalysts, biodiesel, starch, fuel, cosmeceuticals, and biopolymers [8].

2. EXPERIMENTAL

In this work, a systematic literature search was performed in different databases (*Scopus, Web of Science, PubMed, ScienceDirect, Directory of Open Access Journals*) and the nutritional/chemical composition and bioactive potential of each part of the avocado was evaluated, as well as potential applications of avocado byproducts.

3. RESULTS AND DISCUSSION

Information on the nutritional/chemical composition of avocado fruit dates back to 1922. Data on the macro- and micronutrients contained in avocado have been compiled in various food composition tables, although the nutrient content of the edible part of the fruit (pulp or mesocarp) varies greatly depending on factors such as variety, ripeness, and growing conditions. The avocado is a very complex matrix composed of a variety of compounds. It is highly valued as an excellent source of energy, fatty acids, and vitamins [1] (Tables 1 and 2). Extracts from all parts of the avocado fruit have many bioactive properties and many potential applications. Numerous beneficial health-promoting effects of avocado byproduct extracts have been confirmed in scientific studies. Among the most important reports are antioxidant, antimicrobial, antidiabetic, antihypertensive and hypocholesterolemic effects. Due to their numerous health benefits, the consumption and production of avocados have increased greatly in recent years [9, 5].

Table 1. Macronutrient composition of an avocado fruit [5]

Nutritional composition	Amount (%)
Water	73.230
Protein	2.000
Total lipid	14.660
Carbohydrate	8.530
Fiber	6.700
Ash	1.580

Table 2. The most abundant micronutrients in avocado fruit [5]

Minerals	Amount (mg/100g)
Potassium, K	485.000
Phosphorus, P	52.000
Magnesium, Mg	29.000
Calcium, Ca	12.000
Sodium, Na	7.000
Vitamins	
Choline	14.200
Asorbic acid (vitamin C)	10.000
Alpha-tocopherol (vitamin E)	2.070
Niacin (vitamin B_3)	1.738
Panthothenic acid (vitamin B_5)	1.390
Pyridoxine (vitamin B_6)	0.257

The main bioactive compounds determined in avocado are polyphenols, mostly including phenolic acids, flavonoids and tannins. Specifically comparing maximum polyphenol concentrations of dry weight, in seed 1-caffeoylquinic acid occurs with 1122.9 µg/g, in peel chlorogenic acid with 1898.9 µg/g and in pulp 5-feruloylquinic acid with 76.2 µg/g [10]. Considering total concentrations, polyphenols are followed by carotenoids and tocopherols, which are mainly found in pulp [6]. The most important fatty acid in avocado is oleic acid (18:1). Frequent consumption of oleic acid is associated with a lower risk of breast cancer, diabetes, and cardiovascular disease. Its easy digestibility combined with its creamy and smooth texture and richness of flavor and nutrients make avocado pulp in pureed form a suitable food for young children [5]. Although avocado is used by many juice processing industries, oil industries and small juice machines, it generates a large amount of waste that is disposed of in the soil and contributes to environmental pollution. [8].

Table 3. The utilization of avocado seed [8]

Industry	Usage
textile industry	starch from avocado seed replaces commercial starches
biopolymer	bioplastic, biomedical applications, construction, electronics
bioenergy production	biogas, in biodiesel and oil production, ethanol production
leather industry	retaining agent
pharmaceutical and cosmetics	as antioxidant, oil in cosmetic
agro-processing industry	animal feed
food industry	as natural food colorant, in food formulations

The demand for sustainable alternative renewable energy, biomaterials and environmental protection is increasing. Many researchers have tried to explore the conversion of biomass into bio-based materials, which is very important for the sustainable promotion of a green economy [8]. With the right technology, avocado seeds and peel can be used as a source of effective natural ingredients and additives to provide new technological solutions. The physical and chemical properties of lipid components, polyphenols, starch, and fibers, as well as the low cost of avocado residues, make this material a potential source of bioactive ingredients for use in the food, cosmetic, and pharmaceutical industries [9] (Table 3). Due to the high consumption of avocado fruit, there are many of its applications that have already been patented in the fields of food, cosmetics and medicine, but these applications are mainly related to the pulp or avocado oil (Figure 2) [9].

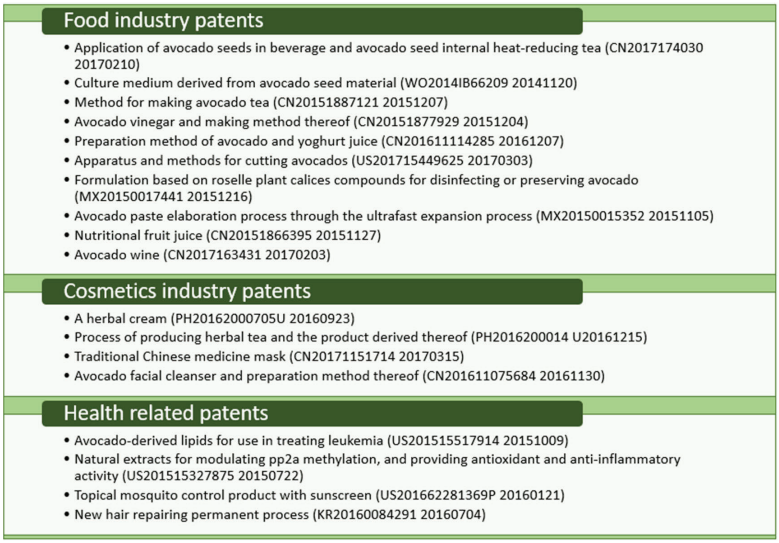

Figure 2. Patents with avocado uses [9]

4. CONCLUSION

Avocado is widely used worldwide and is very often referred to as a superfood because of its high nutritional value. Avocado residues, also known as food waste, has great potential as a source of beneficial nutrients. In particular, high quality starches and oils and a high content of bioactive compounds. Recently, the potential of avocado has been recognized and its application in various industries has led to an exponential growth of the avocado market. This trend will continue in the future.

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PULSED ELECTRIC FIELD-ASSISTED EXTRACTION OF NUTRIENTS AND ANTIOXIDANT BIOACTIVE COMPOUNDS FROM *LENTINULA EDODES*

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ABSTRACT

Pulsed electric fields (PEF) technology has shown favorable results over conventional methods to recover antioxidant bioactive compounds from several food matrices. The mushroom *Lentinula edodes* was chosen in the present study as a target matrix to evaluate the effect of PEF technology in the recovery of high-added-value bioactive compounds, due to its nutritional and therapeutic properties as well as its high consumption. The optimal extraction conditions were applied to the mushroom (50 kJ/kg, 2.5 kV/cm and 6 h) and bioactive compounds content was evaluated by spectrophotometric methods together with high-performance liquid chromatography coupled to mass spectrometry (HPLC-MS/MS) for the characterization and quantification of polyphenols. The results obtained were compared with conventional aqueous extraction (6 h under constant stirring). It was found that PEF-assisted extraction achieved a higher recovery of these compounds, with an increase in extraction of up to 443.48%. In addition, *Lentinula edodes* is a matrix rich in phenolic acids such as cinnamic acid, vanillic acid, and thymol, differing the results when comparing the methods. The results show that PEF technology is a sustainable alternative with higher recovery yield than conventional methods, which is highly useful for application in matrices with high content of bioactive compounds such as *Lentinula edodes*.

***Keywords**: Lentinula edodes; antioxidant compounds; polyphenol; pulsed electric fields; innovative*

1. INTRODUCTION

Global mushroom production is increasing year by year, reaching 57% growth over the last decade (from 7.59 million tons in 2009 to 11.8 million tons in 2019) [1]. *Lentinula edodes* is a mushroom of Asian origin that has been cultivated for thousands of years and its global domestic consumption has increased in recent decades being one of the most popular mushrooms, not only for its good nutritional profile, but also for its therapeutic properties related to its bioactive compounds [2], including cinnamic acid, caffeic acid and ellagic acid. In this sense, the use of sustainable alternative methods such as pulsed electric field (PEF) technology for the extraction of its bioactive components represents a beneficial alternative to conventional methods, reducing thermal degradation and avoiding the use of toxic organic solvents. PEF technology enables cell membrane electroporation and allows a selective extraction of bioactive and nutritional compounds. In fact, the application of 1 kV/cm has been shown to be sufficient to generate micropores [3]. This technology has been applied to different plant matrices obtaining a higher extraction of components such as carbohydrates, proteins and

antioxidant compounds with biological activity: anti-inflammatory, immunomodulatory, antitumor, antibacterial, antiviral, blood pressure and cholesterol regulating properties, among others [2].

The aim of the present study is to evaluate the PEF-assisted extraction in the recovery of bioactive antioxidant and phenolic compounds from the mushroom *L. edodes*, a matrix with a high content of bioactive compounds, and to compare it with the conventional aqueous extraction, characterizing the phenolic profile and the behavior of the different polyphenols in relation to the extraction methods.

2. EXPERIMENTAL

1.1. Sample preparation

Samples of *L. edodes* (shiitake mushroom) were obtained from a supermarket in Valencia (Spain) and stored in the refrigerator at 5 °C for 24 hours until use. The mushroom pileus was sliced to obtain 20 g of fresh sample for each of the three replicates of conventional and pulsed electric field-assisted extraction (PEF). The moisture content of *L. edodes* was 0.92 ± 0.01 g water/100 g sample.

1.2. Extraction conditions

Optimal conditions for mushrooms (50 kJ/kg specific energy, 2.5 kV/cm electric field strength, 6 h) determined by Calleja-Gómez et al. [4] were used with a PEF-Cellcrack III device. 200 mL of water with 20 g of the sample were added in a 900 mL extraction chamber. Electric pulses were applied with a duration of 100 ms and a frequency of 2.00 Hz. The PEF extracts were compared with the 6 h conventional aqueous extractions. All extracts were filtered and stored frozen at -20 °C until analysis.

1.3. Antioxidant activity

To measure total antioxidant capacity, the Trolox equivalent antioxidant capacity (TEAC) assay was performed based on the study published by Miller et al. [5]. 440 µL of $K_2S_2O_8$ (140 mM) was mixed with 25 mL of ABTS (7mM) and the mixture was stored at room temperature for 16 hours. This solution was calibrated to achieve absorbance at 734 nm between 0.680-0.720. Subsequently, 100 µL of sample/standard Trolox and 2 mL of ethanol were added. Results are expressed in µmol Trolox equivalents/g dry matter (µmol TE /g DM). In addition, the oxygen radical absorbance capacity (ORAC) assay was performed according to Cao et al. [6]. Trolox was used as a standard and phosphate buffer (7.2 pH) was used as a blank. In a 96-well microplate, 50 µL blank/Trolox/sample and 50 µL fluorescein were prepared. Measurements were taken at 520 nm on a Wallac 1420 VICTOR 2 plate reader with successive readings every minute. Results are expressed in µmol Trolox equivalents/g dry matter (µmol TE/g DM).

1.4. Total phenolic content

Total phenolic content was determined by the Folin-Ciocalteu method described by Singleton et al. [7]. Na_2CO_3 solution in combination with Folin-Ciocalteu reagent (50% *v/v*) and gallic acid standards at different concentrations or the corresponding sample was used. To each of the tubes, 3 mL of Na_2CO_3, 100 µL of the sample/standard and 100 µL of the Folin-Ciocalteu reagent were added. Samples were then kept in the dark at room temperature for 60 min and measured at 750 nm using a Perkin-Elmer UV/V Lambda 2 spectrophotometer. Results were expressed as mg gallic acid equivalents/g dry matter (mg GAE/g DM).

1.5. Phenolic profile characterization and quantification

For polyphenol characterization and quantification, the method previously described by Roselló-Soto et al. [8] was performed. The phenolic compounds in the samples were separated using a Waters C18 column 1.7 µm (2.1 × 50 mm) Acquity UPLC BEH. C18 and subsequently identified using a TripleTOF™ 5600 LC /MS/ MS system. For high-performance liquid chromatography (HPLC), a mobile phase was used with two solvents: A (water 0.1% formic acid) and B (methanol 0.1% formic acid). From each extract, 5 µL were taken and injected at a flow rate of 0.4 mL/min. The representative polyphenol of each group was used to calibrate and quantify the compounds: apigenin kaempferol, naringenin, catechin, gallic acid, genistein, hydroxytyrosol, resveratrol. Results were expressed in µg/100 g dry matter (µg/100 g DM).

1.6. Statistical analysis

The results were processed using GraphPad Prism 8 software performing an analysis of variance (ANOVA) with a 95% confidence interval. Each analysis was performed in triplicate and the standard deviation was represented graphically by error bars.

3. RESULTS AND DISCUSSION

The antioxidant activity results ranged from 11.50 ± 0.64 to 62.50 ± 8.39 µmol TE /g DM for TEAC and from 56.10 ± 4.58 to 228.07 ± 8.58 µmol TE /g DM for ORAC, based on conventional and PEF-assisted extraction, respectively (Figure 1). In both analyses, significant differences ($p < 0.05$) were observed for the extraction method with an increase in the recovery of antioxidant compounds in the samples pretreated with PEF. The increase in recovery of antioxidant compounds by PEF compared to conventional extraction is 443.48% for TEAC and 306.54% for ORAC.

