



PhD Thesis

Circular economy and olive pomace: economic, food and environmental sustainability strategies.

Adding value to the olive oil processing by-products to promote a sustainable food system.

Maria Antónia da Mota Nunes

*Thesis submitted to Faculty of Pharmacy of the University of Porto
for PhD in Pharmaceutical Sciences, Nutrition and Food Science Speciality*

Supervision

Dr. Rita Carneiro Alves

Prof. Maria Beatriz Prior Pinto Oliveira

Março, 2022

This page was intentionally left in blank.

Circular economy and olive pomace: economic, food and environmental sustainability strategies.

Adding value to the olive oil processing by-products to promote a sustainable food system.

This page was intentionally left in blank.

©Authorised the partial reproduction of this thesis (subject to the approval of the publishers of journals in which the articles were published) only for research purposes through a written declaration of the person concerned that such pledges.

This page was intentionally left in blank.



This work has been supported by Fundação para a Ciência e a Tecnologia (FCT) (Portugal) through a PhD grant (SFRH/BD/130131/2017) financed by Fundo Social Europeu, NORTE2020 Programa Operacional Regional do Norte and national funds from Ministério da Ciência, Tecnologia e Ensino Superior (MCTES).

The research has also received financial support from Portuguese national funds (FCT/MCTES) through UIDB/50006/2020, AgriFood XXI I&D&I project (NORTE-01-0145-FEDER-000041) co-financed by European Regional Development Fund (ERDF), through the NORTE 2020 (Programa Operacional Regional do Norte 2014/2020) and project SYSTEMIC “an integrated approach to the challenge of sustainable food systems: adaptive and mitigatory strategies to address climate change and malnutrition”.

This page was intentionally left in blank.



The work presented in this thesis was developed in the Associated Laboratory for Green Chemistry/Laboratório Associado para a Química Verde (LAQV) of the Network of Chemistry and Technology/Rede de Química e Tecnologia (REQUIMTE), Laboratory of Bromatology and Hydrology, Department of Chemical Sciences, Faculty of Pharmacy of University of Porto, Portugal.

This page was intentionally left in blank.

To my Parents.

This page was intentionally left in blank.

"Se puderes olhar, vê. Se puderes ver, repara."

José Saramago

This page was intentionally left in blank.

Acknowledgements

I would like to express my Special acknowledgement:

To Doctor Rita Alves. Thank you for the strong support, accurate scientific guidance, long discussions, late-night revisions, and by multiplying in numerous tasks but always being present and helpful.

To Professor Beatriz Oliveira for her guidance, availability and, most of all, her contagious and unique characteristic, her enthusiasm with Science.

To all the colleagues at the Laboratory of Bromatology and Hydrology of the Faculty of Pharmacy of the University of Porto for the hard work, support, valuable advice and rich discussions.

To the colleagues from the Laboratory of Applied Chemistry and Laboratory of Microbiology (Faculty of Pharmacy of the University of Porto), NOVA University Lisbon and University of Aveiro that helped go through the work of this thesis contributing with different viewpoints, observations and understandings.

To my Friends. The bond that connects us is so strong that it stretches with distance but never goes away.

To my Family. Thank you for all the support, strength and always wise advice.

To all, even if not expressly referred here, that have contributed to the realization of this work.

Finally, I acknowledge Fundação para a Ciência e a Tecnologia (FCT) for the financial support of PhD grant SFRH/BD/130131/2017. This fellowship made me accomplish the work presented in this thesis.

This page was intentionally left in blank.

Abstract

Olive oil production has been increasing worldwide due to its well-recognized health benefits and organoleptic properties. Olive pomace is the major by-product generated in olive oil processing and is composed of olives pulp, skin and crushed stones. This by-product is a source of added-value bioactive compounds that are benefic for human health and, simultaneously, phytotoxic impairing plants germination and growth.

The main objectives of this thesis were to explore and develop processes to increase the recovery of olive pomace compounds and investigate the potential of olive pomace extracts as functional ingredients for food products addressing key-challenges as food security and environmental sustainability. Additionally, a further objective was to develop an olive oil-based spread with an olive oil content higher than 70%, functionalized with the olive pomace extracts.

Olive pomaces were obtained in Portugal from two-phase extraction olive mills. Four monovarietal pomaces from the Alentejo region (Arbequina, Arbosana, Oliana, Koroneiki) and four olive pomaces composed of a mixture of olive varieties (Cobrançosa, Madural, Verdeal Transmontana, Cordovil, Arbosana), from Trás-os-Montes and Alentejo were investigated.

Methodologies were selected according to the sample in study and the compounds of interest, considering previously raised questions.

Throughout the work, spectrophotometric methods were used to access groups of phytochemicals (i.e., total phenolics and total flavonoids) and antioxidant activity (ferric reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl radical and 2,2'-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid) cation radical scavenging abilities). Chromatographic techniques as liquid chromatography (HPLC-DAD-FLD), liquid chromatography coupled to mass spectrometry (MS) using electrospray ionization interface (ESI) (HPLC-DAD-ESI-MSⁿ) and gas chromatography (GC-FID) were employed to characterize samples. In addition, monovarietal olive pomaces were tentatively discriminated by spectroscopic analysis using Near Infrared (NIR) and Mid Infrared (NIR) spectra. Official methodologies were followed to determine the samples' proximate composition (AOAC) and fatty acid methyl esters (ISO).

Regarding phenolics content and antioxidant activity, Arbosana, Koroneiki, Oliana, and Arbequina pomaces were studied individually and blended. Blends were prepared with different percentages of each pomace, foreseeing possible additive, synergistic or antagonistic effects. Koroneiki and Arbosana monocultivars were the richest in phenolic

compounds and most of the interactions found in the blends were additive or synergistic. It was possible to select the best combinations, which were shown to be advantageous compared to the individual use of monocultivar pomaces.

Lipid- and water-soluble components were determined by an exploratory study using a Trás-os-Montes olive pomace sample. The lipid fraction was especially rich in oleic acid, and the vitamin E profile of the olive pomace comprised mostly α -tocopherol. Also, the extraction efficiency of a new methodology, the Multi-frequency Multimode Modulated (MMM) ultrasonic technique, was compared to a conventional solid-liquid extraction, regarding the obtention of antioxidant compounds. The MMM technique allowed a faster and higher recovery of antioxidants than the conventional process.

The phenolics profile of olive pomace, assessed by HPLC-DAD-FLD and HPLC-DAD-ESI-MSⁿ, showed as major phenolics: hydroxytyrosol > comselogoside > elenolic acid derivative > tyrosol. Among these phenolics, hydroxytyrosol has a particular interest, since it presents strong antioxidant, anti-inflammatory and antimicrobial properties.

One of the guiding principles of this thesis was the development of eco-friendly and cost-effective methodologies to obtain olive pomace extracts. An environmentally friendly integrated approach was studied to concentrate compounds of olive pomace by a membrane processing. The olive pomace compounds were extracted using a developed method (European patent application PCT/IB2017/053422; US Patent 10479958) and three types of membranes were tested. It was concluded that a membrane-assisted process using a BW30 membrane is a recommendable solution to concentrate antioxidant compounds from olive pomace extracts.

A system that uses a combination of time/pressure parameters to physically remove the vegetative water present in olive pomace was idealized and then materialized in a prototype. Since it was an innovative form to process olive pomace, it was protected through patent application (European patent application PCT/IB2018/060111; US patent application 6954060). Pomaces from the two major Portuguese olive oil production regions — Trás-os-Montes and Alentejo - were processed according to that method to obtain an olive pomace functional ingredient (OFI). The extracts were further characterized regarding chemical composition and antimicrobial activity against *Staphylococcus aureus* ATCC® 25923, *Escherichia coli* ATCC® 25922, and *Candida albicans* ATCC® 10231. Those with higher hydroxytyrosol content also showed the best antimicrobial activity against *S. aureus*. Moreover, OFI can be an advantageous ingredient for food products since it comprises specific lipid and hydrophilic bioactive compounds usually not present in other plant extracts. OFI also presents a high potential

to be used as a natural ingredient with a preservative effect. Due to the hydroxytyrosol health effects, its use also can be advantageous as a nutritional profile enhancer. Moreover, this extraction method made it possible to obtain a natural, plant-based, chemical-free functional ingredient.

Hence, OFI was used to develop an olive oil-based spread - Sprelive. Sprelive is an olive oil-based spread with more than 70% of olive oil and functionalized with OFI. Two formulations were selected, and high-pressure processing (HPP) was tested as a preservative method.

Total phenolics, antioxidant activity, hydroxytyrosol contents, and fatty acid profile were determined in Sprelive samples and in three commercial vegetable creams. As expected, Sprelive formulations with OFI presented significant nutritional advantages regarding the antioxidant profile and monounsaturated fatty acid contents compared to control or commercial vegetable creams. Also, HPP can be a suitable method to produce a safe product since no significant differences were found between control and HPP samples.

Throughout the development of new food products, it is important to ascertain the product attributes. Data from a survey confirmed the Sprelive attributes, especially the physical features that consumers search for in a vegetable cream. Also, it reinforced the consumers' preference by the “natural” and supported the use of by-products to develop new products.

Eco-nutrition aims to promote the best nutrient supply for human health while reducing environmental impact. This work presents strategies to upcycle olive pomace supported in sustainability and food and nutrition axis. Innovative products were developed, such as Sprelive, meeting consumers' awareness and preference for natural “clean-label”, simpler and sustainable food products.

Keywords: olive pomace, nutrition, sustainability, spreadable, hydroxytyrosol.

This page was intentionally left in blank.

Resumo

Globalmente, a produção de azeite tem vindo a aumentar devido aos seus efeitos benéficos na saúde e propriedades organoléticas. O bagaço de azeitona, composto pela polpa, pele e caroço das azeitonas, é o principal subproduto gerado no processamento do azeite. Este subproduto é uma fonte de compostos bioativos de valor acrescentado que são benéficos para a saúde humana e, simultaneamente, fitotóxicos comprometendo a germinação e crescimento de plantas.

Os principais objetivos desta tese foram explorar e desenvolver processos para promover a extração dos compostos do bagaço de azeitona e investigar o potencial desses extratos como ingredientes funcionais em produtos alimentares, tendo como desafio a segurança alimentar e a sustentabilidade ambiental. Além disso, um outro objetivo foi desenvolver um produto alimentar barrável com um teor de azeite superior a 70% e funcionalizado com um extrato de bagaço de azeitona.

Os bagaços de azeitona estudados foram obtidos em Portugal em lagares de extração contínua de duas fases. Foram selecionados quatro bagaços monovarietais da região do Alentejo (Arbequina, Arbosana, Oliana, Koroneiki) e quatro bagaços compostos por uma mistura de variedades de azeitonas (Cobrançosa, Madural, Verdeal Transmontana, Cordovil, Arbosana), de Trás-os-Montes e Alentejo.

Os métodos de análise foram selecionados e aplicados de acordo com o tipo de amostra a estudar e os compostos de interesse a analisar, considerando as questões levantadas durante o plano de trabalho.

Assim, foram frequentemente utilizadas metodologias espectrofotométricas para determinar o teor de fitoquímicos (fenólicos totais e flavonoides) e a atividade antioxidante (poder antioxidante por redução do ião férrico, inibição do radical 2,2-difenil-1-picril-hidrazilo e do radical ácido 2,2'-azino-bis (3-etilbenzotiazolina-6-sulfónico). Foram utilizadas técnicas analíticas cromatográficas como a cromatografia líquida (HPLC-DAD-FLD), cromatografia líquida acoplada a espectrometria de massa (MS) de ionização por *eletrospray* (ESI) (HPLC-DAD-ESI-MSⁿ) e cromatografia gasosa (GC-FID) para a determinação de compostos. A análise espectroscópica usando espectros de infravermelho próximo (NIR) e infravermelho médio (MIR) foi utilizada para discriminar bagaços de azeitona monovarietais. A composição proximal e os esteres metílicos de ácidos gordos foram determinados de acordo com metodologias oficiais (AOAC e ISO, respetivamente).

Foram avaliados o teor de fenólicos totais e a atividade antioxidante de bagaços de Arbosana, Koroneiki, Oliana e Arbequina. Além disso, foram preparados *blends* com diferentes percentagens de cada variedade, de forma a verificar possíveis efeitos aditivos, sinérgicos e antagónicos.

Os bagaços Koroneiki e Arbosana apresentaram os teores mais elevados de compostos fenólicos. A maioria das interações encontradas nos *blends* foram aditivas ou sinérgicas. Foi possível selecionar as melhores combinações, permitindo concluir que misturar os bagaços estudados nessas percentagens é vantajoso em relação ao uso individual dos mesmos.

Os compostos lipossolúveis e hidrossolúveis presentes numa amostra de bagaço de azeitona de Trás-os-Montes foram analisados através de um estudo exploratório. A fração lipídica revelou-se especialmente rica em ácido oleico, e o perfil de vitamina E do bagaço de azeitona contém maioritariamente α -tocoferol. Uma nova metodologia, *Multi-Frequency Multimode Modulated* (MMM), foi aplicada e os resultados comparados a um processo convencional de extração sólido-líquido. A técnica MMM permitiu uma extração mais rápida e superior dos compostos.

O perfil fenólico do bagaço de azeitona, avaliado por HPLC-DAD-FLD e HPLC-DAD-ESI-MSⁿ, mostrou que os principais fenólicos identificados foram hidroxitirosol>comselogosídeo> derivado do ácido elenólico> tirosol. Entre estes, o hidroxitirosol tem um interesse particular pois apresenta forte atividade antioxidante, anti-inflamatória e propriedades antimicrobianas.

O desenvolvimento de metodologias ecológicas e economicamente sustentáveis para a obtenção de extratos de bagaço de azeitona foi um dos princípios orientadores desta tese. Através de uma abordagem integrada, foi explorado um sistema de membranas de forma a concentrar compostos do bagaço de azeitona. Os compostos de bagaço de azeitona foram extraídos por um método que está sob pedido de patente na Europa (PCT/IB2017/053422) e patenteado nos EUA (US Patent 10479958) e testados três tipos de membranas. Concluiu-se que a membrana BW30 é uma solução viável para concentrar compostos antioxidantes de extratos de bagaço de azeitona.

Foi idealizado e materializado num protótipo um sistema físico para extração de compostos antioxidantes da água vegetativa do bagaço de azeitona, baseado na combinação de parâmetros de tempo e pressão. Por se tratar de uma forma inovadora de processamento de bagaço de azeitona foi protegida através de pedido de patente na Europa (PCT/IB2018/060111) e nos EUA (pedido de patente 6954060). Bagaços das

duas principais regiões produtoras de azeite de Portugal - Trás-os-Montes e Alentejo - foram processados, segundo o método descrito no pedido de patente, para obtenção de um ingrediente funcional de bagaço de azeitona (OFI). Depois, os respetivos OFI foram caracterizados quimicamente e avaliada a atividade antimicrobiana contra *Staphylococcus aureus* ATCC® 25923, *Escherichia coli* ATCC® 25922 e *Candida albicans* ATCC® 10231.

Os extratos com maiores teores de hidroxitirosol tinham também maior atividade antimicrobiana contra *S. aureus*. Além disso, a utilização de OFI como ingrediente para produtos alimentares pode ser vantajosa, uma vez que inclui compostos bioativos lipofílicos e hidrofílicos específicos que geralmente não estão presentes noutros extratos botânicos.

Considerando os resultados obtidos, o OFI tem potencial para ser utilizado como ingrediente natural com efeito conservante. Ainda, devido aos efeitos conhecidos do hidroxitirosol na saúde humana, a incorporação de OFI em alimentos pode melhorar o seu perfil nutricional. O método de extração utilizado permitiu obter um ingrediente funcional derivado do bagaço de azeitona sem recurso a produtos químicos, natural e vegetal.

O OFI foi utilizado como ingrediente funcional na formulação de um creme barrável à base de azeite (Sprelive) com um teor de azeite superior a 70%. Foram selecionadas duas formulações e foi testado o processamento por alta pressão (HPP) como método conservante.

O teor de fenólicos totais e hidroxitirosol, a atividade antioxidante e o perfil de ácidos gordos foram determinados nas amostras Sprelive e em três cremes vegetais comerciais. As formulações Sprelive com OFI apresentaram vantagens nutricionais significativas em comparação, quer com as formulações controlo quer com os produtos comerciais, considerando o perfil antioxidante e em ácidos gordos. Além disso, o HPP pode ser um método viável para obter um produto seguro, uma vez que não foram observadas diferenças significativas entre as amostras controlo e as submetidas a HPP.

Durante o desenvolvimento de novos produtos alimentares é importante aferir os seus atributos. Os resultados de um questionário e uma mostra ao consumidor confirmaram os atributos do Sprelive, principalmente as características físicas desejadas num creme vegetal. Além disso, reforçou a preferência do consumidor pelo “natural” e suportou a utilização de subprodutos no desenvolvimento de novos produtos.

A eco-nutrição visa promover o melhor provimento de nutrientes para a saúde humana e, simultaneamente, reduzir o impacto ambiental. Este trabalho apresentou estratégias para a valorização do bagaço de azeitona apoiadas nos eixos da sustentabilidade e alimentação e nutrição. Foram desenvolvidos e caracterizados produtos inovadores, como o Sprelive, que vão de encontro ao conhecimento e preferência dos consumidores por produtos naturais com um “rótulo limpo”, mais simples e sustentáveis.