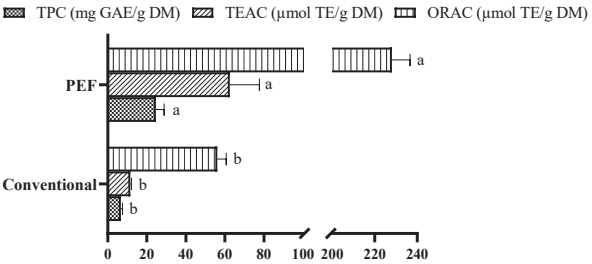

Figure 1. Trolox equivalent antioxidant capacity (TEAC), oxygen radical absorbance capacity (ORAC) and total phenolic content (TPC) obtained by pulsed electric field-assisted extraction and conventional aqueous extraction from *L. edodes*. Different lowercase letters indicate significant differences ($p < 0.05$) between extraction methodologies

In addition, similar results were observed for the extraction of total phenolic compounds with a range from 6.70 ± 0.90 to 24.68 ± 4.19 mg GAE /g DM for conventional and PEF-assisted extraction, respectively. Moreover, PEF-assisted extraction significantly increased the recovery of phenolic compounds from *L. edodes* ($p < 0.05$), with an increase over conventional extraction of 268.36%.

Regarding the phenolic content, the phenolic profile characterization showed that *L. edodes* mushroom contains mainly phenolic acids such as cinnamic and vanillic acids and thymol, which belong to the group of terpenes. The concentrations in each extract are shown in Figure 2: 305.55 ± 29.75 and 859.13 ± 70.34 µg/100g of cinnamic acid, 880.32 ± 81.07 and 315.32 ±

25.1 µg/100g of vanillic acid, and 447.59 ± 44.76 and 366.64 ± 30.11 µg/100g of thymol for the conventional and PEF-assisted extraction, respectively. In this sense, the behavior of the phenolic compounds showed a higher variability with respect to the extraction method, which was significant for all compounds ($p < 0.05$), with an increase in PEF-assisted extraction of 181.21% for cinnamic acid and 179.18% for vanillic acid, but a decrease in PEF recovery of thymol of 18.09%.

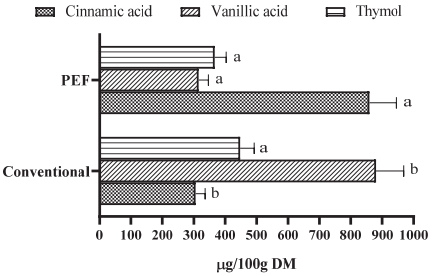

Figure 2. Content of phenolic compounds in conventional and pulsed electric field-assisted extraction from *L. edodes*. Different lowercase letters indicate significant differences ($p < 0.05$) between extraction methodologies

These results are in agreement with those observed by other authors [3,4] who suggested that PEF technology increases the extraction of bioactive compounds in plant matrices and mushrooms, especially in *L. edodes*, which stands out for its high antioxidant capacity. The higher yield of bioactive compounds could be due to the preservation of the compounds by not reaching high extraction temperatures and to the electroporation induced by the methodology. Moreover, according to Kala et al. [9] *L. edodes* is a matrix rich in phenolic acids such as cinnamic and vanillic acid, with higher contents than other mushrooms such as *Agaricus bisporus*. On the other hand, the presence of flavonoids was not observed, as suspected by Gil-Ramirez et al. [10] in which their absence to the lack of enzymes of the metabolic pathway. However, further studies are needed to determine the behavior of the different individual phenolic compounds under different extraction conditions in order to optimize the extraction for specific components.

4. CONCLUSIONS

Based on the obtained results, it can be concluded that PEF-assisted extraction is beneficial for the recovery of antioxidant and phenolic compounds and phenolics, showing significantly greater results compared to the conventional aqueous method. Moreover, it was observed that *Lentinula edodes* is a matrix rich in high nutritional compounds compared to other matrices and other mushrooms such as *Agaricus bisporus*. On the other hand, the predominant phenolic compounds in *L. edodes* were phenolic acids, with high amounts of cinnamic and vanillic acids, and thymol, which belongs to the terpene group. However, the presence of flavonoids was not observed. The behavior of each phenolic compound differed according to its nature, with a strong increase in the recovery of cinnamic and vanillic acid after applying PEF technology, while it decreased in the extraction of thymol compared to conventional extraction. In addition, it is noteworthy that the application of PEF technology is very useful for the selective recovery of compounds with high nutritional value in matrices of high dietary and therapeutic interest, such as *Lentinula edodes.*

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CHEMICAL COMPOSITION OF THE DRIED PULP OF *CUCURBITA MOSCHATA* PUMPKIN

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ABSTRACT

Pumpkin is one of the inexpensive and widely available fruits that can be kept for one to three months after harvest. To extend its shelf life and avoid microbial spoilage, moisture loss and colour changes, convective hot air drying (HAD) can be used. This method enables the use of pumpkin powder as an excellent, nutrient-rich ingredient for the production of various food products. The proposed work shows the results of chemical composition of pumpkin powder obtained after drying the pulp with hot air at temperatures of 50, 60 and 70 °C and airflow velocities of 1.5 and 0.5 m s^{-1}.

The results show that the dried pumpkin pulps have a low moisture content, ranging from 9.62 to 16.59% depending on the temperature and airflow velocity. Ash content ranges from 5.78 to 8.08%, followed by high amounts of dietary fibre (6.98-10.18%) and low fat content (0.32-0.88%), regardless of drying parameters. The protein and sugar content is particularly high, ranging from 8.90 to 10.98% and 50.80 to 66.07%, respectively, depending on the drying parameters.

Overall, the low moisture content indicates that the shelf life of the pumpkin can be extended by HAD. Because of the high ash and low fat content, the dried samples are probably an excellent mineral and fat free source. The high levels of dietary fibre, protein, and sugars indicate that the dried pumpkin pulp may be considered an acceptable and valued food ingredient due to its great nutritional potential.

Keywords: *Curcurbita moschata*, *pumpkin pulp, hot air drying, ultrasound-assisted extraction, chemical composition*

1. INTRODUCTION

Pumpkin is a fruit vegetable that is commonly cooked, baked and processed into various pumpkin products in the food industry. Due to the large global production of pumpkin and its use [1], it is necessary to use preservation methods that allow the chemical properties of pumpkin to be preserved over a long period of time. One effective preservation technology is drying, which prolongs the shelf life of the products and reduces the volume and weight during transportation. Among the various drying methods [2,3], convective hot air drying (HAD) is one of the most commonly used. Although HAD reduces water activity and consequently minimizes microbiological changes, structural and chemical changes may occur in the fresh material during drying that ultimately affect product quality. Therefore, knowledge of the chemical composition of dried pumpkin would be important information to promote its consumption in the off-season. In this context, the present work presents the chemical profile

of *Cucurbita moschata* pumpkin pulp dried using HAD to extend the shelf life of fresh pumpkin and produce high quality dried pulp. Experiments were conducted at three different temperatures (50, 60, and 70 °C) and airflow velocities of 1.5 and 0.5 m s^{-1} using the following combined parameters: 50 °C (1.5 and 0.5 m s^{-1}), 60 °C (0.5 m s^{-1}), and 70 °C (0.5 m s^{-1}). To investigate whether the parameters used affect the chemical profile of pumpkin pulp after drying, the contents of moisture, ash, fat, fibre, protein, and sugar were evaluated. Since there are no comprehensive data in the literature on the main chemical compositions of HAD of *Cucurbita* spp. [4-11], this work provides information on the above samples that can be used as a basis for the choice of HAD parameters for the production of dried pumpkin pulp.

2. EXPERIMENTAL

2.1. Chemicals and reagents

All reagents, standards, and solvents were of analytical grade. Glucose monohydrate and bovine serum albumine used for the determination of sugars and proteins were purchased from Sigma Aldrich (Steinheim, Germany). Petroleum ether, sulfuric acid, hydrochloric acid, copper sulphate, potassium iodide, potassium sodium tartarate, and sodium hydroxide were purchased from Kefo (Zagreb, Croatia). Phenol was provided by Acros Organics (Geel, Belgium). Deionized water used for the preparation of reagents, standards, and solvents was obtained using the Millipore-MilliQ instrument.

2.2. Pumpkin drying

The fresh pumpkin *Cucurbita moschata* was purchased from Exotic king (Šulog, Donja Bistra, Croatia). The fruits were washed with tap water and cut in half lengthwise with a stainless steel knife. The seeds, fibrous strands, and peels were separated from the pulp, and the pulp was cut into 3.5 to 0.5 mm thick slices with an adjustable kitchen knife and air dried.

HAD was performed at temperatures of 50, 60, and 70 °C and airflow velocities of 1.5 and 0.5 m s^{-1} using an ARMFIELD UOP8-MKII dryer (Ringwood, England). Samples were coded as 50 °C/1.5 m s^{-1}, 50 °C/0.5 m s^{-1}, 60 °C/0.5 m s^{-1}, and 70 °C/0.5 m s^{-1} based on the applied temperatures and airflow velocities. At the end of each drying, the pumpkin slices were removed from the dryer and cooled at room temperature for 15 min. They were then placed in polyethylene bags and stored in the refrigerator until further analysis.

2.3. Chemical composition of dried pumpkin

Moisture, ash, total fat, and total fiber in dried pumpkin pulp were determined by AOAC methods [12]. Total proteins (TP) and total sugars (TS) were determined after extraction of 1 g samples with phosphate buffer (pH = 7.4) and deionized MiliporeMiliQ water, respectively, in an ultrasonic bath (Elmasonic, Germany) at a temperature of 50 °C, a frequency of 37 kHz, and a working amplitude of 95%. The sonication times were 20 and 40 minutes for TP and TS, respectively. TP content was determined by the Biuret method using bovine serum albumin as a standard [13], and TS content was determined by the method described by Zouambia et al. [14] using glucose as a standard. Absorbance was measured at 540 nm and 490 nm for TP and TS, respectively, using a Perkin-Elmer Lambda 25 spectrophotometer (Massachusetts, USA). All measurements were performed in triplicate, and results were expressed as g of analyte per 100 g of sample.

3. RESULTS AND DISCUSSION

The effects of temperature (50, 60, and 70 °C) and airflow velocity of 1.5 and 0.5 m s^{-1} used at HAD of fresh *Cucurbita moshata* pulp were studied on water (moisture), ash, total fat, total fibre, total protein, and total sugar content (Figure 1).

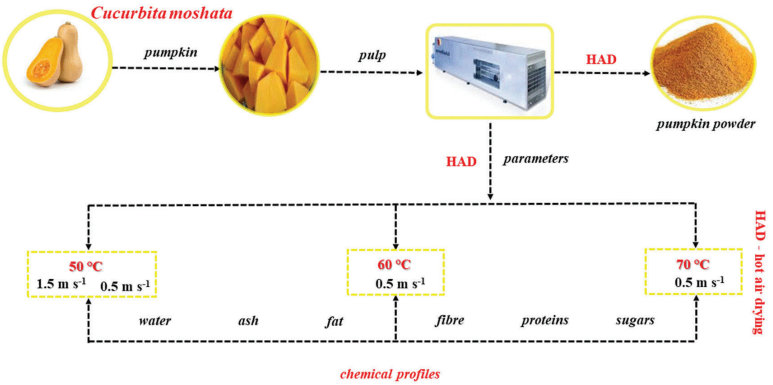

Figure 1. Hot air drying conditions of *Cucurbita moschata* and chemical parameters evaluated

It was found that the water content in fresh pulp ranged from 82.10 to 92.34% depending on the *Cucurbita* variety [4-10]. In this work, it is shown that after HAD, the water content decreases as a function of temperature and airflow velocity (Table 1), confirming the efficiency of HAD. Due to the lower water content (9.62-16.59%), the HAD pumpkin powders have better shelf life than fresh ones. Compared with this work, other studies [4-10] showed that the water content mostly varies (2.79-92.45%) and depends on several factors such as *Cucurbita* variety, temperature, airflow velocity and drying hours.

Table 1. Chemical composition of hot air dried pumpkin pulp of *Cucurbita moschata*.

Analyte	Dried sample			
	50 °C/1.5 m s^{-1}	50 °C/0.5 m s^{-1}	60 °C/0.5 m s^{-1}	70 °C/0.5 m s^{-1}
	w (analyte)/%			
Moisture	16.59 ± 0.36	11.10 ± 0.11	9.62 ± 0.26	13.24 ± 0.06
Ash	8.08 ± 0.13	8.03 ± 0.22	5.78 ± 0.57	7.72 ± 0.32
Total fat	0.88 ± 0,16	0.52 ± 0.06	0.68 ± 0.07	0.32 ± 0.03
Total dietary fibre	8.53 ± 0.03	10.18 ± 1.56	6.98 ± 0.10	9.66 ± 0.27
Total proteins	9.26 ± 0.53	8.93 ± 0.47	8.90 ± 0.54	10.98 ± 0.27
Total sugars	50.80 ± 10.27	48.63 ± 6.36	66.07 ± 7.98	53.64 ± 5.77

Values are mean ± standard deviations of three (n = 3) measurements.

As the results show, the pumpkin pulp HAD (Table 1) contains higher ash content (5.78-8.08%) than fresh pulp [4,7,10,11], suggesting that dried pulp may have an important effect on mineral absorption. For example, Montesano et al. [10] determined an ash content of 0.84% for fresh

pulp of *Cucurbita maxima*, while Guine et al. [10] and Umuhozariho et al. [4] reported values of 1.1 and 3.21%, respectively. The data for fresh pumpkin with the species *Cucurbita pepo* [7] and *Cucurbita moshata* [11] were higher, 5.50 and 6.16%, respectively. The ash content in dried pulps reported by other authors [4-7,10] is highly variable due to the differences among *Cucurbita* species, drying temperature and airflow velocity, and drying time.

The results showed that dried pumpkin pulp contains a low mass fraction of fats (0.32 to 0.88%, depending on the temperature and airflow velocity) and therefore can be used as a fat free food source in a balanced diet. The data obtained in this work are somewhat lower compared to other studies that gave values ranging from 1.40 to 2.06% depending on *Cucurbita* spp. and drying conditions [6,10].

To find out whether the dried pumpkin pulp is a valuable source of dietary fibre, the samples were analysed from HAD, which had contents ranging from 6.98 to 10.18%, depending on the temperature and airflow velocity (Table 1). The total dietary fibre content determined in other works [5,10] differed mostly from this study, with variations due to *Cucurbita* spp. and drying conditions. Values ranged from 1.55 to 9.69% [5,10] and 0.81 to 11.25% [15]. Compared to the dried pumpkin samples, the fresh pulp contained 1.0 to 8.77% [5,10] and 0.29 to 1.13% dietary fibre [15], highlighting that the dried pulp is a promising solution for the intake of dietary fibre from this vegetable.

As for the total protein content, the results showed values ranging from 8.90 to 10.98%, depending on the temperature and airflow velocity (Table 1). For comparison, the review paper by Hussain et al. [15] reported values between 1.30 and 15.50, so the results obtained in this study were within these limits. In addition to the dried samples, the fresh pumpkin samples contained between 0.29 and 1.13% of total proteins [15], indicating that the samples from HAD yielded higher amounts. As shown in Table 1, all combined drying parameters could be used for further protein intensification.

The total sugar content in dried pumpkin pulp ranged from 50.80 to 66.07% regardless of drying parameters. These remarkably high values compared to fresh pulp, which contains 2.15 to 2.90% total sugars (depending on *Cucurbita* cultivar and cultivation method) [16] indicate that HAD has a significant effect on sugar content. For the dried pumpkin pulp, Sojak et al. [17] also reported high total sugar contents, i.e. 58.56 to 79.44%, depending on the type of drying (chamber, tunnel, and fluidized bed dryer) and the temperatures applied (40, 50, 60, 70, and 80 °C). On the other hand, Guine et al. [10] obtained lower values (17.09 to 18.66 %, depending on the drying conditions) compared to the results of this study.

4. CONCLUSION

While HAD led to a reduction in moisture content, which prolonged the shelf life of pumpkins, at the same time it is possible to obtain a high quality dried pulp. Because of their high ash and low fat content, they are likely to be an excellent source of minerals and fat free. Since dried pumpkin pulp is rich in fibre, protein and sugar, it can be considered a valuable food ingredient.

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EFFECT OF SUPERCRITICAL CO_2 EXTRACTION PARAMETERS ON THE FATTY ACIDS COMPOSITION OF GRAŠEVINA GRAPE SEED OIL

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ABSTRACT

Grape pomace, generated during winemaking, represents an important raw material for obtaining valuable products. Among them, seed oil has great potential in food industry as an alternative source of vegetable oils, due to the high content of essential fatty acids. The aim of the proposed research was to investigate the effect of supercritical CO_2 (SC CO_2) process parameters, in comparison to cold pressing, on the fatty acids composition in Graševina grape seed oils. The parameters varied during the SC CO_2 extraction were: pressures (300, 400 and 500 bar), temperatures (35, 45 and 55 °C) and gas flow (15, 30 and 45 g/min). The fatty acids composition was analyzed by GC-FID according to the ISO 5509:2000 method. Dominant fatty acid in all extracted samples was linoleic acid, followed by oleic and palmitic acid. Moreover, saturated fatty acids accounted up to 12.8%, monounsaturated up to 22.6% and polyunsaturated fatty acids up to 66.02%. Although the differences among samples were slight, statistical analysis of variance (ANOVA) showed that most of them were significant ($p < 0.05$). Cold pressed oil was characterized with significantly higher concentration of linoleic acid, and significantly lower concentration of oleic acid. On the other hand, significantly higher concentrations of palmitic acid were detected in SC CO_2 extracted oils. Regarding the treatments applied, significantly higher concentrations of most of fatty acids were obtained after application of pressure at 400 bars, temperature at 55 °C and flow rate of 15 g/min of CO_2.

***Keywords**: supercritical CO_2 extraction, fatty acids, grape seed oil, Graševina*

1. INTRODUCTION

Grape pomace, a by-product of winemaking, has been emphasized as a potential source of nutritionally valuable fractions that could have important applications in food and nutraceutical industries [1]. Seeds represent the important portion of grape pomace (38-52%) that could be used for oil extraction. Generally, grape seeds contain 8-20% of oil, and its quality is due to high level of unsaturated fatty acids (90% poly- and monounsaturated fatty acids), particularly linoleic (18:2) and oleic (18:1), and low levels of saturated fatty acids (about 10%), as well as tocopherols and tocotrienols [2-5].

The extraction technique, type of solvent and operating conditions employed, as well as grape variety and the environmental factors during the harvest affect the oil yield and oil quality [6].

Cold pressing represents a method of oil extraction that involves no heat or chemical treatment and, although the yield is usually lower than with conventional solvent extraction, there is no concern about the solvent residues after its application, making these oils safer and more acceptable by consumers [7]. Besides cold pressing, supercritical extraction (SC CO_2) represents an alternative that can achieve comparable oil yield as traditional solvent extraction techniques using non-toxic, non-flammable, non-polluting and cheap substance (CO_2), and no solvent traces remain in extracted oil [1], which makes this technique highly desirable in food industry.

The aim of this work was to investigate the effect of SC CO_2 process parameters (pressure, temperature and gas flow) on the fatty acids compositions in Graševina grape seed oils, in comparison to the cold pressing procedure.

2. EXPERIMENTAL

2.1. Grape seed preparation

Grape seed pomace from Graševina cultivar was provided from Kutjevo d.d. (sub-region Slavonia, Croatia) during vintage 2021. Seed were immediately manually separated from the rest of the pomace and frozen at -20 °C until the oil extraction. Prior to extraction, seeds were adequately prepared, depending on the extraction technique used: (i) for cold pressing seeds were dried at 48 °C until 6-9% of moisture, while (ii) for supercritical CO_2 extraction were completely dried at 48 °C during 48 h. Dry seeds were ground using electrical grinder (Bühler GmbH, Germany) and the portion of 100 g were sieved using vertical sieve shaker (seven sieved of 2.25, 1.12, 0.67, 0.45, 0.369, 0.282, and 0.18 mm). The average particle diameter (d_p) was calculated to be 0.5446 ± 0.0045 mm.

2.2. Cold pressing

Whole seeds were extracted with a laboratory screw press Komet CA/53 Monforts & Reiners, Rheydt, Germany) without heat treatment. After pressing, the oil was purified from impurities by centifugation at 7800 rpm during 10 min. Purified oil was kept in hermetically sealed bottle at -20 °C until laboratory analyses.

2.3. Supercritical CO_2 extraction (SC CO_2)

The experiment was performed on laboratory scale equipment (Extratex, Neuves-Maisons, France). The grounded grape seeds of 50 g were placed into extractor vessel and extracted for 90 min. The SC CO_2 was performed at different extraction conditions of pressure (300, 400 and 500 bar), temperature (35, 45 and 55 °C) and CO_2 gas flow (15, 30 and 45 g/min) defined by Box-Behnken design. The extracted oil was kept in hermetically sealed bottle at -20 °C until laboratory analyses.

2.4. Fatty acids profile

Fatty acids methyl esters were prepared by transesterification with methanol according to ISO 5509:2000 method [8]. A mass of 60 mg of grape seed oil was dissolved in 4 mL of isooctane and 200 µL potassium hydroxide (2 mol/L). Mixture was vigorously shaken using vortex for 30 s and left for a few minutes at room temperature to react. Then, 1 g of sodium hydrogen sulphate monohydrate was added, mixed and clear supernatant containing methyl esters was transferred into the vial. The prepared methyl esters were injected (1 µL) into an Agilent Technologies 6890N Network GC system (Santa Clara, CA, USA) equipped with flame ionisation detector. Fatty acid methyl esters were separated on a DB-23 capillary column (60 m×0.25 mm×0.25 µm; Agilent Technologies) according to the method in detail described

by Kraljić et al. [9]. Fatty acid methyl esters were identified by comparison of their retention times with those of the commercial standards. The content of each fatty acid is expressed as a percentage of total fatty acids, and the results are presented as the mean value of three parallel determinations.

2.5. Data analysis

Statistical analysis of analytical data was carried out by Analysis of Variance (ANOVA) using Statistica v.10.0 software (Statsoft Inc., Tulsa, OK, USA). The analysis of Variance (ANOVA) was performed on all independent variables of fatty acids. Tukey's HSD test was used as a comparison test when samples were significantly different after ANOVA ($p < 0.05$) of analyzed compounds.

3. RESULTS AND DISCUSSION

The fatty acid profile of analyzed cold pressed oil, as well as of those obtained by SC CO_2 extraction are presented in Table 1. Dominant fatty acid in all oil samples was linoleic acid (18:2n6, 64.67-66.00%) and followed by oleic (18:1n9, 21.12-21.52%), palmitic (16:0, 7.39-8.38%) and stearic (18:0, 4.14-4.35%). Similar values, as well as decreasing order in major fatty acids has been reported previously [2-5, 8, 11, 12]. Other fatty acids detected in analyzed grape seed oils were α-linoleic (18:3n3), palmitoleic (16:1), eicosenoic (20:1), arachidic (20:0) and myristic acid (14:0). The determined values of individual fatty acids are also in accordance within the limits given by Codex [11]. Total saturated fatty acids (SFA) amounted between 11.96 and 12.86%, levels that are slightly higher than that of rapeseed oil, but comparable to most of the other used edible vegetable oils [12]. Furthermore, unsaturated fatty acids amounted up to 88.04%, polyunsaturated fatty acids (PUFA) were predominant (65.11-66.46%), and those levels were also similar to the values reported in previously mentioned studies [2-4, 7, 9-10]. Generally, grape seed oil is similar to sunflower oil which consist of about 60-70% linoleic acid and 12-25% oleic acid, as well as only small amounts of linoleic acid [12].

Although the differences among samples were slight, statistical analysis of variance (ANOVA) showed that most of them were significant ($p < 0.05$). Regarding the oil extraction procedure, it can be observed that cold pressing resulted in oil with significantly higher levels of linoleic and stearic acid, while SC CO_2 extraction resulted in higher levels of oleic, palmitic and myristic acids. Moreover, cold pressed oil is characterized with higher level of total polyunsaturated fatty acids (sum PUFA), and lower levels of total saturated fatty acids (sum SFA) and total monounsaturated fatty acids (sum MUFA), when compared to SC CO_2 extracted oil. Regarding the applied SC CO_2 treatment variations, it can be observed that treatment at 500 bar, 45 °C and 45 g/min gas flow was the most invasive one since it resulted in the lowest level of linoleic acid, as well as of sum PUFA. On the other hand, it resulted in the highest levels of oleic acid, sum PUFA and sum MUFA. Finally, significantly higher concentrations of most of fatty acids after SC CO_2 extraction were obtained after application of pressure at 400 bars, temperature at 55 °C and flow rate of 15 g/min of CO_2.

Nevertheless, all these differences are among 1.5% variation between cold pressed oil and SC CO_2 extracted ones, implying that, despite the statistical significance, SC CO_2 extraction does not importantly affect the level of fatty acids in oils. Fiori and coworkers [1] concluded that no significant differences in the fatty acids composition of oils extracted by mechanical press and SC CO_2 were observed. Similar conclusions regarding the no significant difference between cold pressed and SC CO_2 extracted oils, were also obtained for other plant oils, such as apricot kernel oil [13] and walnut oil [14].