Palavras-chave: bagaço de azeitona, nutrição, sustentabilidade, barrável, hidroxitirosol.

List of Publications

Publications in international peer-review journals

1. **Nunes, M.A.**, Palmeira, J.D., Melo, D., Machado, S., Lobo, J.C., Costa, A.S.G., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. (2021). *Chemical composition and antimicrobial activity of a new olive pomace functional ingredient*. *Pharmaceuticals*, 14, 913.
2. **Nunes, M.A.**, Reszczynski, P., Páscoa, R.N.M.J., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. (2021). *Influence of olive pomace blending on antioxidant activity: additive, synergistic, and antagonistic effects*. *Molecules*, 26, 169.
3. **Nunes, M.A.**, Páscoa, R.N.M.J., Reszczynski, P., Costa, A.S.G., Oliveira, M.B.P.P., Alves, R.C. (2021). *Near Infrared (NIR) spectroscopy as a tool to assess blends composition and discriminate antioxidant activity of olive pomace cultivars*. *Waste and Biomass Valorization*, 12, 4901-4313.
4. **Nunes, M.A.**, Páscoa, R.N.M.J., Alves, R.C., Costa, A.S.G., Bessada, S., Oliveira, M.B.P.P. (2020). *Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: the case of monocultivar pomaces*. *Waste Management*, 103, 378-387.
5. **Nunes, M.A.**, Pawlowski, S., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P., Velizarov, S. (2019). *Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration*. *Science of the Total Environment*, 652, 40-47.
6. **Nunes, M.A.**, Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P. (2018). *Olive pomace as a valuable source of bioactive compounds: a study regarding its lipid- and water-soluble components*. *Science of the Total Environment*, 644, 229-236.

Publications in national journals with scientific peer-review

1. **Nunes, M.A.**, Lobo, J.C., Oliveira M.B.P.P., Alves, R.C. (2021). *Inovar no consumo de azeite*. *Tecnoalimentar*, 28, 28-31.

Book chapters

1. **Nunes, M.A.**, Oliveira, M.B.P.P., Alves, R. (2021). *Nutrigenomics, olive polyphenols and human health*, Molecular Nutrition (AAP/CRP Press, Florida, USA), *in press*.
2. **Nunes, M.A.**, Alves, R.C., Costa, A.S.G., Puga, H., Oliveira, M.B.P.P. (2018). *Olive pomace phenolics extraction: conventional vs emergent methodologies*, in *Wastes solutions, treatments and opportunities II*. Editores: Cândida Vilarinho, Fernando Castro e Maria de Lurdes Lopes, CRC Press, Taylors & Francis. pp. 395 – 401. ISBN: 978-1-138-19669-8.

Intellectual property

1. International Application No. PCT/IB2017/053422

Olive pomace products, method of production and their uses

Nunes, M.A., Costa, A.S.G., Pimentel, F. & Oliveira, M.B.P.P. (2016). Olive pomace products, method of production and their uses. European Patent Application PCT/IB2017/053422 filed 09/06/2017. European Patent Office. Patent pending.

Nunes, M.A., Costa, A.S.G., Pimentel, F. & Oliveira, M.B.P.P. (2016). Olive pomace products, method of production and their uses (US Patent 10479958). U.S. Patent and Trademark Office.

2. International Application No. PCT/IB2018/060111

Foodstuff composition, process and uses thereof

Nunes, M.A., Costa, A.S.G., & Oliveira, M.B.P.P. (2017). Foodstuff composition comprising a derivate of olive pomace. European Patent Application PCT/IB2018/060111 filed 14/12/2018. European Patent Office. Patent pending.

Nunes, M.A., Costa, A.S.G., & Oliveira, M.B.P.P. (2017). Foodstuff composition comprising a derivate of olive pomace. US Patent Application Number 6954060 filed 14/12/2018. U.S. Patent and Trademark Office. Patent pending.

Oral presentations in international scientific meetings

1. Sousa, M.M., **Nunes, M.A.**, Lobo, J.C., Palmeira, J.D., Machado, S., Melo, D., Costa, A.S.G., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. *Heat-treated olive pomace paste: a safe edible ingredient for the food industry*. ISEKI Food e-conferences, Food texture, quality, safety and biosecurity in the global bioeconomy, November 2021, Online, Romania
2. **Nunes, M.A.**, Palmeira, J.D., Correia, J.C., Costa, A.S.G., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. *Influence of the geographical origin on the chemical composition and antimicrobial activity of an olive pomace functional ingredient*. ISEKI Food Association e-conferences, Food Quality and texture in sustainable production and healthy consumption, November 2020, Online, Romania
3. **Nunes, M.A.**, Machado, S., Costa, A.S.G., Alves, R., Oliveira, M.B.P.P. *Olive pomace: a multidimensional approach focusing zero wastes*. XX EuroFoodChem, June 2019, Porto, Portugal
4. **Nunes, M.A.**, Costa, A.S.G., Bessada, S., Santos J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P. *Olive pomace: from an agro-waste to a valuable source of bio-compounds*. 7th International conference on Green Chemistry & Technology, June 2018, Dublin, Ireland

Oral presentations in national scientific meetings

1. Sousa, M.M., **Nunes, M.A.**, Lobo, J., Palmeira, J., Machado, S., Melo, D., Costa, A.S.G., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. *Olive pomace paste: how to obtain a functional food ingredient microbiologically safe*. SIFFUP – Simpósio de Investigação da FFUP, November 2021, Faculty of Pharmacy of University of Porto, Porto, Portugal
2. Lobo, J.C., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Pasta de bagaço de azeitona: um ingrediente saudável para uma indústria alimentar sustentável*. 13.^a Reunião Anual PortFIR, October 2021, Online, Instituto Nacional de Saúde Doutor Ricardo Jorge, Lisbon, Portugal
3. **Nunes M.A.**, Páscoa R.N.M.J., Costa A.S.G., Alves R.C., Oliveira M.B.P.P. *Blending monovarietal olive pomaces: why does it matter for the food industry?* XV Encontro Química dos Alimentos, September 2021, Online, Madeira, Portugal

4. Sousa, M.M., Machado S., **Nunes, M.A.**, Melo, D., Correia J., Nunes, M.A., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Olive pomace paste: impact of processing on antioxidant compound levels*. IJUP21 - 14.º Encontro de Investigação Jovem da Universidade do Porto, Reitoria da Universidade do Porto, May 2021, Online, Portugal

5. **Nunes, M.A.**, Machado, S., Melo D., Correia, J.A., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Olive pomace: a source of a natural ingredient for incorporation in foodstuffs?*
XIX Congress of Food and Nutrition of Portuguese Association of Nutrition, September 2020, Online, Portugal

6. Sousa, M.M., Correia, J.A., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Olive pomace paste: nutritional characterization and development of a topping*. IJUP20 - 13.º Encontro de Investigação Jovem da Universidade do Porto, Reitoria da Universidade do Porto, February 2020, Porto, Portugal

7. **Nunes, M.A.**, Alves, R.C., Oliveira, M.B.P.P. *Empreendedorismo e Inovação: Bagaço de Azeitona*. VII encontro de nutrição e alimentação – Associação Nacional Estudantes de Nutrição, October 2019, Porto, Portugal

8. **Nunes, M.A.**, Alves, R.C., Oliveira, M.B.P.P. *Oliveira: muito para além do azeite*
Fascínio das Plantas, May 2019, Porto, Portugal

9. **Nunes M.A.**, Alves, R., Oliveira, M.B.P.P. *Parâmetros de autenticidade em óleos alimentares*. Seminário Óleos alimentares: mitos e realidades, February 2018, Instituto Politécnico de Beja, Escola Superior Agrária, Beja, Portugal

Poster presentations in international scientific meetings

1. Santo, L.E., Rocca, A.L., Sousa, M.M., Costa, A.S.G., **Nunes, M.A.**, Alves, R.C., Almeida, A., Oliveira, M.B.P.P. *Mineral profile of olive pomace from different crops in Portugal*. ISEKI Food e-conferences, Food texture, quality, safety and biosecurity in the global bioeconomy, November 2021, Online, Romania
2. **Nunes, M.A.**, Palmeira, J.D., Melo, D., Machado, S., Lobo, J.C., Costa, A.S.G., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. *Eco-nutrition and food ingredients development: the case of olive pomace upcycling*. XX Congress of Food and Nutrition | II International Congress of Food and Nutrition, September 2021, Online, Portugal
3. Sousa, M.M., **Nunes, M.A.**, Lobo J.C., Alves, R.C., Oliveira, M.B.P.P. *Nutritional properties of olive pomace*. XX Congress of Food and Nutrition | II International Congress of Food and Nutrition, September 2021, Online, Portugal
4. Oliveira, B. Lobo, J.C., **Nunes M.A.**, Costa, A.S.G.; Alves, R.C., Oliveira, M.B.P.P. *Pasta enrichment with olive pomace: nutritional advantages*. XX Congress of Food and Nutrition | II International Congress of Food and Nutrition, September 2021, Online, Portugal
5. Sousa, M., Melo, D., Correia, J., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Olive pomace paste: effect on vitamin E and fatty acids profiles of different heat-treatments*. ISEKI Food Association E-Conferences, Food Quality and texture in sustainable production and healthy consumption, November 2020, Online, Romania
6. Palmeira J.D., **Nunes, M.A.**, Alves, R., Oliveira, B., Ferreira, H.M.N. *Antimicrobial activity of extracts of natural products – a source of potential antimicrobial active compounds*. 30th ECCMID, April 2020, Paris, France
7. **Nunes, M.A.**, Santos, J., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Chemical characterization of an active ingredient derived from olive pomace for incorporation in foodstuff*. 2nd Food Chemistry Conference, September 2019, Seville, Spain
8. Reszczyński, F., Páscoa, R.N.M.J., **Nunes, M.A.**, Costa, A., Alves, R.C., Oliveira M.B.P.P. *Fourier-transform near infrared spectroscopy as a tool to predict the composition of olive pomace blends*. EuroFoodChem, June 2019, Porto, Portugal

9. Correia, J.A., **Nunes, M.A.**, Senderowicz, E., Bessada, S., Costa, A.S.G., Amaral, M.H., Alves, R.C., Oliveira, M.B. *Olive pomace paste: a promising ingredient for food and cosmetic applications*. EuroFoodChem, June 2019, Porto, Portugal
10. Palmeira, J.D., **Nunes, M.A.**, Correia, J.A., Costa, A.S.G., Alves R.C., Ferreira, H., Oliveira, M.B.P.P. *Antimicrobial activity of a functional ingredient from olive pomace*. EuroFoodChem, June 2019, Porto, Portugal
11. **Nunes, M.A.**, Costa, A.S.G., Bessada S., Alves, R.C., Oliveira, M.B.P.P. *Análise proximal e perfil lipídico de diferentes bagaços de azeitona*. XXIV Encontro Luso Galego de Química, November 2018, Porto, Portugal
12. **Nunes, M.A.**, Santos, J., Costa, A.S.G., Alves, R., Oliveira, M.B.P.P. *Phenolics profile (by HPLC) and antioxidant activity of olive pomace*. XVII Congress of Food and Nutrition and I International Congress of Food and Nutrition, May 2018, Lisbon, Portugal
13. Bessada, S., **Nunes, M.A.**, Alves, R.C., Oliveira, M.B.P.P. *Chemical composition of vegetable oils for food and cosmetic purposes*. XXIV Encontro Luso Galego de Química, November 2018, Porto, Portugal
14. **Nunes, M.A.**, Pawlowski, S., Oliveira, M.B.P.P., Velizarov, S. *Olive pomace treatment by NF and RO membranes*. Euromembrane, July 2018, Valencia, Spain

Poster presentations in national scientific meetings

1. Sousa, M.M., **Nunes, M.A.**, Lobo, J.C., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Análise nutricional de um subproduto oleícola: o bagaço de azeitona*. 13.^a Reunião Anual PortFIR, October 2021, Online, Instituto Nacional de Saúde Doutor Ricardo Jorge, Online, Lisbon, Portugal
2. Lobo, J.C., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Pasta de bagaço de azeitona: um ingrediente saudável para uma indústria alimentar sustentável*. 13.^a Reunião Anual PortFIR, October 2021, Instituto Nacional de Saúde Doutor Ricardo Jorge, Online, Lisbon, Portugal
3. **Nunes, M.A.**, Palmeira, J.D., Machado, S., Melo, D., Lobo, J.C., Costa, A., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. *A novel method to obtain an olive pomace functional ingredient*. 7th Portuguese Young Chemists Meeting, May 2021, Online, Portugal
4. Sousa, M.M., **Nunes, M.A.**, Machado, S., Melo, D., Lobo, J.C., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Strategies for upcycling by-products: the case of olive pomace*. 7th Portuguese Young Chemists Meeting, May 2021, Online, Portugal
5. Oliveira, B.C.C., Lobo, J., **Nunes, M.A.**, Melo, D., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *By-products valorisation: Processing of olive pomace for incorporation into foodstuffs*. VI Jornadas da Bioquímica, "O passaporte para a inovação", May 2021, Online Portugal
6. Rocca, A.L., Cuna, F.S.R.D., **Nunes, M.A.**, Almeida, A., Oliveira, M.B.P.P. *Minerals in olive pomace and relevance for human health and well-being*. IJUP21 - 14.^o Encontro de Investigação Jovem da Universidade do Porto, Reitoria da Universidade do Porto, May 2021, Online, Portugal
7. Sousa M.M., Lobo J.C., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Impact of different heat-treatment processes on the nutritional profile of olive pomace paste*. XIX Congress of Food and Nutrition of Portuguese Association of Nutrition, September 2020, Online, Portugal
8. **Nunes, M.A.**, Santos, J., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Bagaço de azeitona: extração e caracterização química de um ingrediente ativo para a incorporação em produtos alimentares*. 1.^a edição do Simpósio INIAV para a Segurança Alimentar, November 2019, Vila do Conde, Portugal

9. Sousa, M.M., Correia, J.A., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Caracterização nutricional de pasta de bagaço de azeitona e avaliação do seu potencial tecnológico para o desenvolvimento de toppings*. 1.^a edição do Simpósio INIAV para a Segurança Alimentar, November 2019, Vila do Conde, Portugal
10. **Nunes, M.A.**, Costa, A.S.G., Santos, J., Alves, R.C., Oliveira, M.B.P.P. *Extraction of olive pomace antioxidant compounds: sustainability versus yield*. XIV Encontro de Química dos Alimentos Indústria, Ciência, Formação e Inovação, November 2018, Viana do Castelo, Portugal
11. **Nunes, M.A.**, Pawlowski, S., Alves, R.C., Oliveira, M.B.P.P. *Sustainable recovery of antioxidant compounds from olive pomace via a membrane-assisted processing*. XIV Encontro de Química dos Alimentos Indústria, Ciência, Formação e Inovação, November 2018, Viana do Castelo, Portugal

Prizes and Awards

1. Best poster award – 2021

Lobo, J.C., **Nunes, M.A.**, Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Pasta de bagaço de azeitona: um ingrediente saudável para uma indústria alimentar sustentável*. 13.^a Reunião Anual PortFIR, October 2021, Instituto Nacional de Saúde Doutor Ricardo Jorge, Online, Lisbon, Portugal

2. 2nd Prize in Communication - oral communication – 2020

Nunes, M.A., Machado, S., Melo D., Correia, J.A., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Olive pomace: a source of a natural ingredient for incorporation in foodstuffs?* XIX Congress of Food and Nutrition of Portuguese Association of Nutrition, September 2020, Online conference, Lisbon, Portugal

3. "NewFood - Food Technologies Valorization" - 2019

Nunes, M.A., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. *Projeto SPRELIVE - Creme vegetal funcional à base de azeite e um ingrediente ativo extraído do bagaço de azeitona*

Universidade de Trás-os-Montes e Alto Douro, Universidade Católica Portuguesa, Universidade do Minho e Universidade do Porto

4. "Empreendedorismo e Inovação 2018" - Crédito Agrícola - 2018

Nunes, M.A., Costa, A.S.G., Pimentel, F.B., Oliveira, M.B.P.P. *Projeto SOILIFE - processo sustentável para reduzir a fitotoxicidade do bagaço de azeitona.* Cimeira Agro Inovação 2018/Crédito Agrícola, Lisbon, Portugal

5. "Born from Knowledge – BfK awards" -Agência Nacional de Inovação - 2018

Nunes, M.A., Costa, A.S.G., Pimentel, F.B., Oliveira, M.B.P.P. *Projeto SOILIFE - processo sustentável para reduzir a fitotoxicidade do bagaço de azeitona.* Cimeira Agro Inovação 2018/Crédito Agrícola, Lisbon, Portugal

This page was intentionally left in blank.