Table 1. Fatty acids composition (% of total) in cold pressed and SC CO_2 extracted Graševina seed oils

Pressure (bar)	T (°C)	g/min CO_2	C14:0	C16:0	C18:0	C20:0	C16:1	C18:1c	C18:2c	C18:3n3	C20:1	sum SFA	sum MUFA	sum PUFA
Cold pressed oil			0.06^{e}	7.39^{e}	4.35^{a}	0.16^{g}	0.28^{g}	21.12^{j}	66.00^{a}	0.47^{f}	0.17^{a}	11.96^{e}	21.57^{l}	66.46^{a}
300	35	30	0.08^{cd}	8.24^{cd}	4.26^{bc}	0.17^{bcde}	0.37^{de}	21.34^{bcd}	64.87^{d}	0.52^{e}	0.17^{a}	12.75^{bcd}	21.87^{cde}	65.38^{efg}
500	35	30	0.08^{cd}	8.27^{abcd}	4.27^{bc}	0.18^{ab}	0.37^{cde}	21.30^{ef}	64.85^{d}	0.52^{e}	0.17^{a}	12.80^{abc}	21.84^{gh}	65.36^{ef}
300	55	30	0.08^{abc}	8.33^{abc}	4.21^{ef}	0.17^{def}	0.37^{abc}	21.23^{g}	64.92^{d}	0.52^{de}	0.17^{a}	12.79^{abcd}	21.77^{hi}	65.44^{d}
500	55	30	0.08^{ab}	8.38^{a}	4.21^{def}	0.17^{ef}	0.38^{ab}	21.28^{fg}	64.80^{fg}	0.53^{cd}	0.17^{a}	12.84^{a}	21.83^{fgh}	65.33^{fgh}
300	45	15	0.08^{abc}	8.32^{abc}	4.21^{def}	0.17^{cdef}	0.37^{abcd}	21.28^{f}	64.85^{d}	0.53^{bc}	0.17^{a}	12.79^{abcd}	21.83^{gh}	65.39^{def}
500	45	15	0.08^{bc}	8.25^{bcd}	4.25^{bcd}	0.18^{bcde}	0.37^{cde}	21.33^{de}	64.84^{de}	0.53^{bc}	0.17^{a}	12.76^{bcd}	21.87^{efg}	65.38^{def}
300	45	45	0.08^{bc}	8.30^{abcd}	4.27^{bc}	0.18^{bcd}	0.37^{cde}	21.39^{b}	64.69^{h}	0.54^{ab}	0.18^{a}	12.83^{a}	21.94^{b}	65.23^{j}
500	45	45	0.08^{bc}	8.32^{abc}	4.28^{b}	0.18^{bc}	0.37^{bcde}	21.39^{bc}	64.67^{i}	0.53^{c}	0.17^{a}	12.86^{a}	21.94^{bc}	65.20^{ij}
400	35	15	0.08^{bc}	8.25^{bcd}	4.24^{cde}	0.17^{bcde}	0.37^{cde}	21.29^{ef}	64.89^{d}	0.53^{bc}	0.17^{a}	12.74^{bcd}	21.83^{fgh}	65.42^{d}
400	55	15	0.09^{a}	8.37^{ab}	4.14^{g}	0.17^{fg}	0.38^{a}	21.17^{i}	64.98^{c}	0.54^{ab}	0.17^{a}	12.77^{bcd}	21.71^{j}	65.52^{c}
400	35	45	0.08^{cd}	8.23^{cd}	4.28^{b}	0.18^{b}	0.37^{e}	21.37^{bc}	64.80^{ef}	0.53^{cd}	0.17^{a}	12.76^{bcd}	21.91^{bcd}	65.33^{gh}
400	55	45	0.08^{cd}	8.27^{abcd}	4.27^{bc}	0.18^{bcd}	0.37^{cde}	21.36^{bc}	64.77^{fg}	0.53^{bc}	0.17^{a}	12.80^{abc}	21.90^{def}	65.30^{hi}
400	45	30	0.08^{bc}	8.30^{abcd}	4.26^{bc}	0.18^{ab}	0.37^{cde}	21.36^{cd}	64.74^{gh}	0.53^{bc}	0.17^{a}	12.82^{bc}	21.90^{cde}	65.28^{i}
400	45	30	0.08^{d}	8.20^{d}	4.37^{a}	0.19^{a}	0.36^{f}	21.52^{a}	63.58^{j}	0.53^{cd}	0.18^{a}	12.84^{a}	22.05^{a}	65.11^{k}
400	45	30	0.08^{cd}	8.22^{cd}	4.26^{bc}	0.18^{ab}	0.36^{e}	21.31^{ef}	64.86^{d}	0.54^{a}	0.17^{a}	12.75^{bcd}	21.85^{efgh}	65.41^{de}
400	45	30	0.08^{cd}	8.27^{abcd}	4.22^{def}	0.17^{bcde}	0.37^{cde}	21.23^{h}	64.95^{c}	0.54^{a}	0.17^{a}	12.74^{cd}	21.77^{ij}	65.49^{c}
400	45	30	0.08^{cd}	8.26^{abcd}	4.19^{f}	0.18^{bc}	0.36^{e}	21.03^{j}	65.18^{b}	0.53^{bc}	0.17^{a}	12.71^{d}	21.57^{k}	65.72^{b}

sum SFA- sum of saturated fatty acids; sum MUFA- sum of monounsaturated fatty acids; sum PUFA- sum of polyunsaturated fatty acids. Different lowercase letters indicate significant difference between samples (Tukey's test, $p<0.05$)

4. CONCLUSION

The results reported in this work indicate the potential of grape seed oil as a source of unsaturated fatty acids. Cold pressing resulted in oil with significantly higher levels of linoleic and stearic acid, while SC CO_2 extraction resulted in higher levels of oleic, palmitic and myristic acids. Moreover, SC CO_2 extraction parameters slightly affect their final concentration and the highest levels of the most important fatty acids were detected after application of pressure at 400 bars, temperature at 55 °C and flow rate of 15 g/min of CO_2.

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PROSEED: CIRCULAR-BASED ALTERNATIVE PROTEINS EXTRACTION FROM GRAPE SEEDS UNDER ULTRASOUND

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ABSTRACT

The aims of the project PROSEED are the production and commercialization of circular-based environmentally and economically sustainable proteins extracted from grapevine seeds, an abundant by-product of wine production. In a circular-based strategy, PROSEED proteins represent a value-added product with potential application in several production chains as natural ingredient in food industry. This protein-rich product can be considered a healthy phytocomplex containing soluble fibres, oligoelements and some polyphenols. Currently industrial wine clarification is carried out with animal proteins or vegetal proteins of different origin. PROSEED proteins could replace these additives, providing a vegan and gluten-free product within the same production chain.

Moreover, the project will contribute to a better carbon footprint with an estimated 82% reduction of CO_2 emission thanks to a valuable implementation of the wine production chain.

This project is based on the proteins extraction processes in water tested in laboratory scale, an alkaline – acid extraction (AAE), was much improved by ultrasound-assisted extraction (UAE)**.** The investigation plan of PROSEED is aiming to define the most efficient extraction conditions in term of yield and selectivity The main extraction parameters are temperature (30 – 50 °C), water: seeds ratios, and sonication time. The proteins-rich extract from crushed detannificated grape seeds, is expected around 12 w/w % of proteins content respect the dry material. So far the best results achieved about 25% w/w of proteins extract compared to whole proteins content in the seeds. Economic sustainability has been verified through a comprehensive business plan : business model would be confirmed by Process Flow Diagram (PFD) and Process & Instrumentation Diagram (P&I) documents according to the scale up evaluation already performed.

Keywords*: Alternative proteins; Ultrasound-Assisted Extraction; Grape seeds; EIT Food; Circular economy.*

1. Introduction

In the last decades, there has been an increased interest in alternative protein sources such as vegetal biomasses, insects, fungi and algae [1][2]. This growing demand requires special attention to environmental sustainability, high nutritional value and ethical production standards in the context of a circular economy. In this context, the European Institute of Innovation and Technology of Food (EIT Food), which supports the current work, invests extensively in projects, organisations and individuals that share the goals of a healthy and sustainable food system. EIT Food goal is to create valuable networks of stakeholders and research centres. The amended data on global grape production worldwide in 2020 was about 9,211,896 hg/ha, total harvested area was 7,718,443 ha, and vineyard production was 92,877,423 tonnes worldwide (all data updated in 2020) [3]. In the European Union (EU) wine

industry, about 28 million tons of grapes are harvested annually and nearly 5.6 million tons of grape pomace are produced as a by-product [4] Grape seeds, grape stalks and grape pomace are important by-products of the wine industry [4].This extensive waste represents a valuable biomass for the extraction of valuable compounds, such as polyphenols, enocyanines, oil and proteins that can be used as functional food and bioactive ingredients in food, pharmaceutical, cosmetic and nutraceutical formulations.

Currently, the recovery of proteins from seeds is carried out using organic solvents and/or basic/acid extraction [5] Although several excellent results obtained with new technologies at laboratory scale can be found in the literature [6], the industrial implementation remains a complicated task [7]. Low-frequency ultrasound (20 kHz-100 kHz), usually used at high-intensity (10-1000 W/cm^2), has several applications in the food industry due to the cavitation effects [8][9]. The acoustic cavitation phenomena consist of the formation and subsequent collapse of microbubbles in a liquid medium. The mechanical effect produced by such collapse leads to detexturization of the matrix [10], which increases the surface area and penetration ability of the solvent, thus enhancing mass transfer of the target molecules [11].

UAE was therefore investigated in the present work for the extraction of grape seed proteins in order to improve the sustainability of the process, avoid the use of unnecessary organic solvents, and increase the overall extraction yield.

2. Experimental

2.1. Samples

Grape seed samples were supplied by Caviro (Forlì, Italy). In a first step, grape seeds were air dried at a temperature not exceeding 80 °C. Subsequently, the phenolic compounds were recovered using an ethanol-water mixture (50:50, v/v) before extraction of the proteins. Solid-liquid separation was then performed in a decanter and the liquid fraction was concentrated to obtain a polyphenol-rich extract. The remaining solid fraction was then washed with water and ground without drying before the protein extraction step.

2.2. Moisture content determination

To determine the water content of the grape seeds studied, a certain amount of each sample was dried in an oven at 105 °C until constant weight. The difference in weight before (mi) and after (mf) drying gives the moisture content of the sample, as shown in the following equation:

$$X(\%) = \frac{m_i - m_f}{m_i} x100 \qquad (1)$$

2.3. Ash content determination

5 g of grape seeds were weighed in a combustion crucible. The sample was then placed in a Nabertherm muffle furnace (Nabertherm Gmbh, Lilienthal/Bremen, Germany) set at a temperature of 650 °C ± 1 °C for at least 4 h until a carbon particle-free ash was obtained. The crucible was then cooled in a desiccator for 1 h and then reweighed. The total ash content was calculated as follows:

$$\text{Ash content } (\%) = \frac{(W_f - W_0)}{sample\ weight} \times 100 \qquad (2)$$

While W_f is the final weight of the crucible and W_0 is the empty weight of the crucible.

2.4. Ultrasound-assisted extraction of proteins

After extraction of the phenolic compounds, the ground samples were mixed with distilled water at a ratio of 1:5 or 1:10 (w/v). Then, the pH was adjusted to 11 with 6 M sodium hydroxide (NaOH), and the resulting mixture was sonicated for 1 h, 2 h, and 4 h in an ultrasonic bath (200 W, 24 kHz) at a selected temperature (30-50 °C). The pH of the suspension was checked and finally adjusted to the original basic value with 0.1 N NaOH. The resulting mixture was then

centrifuged at 4200 rpm for 20 min, the supernatant was filtered and acidified to pH 3.0 with 0.1 N sulfuric acid, and then kept at a cold temperature (5 °C) overnight. The supernatant was filtered off, and the remaining solids were recovered and freeze-dried to obtain the final extract. The yield of the final extract obtained was determined on a mass basis as a proportion of the amount of grape seed proteins extracted.

2.5. Protein analysis

Kjeldahl method (performed through SpeedDigester K-439 and Kjel Line) was used to determine the amount of nitrogen and thereby proteins in grape seeds, in the supernatant liquid of the extraction, and in proteins extracts. The percentage of total nitrogen (%N) and the percentage of proteins (%P) in samples were determined by using Equations (3) and (4), respectively.

$$\%N = \frac{[V(1)-V(BI)]\cdot c_N \cdot M(N)}{m\cdot 1000} \times 100 \quad (3)$$

With V(1) [mL]: consumption of titrant, sample; V(BI) [mL]: average consumption of titrant, blank; $[c]_N$ [eq/L]: concentration of titrant; M(N) [g/mol]: molecular weight of N; m [g]: sample weight.

$$\%P = \%N \times 6.25 \quad (4)$$

In grape seeds, the temperature ramp set up to digest the proteins extract were: preheating for 15 min to 300 °C; heating at 480 °C (10 min), heating at 550 °C (10 min), digestion at 490 °C (3 h), and, finally, cooling for 80 min until 25 °C. When focusing on the proteins extract, the temperature ramp set up to digest the proteins extract can be summarized as follows: preheating for 15 min to 250 °C; heating at 310 °C (10 min), 370 °C (30 min), 430 °C (10 min), digestion at 490 °C for 2 hours and then cooling for 80 min until 25 °C.