Table of Contents

Acknowledgements.....	xv
Abstract.....	xvii
Resumo	xxi
List of publications	xxv
Table of contents	xxxv
List of figures	xxxvii
List of tables.....	xli
Abbreviations.....	xliv
Thesis outline and organization	xlix
Chapter 1. Circular economy in the olive oil sector. Where do we stand?	1
1.1 Economic, food and environmental sustainability in the olive oil sector	3
1.2 General and specific objectives	7
Chapter 2. Olive, olive-derived products and by-products and human health	9
2.1. Nutrigenomics, olive polyphenols and human health	11
Chapter 3. Olive pomace strategies valorisation	53
3.1. <i>Science to Science</i>	55
3.1.1 Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components	55
3.1.2 Olive pomace phenolics extraction: conventional <i>vs</i> emergent methodologies....	79
3.1.3 Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration.....	91
3.1.4 Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: The case of monocultivar pomaces	115

3.1.5 Near infrared (NIR) spectroscopy as a tool to assess blends composition and discriminate antioxidant activity of olive pomace cultivars.....	143
3.1.6 Influence of olive pomace blending on antioxidant activity: additive, synergistic, and antagonistic effects	165
3.2 <i>Science to Society</i>	183
3.2.1 Olive pomace products, method of production and their uses.....	183
Publication number: WO/2017/212450.....	183
3.2.2 Foodstuff composition comprising a derivate of olive pomace.....	184
Publication number: WO/2019/116343	184
3.2.3 Science and technology transfer process.....	188
Chapter 4. Olive pomace functional ingredient as a potential food ingredient: chemical and antimicrobial assessment.....	191
4.1 Chemical composition and antimicrobial activity of a new olive pomace functional ingredient	193
4.2 Inovar no consumo de azeite.....	215
Chapter 5. Olive pomace functional ingredient: future developments	225
5.1 Chemical analysis of a mixed olive pomace functional ingredient	227
5.2 Olive oil-based spread: preparation and composition	230
Chapter 6. Olive pomace and further valorisation approaches towards the circular economy	243
Chapter 7. Conclusions and future perspectives	249
7.1 Conclusions	251
7.2 Future perspectives and developments	255

List of figures

Chapter 2. Olive, olive-derived products and by-products and human health

2.1 Nutrigenomics, olive polyphenols and human health

Figure 1. Chemical structure of the main olive polyphenols (hydroxytyrosol, oleuropein, oleocanthal and tyrosol). 20

Figure 2. Olive polyphenols beneficial effects. 39

Chapter 3. Olive pomace strategies valorisation

3.1 Science to science

3.1.1 Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components

Figure 1. Ultrasonic extraction set-up: (a) experimental apparatus, (b) numerical modelling, (c) experimental acoustic cavitation zones. (1) Transducer, (2) waveguide, (3) booster, (4) special acoustic radiator. 61

Figure 2. Distribution (in percentage) of the olive pomace phenolic compounds. Hydroxytyrosol, tyrosol, and pinoreosinol were identified by comparison with standards. The other compounds were tentatively identified by HPLC-DAD-MSⁿ. Tentative identification based on accurate mass and literature data. 68

Figure 3. Hydroxytyrosol, tyrosol, and pinoreosinol contents in olive pomace. 69

Figure 4. Total phenolics content and antioxidant activity of the different extracts: (a) TPC - total phenolic compounds; (b) FRAP - ferric reducing antioxidant power; (c) DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) scavenging ability. A, B, C, are extracts obtained by MMM technique and D expresses the results of conventional method. 71

Figure 5. Temperature evolution in the Multi-frequency Multimode Modulated ultrasonic technique. 72

3.1.2 Olive pomace phenolics extraction: conventional vs emergent methodologies

Figure 1. Ultrasonic extraction. (a) Experimental apparatus; (b) Numerical results of acoustic pressure in the sonicated medium (input electrical power of 160 W and $f_0=20.01 \pm 0.03$ kHz). 83

Figure 2. Temperature increase during ultrasonic olive pomace antioxidants extraction. 87

3.1.3 Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration

- Figure 1. A flow chart of the steps from the olive pomace collection to the membrane processing. 95
- Figure 2. OP proximate composition (g/100 g dry weight) before (raw OP) and after pressing process (pressed OP). 100
- Figure 3. Permeate fluxes (J_v) in function of the concentration factor (CF) obtained during filtration of aqueous extracts of olive pomace (IAE) at 20 bar, with the investigated membranes. 101
- Figure 4. Fouling index (I_f) expressing the drop in permeate fluxes, obtained during filtration of aqueous extracts of olive pomace (IAE) at 20 bar, with the investigated membranes. 103

3.1.4 Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: The case of monocultivar pomaces

- Figure 1. Average raw MIR spectra of each olive pomace cultivar. 126
- Figure 2. Average raw NIR spectra of each olive pomace cultivar. 126
- Figure 3. Scores obtained of PC1 versus scores obtained of PC3 considering MIR spectra. 128
- Figure 4. Scores obtained of PC1 versus scores obtained of PC2 considering NIR spectra. 128
- Figure 5. Loadings of PC1 and PC2 obtained with the PCA that showed the best separation between each olive pomace cultivar considering NIR data. 129
- Figure 6. Biplot score map generated with the mean values of all chemical parameters. 130

3.1.5 Near infrared (NIR) spectroscopy as a tool to assess blends composition and discriminate antioxidant activity of olive pomace cultivars

- Figure 1. Raw NIR spectra of olive pomace samples and respective characteristic NIR absorption bands. 151
- Figure 2. Experimental values against PLS cross-validation predictions (filled circle) and independent test set predictions (open square) for the composition determination of olive pomace samples (a Arbosana cultivar, b Arbequina cultivar, c Koroneiki cultivar, d Oliana cultivar) including also the corresponding parameters values of the PLS models. 153
- Figure 3. Regression coefficients vectors squared of the PLS models built for the composition determination of olive pomace samples with spectral region R1, from 4961 to 4035 cm^{-1} (a Arbosana cultivar, b Arbequina cultivar, c Koroneiki cultivar, d Oliana cultivar). 154

- Figure 4. Regression coefficients vectors squared of the PLSDA models built for the discrimination of olive pomace samples based on DPPH $\cdot$, TPC and FRAP assays results' using the entire spectra (a DPPH $\cdot$ assay, b TPC assay, c FRAP assay). 158

3.2 Science to Society

- Figure 1. Major by-products of olive oil processing. 183
- Figure 2. Flow diagram of the olive pomace functional ingredient obtention and olive oil-based spread processing. 187

Chapter 4. Olive pomace functional ingredient as a potential food ingredient: chemical and antimicrobial assessment

4.1 Chemical composition and antimicrobial activity of a new olive pomace functional ingredient

- Figure 1. Obtention of the olive pomace functional ingredients. 205

4.2 Inovar no consumo de azeite

- Figura 1. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Qual a principal ou principais razões por que consome azeite?” e B – “Costuma utilizar azeite como substituto de manteiga, margarina ou creme vegetal?”. 218
- Figura 2. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Consome um produto alimentar que mesmo que não seja “saboroso” é benéfico para a saúde?” e B – “Considera que os produtos mais saudáveis são também os mais caros?”. 219
- Figura 3. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Está satisfeito com os cremes vegetais que, neste momento, se encontram no mercado?” e B – “Qual a característica ou quais as características físicas que mais valoriza num creme vegetal? Espalhamento, consistência à temperatura ambiente, brilho, cor, textura?”. 220
- Figura 4. Distribuição das respostas dos inquiridos (%) às perguntas: A - “Normalmente, prefere produtos alimentares em que o processo de fabrico e os ingredientes são “amigos do ambiente” e que a pegada ecológica é pequena?” e B – “Consumiria um produto alimentar que incluísse ingredientes naturais obtidos ecologicamente de subprodutos da indústria agroalimentar?”. 221
- Figura 5. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Valoriza mais um produto se tiver aditivos naturais em vez de artificiais?” e B – “Compraria um creme vegetal para barrar à base de azeite?”. 222
- Figura 6. Distribuição das respostas dos inquiridos (%) à pergunta: “Qual o fator ou fatores de decisão mais importante(s) para si no momento de compra? Indique um ou vários.” 222

Chapter 5. Olive pomace functional ingredient: future developments

5.1 Chemical analysis of a mixed olive pomace functional ingredient

Figure 1. Lyophilized olive pomace functional ingredient (OFIx). 228

5.2 Olive oil-based spread: preparation and composition

Figure 2. Sprelive with olive pomace functional ingredient (OFIx). A, formulation A; B, formulation B. 232

Figure 3. Total phenolics, hydroxytyrosol content and antioxidant activity (FRAP and DPPH[•] scavenging ability): comparison between Sprelive formulations A and B with OFIx (SAO and SBO) and the commercial vegetable creams CVC1, CVC2 and CVC3. 236

Figure 4. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (relative %) profiles of Sprelive formulations A and B without the addition of OFIx (SAC and SBC, respectively) and formulations with OFIx (SAO and SBO, respectively); comparison with selected commercially available vegetable creams (CVC1, CVC2, CVC3). 239

List of tables

Chapter 3. Olive pomace strategies valorisation

3.1 Science to Science

3.1.1 Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components.

Table 1.	Extraction conditions used in the present study.	60
Table 2.	Chemical characterization of olive pomace.	64
Table 3.	Phenolic compounds identified (or tentatively identified) in olive pomace.	67
Table 4.	Pearson correlation coefficients (r) between antioxidant parameters.	72

3.1.2 Olive pomace phenolics extraction: conventional vs emergent methodologies

Table 1.	Total phenolics content and antioxidant activity of differently prepared extracts.	86
Table 2.	Pearson correlations coefficients (r) between the analyzed parameters.	86

3.1.3 Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration

Table 1.	Water quality for irrigation considering only the sodium adsorption ratio (SAR) and electrical conductivity (EC) taking together (FAO, 1985).	99
Table 2.	Physicochemical characteristics of the IAE (feed), permeate and concentrate streams obtained using the selected membranes (NF90, NF270, BW30) and their apparent rejection (R_i) values toward total organic carbon (TOC), phenolics and flavonoids at CF = 10.	104
Table 3.	Elemental composition of the permeate obtained with the BW30 membrane.	106

3.1.4 Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: The case of monocultivar pomaces

Table 1.	Chemical composition of olive pomaces from Arbequina, Arbosana, Oliana, and Koroneiki cultivars.	124

3.1.5 Near infrared (NIR) spectroscopy as a tool to assess blends composition and discriminate antioxidant activity of olive pomace cultivars

Table 1.	Total antioxidant activity through FRAP and DPPH• scavenging ability assays and total phenolics values obtained for olive pomace samples.	155

Table 2.	Confusion matrices obtained through PLSDA for the discrimination of olive pomace samples with high values of DPPH• scavenging ability, TPC and FRAP with an overall percentage of correct predictions of 82, 90 and 61%. Values are expressed in %.	157
<i>3.1.6 Influence of olive pomace blending on antioxidant activity: additive, synergistic, and antagonistic effects</i>		
Table 1.	Total phenolics and antioxidant activity (FRAP and DPPH• scavenging ability) of the monovarietal olive pomaces and mixtures. Experimental, predicted results, and type of interaction.	169
Chapter 4. Olive pomace functional ingredient as a potential food ingredient: chemical and antimicrobial assessment		
<i>4.1 Chemical composition and antimicrobial activity of a new olive pomace functional ingredient</i>		
Table 1.	Predominant olive varieties in the olive pomaces from the northeast region (Trás-Os-Montes) and south (Alentejo) of Portugal.	196
Table 2.	Total phenolics; flavonoids; hydroxytyrosol content and antioxidant activity (FRAP, DPPH and ABTS) of the olive pomace extracts (dry weight).	197
Table 3.	General results of antimicrobial activity of the olive pomace extracts.	197
Table 4.	Results of the antimicrobial activity against <i>E. coli</i> and <i>S. aureus</i> of the olive pomace extracts.	197
Table 5.	Chemical analysis of the olive pomace ingredients (total fat, fatty acid and vitamin E profiles, total protein, ash and pH) (dry weight).	198
Table 6.	Olive pomace bioactive compounds and proposed mechanisms of action.	200
<i>4.2 Inovar no consumo de azeite</i>		
Tabela 1.	Perfil demográfico dos participantes.	217
Chapter 5. Olive pomace functional ingredient: future developments		
<i>5.1 Chemical analysis of a mixed olive pomace functional ingredient</i>		
Table 1.	Chemical analysis of OFIx: total phenolics, flavonoids, hydroxytyrosol content, antioxidant activity (FRAP, DPPH• and ABTS•+ scavenging abilities), fatty acids and vitamin E profiles, total fat, total protein and ash (dry weight).	229
<i>5.2 Olive oil-based spread: preparation and composition</i>		
Table 2.	Samples denomination.	232
Table 3.	Ingredient's list of commercial vegetable creams.	233

Table 4.	Proportion (%) between the aqueous and lipidic phases of Sprelive formulations A (SAO) and B (SBO) with OFIx and the commercial vegetable creams.	233
Table 5.	Total phenolics, hydroxytyrosol content and antioxidant activity (FRAP and DPPH• scavenging ability): comparison between Sprelive formulations A and B with and without the addition of OFIx; and influence of HPP on Sprelive formulations with OFIx.	235
Table 6.	Fatty acids profile (relative %) of Sprelive formulations A and B without the addition of OFIx (SAC and SBC, respectively) and formulations with OFIx (SAO and SBO, respectively); comparison with selected commercially available vegetable creams (CVC1, CVC2, CVC3).	238

This page was intentionally left in blank.

Abbreviations

ABTS	2,2'-azino-bis(3-ethylenbenzothiazoline-6-sulfonic acid)
ANOVA	One-way analysis of variance
AOAC	Association of Official Analytical Collaboration
CE	Catechin equivalents
CS	Cell suspensions
dw	Dry weight
DAD	Diode array detector
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC	Electrical conductivity
EE	Epicatechin equivalents
EOP	Extracted olive pomace
ESI	Electrospray ionization
EU	European Union
EUA	Estados Unidos da América
FA	Fatty acids
fw	Fresh weight
FAME	Fatty acid methyl esters
FAO	Food and Agriculture Organization
FC	Folin-Ciocalteu
FLD	Fluorescence detector
FRAP	Ferric reducing antioxidant power
FSE	Ferrous sulfate equivalents
GAE	Gallic acid equivalents
GC-FID	Gas chromatography - flame ionisation detector
HE	Hydroxytyrosol equivalents
HPLC	High performance liquid chromatography

HPLC-DAD-ESI-MS ⁿ	High-performance liquid chromatography-diode array detector-electrospray ionization interface-mass spectrometry
HPLC-DAD-FLD	High-performance liquid chromatography-diode array detector-fluorescence detector
HPP	High pressure processing
ICP-AES	Inductively coupled plasma atomic emission spectroscopy
INPI	Instituto Nacional da Propriedade Industrial
ISO	International Organization for Standardization
IZ	Inhibition zone
MIC	Minimal inhibitory concentration
MIR	Mid infrared
MMM	Multi-frequency multimode modulated
MUFA	Monounsaturated fatty acids
NIR	Near infrared
OFI	Olive pomace functional ingredient
OFI _x	Mixed olive pomace functional ingredient
OP	Olive pomace
PCA	Principal component analysis
PCT	Patent cooperation treaty
PLS	Partial least squares
PLSDA	Partial least square discriminant analysis
PUFA	Polyunsaturated fatty acids
R&D	Research and development
RER	Range error ratio
RMSEC	Root mean square error of calibration
RMSECV	Root mean square error of cross-validation
RMSEP	Root means square error of prediction

ROS	Reactive oxygen species
RPD	Residual predictive determinations
rpm	Revolutions per minute
SAR	Sodium adsorption ratio
SD	Standard deviation
SFA	Saturated fatty acids
TE	Trolox equivalents
TFC	Total flavonoids content
TOC	Total organic carbon
TPC	Total phenolics content
TPTZ	2,4,6-tris(1-pyridyl)-5-triazine
TSB	Tryptic soy broth
UHPLC-ESI-QTOF- MS	Ultra-high-performance liquid chromatography- electrospray ionization-quadrupole time-of-flight-mass spectrometry
US	United States
UV	Ultraviolet
WOP	Wet olive pomace

This page was intentionally left in blank.

Thesis outline and organization

This thesis is divided into 7 chapters following the line of the developed work.

Chapter 1 describes why this work intended to put the olive oil sector in Portugal under the circular economy perspective and why open innovation is essential. Also, the general and specific objectives of the thesis are presented.

Chapter 2 includes a comprehensive analysis of the main phenolics present in olive products and by-products and their impact on gene regulation. Overall, the nutrigenomic effects of olive polyphenols, especially hydroxytyrosol, tyrosol, oleocanthal and oleuropein, on intracellular molecular mechanisms are highlighted, especially those related to the cardiovascular system, lipids metabolism, obesity, type 2 diabetes, inflammatory bowel diseases and microbiota, osteoarthritis, Alzheimer's disease and cancer.

Based on the current state of the art, strategies were studied to add value to olive pomace, the major olive oil processing by-product. Olive pomace has a great ecological pollution load but is, at the same time, an outstanding source of bioactive compounds. Therefore, **Chapter 3** describes the strategies developed to valorise olive pomace, such as the utilization of an ultrasound system to extract bioactive compounds from olive pomace and comparison to conventional methodologies and a membrane-assisted concentration process of the olive pomace bioactive compounds. Also, non-invasive tools were explored, and an attempt to find synergistic olive pomace combinations experimented. The experimental work aimed to find methodologies that balance efficient bioactive compounds recovery, green chemistry application and innovation. This chapter also presents non-confidential outputs about two olive pomace patent applications processes that were developed and managed during this thesis.

The obtention and chemical characterization of an olive pomace functional ingredient is approached in **Chapter 4**, as well as its potential to be used as a natural food ingredient. Moreover, the results of a survey developed during the prototyping phase of an olive oil-based spread functionalized with the olive pomace extract are presented.

Chapter 5 relates to future developments of the olive pomace functional ingredient, namely its utilization in food products, presenting preliminary results about the chemical profiles of two olive oil-based spread formulations (Sprelive).