Proteins extract samples were weighed in the range of 1 to 1.5 g inside the 12 tubes adding 10 mL of 95% H_2SO_4, 1 catalyst tablet (5 g, 98% K_2SO_4, 2% Na_2SO_4). Grape seeds weight was 0.500 g inside the 12 tubes adding 10 mL of 95% H_2SO_4, 1 catalyst tablet (5 g, 98% K_2SO_4, 2% Na_2SO_4). Once the digestion is accomplished, the tubes were left air-cooling to room temperature. Afterwards, the distillation step was conducted using 40 mL of water and 60 mL of NaOH 30% w/w %. The distillation time used was 300 seconds. The drops of condensed NH_3 solution fall to a 60 mL buffer solution of 4% Boric acid with indicator. Finally, the titration was carried out by H_2SO_4 0.1M.

2.6. Statistical analysis

The obtained results were expressed as mean values ± standard deviation (SD). One-way ANOVA and Fischer test were determined by using Excel (Microsoft Office, 2020).

3. Results and discussions

3.1. Grape seeds characterization

Detannified grape seeds were characterized in terms of moisture content, organic and inorganic content and proteins content (Table 1).

Table 1. Characterization of detannified grape seeds

Moisture content	Organic content	Inorganic content	Proteins content
41.91% ± 2.31%	56.06% ± 3.12%	2.03 ± 0.81%	12 ± 0.9%

3.2. Extraction conditions screening

Extraction conditions screenings were carried out considering three different variables, namely time, temperature and solid to liquid ratio, as already mentioned. Two variables were considered fixed for each screening, to investigate the contribution of each parameter.

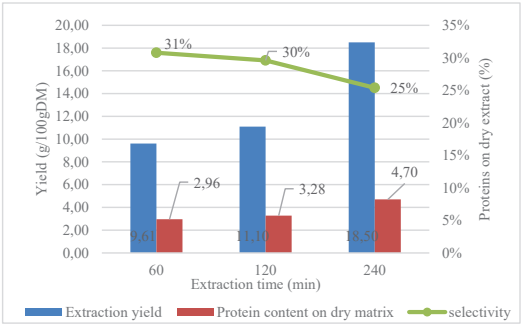

Figure 1. Effect of extraction time on proteins extraction.

Proteins extraction yield was considered as the main parameter for conditions optimization. A first screening considered the effect of time on proteins yield, while solid to liquid ratio was fixed at 1:10 and temperature was set at 40 °C (*Figure 1*).

As can be noticed in the abovementioned figure, a prolonged extraction time induced an increase in both proteins yield and overall extract yield, with a higher growth rate for the latter. This in turn marks a decrease in the selectivity, which should be considered for its influence on the quality of the final product.

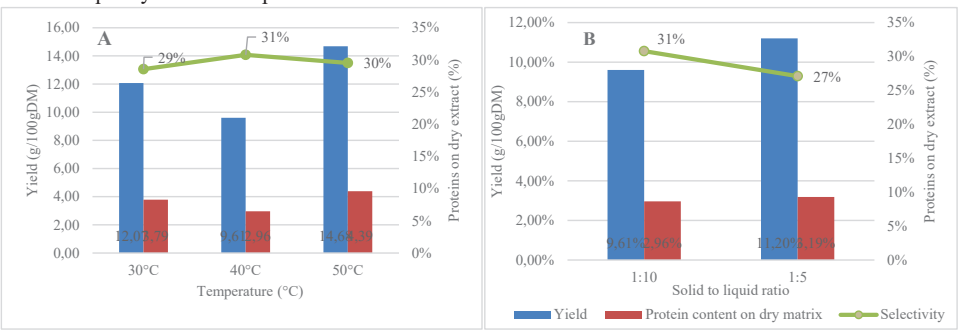

Figure 2. Effect of temperature and solid to liquid ratio on proteins extraction. A: effect of temperature; B: effect of solid to liquid ratio.

A second screening involved the extraction temperature, fixing the extraction time to 1 h and the solid to liquid ratio at 1:10 (*Figure 2.A*). An increase in temperature from 30 °C to 50 °C leads to an increase both in overall and proteins yield of 21% and 15% respectively (*Figure 2.A)*. A higher temperature is not only favourable in terms of yields, but also advantageous in an economic point of view since a higher amount of energy is required to keep the temperature at 30 °C.

Finally, two different solid to liquid ratios were considered, namely 1:5 and 1:10. Extraction time and temperature were fixed at 1 h and 40 °C respectively (*Figure 2.B*). An increase of 16%

in overall extraction yield and a 7% increase in proteins extraction yield was noticed when reducing the solid to liquid ratio from 1:10 to 1:5. While decreasing the solid to liquid ratio marks a slight decrease in selectivity, the advantages of a reduced water consumption should be considered in a scale-up perspective.

4. Conclusion

UAE in water at modified pH was applied for the recovery of proteins from crushed grape seeds. Several tests were performed, changing three parameters: extraction time, temperature and grape seeds/solution ratio. 40 °C was chosen as the best working temperature, since the yields obtained at the different temperatures (30 °C/40 °C/50 °C) did not show significant differences. Setting the temperature at 30 °C requires higher energy consumption for cooling, while a temperature of 50 °C is associated with a higher proteins denaturation. Since the yield did not justify the higher energy and time consumption, 1 h of UAE was defined as the optimal time. Finally, the seed/solution ratio 1:5 gave the higher proteins yield with lower water consumption.

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EVALUATION OF DIFFERENT ACRYLAMIDE GREEN EXTRACTION METHODS

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ABSTRACT

Acrylamide (AA) is a processing-based contaminant produced in different foods, (i.e., bread, coffee, and cookies) as a derived product of the Maillard Reaction (MR) after processing at high temperatures (>120 °C). It is classified by the International Agency for Research on Cancer (IARC) as a probably carcinogenic to humans (Group 2A) and presents others adverse health effects which are related to its neuro and reproductive toxicity. This work aims to compare several green extraction methods to recover AA and subsequently identify and quantify its levels using high performance liquid chromatography equipment with an ultraviolet detector (HPLC-UV). Coffee, corn, cookies, and toasted bread were the food matrices studied. The green extraction techniques employed were Accelerated Solvent Extraction (ASE), Solid Phase Extraction (SPE), Supercritical Fluid Extraction (SFE) and, QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe). ASE and QuEChERS allowed high recoveries of AA in those samples with high AA contents. SPE was a useful technique to concentrate the AA while SFE did not get successful results.

***Keywords**: Acrylamide, Accelerated solvent extraction, Solid phase extraction, Supercritical fluid extraction, QuEChERS, UV detection*

1. INTRODUCTION

Acrylamide (AA) is a food processing contaminant mainly formed during the Maillard Reaction (MR) at temperatures above 120 ºC. MR provides desirable browning and flavor in the food, but also produces toxic substances such as AA. Its precursors are amino acids (primarily asparagine) and reducing sugars [1]. The main average of some foodstuffs susceptible to forming AA are listed in Table1.

Table 1. Average AA contents of foodstuffs.

Food	Bread	Breakfast cereals	Cookies	Potato chips	Coffee powder
Mean ± SD[1] (µg/kg)	10.46 ± 15.64	207.3 ± 275.4	246.6 ± 224	753.1 ± 466.6	272.4 ± 118
Reference	[2]	[3]	[2]	[2]	[3]

[1]Standard deviation

AA's toxic effects are well-known. For instance, The International Agency for Research on Cancer (IARC) classifies AA as a "probable human carcinogen"(group 2A). AA is genotoxic and in small amounts may produce DNA damage. In 2005, European Food Safety Authority (EFSA) published a scientific opinion linking AA exposure to the risk of having several kinds of cancer, making it not possible to establish a Tolerable Daily Intake (TDI). EFSA does not provide AA maximum legal level on food, but it issued guidance to reduce the amount of AA in food industry and reference values [4,5].

Over the last years, the most common techniques used for AA extraction have been SPE, QuEChERS (quick, easy, cheap, effective, rugged, and safe) and DLLME (Dispersive Liquid-Liquid Microextraction), being the detection usually carried out by liquid and gas chromatography coupled to tandem mass spectrometry, but the current need for faster, cleaner, and sustainable analytical tools has promoted the interest of researchers to explore innovative alternatives and/or optimizing conventional tools [6,7].

In this sense, Accelerated Solvent Extraction (ASE) technology is an extraction technique of polar compounds through organic solvents and aqueous phases. The main difference with other methods (QuEChERS or DLLME) is the possibility of making use of controlled temperatures and pressures. It has been widely employed in the extraction of pesticides but the scientific literature available on the extraction of AA in ASE is limited [8,9]. On the other hand, in previous studies, SPE columns were used for two purposes: to clean up the sample and to concentrate the analyte. Clean-up columns retain part of the AA and do not retain other polar compounds that may interfere with AA detection [7,10]. Moreover, another interesting tool is the supercritical fluid extraction (SFE), which is based on subjecting a fluid to pressure and temperature conditions that make it impossible to distinguish whether it is a liquid or a gas (critical point). When the fluid is under supercritical conditions it is useful for the extraction of several compounds. Usually, CO_2 is used as a solvent because it is non-toxic and does not alter the sample [11]. QuEChERS is a dispersive solid phase extraction (dSPE) which has been employed in AA extraction before mass spectrometry determination [12,13]. The aim of this study is to evaluate the potential of these techniques (ASE, SPE, SFE and QuEChERS) as effective tools to extract AA on food samples to facilitate its identification and quantification by high performance liquid chromatography equipment with an ultraviolet detector (HPLC-UV).

2. EXPERIMENTAL

2.1. Reagents and chemicals

Acetonitrile (ACN) and methanol (MeOH) (HPLC grade) used for the extraction were purchased from Merck (Darmstadt, Germany). Methanol used for the mobile phase was the same. The deionized water (resistivity > 18 MΩ cm) was prepared using a Milli-Q SP® Reagent Water System (Millipore Corporation, Bedford, USA). Diatomaceous earth was provided by Thermo scientific (USA) and sea sand by Panreac química (Barcelona, España). Sodium chloride (NaCl) was supplied by VWR Chemicals (Leuven, Belgium). Zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (≥ 99%) and Magnesium oxide (MgO) (98%) were obtained from Thermo Fisher (Loughborough, Leics, UK). Primary secondary amine (PSA) was purchased from Agilent Technologies (USA). Strata® Activated Carbon, 400mg/Pass Through Cartridge, 50/pk cartridges were purchased from Phenomenex (China). Acrylamide (≥ 99%, HPLC grade) was supplied by Sigma (St. Louis, USA).

2.2. Samples

Coffee, corn, cookies, and toasted bread samples were purchased from a local supermarket (Valencia, Spain). Samples were homogenized and powdered. Prior to the treatment, 1 g of sample was weighted and spiked with AA. Extracts were stored at 5 ºC until their analysis.

2.3. Accelerated solvent extraction (ASE)

For the ASE treatment, the Dionex ASE 150 purchased from Thermo Scientific, with a 34 mL cell extraction volume, was used. Diatomaceous earth and sea sand were used to fill the cell. The extraction parameters were set at 40 ºC, 1700 psi, 3 min of static time and 3 static cycles, 60 s of purge and 3 flushes were used at 100%, 50%, and 30%. The solvents used were water and methanol. The cell was contaminated with 200 µL of 1000 ppm of AA. 0.5 g of $ZnSO_4$, and 0.5 g of MgO were also added to the cell. To concentrate the analyte to 5 mL, the rotary evaporator was used at a pressure of 280 mbar and a temperature of 50 ºC for 4 min.

2.4. Solid phase extraction

Strata® Activated Carbon (400 mg) was conditioned with 5 mL of water followed by 5 mL of methanol, both were discarded. Subsequently, 10 mL of sample were loaded and discarded. Finally, 3 mL of methanol, as an elution solvent, were loaded and 2 mL were collected for analysis. Vacuum Manifold for SPE at was used. The concentration of the working standards solutions were 100 ppb and 1000 ppb of AA.

2.5. Supercritical fluid extraction

The SFE was carried out using a supercritical extraction system (JASCO, Tokyo, Japan) composed of a main isocratic CO_2 pump (PU-4387). The method was set at 30 min, 10 MPa of pressure, 40 ºC of temperature, 10 mL/min of flow and a combination of solvents: metanol:CO_2 (10:90) and Water:CO_2 (10:90).

2.6. QuEChERS

Two milliliters of water and two mililiters of methanol followed by 1 g of $MgSO_4$ and 0.5 g of NaCl were added to 1 g of spiked coffee at 200 ppm, 500 ppm and 50 ppm. The tube with the mixture was vortexed for 1 min and centrifuged for 5 min at 4000 rpm. 1 mL of the supernatant was placed in a 5 mL tube containing 0.15 g of $MgSO_4$ and 0.05 of PSA. After 1 min of vortexing and 10 min of centrifuging at 4000 rpm, the supernatant was ready to be analyzed.

2.7. HPLC-UV determination

For AA identification, an HPLC system and PU-2089 PLUS (Jasco) equipped with UV-1570 detector system (Jasco) was employed. The mobile phases consisted of water:ACN (90:10) used in isocratic mode. The injection volume was 10 µL and the flow rate 0.8 mL/min. The length wave set at 200 nm. A Luna C8 LC column (250 x 4.6 mm, 5 µm) by Phenomenex was used for the separation. The method time was 20min and the retention time of AA was 2.56 min.

3. RESULTS AND DISCUSSION

3.1. Accelerated solvent extraction (ASE)

The results showed that diatomaceous earth, commonly used to fill the excess space in the cell, retained the AA and did not allow the solvent to extract it. Instead of diatomaceous earth, sea sand was used, which does not interact with the samples and allows AA extraction. Moreover, the solvents studied for extraction were water and methanol, being water the solvent with a higher AA solubility, even methanol allowed a further concentration of the analyte. Both showed recoveries above 90%. Finally, methanol was chosen as the extractant because of its ability to be easily evaporated. Subsequently, different flush or rinse volumes (the amount of solvent flushed through the cell, expressed as a percentage of the cell volume) were studied and it was observed that 100% was optimal. Lower recoveries were obtained with a rinse volume of 50 and 30%. About 65 mL of solvent with the dissolved analyte was obtained and concentrated. Different studies show that concentrating to dryness leads to the loss of the analyte[14], therefore, it was concentrated to a final volume of 5 mL.

ASE extraction was useful at concentrations around 2000 ppb but decreased to 50% at concentrations of the order of 500 ppb and below 100 ppb was less than 7%. AA was extracted from coffee, corn, cookie, and toasted bread samples. In all of them, chromatograms with fewer interferences were observed when 0.5 g of $ZnSO_4$ and 0.5 g of MgO were added to the cell. The following chromatogram shows a sample of coffee extracted by ASE with and without the salts (Figure 1).

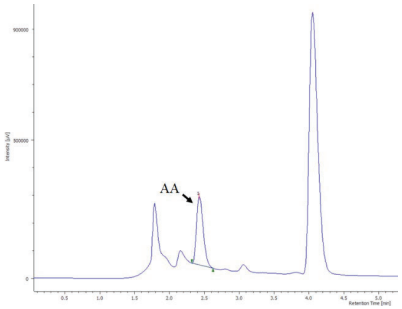

Figure 1. HPLC-UV chromatograms of ASE extraction with salts of roasted coffee spiked at 100 ppm

3.2. Solid phase extraction

To concentrate AA, Strata® Activated Carbon (400 mg) column was studied, being adsorption (surface phenomenon) the main mechanism of action of these columns. The retention of the analyte was intense, as shown in Table 2, and remained at the head of the column; for this reason, it was studied whether flow direction of elution increases recovery. In parallel, the volume of the elution and a second elution was studied to evaluate the behavior of the column. The use of 3 mL of methanol resulted in the optimal extraction volume. For this purpose, 10 mL of water contaminated at 1000 ppb and 100 ppb were loaded in the column. The results showed that reverse flow increased the recovery from 69.2% to 89.6% at 1000 ppb. A second elution was necessary to achieve recoveries above 80% at 100 ppb concentrations (Table 2).

Table 2. AA recovery percentages obtained by SPE

	Reverse flow		Normal flow	
Concentration	**First elution**	**Second elution**	**First elution**	**Second elution**
10 mL 1000 ppb	89.6 %	6.8 %	69.2 %	15.5 %
10 mL 100 ppb	80.65 %	28.47 %		

3.3. Supercritical fluid extraction

The use of SFE, combing CO_2 and methanol as solvents did not show any significant AA recoveries. Rather than methanol, water was evaluated as a solvent for its high solubility [15]. AA could not be recovered at rates. The water was frozen in the system pipes not allowing AA collection. The extraction of AA by SFE was not possible due to SFE aimed at extracting mainly nonpolar compounds and AA is an extremely polar molecule [11].

3.4. QuEChERS

The results revealed that the recovery percentages depend on AA concentration, so that, recovery percentage decrease when AA content is lower. The recoveries obtained were 87.3% at 2000 ppb, 54.5% at 500 ppb and 5.67% at 50 ppb.

4. CONCLUSION

ASE set at 100% of rinse volume and in combination of $ZnSO_4$ and MgO and QuEChERS are useful methods to extract high amounts (2000 ppb) of AA. SPE is a good technique to concentrate AA using reverse flow direction in the elution. SFE is not an effective tool to AA extraction because AA is an extremely polar molecule. AA extraction is difficult; therefore, it is necessary to use internal standards and high-precision detection instruments.

A. Author, B. Author, Paper title, Journal title, Volume (Year of publication) Issue, pp. from – to.

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RECOVERY OF POLYPHENOLS, ANTIOXIDANT COMPOUNDS AND MINERALS FORM SPIRULINA: INFLUENCE OF SUPERCRITICAL FLUID EXTRACTION

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ABSTRACT

Microalgae represent a new source of nutrients and bioactive compounds, with a more sustainable production. In particular, spirulina is the most widely known and consumed microalgae worldwide. On the other hand, conventional extraction of these compounds involves long extraction times, as well as the use of toxic solvents and a large amount of energy. In this sense, extraction with supercritical fluids represents a sustainable alternative, with high process efficiency and in an environmentally friendly way. In the present work, a supercritical fluid extraction (SFE) is compared with a conventional stirred extraction (used as a control). For this purpose, 5 g of spirulina were used for each process. The supercritical fluid extraction (SFE) was carried out for 1 h, using a temperature of 50 °C, a pressure of 25 MPa and a 90:10 CO_2:ethanol ratio, with a flow rate of 16 mL/min. For conventional extraction, 5 g of microalgae were suspended in 50 mL of pure ethanol. The mixture was stirred at 500 rpm for 30 minutes at room temperature and protected from darkness. The process was done twice. Finally, the solvent was evaporated, and the dried residue of the SFE extract and the conventional extract were resuspended to a final concentration of 1 mg/mL. Antioxidant capacity was measured by TEAC assay. The phenolic profile was analysed by Triple TOF-LC-MS-MS. Minerals and heavy metals were analysed by ICP-MS. The results obtained for antioxidant capacity revealed that no differences were obtained between the two extracts when measuring antioxidant capacity by TEAC assay. Regarding the phenolic profile, the extract obtained by SFE mainly detected 4-hydroxybenzaldehyde and benzoic acid. On the other hand, in the conventional extraction, these polyphenols were also detected in addition to glycitin. Concerning the minerals, the SFE extraction recovered 77% more Mg than the conventional extraction. However, the conventional extraction obtained a higher yield for the recovery of P and Ca. Finally, for heavy metals, a higher Pb extraction was observed for the SFE extract, while for Hg there are no significant differences between both extractions. These results demonstrate that SFE technology can be a valuable tool to sustainably extract bioactive and health-relevant compounds. However, potential contaminants such as Pb, which would also be increased after the process, need to be controlled.

***Keywords**: microalgae, supercritical fluid extraction, polyphenols, minerals, heavy metals*

1. INTRODUCTION

Spirulina is a cyanobacterium considered blue microalgae, widely found in South America. This microalga constitutes a source of bioactive compounds, as well as unsaturated fatty acids and essential amino acids, which contribute to basic human nutrition and can be used as a protein source. Moreover, it is rich in chlorophylls, carotenoids, phycocyanins, and phenolic compounds which can be used as colorants and natural antioxidants [1,2].
Conventional extraction techniques such as maceration, percolation, counter-current extraction and Soxhlet have been used to extract lipids and pigments from plant materials and microalgae. However, they present some disadvantages, because they imply the use of large amounts of solvents, and the risk of thermal denaturation or transformation of molecules of interest [3]. The use of environmentally friendly technologies such as supercritical fluid extraction, pulsed electric fields, high-voltage electric discharges, high-pressure homogenization, ultrasound and microwave-assisted extraction have emerged as alternative processes that can extract valuable compounds from microalgae. These technologies are aligned with green chemistry concepts and sustainability [4,5].
Supercritical fluid extraction (SFE) constitutes an environmentally friendly and cost-effective extraction. It allows to obtain greater selectivity and extraction efficiency achieved by tailoring the operative parameters such as temperature, pressure, and flow rate. Moreover, SFE extraction is effective for the recovery of thermolabile compounds, due to the low extraction temperature and pressure. The major limitation is represented by the CO_2 chemical behavior in supercritical conditions. In order to overcome this disadvantage, the addition of appropriate polar co-solvent is an advantageous strategy for modifying SFE-CO_2 polarity and increasing the solubility of bioactive compounds [6]. This technique has been applied previously in the recovery of bioactive compounds form spirulina achieving good results [7,8].
The present study explores the potential of SFE extraction in the recovery of phenolic compounds and molecules with antioxidant capacity from spirulina. Moreover, the recovery of mineral compounds has also been investigated, as well has been controlled the presence of heavy metals in the resulting extracts.

2. EXPERIMENTAL

2.1. Samples

The spirulina samples were purchased to Ecospirulina (Serra, Valencia, Spain) as freeze dried powder, and were kept freeze dried at room temperature.

2.2. Extraction process

The supercritical fluid extraction (SFE) was carried out for 1 h, using a temperature of 50 °C, a pressure of 25 MPa and a 90:10 CO_2:ethanol mixture, with a flow rate of 16 mL/min. For conventional extraction, 5 g of microalgae were suspended in 50 mL of pure ethanol. The mixture was stirred at 500 rpm for 30 minutes at room temperature and protected from darkness. The process was done twice. Finally, the solvent was evaporated, and the dried residue of the SFE extract and the conventional extract were resuspended to a final concentration of 1 mg/mL.

2.3. Measure of antioxidant capacity and polyphenols

The measure of antioxidant capacity was carried out by Trolox Equivalent Antioxidant Capacity (TEAC) method. This assay was carried out following the method described by Khawli et al.

[5]. The identification of phenolic profile was done using a Triple-TOF-LC-MS-MS characterization, based on the method reported by Zhou et al. [9].

2.3. Evaluation of minerals and heavy metals content

The minerals analysed were Mg, P, Ca, Fe, Zn and Se. The heavy metals studied were As, Cd, Hg and Pb. All of them were identified and quantified by ICP-MS, following the method previously used by de la Fuente et al. [10] and Wang et al. [11].

2.4. Statistical analysis

The statistical analysis was performed using GraphPad Prism 8. A *t*-student was used to compare SFE vs. Control, considering significant $p<0.05$. All the experiments were performed in triplicate. The results are presented as mean ± SD.

3. RESULTS AND DISCUSSION

3.1 Antioxidant capacity

The results obtained for antioxidant capacity revealed that no differences were obtained between the two extracts when measuring antioxidant capacity by TEAC assay (Figure 1).

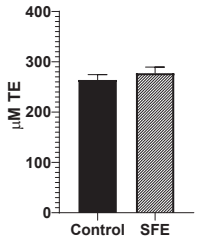

Figure 1. TEAC antioxidant assay, comparing SFE vs. Control

3.2 Phenolic profile

Regarding the phenolic profile, the extract obtained by SFE mainly detected 4-hydroxybenzaldehyde and benzoic acid. On the other hand, in the conventional extraction, these polyphenols were also detected in addition to glycitin.

3.3 Mineral content

Concerning the minerals (Mg, P, Ca, Fe, Zn and Se), the SFE extraction recovered 77% more Mg than the conventional extraction. However, the conventional extraction obtained a higher yield for the recovery of P and Ca (Figure 2).

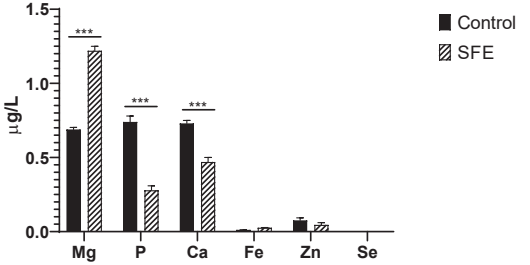

Figure 2. Content of Mg, P, Ca, Fe, Zn and Se in both SFE and Control extract. ***=p<0.001

3.4 Heavy metals content

For heavy metals (As, Cd, Hg and Pb), a higher Pb extraction was observed for the SFE extract, while for Hg there are no significant differences between both extractions (Figure 3).

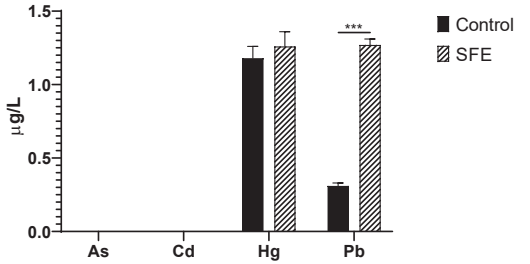

Figure 3. Content of As, Cd, Hg and Pb in both SFE and Control extract. ***=p<0.001

4. CONCLUSION

These results demonstrate that SFE technology can be a valuable tool to sustainably extract bioactive and health-relevant compounds. However, potential contaminants such as Pb, which would also be increased after the process, need to be controlled.