Chapter 6 presents work of students from different areas, with the collaboration of the PhD candidate, that contributed to the state of art in the olive pomace scientific field,

based on food security and environmental concerns, addressing circular economy. They are briefly presented.

In the last chapter (**Chapter 7**), the partial and general conclusions are listed. It also provides future perspectives and developments for the work presented in this thesis.

Chapter 1 – Circular economy in the olive oil sector. Where do we stand?

Chapter 1 analyses why this work intended to put the olive oil sector under the circular economy perspective and why open innovation and universities are significant for that progress. As a result of that considerations, the general and specific objectives of the work are outlined.

This page was intentionally left in blank.

1.1 Economic, food and environmental sustainability in the olive oil sector

The worldwide population is increasingly growing. Agriculture intensification has been the answer to the high food demand contributing significantly to land degradation [1]. Therefore, agroecology systems are urgently required to transform agricultural production into a more sustainable, productive, economically fair, and attractive practice [2].

Over the last years, science, industrial and governmental players have been exploring sustainable agricultural solutions. New technologic systems have emerged to increase the production of various types of crops. Nevertheless, green growth of the sector is now mandatory, and the management of the life cycle of the natural resources should be lined up with eco-innovation and circular economy practices as recently promoted in the Green Deal package of the European Commission. A healthy food system for people and the planet is now targeted [3].

Particularly, olive oil production has a relevant socio-economic impact in the European Union (EU), the leading global producer, consumer, and exporter of olive oil; in fact, the EU is responsible for $\approx 69\%$ of the olive oil world's production. Olive oil production is increasing due to its organoleptic properties and well-known health benefits. In the EU, in 2020, more than 1 900 thousand tonnes of olive oil were produced. Spain was the leading producer (1125 thousand tonnes), followed by Italy (366), Greece (275), and Portugal (141). In the 2014/2015 campaign, Portugal produced 61 thousand tonnes, meaning that in 5 years the production of olive oil doubled [4]. This results from the global intensification that occurred over the last decade in olive oil production, particularly in some countries like Portugal, with a great sector innovation, new olive tree plantations, and national and European investments [5]. Furthermore, the sector is still expanding in the EU. It is expected that olive oil production will increase 1.1%/year until 2030 [6].

In Portugal, the main olive producer region is Alentejo, followed by Trás-os-Montes. Alentejo presents a more extensive olive groves area and the major industrial players. In Trás-os-Montes, olive groves still follow the small-scale/familiar traditional olive oil production. Hence, the olives are processed in small olive mills or cooperatives. The number of olive mills in Alentejo is lower (95) than in Trás-os-Montes (113) [7], which agrees with areas' advanced technology, type of olive cultivation and olive oil production.

Usually, the harvest season takes place between October and January. After harvesting, olives must be processed within 24 hours to olive oil attain good chemical/sensory

features. During this period, olive mills are producing olive oil intensively (24 hours/7 days a week). Considering that the olive oil yield ranges between 15 and 20%, olive pomace is produced in high amounts (80-85%). Olive pomace has an environmental pollution load due to its phytochemicals which are, at the same time, an outstanding source of bioactive compounds with human health benefits.

Olive pomace, that is generated in olive mills, is continuously transported to other companies (olive pomace oil extractors), where it is stored in large open-air containers throughout the rest of the year. Considering the intensive olive pomace generation in a short period and the limited storage capacity of olive pomace oil extractors, the continuous production of olive oil with high-quality standards can be difficult. In olive pomace oil extractors, the remaining oil is chemically removed to obtain olive pomace oil. These companies are the end-of-line stakeholders in the olive oil sector, and the waste that results from this process is the extracted olive pomace. This raw material has low economic value and is essentially used as biomass for heat and energy production. The increase of competition, especially regarding substitute products, has led to a severe decrease in its profitability.

At this point, scientific institutions are strategic partners in building viable solutions with companies, saving natural resources and ensuring food security for future generations. The eco-nutrition framework supports the upcycle of olive pomace, a by-product with a reduced economic value, high environmental load and advantageous nutritional properties, into health evidence-based functional ingredients suitable for human consumption.

The benefits of olive products and by-products compounds on human health have been highlighted continuously. Also, consumers' awareness about the association between food and health is increasing, including frequently an ecological concern. Likewise, "natural" designation is perceived as beneficial [8]. Therefore, it seems clear-cut to use a nutritional rich raw material produced in high amounts and obtained at low cost and converted it into food products.

Olive pomace valorisation addresses the fundamental axis of the eco-nutrition framework. First, considering the environment and agricultural practices, its use can add value to an agro-food waste by recovering the bioactive compounds that have human health benefits while simultaneously reducing its environmental load and preserving the soil and natural resources. Then, it allows the economic development of the olive oil industry by bringing new industrial players to the sector and expanding the market by introducing new food products. Regarding human health, olive pomace is rich in

polyphenols with anti-atherogenic and cardioprotective effects, anticarcinogenic, neuroprotective, antidiabetic, lipid-regulating, and lipogenesis inhibition, antimicrobial and anti-inflammatory effects [9]. Finally, regarding nutrition and food technology, olive pomace addresses several applications as a natural additive or the improvement of foods profile [8].

However, despite intensive research in the field, olive pomace is still a problem for the olive oil sector. Therefore, the first question raised in this work was “How to add innovation to an “old” raw material that can be, at the same time, a breakthrough in science, olive oil supply chain and food industry?”. Accordingly, and considering the high and growing amounts of olive pomace produced, another question emerged: “How to develop robust valorisation strategies with a positive impact on outcomes to the olive oil sector, the community around this sector and the national socio-economic activity?”.

The Farm to Fork Strategy, a central point in the European Green Deal, encourages the combination of scientific results, new technologies and public awareness to safeguard nutritious and sustainable foods [10].

The development of new food products with the addition of bioactive extracts from the olive pomace as a natural way to improve their nutritional profile and/or shelf-life is a challenge and follows the sustainable food systems path. Hence, “Is it possible to develop a sustainable and functional food product that contains an active ingredient derived from olive pomace?” was the proposal.

The olive supply chain can negatively impact the environment. The major effects are land degradation and exhaustion of resources, air emissions and waste production. The intensity of the impact depends on the agricultural techniques used in olives cultivation, the selected olive oil extraction system, and waste management [11]. Circular economy approaches can reduce the impact of by-products generated within the olive oil production by adding value to them and allowing the nutrient recovery. Hence, at this moment, it is emergent to focus on upcycling olive pomace and move from a linear to a circular economy by maximizing the use of a natural and rich resource.

Open science is part of the research process as scientific knowledge is a product of social collaboration and a contribution to the community evolution. According to European Commission, “*Open science is a policy priority for the European Commission and the standard method of working under its research and innovation funding programmes as it improves the quality, efficiency and responsiveness of research.*” [12]. Ultimately,

Science must be applied to the community to ensure the next generation's sustainable growth.

A key challenge is finding ways to integrate, develop, and connect scientific knowledge to the know-how that comes from experience and industrial practice, the experimental knowledge [13]. Collecting information, data, experience, and concerns of several societal sectors and engaging with project partners is valuable. More focused, applied, creative and integrative research can be achieved as these are listened to, and academic projects are discussed.

This thesis follows research previously developed in the Faculty of Pharmacy of the University of Porto, focused on the olive pomace valorisation. It intends to contribute to expanding the state of the art in the olive oil sector and transfer the acquired knowledge to the community where the University is integrated.

1.2 General and specific objectives

Several works and intensive research have been developed in the field of olive by-products valorisation. However, it is still critical to enhance scientific knowledge since more and more sustainability and food security issues impact our society.

The **general objective** of this work was to develop science-based and sustainable strategies to obtain olive pomace functional ingredients able to be used in the research and development of new food products.

Thus, to successfully attain this purpose, interconnected steps and activities were implemented to answer the **specific objectives**:

- To characterize olive pomaces from different regions of Portugal by assessing their nutritional and chemical composition.
- To discriminate olive pomaces using rapid, green and non-destructive techniques.
- To apply and optimize methods to increase the extraction yield of bioactive compounds, such as phenolic compounds.
- To explore environmentally friendly integrated processes to concentrate antioxidant-rich extracts derived from olive pomace.
- To assess the chemical profile of olive pomace extracts, focusing especially on hydroxytyrosol content, foreseeing its functionality in food products.
- To evaluate antimicrobial activity of olive pomace extracts, expecting their use as a natural food preservative.
- To assess the chemical profile of a food product (olive oil-based spread) fortified with an olive pomace functional extract.

References

- [1] van der Ploeg JD. The political economy of agroecology. *J Peasant Stud* 2020; 1-24.
- [2] Rotz S, Duncan E, Small M, Botschner J, Dara R, Mosby I, et al. The politics of digital agricultural technologies: A preliminary review. *Sociol Ruralis* 2019; 59(2): 203-229.
- [3] European Commission: A European Green Deal. Accessed in 10/10/21: https://ec.europa.eu/info/strategy/priorities-2019-2024/european-green-deal_en
- [4] European Commission: Olive oil - Detailed information on the market situation, price developments, balance sheets, production and trade. Accessed in 09/10/21: https://ec.europa.eu/info/food-farming-fisheries/farming/facts-and-figures/markets/prices/price-monitoring-sector/plant-products/olive-oil_en
- [5] Neves B, Pires, IM. The Mediterranean diet and the increasing demand of the olive oil sector: Shifts and environmental consequences. *Region* 2018; 5(1).
- [6] European Commission: EU agricultural outlook 2019-30: olive oil consumption to rise in non-producing EU countries. Accessed in 09/10/21: https://ec.europa.eu/info/news/eu-agricultural-outlook-2019-2030-olive-oil-consumption-rise-non-producing-eu-countries-2019-dec-10_en
- [7] Statistics Portugal: Produção de azeitona (t) por Local de proveniência da azeitona (Região agrícola), 2020. Accessed in 09/10/21: https://www.ine.pt/xportal/xmain?xpid=INE&xpgid=ine_base_dados
- [8] Nunes MA, Pimentel, FB, Costa ASG, Alves RC, Oliveira MBPP. Olive by-products for functional and food applications: Challenging opportunities to face environmental constraints. *Innov Food Sci Emerg Technol* 2016; 35: 139-148.
- [9] Karković MA, Torić J, Barbarić M, Jakobušić BC. Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. *Molecules* 2019; 24: 2001.
- [10] European Commission: Farm to Fork strategy. Accessed in 09/10/21: https://ec.europa.eu/food/horizontal-topics/farm-fork-strategy_en
- [11] Maffia A, Pergola M, Palese AM, Celano G. Environmental impact assessment of organic vs. integrated olive-oil systems in Mediterranean context. *Agronomy* 2020; 10(3):416. <https://doi.org/10.3390/agronomy10030416>
- [12] European Commission: Open Science. Accessed in 10/10/21: https://ec.europa.eu/info/research-and-innovation/strategy/strategy-2020-2024/our-digital-future/open-science_en#future-of-open-science-under-horizon-europe
- [13] Scholz RW. Transdisciplinarity: science for and with society in light of the university's roles and functions. *Sustain Sci* 2020; 15: 1033–1049.

Chapter 2 – Olive, olive-derived products and by-products and human health

An optimal nutritional status is required for the human body to function at a high-performance level. However, the concept of human nutrition should be integrated with a holistic framework that comprises external (food security/insecurity, socio-economic conditions, lifestyle, politics, agriculture and health services) and internal factors. The latter considers the human body system as cells, tissues, organs, and genetics, particularly nutrigenetics and nutrigenomics. “We are what we eat” can be adjusted to “We are what (each one of us) eat” and reveals the fundamental principle associated with nutrigenomics: diet modulates the gene expression that influences the individual health status and predisposition to nutrition-related diseases.

Chapter 2 focuses on the interaction of the main olive polyphenols (hydroxytyrosol, tyrosol, oleocanthal and oleuropein) on genes and how it is translated into health outcomes.

This page was intentionally left in blank.

2.1 Nutrigenomics, olive polyphenols and human health

M. Antónia Nunes, Beatriz P.P. Oliveira, Rita C. Alves

REQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy,
University of Porto, R.J. Viterbo, 4050-313 Porto, Portugal

- Nunes, M.A., Oliveira, M.B.P.P., Alves, R. (2021). *Nutrigenomics, olive polyphenols and human health*, *Molecular Nutrition (AAP/CRP Press, Florida, USA)*, in press.

Abstract

Nutritional ecology focuses on the dynamic relationship between humans, nutrition and the environment. Diet is one of the most critical environmental factors that individuals are exposed to and strongly impact gene-nutrient interactions. Olives are a valuable product for the Mediterranean basin countries, and their health benefits have been highly valorized over the years. The drupes are processed mainly to obtain two food products: table olives and olive oil. Olive tree leaves can be directly picked or can also be considered a by-product resulting from farming or processing. Olive oil production generates by-products such as olive pomace and olive mill wastewaters. Nutraceuticals and dietary supplements using olive products/by-products have also emerged. The main olive polyphenols are hydroxytyrosol, tyrosol, oleocanthal and oleuropein and experimental studies have identified molecular targets for these compounds. This chapter highlights the nutrigenomic effects of olive polyphenols, especially hydroxytyrosol, tyrosol, oleocanthal and oleuropein, on intracellular molecular mechanisms related to the cardiovascular system, lipids metabolism, obesity and type 2 diabetes, inflammatory bowel diseases and microbiota, osteoarthritis, Alzheimer's disease and cancer.

Keywords: Epigenetics, olives, by-products, hydroxytyrosol, oleuropein.

1. Introduction

The dynamic interaction between humans and the environment occurs at several levels, from immediate and short-term responses to the environment to long-term ones, such as natural selection through gene modifications (Raubenheimer and Simpson 2016).

Diet is a critical environmental factor since individuals are exposed to dietary components, able to modulate genes expression (Peña-Romero et al. 2017). In addition, foods are composed of several nutrients, bioactive compounds, and sometimes anti-nutrients and toxins, involved in a matrix, and each compound has different bioactions and targets. Currently, most of the consumed products came from a process that comprises collecting, packaging and distribution or increasingly often came from extensive processing, leading to a profound modification of the initial nutritional profile (Raubenheimer and Simpson 2016).

For a good maintenance of human health, nutrients are required at a particular level that changes according to age, physical activity, diseases, and reproductive state. However, general recommendations comprise macro and micronutrients but not compounds such as polyphenols known for their role in diseases prevention (Durazzo et al. 2019). Finally, the intestinal microbiota differs among individuals, changes during the life cycle and is strongly related to the host and diet (Ferguson et al. 2016).

Regarding the olive fruit, the quantity and type of polyphenols may be dependent on the olive cultivar, edaphoclimatic conditions, agricultural practices and maturation degree. Moreover, olive processing can likewise modify the profile and amount of compounds of the final products obtained. For example, the extraction process used to produce olive oil can influence the polyphenols profile of the virgin olive oil obtained (Klen and Vodopivec 2012).

Virgin olive oil is considered a simple, pressed and cold-treated juice, as olives are only milled, and the obtained pasta is mixed and centrifuged. Despite not being used any technological adjuvant, or biological, chemical or thermal processing, the olive bioactive compounds suffer natural modifications and partition during processing. Therefore, olive oil polyphenols include those initially present in the drupe and others that are newly formed by enzymatic and chemical reactions (Klen et al. 2015). Moreover, polyphenols fractioning between the olive oil and the by-products generated occurs. Most of the polyphenols are retained in the olive pomace or extracted with the olive mill wastewaters (~98%) and only a lower amount is transferred (1-2%) to olive oil (Rodis et al. 2002).

Therefore, olive products and by-products have been largely explored as sources of polyphenols that impact human health. Moreover, their potential as food products/ingredients, food supplements and nutraceuticals have been increasingly recognized (Gorzynik-Debicka et al. 2018; Rodríguez-López et al. 2020).

Nutrition sciences have arisen over the last years and successfully treated, and even made disappear, some diseases associated with micronutrient deficiencies such as scurvy, beriberi and rickets. In spite of that evolutive impact on public health, some chronic diseases present high prevalence and are still increasing despite all the health public measures and recommendations. Obesity and cancer are some examples. As we look at non-communicable chronic diseases, it is even more relevant to contextualize each person's environment and genetics background. Thus, the role of the biology-environment interface for humans is complex and, currently a great challenge (Raubenheimer and Simpson 2016).

Hydroxytyrosol, tyrosol and oleuropein are phenolic compounds almost exclusively from olives, being rare to find it in other plants. Consequently, olive products and by-products are also sources of those polyphenols with human health benefits.

This chapter presents a comprehensive overview on the polyphenols of the olive fruit and their derived products and by-products, focusing on their classic uses in human nutrition as foods (olive oil, leaves and table olives) and upcycled by-products (olive mill wastewaters and olive pomace). The analysis of the interaction gene-olive polyphenol will explore how these products can lead to the known health outcomes under the focus of nutrigenomics.

2. Olive polyphenols: classification and bioactivity

Polyphenols are secondary plant metabolites with strong antioxidant activity. They are widely distributed in nature in vegetables, fruits, nuts, seeds, roots, bark, plant leaves, herbs and whole-grain products. More than 8000 phenolics were identified with a variable chemical structure. They are the response of plants to pests, bacterial infections and environmental stressors and are involved in the growth, lignification, pollination and pigmentation processes (Fraga et al. 2010; Gorzynik-Debicka et al. 2018).

Phenolics present an aromatic ring linked to one hydroxyl group, whereas polyphenols are characterized by having at minimum two phenyl rings and one or more hydroxyl groups. In fact, "polyphenols" is a collective denomination for numerous subgroups of compounds that should be strict to the structures presenting at least two phenolic moieties. Nevertheless, the denominations "phenolic" and "polyphenol" are often found

in scientific literature as a similar designation for the same compound (Durazzo et al. 2019). In this chapter, the term "polyphenol" will be recurrent throughout the text.

Also, the classification of polyphenols in different groups is controversial. A more straightforward classification divides polyphenols into flavonoids and non-flavonoid polyphenols (Durazzo et al. 2019). Other often used classification, groups polyphenols in phenolic acids (hydroxycinnamic and hydroxybenzoic acids) and flavonoids (isoflavones, flavanones, flavanols, flavones, flavonols, anthocyanins), curcuminoids, lignans and stilbenes (Milenkovic et al. 2013).

Oxidation is naturally occurring process in the human body, which originates reactive oxygen species (ROS), reactive sulfur species (RSS) and reactive nitrogen species (RNS), as superoxide, hydrogen peroxide, singlet oxygen and nitric oxide (NO) radicals. In physiological conditions, the endogenous antioxidant system, which includes enzymatic (catalase, glutathione peroxidase and superoxide dismutase) and non-enzymatic compounds (bilirubin and albumin), can scavenge radicals and maintain homeostasis (Santos-Sánchez et al. 2019). However, when the antioxidant enzyme system cannot eliminate efficiently radicals over-production, oxidative stress occurs, leading to chronic and degenerative diseases (Ji et al. 2020). In this case, dietary sources are crucial to provide sufficient antioxidant compounds, such as polyphenols, to complement the endogenous system.

The accumulation of reactive species in the body is deleterious and can lead to pathologies as cardiovascular diseases and cancer. Superoxide is one of the most reactive radical species. It contains at least one unpaired electron and is generated by the one-electron reduction of oxygen by transition metals, reducing radicals or UV radiation. Superoxide is produced by endogenous biological sources purposely or as an outcome of metabolic processes in mitochondria, multiprotein complexes [e.g., nicotinamide adenine dinucleotide phosphate (NADPH) oxidases] or enzymes (e.g., xanthine oxidase) (Winterbourn 2020). Autoxidation reactions are also responsible for superoxide generation. For example, hemoglobin (Hb) in erythrocytes constantly undergoes autoxidation leading to a considerable presence of superoxide in these cells (Nishida et al. 2016).

Indeed, the aerobic human conditions demand oxygen to maintain the cell metabolism and life. Partially oxidized intermediates, like ROS, are formed and present high reactivity (Santos-Sánchez et al. 2019). Superoxide is, hence, the result of physiological functions (Winterbourn 2020).

ROS and RSS are chemically similar. RSS, produced from sequential one-electron oxidations, are described as even more reactive than ROS and have been mainly studied lately (Kolluru et al. 2020; Olson 2020). RSS can result from the oxidation of sulfite or sulfate molecules or a by-product of major thiols and can damage cellular components (Bora et al. 2018).

NO is the most active radical of the RNS resultant from L-arginine through the NO synthase enzyme. Other RNS are nitrogen dioxide, nitrosoperoxycarbonate, nitronium ions, peroxyxynitrite, and peroxyxynitrous acid and dinitrogen trioxide. NO at physiological levels contributes to the blood vessels smooth muscle relaxation (Santos-Sánchez et al. 2019).

Excessive free radicals can act as biological oxidants and deleteriously by damaging cells. When the human body's response is not sufficient to oxidative stress, several pathophysiological processes are triggered. Among them, cell ageing, inflammation, atherosclerosis, mutagenesis and cancer are prevalent (Santos-Sánchez et al. 2019).

However, not only physiological conditions are linked to the generation of radicals. Current lifestyle habits, pollution and stress, can contribute increasingly to radical production. Therefore, it is important to reinforce the natural endogenous antioxidant system with exogenous antioxidants such as carotenoids vitamin C, minerals (selenium and zinc) and polyphenols. Because of their chemical structure, polyphenols act as antioxidants by reactive-free radicals scavenging and metal sequestration activities (Santos-Sánchez et al. 2019).

The interactions of polyphenols with membrane components as lipids and proteins can also mediate some biological effects by modifying the plasma membrane structure and the electrical and fluidity properties resulting in occurrences such as enzymes activity, interactions between ligand and receptor, ion and/or metabolite fluxes and modulation of signal transduction. Hence, polyphenols also provide antioxidant protection through mechanisms besides free radical scavenging or metal chelating activities (Fraga et al. 2010).

2.1 Olive fruit, virgin olive oil and table olives

The olive tree is an evergreen tree belonging to the family Oleaceae (*Olea europaea* L.) and its fruit is the olive. The fruit is classified as a drupe. It is a fleshy fruit with a central stone containing the seed. Compared to other drupes, olive contains less sugar and presented a high quantity of oil, varying between 3 and 38% depending on cultivar, agricultural practices, and maturity. Olive is constituted by the skin (epicarp), pulp

(mesocarp) and the stone wall around kernel (endocarp) and kernel (Charoenprasert and Mitchell 2012).

Olive oil and olive tables are the main products of olives processing. These are great sources of simple phenolics such as phenolic acids and phenolic alcohols (hydroxytyrosol and tyrosol), lignans, secoiridoids and flavonoids (Ghanbari et al. 2012). Olive polyphenols are usually more distributed in the skin and around the seed structure (Charoenprasert and Mitchell 2012).

Olive polyphenols are chemically varied, but most of them can present hydrophilicity due to sugar moieties and multiple hydroxylation sites, making them move and remain in by-products as olive pomace (with a high-water content) and olive mill wastewaters (Charoenprasert and Mitchell 2012).

Olive oil processing is mainly composed of three sequential steps: crushing or milling, malaxing and centrifugation. Throughout the process, physical and chemical events lead to modifications on the quantitative and qualitative polyphenols profiles. The crushing step decreases polyphenols initial amount by 46%, reinforcing crushing as a critical step in all process. Industrial-scale assays already reported a loss of half of the polyphenols (Klen and Vodopivec 2012). The modifications observed result from the exposure of the pulpous material and from malaxation, the mechanical mixing of the olives paste. Hence, during both processes (crushing and malaxation), chemical, enzymatic and non-enzymatic hydrolyses and oxidation occurs (Parenti et al. 2008), leading to the compound's differences found in olives and virgin olive oil. Crushing enhances the presence of simple phenols and benzoic acids, whereas lignans are not affected by the milling process. Other compounds decrease or even disappear during this step due to transformation events, and others increase by liberation and formation of new derivatives. The most affected compounds are secoiridoids such as oleuropein, demethyleuropein and ligstroside, converted first into their respective aglycones (oleuropein or ligstroside aglycones) and then to their decarboxymethylated forms (hydroxytyrosol or tyrosol). Hydroxytyrosol and tyrosol increase significantly during this step mostly due to endogenous esterases cleavage of the ester bond. Also, glucosidases can be responsible for releasing the fruit hydroxytyrosol glucoside and/or verbascoside, and tyrosol could be obtained from nüzhenide and esters. Crushing can impact most polyphenols. However, it is reported that a small fraction of those compounds do not suffer any transformation, as is the case of comselogoside, caffeoyl-6'-secologanoside and luteolin-3'-O-glucoside, meaning that these compounds present some type of technological resistance. During malaxation, the components present the same

quantitative and qualitative behavior observed in the crushing phase, suggesting a balance between the degradation and formation of polyphenols. Therefore, the phase where the losses are more significant is crushing rather than malaxation. Compounds with particular relevance for health, such as hydroxytyrosol, increase during both olive oil processing stages (Klen and Vodopivec 2012). In the Klen and colleague study (2012), only ~0.5% of the olive polyphenols finished in the olive oil at the end of the olive processing. In this way, it is possible to recognize that along with olive oil, the by-products generated are also a great source of polyphenols.

On the other hand, it is not possible to consume olives as raw fruit due to their astringent flavor attributed to oleuropein, and they should be processed into table olives to be organoleptically acceptable. The most common methods to process table olives are salt curing and alkaline hydrolysis resulting in oleuropein-derived compounds such as hydroxytyrosol (Charoenprasert and Mitchell 2012).

Table olives are highly appreciated and part of the Mediterranean diet. Olive debittering methods are the primary factor that affects the olives polyphenols content. The major phenolic found in raw olives is the secoiridoid oleuropein that confers the bitterness to raw olives. The fruits processing allows to reduce the oleuropein levels and increases hydroxytyrosol and tyrosol contents (Charoenprasert and Mitchell 2012).

There are three predominant types of olive processing methods to obtain table olives: “California-style black ripe”, “Spanish-style green” and “Greek-style naturally black olives”. All methods have a common purpose: debittering olives but lead to different final amounts of phenolics. Usually, the California-style processed olives present the lowest amount of polyphenols, especially hydroxytyrosol, compared to the other two methods (Charoenprasert and Mitchell 2012). Also, the profile is distinct. For example, in the Spanish processing method, the major compound identified is hydroxytyrosol, followed by tyrosol, vanillic acid and oleoside-11-methyl ester. In the Greek processed olives, it was possible to find: hydroxytyrosol>tyrosol>oleoside-11-methyl ester>vanillic acid>oleuropein=3,4-dihydroxyphenylglycol (Charoenprasert and Mitchell 2012).

Table olives are considered part of a healthy pattern as the Mediterranean diet contributes with large amounts of phenolics, namely hydroxytyrosol and monounsaturated fatty acids, both protective against chronic diseases.

After olive oil obtention, the olive pomace is transported to other industries, the olive pomace oil extractors, which use organic solvents (*n*-hexane) to remove the remaining oil. The olive pomace oil corresponds to crude oil. To eliminate the compounds

responsible for undesirable flavors and colors, the oil is refined, which significantly reduces the content of polyphenols. Then, the refined oil is blended with virgin olive oil, being commercialized as olive pomace oil (Mateos et al. 2020). As it is mixed with virgin olive oil, the polyphenols found are mainly those characteristics of olive oil which were previously described.

2.2 Olive by-products: leaves, pomace and olive mill wastewaters

The main factors that affect the olive polyphenols content are variety, ripening stage, agricultural practices and edaphoclimatic conditions. These have an impact on the olive fruit but also on the olive oil and, subsequently, on the by-products (Covas et al. 2009).

The major source of olive leaves is tree pruning. Nevertheless, they are also separated in olive mills from the fruits and represent ~10% of the weight of the delivered products in olive mills. Olive leaves, prepared for consumption as infusion or decoction, have been used for a long time as preventive or the treatment of several diseases by folk medicine (Roselló-Soto et al. 2015; Acar-Tek and Ağagündüz 2020).

The phenolics found in olives are also present in the leaves. Oleuropein is the major compound described. Other reported phenolics are demethyloleuropein, oleuropein diglucoside, verbascoside, ligstroside, hydroxytyrosol glucoside, hydroxytyrosol, tyrosol glucoside, tyrosol, oleoside-11-methyl ester, oleuroside, nuzhenide, caffeic acid, chlorogenic acid, p-coumaric, vanillic acid and homovanillic. Moreover, the following flavonoids have been also reported: apigenin-7-glucoside, apigenin-7-rutinoside, luteolin-4-glucoside, luteolin-7-rutinoside, luteolin-7-glucoside, luteolin, diosmetin, quercetin, hesperidin and rutin (Charoenprasert and Mitchell 2012). Figure 1 shows the chemical structure of hydroxytyrosol, oleuropein, oleocanthal and tyrosol.

The health effects described for leaves are mainly associated with their content in oleuropein. Substantial health-promoting effects have been reported, such as antioxidant, hypotensive, cardioprotective, anti-inflammatory, stimulant of the immune system, anti-inflammatory, antiarrhythmic. Also, antihyperglycemic and lipid-regulating effects were reported (Acar-Tek and Ağagündüz 2020).

Most olive oil is currently extracted using a two- or three-phases system. The two-phases system generated a waste called wet olive pomace or *alperujo*, whereas, in the three-phases, it is generated a pomace with a very low amount of water and olive mill wastewaters (Višnjevec et al. 2021).

Wet olive pomace is an heterogenous biomass composed of olives skin, pulp, stone and kernel. It contains the residual oil and a high-water content that can attain 70%. The

pomace is rich in water-soluble (e.g., hydroxytyrosol, tyrosol and comsegolosite) and liposoluble (e.g., tocopherols and oleic acid) bioactive compounds (Nunes et al. 2018).

Other functional compounds have been reported as dietary fiber, sterols and hydrocarbons, β -carotene and minerals (Difonzo et al. 2021). Nevertheless, polyphenols are the main bioactive compounds present in wet olive pomace. Several studies explored the profile and amount of polyphenols.

The main polyphenols in olive pomace identified are hydroxytyrosol, hydroxytyrosol glucoside, hydroxytyrosol diglucoside, hydroxytyrosol rhamnoside, tyrosol, tyrosol glucoside, loganin, loganin glucoside, loganic acid, loganic acid glucoside, 7-deoxyloganic acid, secologanic acid, secologanoside, secologanin, eleanolic acid, oleosid, oleosid glucoside, oleosid diglucoside, oleoside riboside, oleoside-11-methylester, oleoside dimethylester, shikimic acid, phenylalanine, cinnamic acid, p-coumaric acid, caffeic acid, protocatechuic acid, vanillic acid, ferulic acid, gallic acid, pinoresinol, hydroxypinoresinol, acetoxypinoresinol, rutin, apigenin, luteolin, apigenin glucoside, luteolin glucoside, taxifolin, diosmetin, quercetin, oleuropein, 10-hydroxy-oleuropein, oleuropein aglycone, verbascoside and oleuropein derivatives (Olmo-García et al. 2018; Peralbo-Molina et al. 2012).

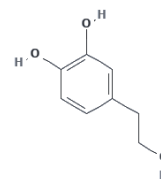
Different amounts of polyphenols and profiles can be found in pomaces accordingly to the process used (two-phases *versus* three-phases). These compounds were found in higher levels in the two-phases system pomace when compared with three-phases. In the latter, a dilution factor must be considered since it is added variable amounts of water in this process. Additionally, differences in the secoiridoids group occur. The pomace from two-phases presented a higher amount (~2000 mg/kg, dry weight), while the three-phases pomace had ~1300 mg/kg (dry weight). Vanillin followed the same tendency with significant differences between the two- and the three-phases system (43 and 6 mg/kg dry weight, respectively). No differences were found on flavonoids, cinnamic acids and simple phenolic compounds contents. Hence, the two most common and used technological processing methods can also affect the bioactive compounds profile of the by-products (Višnjevec et al. 2021).

Compound

Hydroxytyrosol

IUPAC name:

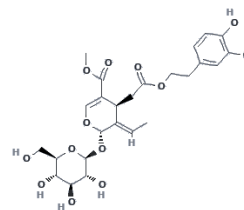
4-(2-hydroxyethyl)benzene-1,2-diol



Oleuropein

IUPAC name:

methyl (4S,5E,6S)-4-[2-[2-(3,4-dihydroxyphenyl)ethoxy]-2-oxoethyl]-5-ethylidene-6-[(2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxy-4H-pyran-3-carboxylate

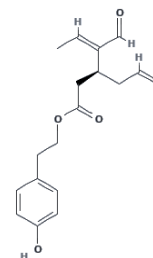


Oleocanthal

IUPAC name:

2-(4-hydroxyphenyl)ethyl
oxoethyl)hex-4-enoate

(E,3S)-4-formyl-3-(2-



Tyrosol

IUPAC name:

4-(2-hydroxyethyl)phenol

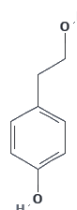


Figure 1. Chemical structure of the main olive polyphenols (hydroxytyrosol, oleuropein, oleocanthal and tyrosol).

National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 82755, 5281544, 11652416, 10393. Retrieved from <https://pubchem.ncbi.nlm.nih.gov>

The olive mill wastewaters derived from the three-phases system is a mixture of lipids, sugars, pectin minerals, tannins and polyphenols. It is particularly rich in hydroxytyrosol and potassium. Due to its composition, the olive mill wastewaters are considered phytotoxic and an environmental burden (Bellumori et al. 2018). The water-saving and ecological two-phases olive oil processing system has been progressively replacing the three-phases system (Višnjevec et al. 2021).

3. Bioavailability of olive polyphenols

Health benefits associated with polyphenols are strongly influenced by bioavailability in the gastrointestinal and circulatory system. The amount of a compound ingested, digested and absorbed into the systemic circulation are related to bioavailability. Overall, bioavailability refers to the ingested fraction that attains organs and tissues and participates in the essential metabolic processes. Bioaccessibility comprises the digestive biotransformations, the absorption into the intestinal epithelium cells, presystemic intestinal and hepatic metabolism followed by tissue distribution and bioaction. Together with absorption and transformation influences the bioavailability (Nunes et al. 2016; Dima et al. 2020).

Several factors affect the bioaction of polyphenols, such as i) the structural features of each compound, including the functional groups and degree of polymerization or glycosylation; ii) the interaction with other food matrix components; iii) food processing/cooking, and iv) the individual physiological conditions. Additionally, higher amounts of polyphenols evaluated in *in vitro* studies do not necessarily mean a positive correlation with those compounds' best bioavailability and bioaccessibility compared with others present in lesser quantity (Shahidi and Peng 2018).