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PRELIMINARY STUDIES FOR THE OPTIMIZATION OF ULTRASOUND-ASSISTED EXTRACTION OF MICROALGAL CAROTENOIDS

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ABSTRACT

The interest in sustainable food has increased in the last years due to climate change, and microalgae have been recently added to the sustainable food system report. Microalgae are the principal supplier of carotenoids in the marine trophic chain, and those compounds also have many benefits for human health, such as antioxidant and anti-inflammatory properties. Moreover, the food industry also requires the use of sustainable technologies, such as ultrasound-assisted extraction (UAE), which allows a higher yield extraction in less time and less use of solvents than conventional methods. Usually, the extraction of carotenoids from food plants is performed with organic solvents such as ethanol, methanol, or dimethylsulfoxide (DMSO), which are responsible for environmental contamination. The demand for bio-based solvents such as 2-methyloxolane (2-MeOx) or ethyl lactate has increased. Thus, the objective of this study was to investigate the effects of bio-based and organic solvents and the application of different green treatments in carotenoid extraction. The matrix used for this study was control and phytoene-enriched microalga *Chlorella sorokiniana* (fresh, freeze-dried, and encapsulated). The UAE was performed at 30% amplitude for 2 minutes in all samples. Five different solvents were tested: ethanol, methanol, ethyl lactate, 2-MeOx, and DMSO. The effect of a pre-treatment with a ball mill (30 kHz, 5 min) was also studied. The application of the mill in freeze-dried and encapsulated *C. sorokiniana* resulted in a significant ($P < 0.05$) increase in the extraction of total carotenoids in all samples, but it was not significant in the control and rich-phytoene-fresh matrix ($P > 0.05$). The bio-based solvent 2-MeOx resulted as efficient as ethanol and methanol for carotenoid extraction in all samples, finding no significant differences between them in most of the matrices ($P < 0.05$). Hence, 2-MeOx could be a great bio-based solvent to replace organic solvents for the extraction of carotenoids from microalgae. application of the mill treatment before the extraction has a variable effect in carotenoid release, depending on the matrix structure.

***Keywords**:* Carotenoids, microalgae, bio-based solvents, ball-mill, ultrasound.

1. INTRODUCTION

The interest in sustainable food has increased in the last few years. Microalgae have high productivity as their production is during the whole year and they contribute to the sequestration of CO_2. These microorganisms are also able to grow under stressful conditions (being able to modify biosynthesis of valuable compounds), with low needs of water and nutrients [1]. Microalgae have already been included in the sustainable food system for healthy diets report from the EAT-Lancet Commission [2]. More than 40.000 microalgae species have been investigated for commercial purposes [3]. Recently, the incorporation of different microalgae compounds into several types of food has been highlighted [1], as microalgae is a sustainable source of bioactive compounds and they could play an essential role in reducing hunger and

improving well-being. *Chlorella sorokiniana* is a rich source of carotenoids such as lutein, α-carotene, and β-carotene. Carotenoids are usually colored lipophilic compounds, however, there are also colorless carotenoids, such as phytoene or phytofluene. Phytoene is the precursor carotenoid in the pathway of carotenogenesis. Carotenoids have been widely studied due to their benefits for human health as they are active antioxidants, and can decrease the risk of numerous diseases such as cardiovascular diseases, eye disorders, diabetes, different types of cancer, etc. [4]. The most common carotenoids found in microalgae are lutein, β-carotene, zeaxanthin, fucoxanthin, astaxanthin, canthaxanthin, and violaxanthin [5]. Carotenoids also have an important role in microalgae, contributing to the stabilization of the membrane, capture of light, and dissipation of energy.

The demand for the use of bio-based solvents is increasing due to the development of green analytical chemistry, not only related to the damage to humans but environmentally [6]. Ethyl lactate is a bio-based solvent that was approved by the European Food Safety Authority (EFSA) as it has low toxicity. Another bio-based solvent is 2-MeOx, which could be attractive to industries due to its low boiling point and low enthalpy of vaporization [7].

Besides the use of sustainable extraction solvents, industries and society also demand green extraction methods, such as UAE. UAE leads to an increase in the extraction of different compounds reducing time and use of solvents compared to conventional extraction, due to the power of the sonication that produces cell breaking of the matrix, allowing a higher compound release [8]. The main objective of this study was to evaluate the capacity of bio-based and organic solvents for the extraction of carotenoids by UAE and assess the effect of the ball-mill pre-treatment in the extraction.

2. EXPERIMENTAL DESIGN

2.1 Sample

The microalgae *Chlorella sorokiniana* 211-32 was used and cultured in liquid Tris-acetate phosphate (TAP) medium. Biomass was harvested by centrifugation at 6000 rpm and either frozen at -20 ºC, lyophilized, or encapsulated. For the encapsulation, cultures of *C. sorokiniana* were mixed with sterile alginate solution and dropped into a 0.1 M $CaCl_2$ solution at 4 °C, obtaining spheres of approximately 3 mm diameter. Finally, for the phytoene enrichment, *C. sorokiniana* culture was resuspended in TAP medium and incubated with norflurazon (1 µg/mL).

2.2 Experimental design

Two groups were different: the ultrasound group (UAE), and the mill and ultrasound group (Mill + UAE). Five extraction solvents were tested: ethanol and methanol, which are authorized for food use; ethyl lactate and 2-methyloxolane, which are emerging sustainable solvents, and DMSO, which is a common solvent for carotenoid extraction.

In a 15 mL tube, 2 mL of each solvent was added to approximately 0.1 g of sample, and sonicated (Q500, Qsonica, EE.UU.) for 2 min, with an amplitude of 30%, and a frequency of 20 kHz. Ultrasound was applied with a probe of 1.6 mm. To avoid the degradation of carotenoids, the samples were immersed in an ice bath during the process. It was repeated until the sample showed no color. Prior treatment with a ball mill (MM 400, Retsch, Germany) was also assessed before the ultrasound application (Mill + UAE). Approximately, 0.1 g of each matrix was introduced in an Eppendorf with three stainless steel balls of 3 mm in diameter. The milling was applied for 5 min with a frequency of 30 Hz, and after that, the solvent is added and the UAE applied. A rotatory evaporator (<30 °C) was used to eliminate solvents. As ethyl lactate and DMSO are no volatile compounds, 3 mL of trichloromethane and NaCl (5% w/v) were added, water was removed and the trichloromethane was evaporated.

3. RESULTS AND DISCUSSION

3.1 Carotenoid profile

The main carotenoids detected in control *C. sorokiniana* (both fresh, freeze-dried, and encapsulated matrices) were lutein, α-carotene, β-carotene, and 9-*Z*-β-carotene. Violaxanthin and zeaxanthin were detected in small amounts. In other studies, a similar carotenoid profile in freeze-dried *C. sorokiniana* was found [9]. Phytoene was also found in the fresh and freeze-dried rich-phytoene-*C. sorokiniana*, while in the encapsulated matrix only lutein and phytoene were found. Thus, the norflurazon treatment blocked the carotenogenesis pathway at the level of phytoene desaturase, allowing a higher phytoene accumulation in the treated matrices.

3.2 Effect of the ball-mill

The pre-treatment of the ball-mill significantly increases total carotenoid release in both, control freeze-dried, rich-phytoene-freeze-dried, control encapsulated, and rich-phytoene-encapsulated (1.25, 1.48, 1.81, and 5.15-fold, respectively), however, no significant differences were found in the fresh matrix (control and rich-phytoene) (Table 1). The pre-treatment of the mill allowed a higher cell disruption of the complex cell wall of *C. sorokiniana*, producing a higher yield extraction [10]. In other studies where the application of the mill has been assessed, it has also been seen a significant increase in the yield of *Chlorella* [11]. In fresh samples, both control and rich-phytoene, the mill did not have the same effect, which could be related to the water content and structure of the matrix.

3.3 Effect of the extraction solvent

For solvent comparison, the ball-mill effect was taken into account. In the fresh matrix (control and rich-phytoene), the UAE group was compared as the mill did not affect carotenoid extraction, in the freeze-dried and encapsulated matrix (control and rich-phytoene), the Mill + UAE group was used. In the fresh control matrix, the best solvent for the extraction of total carotenoid content (TCC) in UAE was methanol and ethanol, with no significant differences between them, followed by 2-MeOx. In the freeze-dried matrix, the best solvents in Mill + UAE were methanol, ethyl lactate, and 2-MeOx, showing no significant differences between them. Finally, in the control encapsulated matrix, the best solvents in Mill + UAE were ethanol, methanol, ethyl lactate, and 2-MeOx, showing no differences between them (Table 1). The best solvents for the extraction of phytoene in the fresh rich-phytoene matrix were ethanol, methanol, and 2-MeOx, showing no significant differences between them ($P < 0.05$). In the freeze-dried rich-phytoene-matrix, both methanol and 2-MeOx were the best extraction solvent showing no significant differences between them ($P > 0.05$). Finally, in the encapsulated rich-phytoene-*C. sorokiniana* the solvents that extracted the highest amount of phytoene were ethanol, ethyl lactate, and 2-MeOx ($P > 0.05$).

One of the best solvents for carotenoid extraction in all the matrices, both control and rich-phytoene, fresh, freeze-dried, and encapsulated, was 2-MeOx compared to the other four solvents evaluated.

Ethanol and methanol also resulted in high carotenoid recovery compared to the other solvents in all the matrices. Those two solvents are already accepted by EFSA for extraction of food compounds, however, if methanol is ingested in high amounts, can produce toxicity in humans [12]. EFSA has recently established a tolerable daily intake of 1 mg of 2-MeOx per kg of body weight [13]. DMSO has been also used for carotenoid extraction from microalgae as it presents advantages as an extraction solvent such as fast extraction, stability of microalgae components, and low toxicity [14]. In the present study, DMSO has consistently been found to be one of the worst extraction solvents among those evaluated. Ethyl lactate has been also proposed as an extraction solvent of carotenoids from tomato by-products [15]. In our study, ethyl lactate resulted in one of the best solvents for TCC in the control freeze-dried, control encapsulated,

and rich-phytoene-encapsulated matrices, however, ethyl lactate owns a high boiling point (154 °C) and a high enthalpy of vaporization (49.2 kJ/mol), and this makes it less desirable for the industry as the cost for solvent elimination will be high [7].

Table 1. Effect of mill pre-treatment on TCC of *C. sorokiniana* extracted by UAE, and assessment of the extraction capacity of five solvents.

Matrix	Solvent	UAE (μg/g)				Mill + UAE (μg/g)				*P*-value
Control-Fresh	Ethanol	1803.18	±	55.58	D	1806.91	±	138.41	D	ns
	Methanol	1874.38	±	133.52	D	1806.46	±	13.67	D	ns
	Ethyl lactate	1260.40	±	53.00	B	1227.17	±	46.89	B	ns
	2-MeOx	1586.14	±	47.55	C	1502.52	±	90.50	C	ns
	DMSO	988.24	±	15.97	A	976.57	±	8.21	A	ns
Control-Freeze-dried	Ethanol	2806.35	±	121.09	BC	3626.65	±	223.19	B	**
	Methanol	2793.33	±	190.86	BC	3770.21	±	295.60	B	**
	Ethyl lactate	2604.32	±	46.31	B	3446.48	±	119.03	B	***
	2-MeOx	2941.17	±	76.19	C	3426.91	±	71.85	B	**
	DMSO	965.64	±	22.89	A	1085.78	±	37.17	A	**
Control-Encapsulated	Ethanol	2.82	±	0.07	C	3.88	±	0.17	A	***
	Methanol	3.14	±	0.08	C	4.43	±	0.26	AB	**
	Ethyl lactate	3.04	±	0.14	C	4.85	±	0.25	B	***
	2-MeOx	1.65	±	0.02	A	4.75	±	0.28	AB	***
	DMSO	2.21	±	0.28	B	3.90	±	0.20	A	***
Rich-phytoene-Fresh	Ethanol	1098.29	±	35.36	C	1082.89	±	62.22	C	ns
	Methanol	1055.56	±	36.89	B	1088.81	±	56.83	C	ns
	Ethyl lactate	877.79	±	11.37	A	916.54	±	60.66	AB	ns
	2-MeOx	1010.84	±	29.78	B	1024.96	±	20.32	BC	ns
	DMSO	863.88	±	14.32	A	806.60	±	88.21	A	ns
Rich-phytoene-Freeze-dried	Ethanol	5273.04	±	69.60	D	5714.53	±	19.80	C	***
	Methanol	5680.41	±	118.64	E	6357.98	±	158.96	D	**
	Ethyl lactate	3593.55	±	204.44	C	4819.32	±	283.67	B	**
	2-MeOx	2270.23	±	40.13	A	5546.96	±	31.07	C	**
	DMSO	2864.76	±	67.36	B	4083.43	±	28.15	A	***
Rich-phytoene-Encapsulated	Ethanol	176.14	±	5.61	D	200.60	±	2.71	B	**
	Methanol	125.46	±	4.71	C	147.60	±	1.31	A	**
	Ethyl lactate	110.09	±	11.27	C	201.00	±	13.07	B	***
	2-MeOx	13.65	±	1.08	A	202.74	±	4.27	B	***
	DMSO	81.85	±	6.10	B	158.50	±	8.98	A	***

P-values (ns: not significant; *: $P < 0.05$; **: $P < 0.01$; ***: $P < 0.001$) refer to the comparison among UAE and Mill + UAE. Different capital letters indicate differences between TCC using different solvents ($P > 0.05$).