The food matrix is essential in defining the compound release as polyphenols can be found as free, conjugated, and insoluble-bound phenolics. Free phenolics occur as phenolic aglycones, while conjugated phenolics are present in phenolic glycosides form. Most of the phenolic glycosides are rapidly released to the digestive juice by the effect of mechanical and chemical digestions. Usually, the insoluble phenolics are bound to polysaccharides, proteins, and high-polymerized phenolics like lignin and condensed tannins, meaning that considering the total of phenolics ingested in a food matrix, some can be partially or even vaguely released, and few will penetrate the intestinal epithelium and reach the blood system. Other phenolics continue to the colon, where a metabolization and release via fermentation can occur (Shahidi and Peng 2018).

Hydroxytyrosol is the major phenolic in olive by-products (e.g., olive pomace) and the most biologically active one. Several research projects and studies have been focusing on the potential of this compound on the human metabolic system and the possibility of its use in other products beyond olive oil (González-Santiago et al. 2010; Robles-Almazan et al. 2018). Because hydroxytyrosol is an amphipathic compound, it can be detected not only in olive oil but mostly in olive pomace, table olives, and olive tree leaves as a result of oleuropein hydrolysis. It can be found in the free form, as acetate or as part of complex

compounds like oleuropein, verbascoside and oleacein, being partially released during digestion (Robles-Almazan et al. 2018; Fabiani et al. 2021).

Hydroxytyrosol is a potent antioxidant, due to its ortho-diphenol moiety, with cardio and neuroprotective properties, antimicrobial, anti-inflammatory and anticarcinogenic actions. These are dependent on the absorption and metabolization of the compound (Boronat et al. 2019). For example, Vissers et al. (2004) reported that, in humans, the absorption of hydroxytyrosol, tyrosol, oleuropein aglycone and ligstroside aglycone ranged between 55 and 66%; 5% was eliminated in urine as hydroxytyrosol and tyrosol.

Though hydroxytyrosol is associated with the beneficial effects described and reduced attention has been paid to tyrosol due to its low antioxidant activity, pre-clinical studies identified the bioconversion of tyrosol into hydroxytyrosol via cytochrome P450 isoforms CYP2A6 and CYP2D6 (Boronat et al. 2019). Considering the current data about the endogenous conversion of tyrosol into hydroxytyrosol, both are health-related compounds of interest.

Olive leaves are rich in secoiridoids as oleuropein and ligstroside, flavonoids (luteolin, kaempferol, apigenin) and phenolics such as hydroxytyrosol, tyrosol and caffeic acid. Oleuropein is the most abundant phenolic in leaves, but it is found in reduced amounts in olive oil. Due to their richness in such compounds, olive leaves are used in cosmetics, nutraceuticals and food supplements. Regarding the metabolization and bioavailability of the olive tree leaves compounds, human studies report that they could be affected by interindividual variations in absorption and metabolism, and the effects are also dose-dependent (51.1 mg oleuropein; 9.7 mg hydroxytyrosol versus 76.6 mg oleuropein; 14.5 mg hydroxytyrosol) (de Bock et al. 2013).

Olive polyphenols resist acidic stomach conditions and are rapidly absorbed. Oleuropein is rapidly absorbed via oral and has a plasma peak 2 hours after administration. In hydroxytyrosol, the concentration peak in plasma occurs after only 5 – 10 min of ingestion. The renal clearance time occurs during the first hour after administration (Piroddi et al. 2017).

After being absorbed in the bowel, polyphenols are subjected to hepatic metabolism. Polyphenols undergo phases I and II of metabolism, where they are transformed into glucuronide, methylated and sulphate by-products. In the case of hydroxytyrosol, it can be detected in plasma and urine in conjugated forms (glucuronoconjugates) (98%), whereas only 2% is found in free form (Miro-Casas et al. 2003).

As soon as absorbed, hydroxytyrosol becomes part of the plasmatic high-density lipoproteins acting as an antioxidant. The plasma half-life of this compound is about 1 – 2 min, and the rapid and optimal distribution in tissues as muscle and liver leads to the recognized health properties of hydroxytyrosol. Moreover, hydroxytyrosol can cross the blood-brain barrier and be present in the brain tissue reinforcing its neuroprotective benefits (Robles-Almazan et al. 2018). The conjugated metabolites are mainly excreted by the kidneys being 6 hours the time required to complete the human body's elimination (Rodríguez-Morató et al. 2016; Robles-Almazan et al. 2018).

Hydroxytyrosol was already classified as non-mutagenic, non-genotoxic and with No Observed Adverse Effects Level (NOAEL), indicating a safety profile and a promising utilization in functional foods and pharmaceutical applications (Rodríguez-Lara et al. 2019; Bertelli et al. 2020).

4. Epigenetic effects of olives and olives derivative polyphenols

Olive products and by-products polyphenols can impact the outcomes of metabolic syndrome as central obesity, hyperglycemia, hypertension and dyslipidemia (Saibandith et al. 2017). Other effects as neuroprotection, anti-ageing, anti-carcinogenesis and anti-inflammatory have been reported. Three main epigenetics modifications can be responsible for those effects: post-transcriptional gene regulation by non-coding microRNAs (miRNAs), DNA methylation and histone modifications (acetylation and methylation) (Fabiani et al. 2021).

miRNAs have an important role in regulating the gene expression by directly binding to the target mRNAs and inhibiting translation or stability. They are involved in several fundamental physiologic processes and can be biomarkers for dysfunctions such as obesity since associations between circulating miRNAs levels and obesity in adults have been reported (Ortega et al. 2013; Scoditti et al. 2019).

The regulation of the "epigenome" defines if a gene is expressed or silenced. The disruption of the epigenetic mechanisms has been associated with chronic diseases, namely cancer (Bishop and Ferguson 2015).

The effects of olive compounds are exerted at a genomic level by modulating the expression of key genes related to some disease's onset and progression. Often, the beneficial effects are reported not for a single compound but for a food matrix such as olive oil that contains polyphenols, fatty acids, squalene and other triterpenes, tocopherols, pigments and sterols. Like polyphenols, the amount of those minor compounds varies according to the olive's cultivar and ripeness, agricultural practices,

and edaphoclimatic conditions. Also, it depends on the olive oil processing system (Covas et al. 2009).

The bioactivity of olive compounds, especially polyphenols have been studied, being hydroxytyrosol and oleuropein, the most focused phenolics (Kiritsakis et al. 2020). Figure 1 shows the chemical structure of the main olive derivatives polyphenols (hydroxytyrosol, oleuropein, oleocanthal and tyrosol).

Several health benefits, including immunostimulant and antimicrobial activities, were reported, as well as beneficial effects on several diseases due to their anti-inflammatory and antioxidant activities.

The effects of major olive polyphenols on the expression of genes will be presented considering the major and most representative organic systems dysfunctions (atherosclerotic process, hypertension, dyslipidemia, obesity type 2 diabetes, inflammatory bowel diseases, osteoarthritis, Alzheimer's disease and cancer) that have an impact in the modern society and human well-being. Figure 2 is a schematic presentation of some of the olive polyphenols benefits under the nutrigenomics approach.

4.1 Cardiovascular system: heart and vessels

Modifiable factors such as smoking, physical activity and optimal nutrition can prevent 80% of heart diseases (angina, heart attack, coronary heart disease, congestive heart failure). Despite the increase in diagnostic tests accuracy and the prevention and treatment strategies, the main cardiovascular factors (dyslipidemia, obesity, type 2 diabetes, smoking and hypertension) persists. Moreover, 50% of the individuals who normalize the levels of the top five risk factors mentioned continue to have coronary heart disease or myocardial infarction. This is referred to as the "coronary heart disease gap" since those factors do not explain the currently limited reduction of cardiovascular diseases. The cardiovascular system's response to risk factors involves oxidative stress, vascular immune dysfunction, and inflammation. Therefore, other risk factors and biomarkers can start to be considered for cardiovascular pathogenesis, such as micronutrient testing, genetic evaluation, metabolomics and nutrigenomics (Houston 2018).

The impact of olive-derived compounds in preventing cardiovascular diseases, mainly in the context of Mediterranean diet pattern or olive oil consumption, has been extensively reviewed (Nocella et al. 2018; Ditano-Vázquez et al. 2019; Marcelino et al. 2019). However, few studies explored the nutrigenomics of those benefits. It is known that

oxidative stress is associated with endothelial dysfunction, inflammation, hypertrophy, cell migration, fibrosis and angiogenesis, which can be a leading factor of atherosclerosis. Atherosclerosis is a vascular disease that causes thickening and inelasticity of arteries. Hence, oxidative stress is also a key factor in hypertension onset and, then in cardiovascular disease. The atherosclerotic risk can be reduced by managing a lower ROS production and restoring cellular antioxidant defenses (D'Angelo et al. 2020).

Hydroxytyrosol has the capacity of scavenging oxidant species. Moreover, it can also promote the synthesis and activity of several antioxidant enzymes like glutathione reductase, catalase, superoxide dismutase, glutathione peroxidase and NO synthase while preserving the cellular reduced glutathione high levels (D'Angelo et al. 2020). It can induce the synthesis and translocation to the cell nucleus of Nrf2, promoting the transcription of encoding genes of the antioxidant response as phase II detoxifying enzymes and DNA-repair proteins (Bertelli et al. 2020).

The inflammatory response is generally fast and self-limited. In these conditions, it is beneficial and protective. However, chronic inflammation is detrimental if endothelium, smooth muscle and peripheral blood cells are subjected to continuous and high levels of pro-inflammatory cytokines, chemokines, prostaglandins and leukotrienes, and potentially toxic molecules (D'Angelo et al. 2020).

The inflammation process is complex and involves numerous mediators with cells and plasma origins. The development and severity of several chronic diseases is linked to a chronic inflammation state. Hydroxytyrosol can effectively inhibit the pathways that stimulate NO production, the cytokines IL-1a, IL-1b, IL-6, IL-12, TNF-a, the chemokines CXCL10/IP-10, CCL2/MPC-1 and the eicosanoid PGE2. Equivalent repression effects occurred in genes such as the inducible NO synthase, IL-1a, CXCL10/IP-10, MIP-1b, MMP-9, and PGE2 synthase (Piroddi et al. 2017).

The anti-inflammatory effect of hydroxytyrosol and oleuropein was studied using a cell line of murine macrophages and in human granulocytes and monocytes. Nutritionally low relevant doses of hydroxytyrosol, oleuropein and resveratrol were tested. In concentration-dependent doses (10 μ M), the three compounds inhibited the granulocytes oxidative burst and the CD11b expression (resveratrol and hydroxytyrosol). Overall, an anti-inflammatory effect of resveratrol and hydroxytyrosol was showed by the inhibition of the activation of granulocytes and monocytes. Also, it was observed a modulation of miR-146a expression and the activation of the transcription factor Nrf2 (Bigagli et al. 2017). Hence, the anti-inflammatory preventive actions of hydroxytyrosol have also a relevant impact on the atherosclerotic process.

In human umbilical vein endothelial cells treated with serum obtained from individuals two hours after the intake of olive oil rich in polyphenols decreased the p65 and MCP-1 gene expression and increased MT-CYB, SDHA and SOD1 gene expression. The same treatment applied after four hours decreased MCP-1 and catalase gene expression and increased MT-CYB gene expression. p65 and MCP-1 are anti-inflammatory genes, MT-CYB and SDHA are prooxidant enzymes and respiratory chain genes, and SOD1 and catalase are antioxidant enzymes. Olive oil reduced inflammation and increased the expression of oxidative stress-related genes in the vascular endothelium reducing the atherosclerotic risk (Meza-Miranda et al. 2016). Therefore, olive oil metabolites, particularly hydroxytyrosol metabolites, can prevent endothelium dysfunction (Catalán et al. 2015; D'Angelo et al. 2020).

Hypertension is a marker for vascular endothelium dysfunction. It can develop silently and can be a severe cause or aggravate cardiovascular diseases or chronic heart failure. The risk of cardiovascular diseases increases progressively as the blood pressure deregulates. The effect of extra-virgin olive oil with high polyphenols content on the expression of the blood pressure-related genes (ACE, ADRB2, ECE2, IL8RA, MPO, NR1H2, OLR1 and PPARG) involved in the regulation of the renin-angiotensin-aldosterone system (RAAS) was studied. RAAS regulates blood volume and systemic vascular resistance, influencing cardiac output and arterial pressure. Data showed that the daily consumption of virgin olive oil (25 mL) for three weeks decreased the ACE, NR1H2 and IL8RA gene expressions that reflected in a decrease in the systolic blood pressure. In this case, olive oil presented an oleuropein content of 65%, followed by 18% of ligstroside aglycones and 18% hydroxytyrosol (Martín-Peláez et al. 2017). A blood pressure-lowering effect has been reported to oleuropein, which goes in agreement with the deep-rooted traditional use of olive leaves in decoctions and infusions to treat mild to moderate hypertension. A daily dosage of oleuropein (60 mg) in an animal model resulted in a significant decrease in systolic blood pressure (Khalili et al. 2017), and 30 mg of polyphenol-rich olive oil reduced both systolic and diastolic blood pressures in young women with mild hypertension (Moreno-Luna et al. 2012).

4.2 Liver and biliary tract: lipids metabolism

Liver, biliary tree and gallbladder structures are considered together due to their anatomic proximity and functions. The liver is exposed to various metabolic, toxic, microbial, circulatory and neoplastic attacks. This organ acts as a large functional reserve and regulates most chemical levels in the blood. More than 500 vital functions were identified until now. Some of the principal ones and well known are (i) production of

cholesterol and proteins to carry fats through the bloodstream, (ii) conversion of excess glucose into glycogen and storage, (iii) processing of hemoglobin as the liver stores iron, (iv) regulation of blood clotting, and (v) clearing the blood of drugs and other toxic substances. Overall, liver functions comprise carbohydrates, proteins and lipids metabolism. The main processes related to lipids metabolism in the liver are lipogenesis, fatty acid oxidation, lipoprotein synthesis, phospholipids and cholesterol synthesis (Mitra and Metcalf 2012).

High levels of total cholesterol and low-density lipoprotein (LDL) and low levels of high-density lipoproteins (HDL) are associated with the onset of certain diseases such as cardiovascular ones, mainly related to atherosclerotic plaque formation. Therefore, improving the lipid profile and dyslipidemia prevention can have a protective effect (D'Angelo et al. 2020).

The positive impact of extra-virgin olive oil (richer in polyphenols than olive oil) and olive leaves extracts on dyslipidemia is being corroborated and reviewed (Saibandith et al. 2017). As a ROS scavenger, hydroxytyrosol reduces oxidized LDL and platelets aggregation, seeming to be a key mechanism associated with the regulation of dyslipidemia. Hence, potential effects of olive products on lipid metabolism and inflammation arise as linked to metabolic pathways and inflammation-related genes (Correa et al. 2009).

The oxidation of the lipid fraction of LDL results in a modification of the lipoprotein conformation, enabling the atherosclerotic process. Olive polyphenols, mainly hydroxytyrosol, protects LDL from oxidation. Based on this effect, the European Food Safety Authority claimed that daily consumption of 5 mg of hydroxytyrosol and its derivatives protect blood lipids from oxidation (Peyrol et al. 2017). Several other studies, such as the EUROLIVE (Effect of Olive Oils on Oxidative Damage in European Populations), also demonstrated that olive oil with different polyphenols content reduces LDL in a dose-dependent manner. It was observed, in rodent and murine models, that hydroxytyrosol reduces the accumulation of triglycerides in tissues such as liver and skeletal muscle and low serum cholesterol in high-fat-diet animals (Peyrol et al. 2017).

Molecular targets for hydroxytyrosol, related to its beneficial effects in the lipid metabolism, were identified in experimental studies. Recently, López de las Hazas et al. (2019) reviewed and, afterwards, experimentally validated molecular hydroxytyrosol targets. Four miRNAs responded to hydroxytyrosol supplementation (miR-802-5p, miR-423-3p, miR-30a-5p and miR-146b-5p), and Fgf21 and Rora were newly identified as potential hydroxytyrosol targets in this study. Also, miR-802-5p responded

consistently to dietary hydroxytyrosol supplementation in two different tissues (liver and intestine), supporting a miRNA modulating action by hydroxytyrosol (López de las Hazas et al. 2019).

Nevertheless, most studies performed in humans or animal models have reported a modest or positive effect on circulating lipids and lipid peroxidation (Visioli et al. 2020). Likewise, despite transcriptomics and proteomics greatly contributed to the knowledge of olive compounds effect on the expression of a vast number of genes related to dyslipidemia, inflammation, endothelial dysfunction and cardiovascular diseases, the evidence about those mechanisms is far from complete comprehension. The limited number of human studies and the heterogenicity of the doses administered leads to an increasing requirement of transcriptomics and proteomics tools to identify and validate hydroxytyrosol and oleuropein targets (López de las Hazas et al. 2019).

Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease that comprises several conditions, from simple steatosis to a severe degree of the diseases characterized by inflammation, non-alcoholic steatohepatitis that can turn into cirrhosis, hepatocarcinoma and organ failure (Priore et al. 2015). It was reported in rats with NAFLD that olive oil decreased the storage of hepatic triglycerides, increased the secretion of triglycerides (as very-low-density lipoprotein) and decrease the occurring lipolytic flux from the peripheral adipose tissue to the liver. Thus, olive oil can provide protection against liver steatosis (Hussein et al. 2007).

In human studies, olive oil decreased the serum triglycerides by increasing the expression of hepatic fatty acid β -oxidation enzymes through the corresponding genes by the transcription factor peroxisome proliferator-activated receptor- α (Assy et al. 2009; Priore et al. 2015).

4.3 Endocrine system: obesity and type 2 diabetes

Adipose tissue was formerly considered a non-dynamic tissue which function was limited to the body energy storage. Currently, it is known that adipose tissue is highly active, with immunological and hormonal regulatory functions (González-Casanova et al. 2020). In obesity, ghrelin, an orexigenic hormone, is increased, and the appetite-suppressing or anorexigenic hormones (insulin, amylin, glucagon-like peptide-1, cholecystokinin, peptide YY and leptin) are decreased. Hormone's regulation is essential to regulate food intake. Hence, appetite and satiety are controlled by a complex communication network comprising adipose tissue, endocrine organs, gastro-enteric peptides and central nervous system (Poddar et al. 2017)

Obesity is a multifactorial and chronic disease. Particularly, the chronic inflammation of adipose tissue contributes largely to cardiometabolic disease. The occurring abnormal adipose tissue expansion in obesity associated with adipocyte hypoxia and death leads to an increased production of ROS and lipid mediators. Also, adipokines like prostaglandins are produced, as well as peptide hormones, and cytokines enrolled in the local/systemic chronic inflammation, being insulin resistance and vascular dysfunction also observed. The hypertrophied adipocytes induce the recruitment, infiltration and pro-inflammatory activation of macrophages and lymphocytes (Scoditti et al. 2019; Wadey et al. 2019). TNF- α , a secretory product of those cells, activates the inflammatory signaling, with an abnormal expression and release of pro-inflammatory, -atherogenic, -thrombotic adipokines and factors associated with the metabolic deregulation, angiogenesis, matrix remodeling and fibrosis. Overall, the cell products will aggravate systemic inflammation and resistance to insulin (Wadey et al. 2019). The insulin resistance syndrome leads to several other pathologies, such as increased triglyceride levels, blood pressure, and serum glucose, which enhance the severity of type 2 diabetes and cardiovascular disease. Due to the strong relationship between obesity and diabetes, the combination of the two diseases is often named "diabesity" and are treated together (Poddar et al. 2017).

miRNAs can regulate the obesity pathophysiological processes by an active regulation of adipokine production, inflammation and intercellular communication. Along with inflammation, miRNAs can be unregulated. For example, in adipose tissue, miR-34a leads to the pro-inflammatory macrophages activation or miR-155, promoting lipid cells dysfunction and inflammation (Scoditti et al. 2019). Considering the strong hydroxytyrosol anti-inflammatory activity, its application to treat adipocyte inflammation in adipose tissue is appealing. Hydroxytyrosol and oleuropein prevented 3T3-L1 (preadipocytes) differentiation, with the most potent effects in early stages, at 150 and 300 $\mu\text{mol/L}$, respectively and without affecting the cell viability. Also, both compounds downregulate the expression of adipogenesis genes (Drira et al. 2011).

Recently, an olive leaves extract with 12% of polyphenols, in which oleuropein represented 10%, reduced the body weight gain in mice and improved the expression of the adipogenic genes PPARs, adiponectin and leptin receptor in adipose tissue. The extracts also ameliorated the anti-inflammatory status in the adipose tissue by reducing the RNA expression of IL6, IL1 β and TNF- α (Vezza et al. 2019).

Diabetes has two main forms of presentation: immune-mediated β -cell destruction (type 1 diabetes) and loss and death of β -cell functional mass that leads to the development of

type 2 diabetes. The effect of olive oil commercial standard polyphenols (10 μ M) on β -cell function and survival was studied for each compound for 24h showing that hydroxytyrosol, tyrosol and apigenin preserve β -cells function and survival, meaning that those compounds may ameliorate the insulin secretion and control glycaemia (Marrano et al. 2021). In overweight type 2 diabetes individuals, the daily consumption of olive oil enriched in polyphenols (~580 mg/kg) improved the circulatory inflammatory adipokines (Santangelo et al. 2016) and supplementation with 10 mg hydroxytyrosol/kg (daily) for five weeks improved glucose tolerance and insulin sensitivity in rat models (Pirozzi et al. 2016). Also, after ingestion of 50 mL of olive oil, modest changes were observed in the expression of insulin sensitivity-related genes: after 1h it was observed downregulation in OGT and ALOX5AP genes; OGT was upregulated at 6h; CD36 was upregulated at 1h; LIAS, PPARBP, ADAM17 and ADRB2 genes were upregulated at 6h (Konstantinidou et al. 2010).

Anti-diabetic properties have been attributed to oleuropein and one of the proposed mechanisms - reduction of serum glucose – seems to be related to its chemical structure. As oleuropein exists in a glycoside form, it can compete with glucose in the intestinal epithelial cells to be absorbed through sodium-dependent glucose transporter 1 (Kerimi et al. 2019). The inhibition of digestive enzymes and the increase of the glucagon-like peptide 1 levels, which attenuate the postprandial glycaemia, are other proposed actions (Carnevale et al. 2018).

The studies that address the nutrigenomics effect of hydroxytyrosol or oleuropein in type 2 diabetes are scarce. These compounds likely have biological properties and mechanisms of action that lead to the positive effects observed, such as inhibition of carbohydrate digestion and glucose absorption, activation of insulin receptors and glucose uptake in the tissues and effects related to the capacity of modulating the antioxidant and anti-inflammatory status (Alkhatib et al. 2018). Additionally, oxidative stress and inflammation are ameliorated with oleuropein or hydroxytyrosol administration or exposition, indicating that the potential effects of the olive biophenols on type 2 diabetes occur through metabolic pathways and inflammation-related genes. Most of the studies are performed in cell cultures or animal models, and the studies conducted on humans are still limited.

4.4 Gastrointestinal tract: inflammatory bowel diseases and microbiota

Ulcerative colitis and Crohn's disease are the two main types of inflammatory bowel disease characterized by an extensive and chronic inflammation process. Usually, they are controlled with massive pharmacological agents. Ulcerative colitis is a colonic

mucosal inflammation related to the deregulation of the immune system. In a mouse model with induced colitis, oleuropein reduced eosinophil, neutrophil and macrophage in colon tissue and inhibited the expression of COX-2 (Giner et al. 2013). Moreover, oleuropein treatment using the same model reduced the intestinal IL-6, IL-17A and TNF- α concentrations and was able to downregulate the Th17 response, a pro-inflammatory pathway activated in intestinal bowel disease (Giner et al. 2016). In the colonic mucosa from patients with active ulcerative colitis, oleuropein ameliorates the inflammatory state. The expression of COX-2 and IL-17 was lower in the oleuropein-treated samples when compared with those without oleuropein (Larussa et al. 2017). Also, hydroxytyrosol showed an anti-inflammatory effect by suppressing inducible NO synthase and COX-2 in a dose-dependent manner (Zhang et al. 2009).

Hydroxytyrosol acetate supplementation in an animal model also improves histological damage and reduces the COX-2 and inducible NO synthase expression. Due to the presence of an ester group, this has higher solubility in lipophilic phases than free hydroxytyrosol, meaning that hydroxytyrosol acetate can be better absorbed across intestinal epithelial cell monolayers compared to free hydroxytyrosol (Sánchez-Fidalgo et al. 2015). Considering this, the impact of hydroxytyrosol acetate vehiculated by a lipidic format as olive oil or lipid-based nutraceuticals on acute ulcerative colitis can be more significant than free hydroxytyrosol. However, new studies, especially in humans and regarding both compounds, are still necessary. Notwithstanding the promising *in vivo* results observed for bowel inflammatory diseases treatment, most studies are performed with extra virgin olive oil since it is the most consumed olive tree product and the base of the Mediterranean diet (Larussa et al. 2019). Moreover, extra virgin olive oil presents many beneficial compounds that can act synergistically (Piroddi et al. 2017). Hence, more research is needed regarding the interconnection between bowel inflammation, olive compounds and epigenetics in humans using clinical trials and a higher number of individuals.

The microbes that comprise the gut microbiota play an important role in the human body physiological maintenance. Dysbiosis, the microbiota dysregulation, impairs its beneficial function, modify intestinal permeability and trigger inflammation. This process could lead to severe pathological conditions such as obesity, insulin resistance, and inflammatory status. Therefore, a positive association between gut microbiota and health and its maintenance is required (Marcelino et al. 2019).

Most of the olive polyphenols are rapidly absorbed in the small intestine. Nevertheless, some simple and complex compounds can reach the large intestine and will be

metabolized by microbiota. For example, it is known that oleuropein is catabolized in hydroxytyrosol *in vitro*. *Lactobacillus*, *Bifidobacteria*, and *Enterococcus* hydrolyze oleuropein into hydroxytyrosol (Santos et al. 2012).

The gut microbiota profile is affected by environmental and genetic factors. Diet is the one with the highest dynamic impact. Olive oil (20% of the diet) can increase the microbial diversity in spontaneously hypertensive rats. These authors also suggested that the changes in gut microbiota are linked to a reduction in systolic blood pressure (Hidalgo et al. 2018). Specifically, hydroxytyrosol showed to modulate the microbiota composition by enhancing the microbiota richness and restraining the pathogenic bacteria (Wang et al. 2019). Moreover, in male mice, it improved obesity and insulin resistance by modifying the microbiota, reduced the release of endotoxins into the bloodstream and reduced inflammation at a dose of 50 mg/kg/day (8 weeks) (Liu et al. 2019).

To sum up, olive hydroxytyrosol and oleuropein influence the microbiota composition regarding its profile and bacteria content and act as a modulator by controlling pathogenic bacteria's growth and stimulating the proliferation of beneficial ones.

The effects of nutrients on the human body, particularly on gene expression, should be studied under the scope of the microbiome perspective since the axis genome-food-microbiota influence gene expression locally or at a systemic level. Moreover, the mechanisms involved in these processes and the study of isolated compounds are a great research area and essential to complement these recent findings.

4.5 Musculoskeletal system: osteoarthritis

Oleuropein, hydroxytyrosol and oleocanthal are being pointed out as compounds of interest in the management of osteoarthritis, an inflammatory and degenerative disease. Oleuropein (10, 50 and 100 μM) was able to inhibit the IL-1 β -induced production of NO and PGE₂ as well as the gene expression of the aggrecanase ADAMTS-5, inducible NO synthase, MMP-1, MMP-13 and COX-2, leading to the degradation of aggrecan (aggregates of proteoglycans and hyaluronic acid) and collagen-II in human primary chondrocytes. The therapeutic effect found was due to the inhibition of NF- κ B and MAPK signaling pathways (Feng et al. 2017).

In osteoarthritis pathogenesis, autophagy is considered a cellular housekeeping system able to recycle and remove impaired macromolecules and organelles. This is a protective mechanism in normal cartilage. Its ageing-related loss is associated with chondrocyte death and pathogenesis of osteoarthritis. Hydroxytyrosol modulates this homeostatic

process by stimulating the induction and flux of the mechanism autophagy. Moreover, when oxidative stress is induced, hydroxytyrosol protects chondrocytes from DNA damage and cell death. SIRT-1, a protein deacetylase that can regulate oxidative stress, inflammation, apoptosis, metabolism and autophagy, modulates the expression of genes associated with autophagy by deacetylating key transcription factors. Hydroxytyrosol also can enhance the SIRT-1 expression in human chondrocytes, exerting a protective role in cell survival and cartilage homeostasis (Facchini et al. 2014; Cetrullo et al. 2016). Hydroxytyrosol affects several genes expression, such as SQSTM1/p62 (Cetrullo et al. 2016) and miR-9 (D'Adamo et al. 2017). Particularly, miR-9 is overexpressed when chondrocytes are exposed to hydrogen peroxide, one of the major ROS in chondrocytes. Hydroxytyrosol could counteract hydrogen peroxide-induced miR-9 expression, restoring the protein translation of SIRT-1 (D'Adamo et al. 2017).

SIRT6 is also involved in osteoarthritis protection due to its ability to suppress the inflammatory response and chondrocyte senescence. Likewise, in this case, hydroxytyrosol promotes the autophagy flux and inhibit the chondrocytes inflammatory response by increasing the gene expression of SIRT6 (Zhi et al. 2018).

Oleocanthal suppresses the expression of inflammatory cytokines and mediators as TNF- α , inducible NO synthase, COX-2, IL-6, IL-8, among others, as well as the catabolic enzymes MMP13 and ADAMTS-5 (Scotece et al. 2018).

4.6 Nervous system and cognition: Alzheimer's disease

The normal function of nervous system is dependent on the maintenance of its structural integrity and metabolic processes. The term neurodegenerative diseases comprises a heterogenous group of diseases that can impair cognitive function. Such examples are Parkinson's and Alzheimer's diseases. Neurodegenerative diseases do not have a cure. However, prevention and treatment in the early stages can manage the symptoms and delay the progression (Agnihotri and Aruoma 2020).

Alzheimer's disease is age-related and gives rise to dementia, causing disability. This disease is characterized by a gradual cognitive decline and memory impairment, and it represents the major form of dementia worldwide. Although it is not entirely understood what causes Alzheimer directly, two factors are pointed as putative causes: gene mutations in the amyloid precursor protein (APP) and presenilin 1 and 2 genes, which are related to the amyloid-beta (A β) peptide synthesis; and processes related to the hyperphosphorylation of the Tau protein and its consequent deposition. There is a positive correlation between Alzheimer's disease symptoms and the presence of Tau and

A β protein in their aggregated forms (neurofibrillary tangles and β -amyloid plaques). Nevertheless, a combination of age-related alterations in the brain and genetics, lifestyle, and environmental factors is probably behind this disease's high incidence. Moreover, most of the knowledge about Alzheimer's has been obtained post-mortem, and, thus, the exact mechanisms of the disease are still poorly understood (Mondragón-Rodríguez et al. 2017; Agnihotri and Aruoma 2020).

Chronic oxidative stress has been demonstrated as detrimental in the pathogenesis of age-related diseases such as Alzheimer's. Oxidative stress can induce several disturbances in mitochondria like DNA mutations, impairment of the mitochondrial respiratory chain and membrane permeability functions and disturbance in Ca²⁺ homeostasis. Hence, antioxidant therapy has been suggested. Nevertheless, good nutrition is assumed as crucial to prevent or delay the neurodegeneration evolution as it provides, among other nutrients, polyphenols that impair the chronic oxidative stress (Agnihotri and Aruoma 2020).

Regarding olive compounds, it was reported that oleocanthal enhances β -amyloid clearance by upregulating major β -amyloid transport proteins in mice brain endothelial cells, more specifically the P-glycoprotein and LDL lipoprotein receptor-related protein-1 and inhibits the Tau protein aggregation into neurofibrillary tangles (Monti et al. 2012; Abuznait et al. 2013).

Oleuropein is an epigenetic modulator. Oleuropein aglycone impairs the β -amyloid and Tau proteins aggregation as well as neuroinflammation and induces autophagy, decreasing aggregated proteins and a reduction of cognitive loss. This effect is related to the capacity of oleuropein to activate the gene expression mediated by sirtuins and histone acetylation or EB transcription factor. The gene expression is inhibited by the increase of histone deacetylase 2 (HDAC2). Its overexpression, in mice, impairs memory. Hence, downregulation or inhibition of HDAC2 is suggested as a therapeutic target for memory-related disturbance. Administration of oleuropein (50 mg/kg; 8 weeks) decreased the levels of HDAC2 in mice and synaptic function amelioration (Luccarini et al. 2015; Cordero et al. 2018).

Also, a polyphenol extract obtained from a concentrated olive mill wastewater (verbascoside>oleuropein di-aldehyde>hydroxytyrosol>tyrosol) improve significantly cognitive function of mice. The number and area of A β ₄₂ and pE-3A β (isoform and modified form of A β) plaques were significantly reduced in the cortex by oleuropein and in the cortex and hippocampus by the polyphenol extract (Pantano et al. 2017).

An aqueous extract of olive with 40–50% hydroxytyrosol, 5–10% of oleuropein and 0.3% of tyrosol (Hidro[®]), administered to rats, promoted a reduction in toxic oxidative stress and neuroinflammation. Moreover, hydroxytyrosol maintained the redox balance and homeostasis by activating the Nrf2 pathway and increasing the expression of phase II detoxifying enzymes, reducing neurodegeneration. Also, the treatment improved memory impairment and cognitive dysfunction. This extract decreased the markers associated with Alzheimer's disease as the β -amyloid accumulation and APP, and Tau overexpression (Cordaro et al. 2021).

It was also reported that hydroxytyrosol acetate improved cognitive function, in animal models, by decreasing inflammatory cytokine levels and improving neuronal apoptosis. The compound was able to induce the transcription of the Er β receptor (abundant in areas such as hippocampal formation, cerebral cortex, and thalamus), increasing the neuronal viability (Qin et al. 2021).

Recently, to investigate the cause-effect of olive leaves, individual compounds (oleocanthal, oleuropein, hydroxytyrosol, oleuroside aglycon, ligstroside, ligustalosite B, ligstroside aglycone, oleuroside, tyrosol and oleacein) were tested on mitochondrial function in SH-SY5Y-APP695 cells. Then, as only oleocanthal and ligstroside were able to enhance the capacity of mitochondrial respiratory chain complexes, their molecular mechanisms on the expression of several genes related with mitochondrial biogenesis, respiration and antioxidative capacity (PGC-1 α , SIRT1, CREB1, NRF1, TFAM, complex I, IV and V, glutathione peroxidase 1, superoxide dismutase 2 and catalase) were explored. It was shown that only ligstroside enhanced the mRNA expression of SIRT1, CREB1, complex I, and glutathione peroxidase 1. On the other hand, just oleocanthal decreased the SH-SY5Y-APP695 cells A β 1–40 after 24h incubation. Additionally, these authors tested oleocanthal and ligstroside (supplemented diet) in a mouse model of ageing for 6 months. The mice fed with a diet enriched with ligstroside presented an improvement in spatial working memory. Also, in aged mice, ligstroside restored the brain ATP levels and significantly extended lifespan compared to the aged control mice (Grewal et al. 2020).