4. CONCLUSION

The mill pre-treatment does not affect carotenoid release in fresh *C. sorokiniana*, however, significantly increases carotenoid release in freeze-dried and encapsulated matrices ($P < 0.05$). 2-MeOx resulted in high lutein, α-carotene, β-carotene, and phytoene extraction from *C. sorokiniana* both, fresh, freeze-dried, and encapsulated. Ethyl lactate showed high carotenoid release in some matrices; however, its elimination cost will be high, and 2-MeOx could be a bio-based solvent alternative to organic solvents. Further studies about the extraction of carotenoids with 2-MeOx are necessary.

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OPTIMISATION OF SARDINELLA LEMURU WASTE OIL EXTRACT USING SUPERCRITICAL FLUID EXTRACTION ($SCCO_2$) METHOD WITH RESPONSE SURFACE METHODOLOGY (RSM)

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ABSTRACT

In this work, the application of Response Surface Methodology (RSM) was applied to optimise the extraction conditions and valorise *Sardinella lemuru* (*S. lemuru*) waste with higher contents of DHA and EPA. This research aimed to determine the optimum total oil yield extracted from sardine waste using Supercritical fluid extraction ($SCCO_2$) with soaking technique prior to waste extraction. The utilisation of fish waste could be valorised as a cheap raw material for the generation of omega three (ω3) concentrates. Central Composite Design (CCD) was applied to optimise various parameters, such as soaking (X1) ranged 1 to 4 h and extraction time (X2) ranged 3 to 5 h to maximise the oil with the highest amount of EPA and DHA recovery while maintaining constant conditions of 350 bars, 60 °C and a 5 mL/min flow rate. $SCCO_2$ with soaking technique was successfully optimised at 1.7 h (X_1) and 3.75h (X_2) soaking and extraction time, respectively, with a pressure of 350 bars, a temperature of 60 °C and a 5 mL/min flow rate remaining constant. The optimum condition of $SCCO_2$ with soaking technique reported with higher total oil yield, 3.3% and higher polyunsaturated fatty acids (PUFAs) recovery as compared to $SCCO_2$ continuous technique, 3.0%. Furthermore, soaking step prior to the extraction had a significant effect ($p<0.05$) on a yield and total oil can be increased as CO_2 solubility increased. This study verifies that the combination of soaking and extraction time (X_1X_2) as variables were highly significant to the oil yield. This is showed by the experimental results fitted the predicted values with a coefficient value of $R^2 = 98.47\%$.

Keywords: *Supercritical fluid extraction, soaking technique, optimisation, Eicosapentaenoic acid; docosahexaenoic acid*

1. INTRODUCTION

S. lemuru is an excellent source of lipids that contain the important eicosapentaenoic acid (EPA; C20:5ω3) and docosahexaenoic acid (DHA; C22:6ω3). Supercritical fluid extraction (SC-CO_2) has been studied as a promising process for the extraction and fractionation of edible oils containing labile PUFAs. Carbon dioxide (CO_2), the extracting solvent for fish oil extraction, has been widely employed due to its good solubility [7, 8]. A relatively low critical point, with a critical temperature of 31 °C and a critical pressure of 75 bars also makes CO_2 desirable for compounds that are sensitive to extreme conditions. Moreover, SC-CO_2 is also non-toxic, non-flammable, inexpensive, and available in higher purity [7], achieved 93.5% oil yield under optimum conditions (250 bar, temperature 40 °C) with a 3 h extraction time from hake (*Merluccius capensis*) by-products. After determining the best level for each parameter, it was

reported that 10.36% oil yield has achieved at 300 bars, 75 °C and 2.5 mL/min flow rate during a shorter extraction time [4]. A systematic understanding of how soaking and extraction times contribute to the extraction yield with the greatest amount of EPA and DHA from sardine (*S. lemuru*) fish wastes is still lacking. Therefore, this study presents optimum oil yield extracted conditions using RSM study design in which extraction and soaking times were selected as factors and total oil yield as a response. Other parameters, such as pressure (350 bars), a flow rate of 5 mL/min and a 60°C temperature, remained constant as adopted from [9].

2. EXPERIMENTAL

Fish waste collected from Pasar Borong Selangor, Malaysia were extracted using supercritical fluid extraction (SC-CO_2) performed with two extraction techniques namely continuous and soaking techniques [9]. Central Composite Design (CCD) was employed for response surface optimisation of the operating conditions. Fourteen experimental runs were design by MINITAB v16 whereby two independent variables, namely, soaking (X_1) and extraction time (X_2) were selected against total oil yield (Y) as a response variable. Soaking and extraction time ranged from 1 to 4 h and 3 to 5 h respectively while of 350 bars, 60 ºC and a 5 mL/min flow rate were constant.

3. RESULTS AND DISCUSSION

3.1 Total Oil Extracted using Supercritical Fluid Extraction (SC-CO_2)

Total oil extracted using continuous and soaking techniques was 3.11± 0.37% and 3.30± 0.15%, respectively, and they were not significantly difference. These values however were comparatively low relative to the total oil extracted using the Soxhlet extraction method [1]. Although the lipid extraction technique via a solvent is significant and allows for the extraction of all lipids, such as polar lipids, phospholipids and likely lipids bound with other components from cellular membranes, the toxicity of the solvents used makes their application in food supplementation impossible. SC-CO_2 soaking technique, is believed to improve equilibrium and solubility of the sample. Thus, despite its yield, the use of SC-CO_2 is still desirable and the CO_2 extraction permits an average yield of 10.5% of the dried material under optimal conditions [4]. The discrepancy of total oil yield in fish can be affected by various factors such as feeding habits, environmental and climatic changes, age and species [5, 8]. A number of extraction techniques have been developed on fish wastes using SC-CO_2 that produce fish oil with high-quality PUFAs [1-3].

General indicators of the nutritional value of fish include moisture, protein, ash and lipid contents. A high-water content in fish is correlated with low protein and lipid contents. The oil extracted under optimum conditions (1.75 h soaking and 3.75 h extraction times) was analysed for its proximate. The determination of proximate composition was used to study the variations in the chemical compositions of S. lemuru waste, which are attributed to the species, maturity, dissected body parts and processing conditions. The total moisture and protein content of dry ground *S. lemuru* waste.

3.2 Solubility

Oil solubilised by CO_2 influences the high recovery of PUFAs. This solubility, however, is limited to the fish's moisture content and low moisture content attains a high solubility and is able to increase the extraction yield [7, 9]. Oil solubility is affected by the water content or fluid phase in the sample and, for that reason, a negative interaction between water content and oil was proposed in this study. Table 1 shows the CO_2 consumption and its solubility required to extract *S. lemuru* waste oil. The oil recovery increased from 1.7% to 2.8% when the solubility increased from 4.09% to 10.50% with soaking time and decreased with extraction time.

Table 1. Solubility and total CO_2 used for the SC-CO_2 soaking technique from *S. lemuru* oil

Run No.	Soaking (hours) (X_1)	Extraction (hours) (X_2)	CO_2 Consumption (g)	Solubility (%)	Total Oil Extracted (%)
1	1.75	3.8	72.8	10.40	3.1
2	3.25	3.8	72.8	8.39	2.5
3	1.75	5.3	101.6	4.09	1.7
4	3.25	5.3	101.6	5.53	2.3
5*	2.5	4.5	86.2	7.09	2.5
6*	2.5	4.5	86.2	6.80	2.4
7*	2.5	4.5	86.2	7.09	2.5
8	1.4	4.5	86.2	7.37	2.6
9	3.6	4.5	86.2	7.09	2.5
10	2.5	3.4	65.1	10.50	2.8
11	2.5	5.6	107.3	4.10	1.8
12*	2.5	4.5	86.2	7.09	2.5
13*	2.5	4.5	86.2	7.09	2.5
14*	2.5	4.5	86.2	7.37	2.6

**The experiment was performed at constant 350 bars, 60°C and a 5 mL/min flow rate*

On another note, linear interaction between the extraction rate and solubility in hake by-products. Primarily, constant internal mass transfer occurred in a solid matrix of fish structure. However, after a certain duration, internal mass transfer tends to be resistant and affects the lipid recovery due to the neutral lipid composition in fish. Lipids in fish by-products are mostly bound to the protein matrix [7].

John and Anthony [8] suggested that the physicochemical properties of SC-CO_2, including density, diffusivity and viscosity, can be controlled by various conditions such as product solubility, pressure, temperature or a combination of these parameters. In 2013, Sahena and co-workers [9] successfully optimised SC-CO_2 at 400 bars, 65 °C and a 3 mL/min flow rate for *Thunnus tonggol* to increase the oil yield from 4.5% to 35.6% with a 22.3% recovery of ω3 fatty acids. These results are likely related to the oil solubility that increased as the temperature and pressure increased. This is confirmed by previous finding [9] that confirmed an increase from 20% to 42% of total crude oil, which consisted primarily of triacylglyceride (TAG) with different lengths and degrees of unsaturated fatty acids. Therefore, the application of supercritical CO_2 with the soaking technique can extract such valuable molecules from fish

wastes. It can therefore be assumed that the extraction time is not important in oil extraction with high PUFA recovery and can be proposed as a negative interaction.

3.3 Fatty Acids Compositions

Based on the data obtained, the extraction technique using SC-CO_2 continuous and soaking techniques preserve the fatty acid composition, especially EPA, which were 13.06% and 15.47%, respectively, and DHA, which were 12.16% and 15.65%, respectively. DHA and EPA recovery was the highest in SC-CO_2 soaking technique as compared to the oil yield extracted with continuous techniques. Based on the data obtained, the extraction technique using SC-CO_2 continuous and soaking techniques preserve the fatty acid composition, especially EPA, which were 13.06% and 15.47%, respectively, and DHA, which were 12.16% and 15.65%, respectively [2, 3]. DHA and EPA recovery was the highest in SC-CO_2 soaking technique as compared to the oil yield extracted with continuous techniques.

The addition of a soaking step prior to extraction was previously reported [7, 8] the optimised SC-CO_2 extraction conditions on *Silurus glanis* (catfish), with a 2.5h soaking time significantly increased the oil yield (67% in a 100 g fish sample). Moreover, 10 h soaking time using SC-CO_2 was highly effective at extracting fish oil for DHA and EPA recovery in mackerel body parts, including arachidonic acid (C20:4ω6). As previously described via interaction terms, extraction and soaking (X_1X_2) times significantly ($p<0.05$) and positively affect *S. lemuru* extracted oil. Higher solubility can be achieved by increasing the density and the interaction between oil and CO_2 can be enhanced to lead to higher mass transfer rates and property changes [2]. This finding agrees with our observations when the temperature and pressure were constant, the extraction yield increased with soaking time due to an increase in lipid solubility in SC-CO_2 [7, 8].

4. CONCLUSION

Various SC-CO_2 techniques, including continuous and soaking, were done to test their efficiency of extracting fish oil from *S. lemuru* waste and the PUFA from these techniques were compared. This method was successfully optimised at 1.7 h and 3.75 h soaking and extraction time, respectively, with a pressure of 350 bars, a temperature of 60 °C and a 5 mL/min flow rate remaining constant. The optimum condition of SC-CO_2 with soaking technique reported with higher 3.3 % of total oil yield recovery compared to SC-CO_2 continuous technique. The extraction of *S. lemuru* oil with SC-CO_2 soaking technique reported with significantly higher PUFA recovery, 59.81 %, through the following proportions: EPA (15.47 %) and DHA (15.65 %), as compared to SC-CO_2 continuous technique which obtain PUFA (53.38 %), EPA (13.06 %) and DHA (12.16 %). Additionally, soaking time also increased total oil from 1.7% to 2.8% as the solubility increased from 4.09% to 10.50% are highly effective at extracting fish oil to recover DHA and EPA. Therefore, soaking step is worth prior step to be applied before extraction process using SC-CO_2.

The application of RSM for the experimental study design was an effective statistical tool to optimise the extraction conditions and valorise *S. lemuru* waste with higher contents of DHA and EPA. This study shows that the experimental results fitted the predicted values with a coefficient value of R^2 = 98.47% verify that the combination of soaking and extraction time (X_1X_2) time were highly significant to the oil yield as per quadratic term. On the contrary, fish oil extracted still subjected to its stability for further application in the food system. Thus, with this limitation, fish oil extracted from *S. lemuru* waste should be further investigated and determine its microencapsulation potential to further characterise its properties before further

application. This combination of soaking and extraction time (X_1X_2) would be useful for future work to incorporate DHA and EPA into the food system.

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