Because olive derivative compounds such as hydroxytyrosol have been proposed to be included in a food pattern as functional ingredient/products or via dietary supplements, or even included in personalized nutritional models, in the case of neurodegenerative diseases, it is still necessary more studies in other animal models and as possible in humans, to evaluate the potential neuroprotective role.

4.7 Chemoprevention

Cancer is a multifactorial genetic disease. Diet is one of the factors that have been highlighted on cancer's predisposition, development and prognosis. The knowledge of the effect of bioactive compounds on genome and transcriptome patterns is of great interest to the oncology field since certain compounds specifically activate cancer inhibitory mechanisms, affecting key cancer hallmarks as apoptosis or the impairment of angiogenesis (Irimie et al. 2019).

The anticancer effect of many plant-derived polyphenols has been explored over the last years. Polyphenols can interact with other food components or drugs, resulting in additive, synergistic or antagonistic effects. The combination of polyphenols with chemotherapeutics can enhance the anticancer efficacy, diminish the side effects of chemotherapy and overcome the radio- or chemo-resistance of cancer cells (Torić et al. 2019).

Olive polyphenols can exert chemoprotective and anticancer actions in breast cancer, prostate, melanoma, promyelocytic leukemia, colon, among others (Torić et al. 2019).

Oleuropein, hydroxytyrosol, oleacein and oleocanthal as olive derivative compounds showed to exert antioxidant and anti-inflammatory effects and also act on the expression of genes that control the proliferation, apoptosis and differentiation of cancer cells. It is hypothesized that the targets of these anticancer effects could be the genes associated with growth factors (GF) production or growth factor receptors (GFR). The overexpression of GF and GFR in cancer cells results in uncontrolled proliferation and production of signaling molecules that, ultimately, lead to tumor growth. Olive polyphenols can, thus, have an anticancer potential (Torić et al. 2019).

Oleuropein, *in vitro*, inhibited thyroid cancer cell proliferation by acting on growth-promoting signal pathways (Bulotta et al. 2013) and reduced UVB radiation-induced tumor volume possibly by inhibition of the expression of VEGF, MMP-2, MMP-9 and MMP-13 through a reduction in COX-2 levels (Kimura and Sumiyoshi 2009). Several *in vitro* studies have shown that oleuropein inhibits proliferation and induces apoptosis in different cancer-related cell lines such as breast, colon, liver, pancreatic, thyroid cancers as well as in osteosarcoma, leukemia, neuroblastoma and mesothelioma cells (Shamshoum et al. 2017).

Although the promising outputs from the *in vitro* studies, few animals' studies assessed the effects of oleuropein on tumor progression *in vivo*. In breast cancer, it was found that oleuropein (125 mg/kg) in mice inhibited tumor growth and metastases and prevented the peripulmonary dissemination of the tumor. On the other hand, it was also reported

that 0.3 mg/kg of oleuropein in an animal model of glioma did not inhibit the tumor growth (Martínez-Martos et al. 2014).

Also, in colon cancer, administration of oleuropein (50 or 100 mg/kg) in mice led to a reduction of the tumor progression, a decrease of inflammation markers, a reduction of the number and dimension of tumors, and an impairment of neoplastic cells proliferation. The effects are related to a reduced expression of Ki-67, upregulation of BAX and inhibition of NF- κ B phosphorylation, Wnt/b-catenin, Akt and STAT-3 (Giner et al. 2016).

In skin cancer, effects related to the inhibition of VEGF expression, MMP-2, MMP-9, and MMP-13 were found. In this case, the treatment with oleuropein (male mice) (10 and 25 mg/kg; 30 weeks; twice daily) reduced the tumor incidence and volume. The positive results observed were dose related. Overall, it was shown that the olive leaves extract administered (300 and 1000 mg/kg; 30 weeks; twice daily) and oleuropein inhibited the increase of skin thickness and reduction in skin elasticity, skin carcinogenesis, and tumor growth in UVB irradiated mice (3 times/week; 30 weeks) (Kimura et al. 2009).

Data also showed that oleuropein could have a preventive effect on tongue cancer. The administration of an oleuropein extract (3 mg/kg; 31 weeks) in a rat model of 4-NQO-induced tongue cancer lead to a reduced tumor incidence and volume due to a decrease of c-Met and Ki67 expression. C-Met is an oncogene able to induce cell proliferation and survival, whereas Ki-67 is a protein required through the cell cycle and which expression inhibits cell proliferation (Grawish et al. 2011).

Likewise, studies with hydroxytyrosol are promising. As hydroxytyrosol is a metabolite of oleuropein, some of the results reported to oleuropein could be effectively due to metabolized hydroxytyrosol (Shamshoum et al. 2017). The administration of 10 mg/kg of hydroxytyrosol for 14 days (female mice) reduced tumor size, vessel lumina and blood perfusion to the tumor in colon cancer cells. These effects resulted from the downregulation of HIF-1 α , VEGF and mPGEs-1 (Terzuoli et al. 2010). Also, mammary tumor growth was inhibited when hydroxytyrosol was administered (0.5 mg/kg; 6 weeks). It was showed that hydroxytyrosol modifies genes related to cell proliferation, apoptosis and the Wnt signaling pathway, promoting a high expression of Sfrp4, a major isoform able to chemo-sensitize tumor cells to chemotherapeutics (Granados-Principal et al. 2011).

Using a human cell line, it was also shown that cholangiocarcinoma, a digestive tumor with a high rate of mortality, growth was reduced by hydroxytyrosol administration (500

mg/kg; daily; 3 weeks). Moreover, *in vivo* assays revealed that, after hydroxytyrosol treatment, tumor growth was significantly suppressed (Li et al. 2014).

The anticancer actions of hydroxytyrosol may be due to its capacity to suppress cell survival and/or to activate pro-apoptotic mechanisms. Another proposed action of hydroxytyrosol is the induction of ubiquitination followed by lysosomal degradation of EGFR, which reduced expression is associated with the decrease of cell proliferation in colon cancer cells (Emma et al. 2021). Recently, in a human colorectal cancer cell line, it was demonstrated that hydroxytyrosol increased the expression of CASP3 and BAX, both proapoptotic genes, and downregulated the expression of the NFE2L2 gene (Hormozi et al. 2020).

In breast cancer, cells treatment with hydroxytyrosol and oleuropein reversed 3-MA-dependent suppression of autophagic flux (3-MA is an inhibitor of autophagy), increasing LC3II/LC3I and reducing p62 expression. In this way, it was observed the reduction of tumor cell migration and invasion by activated autophagy (Lu et al. 2019).

Data obtained from *in vivo* animal and *in vitro* studies regarding the effect of oleuropein and hydroxytyrosol on carcinogenesis in several cell lines is already extensive and suggested that both compounds can inhibit cancer initiation and metastasis. However, the results should be ascertained in human studies (Shamshoum et al. 2017; Torić et al. 2019). Different conditions (isolated compounds versus extracts) and, mainly, different concentrations have led to the opposite and even controversial effects (Emma et al. 2021).

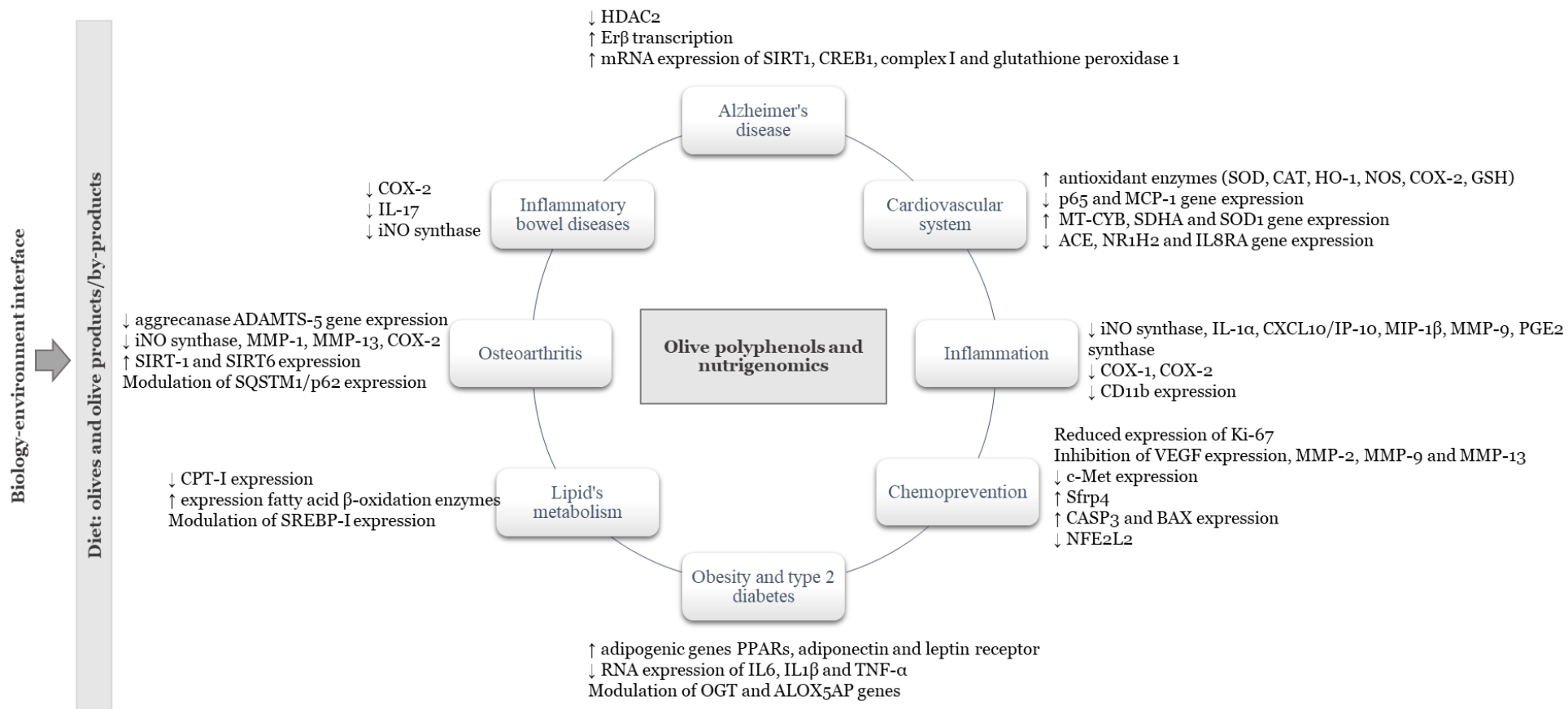


Figure 2. Olive polyphenols beneficial effects.

5. Conclusion

Nutrition is a multidisciplinary field aligned with physiology and food technology but increasingly including environmental, socio-economic and political sciences. Also, agro-food sustainable policies and practices have focused on nutrition to base its practices on food security (Raubenheimer and Simpson 2016). The axis nutrition-environment-gene should not be overlooked since, increasingly, other products beyond the traditionally consumed ones start to be part of dietary patterns and consumed as functional foods.

Most of the nutrigenomic studies are focused on olive oil or olive leaves rather than on their individual compounds, namely polyphenols. The emerging R&D of new products using the olive by-products and claiming health benefits based on individual compounds should be validated. Moreover, extraction and bioactives compounds processing change the way they naturally exist and imbalance food composition, turning the investigation on the bioavailability a challenge.

New food presentations using olive polyphenols will arise on the market. Olive leaves are an example. Olive leaves have been traditionally used mainly to prepare infusions but, nowadays, have also been increasingly used to produce food additives, dietary supplements, and nutraceuticals. Thus, epigenetic effects regarding by-products as foodstuff models could also contribute to the development of the nutrigenomics field.

Overall, eco-nutrition principles promote ethical and sustainable food consumption, considering the environmental and agricultural resources, but above all, support healthy dietary patterns (Bisht 2021). These bounding lines emerge and should be considered by the food industry and research institutions in a joint effort to contribute to nutrigenomics by investigating if a specific olive compound, incorporated or not in a food matrix, prevents the onset of disorders or diseases.

An integrated approach between genomics and nutrition supports personalized diets, a key factor to prevent diseases and, above all, to maintain optimal global health status. Olive compounds will be one of the most future contributors to functional food products, and their impact in genomics ought to be explored.

This chapter presented the main phenolics present in olive products and by-products and their impact on gene regulation and subsequent consequences. Hydroxytyrosol and oleuropein are the compounds most described and bioactive. Although tyrosol, oleocanthal and ligstroside are present in minor amounts, significant beneficial effects have been reported. The nutrigenomic study associated with cancer and cardiovascular

diseases is in great advancement, but lipids metabolism or inflammatory bowel diseases fields are now exciting and promising areas for nutrigenomics research.

References

- Abuznait, A. H., Qosa, H., Busnena, B. A., El Sayed, K. A. and Kaddoumi, A. 2013. Olive-oil-derived oleocanthal enhances β -amyloid clearance as a potential neuroprotective mechanism against Alzheimer's disease: *in vitro* and *in vivo* studies. *ACS Chemical Neuroscience* 4(6):973-82.
- Acar-Tek, N. and Ağagündüz, D. 2020. Olive leaf (*Olea europaea* L. *folium*): potential effects on glycemia and lipidemia. *Annals of Nutrition and Metabolism* 76(1):10-5.
- Agnihotri, A. and Aruoma, O. I. 2020. Alzheimer's disease and Parkinson's disease: a nutritional toxicology perspective of the impact of oxidative stress, mitochondrial dysfunction, nutrigenomics and environmental chemicals. *Journal of the American College of Nutrition* 39(1):16-27.
- Alkhatib, A., Tsang, C. and Tuomilehto, J. 2018. Olive oil nutraceuticals in the prevention and management of diabetes: from molecules to lifestyle. *International Journal of Molecular Sciences* 19(7):2024.
- Assy, N., Nassar, F., Nasser, G. and Grosowski, M. 2009. Olive oil consumption and non-alcoholic fatty liver disease. *World Journal of Gastroenterology* 15(15):1809-15.
- Bellumori, M., Cecchi, L., Romani, A., Mulinacci, N. and Innocenti, M. 2018. Recovery and stability over time of phenolic fractions by an industrial filtration system of olive mill wastewaters: a three-year study. *Journal of the Science of Food and Agriculture* 98(7):2761-9.
- Bertelli, M., Kiani, A. K., Paolacci, S., et al. 2020. Hydroxytyrosol: a natural compound with promising pharmacological activities. *Journal of Biotechnology* 309:29-33.
- Bigagli, E., Cinci, L., Paccosi, S., Parenti, A., D'Ambrosio, M. and Luceri, C. 2017. Nutritionally relevant concentrations of resveratrol and hydroxytyrosol mitigate oxidative burst of human granulocytes and monocytes and the production of pro-inflammatory mediators in LPS-stimulated RAW264.7 macrophages. *International Immunopharmacology* 43:147-55.
- Bishop, K. S. and Ferguson, L. R. 2015. The interaction between epigenetics, nutrition and the development of cancer. *Nutrients* 7(2): 922-47.
- Bisht, I. S. 2021. Agri-food system dynamics of small-holder hill farming communities of Uttarakhand in north-western India: socio-economic and policy considerations for sustainable development. *Agroecology and Sustainable Food Systems* 45(3):417-49.
- Bora, P., Chauhan, P., Pardeshi, K. A. and Chakrapani, H. 2018. Small molecule generators of biologically reactive sulfur species. *RSC Advances* 8(48):27359-74.

- Boronat, A., Mateus, J., Soldevila-Domenech, N., et al. 2019. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. *Free Radical Biology and Medicine* 143:471-81.
- Bulotta, S., Corradino, R., Celano, M., et al. 2013. Antioxidant and antigrowth action of peracetylated oleuropein in thyroid cancer cells. *Journal of Molecular Endocrinology* 51(1):181-9.
- Carnevale, R., Silvestri, R., Loffredo, L., et al. 2018. Oleuropein, a component of extra virgin olive oil, lowers postprandial glycaemia in healthy subjects. *British Journal of Clinical Pharmacology* 84(7):1566-74.
- Catalán, Ú., López de las Hazas, M.-C., Rubió, L., et al. 2015. Protective effect of hydroxytyrosol and its predominant plasmatic human metabolites against endothelial dysfunction in human aortic endothelial cells. *Molecular Nutrition and Food Research* 59(12):2523-36.
- Cetrullo, S., D'Adamo, S., Guidotti, S., Borzì, R. M. and Flamigni, F. 2016. Hydroxytyrosol prevents chondrocyte death under oxidative stress by inducing autophagy through sirtuin 1-dependent and -independent mechanisms. *Biochimica et Biophysica Acta (BBA) - General Subjects* 1860(6):1181-91.
- Charoenprasert, S. and Mitchell, A. 2012. Factors influencing phenolic compounds in table olives (*Olea europaea*). *Journal of Agricultural and Food Chemistry* 60(29):7081-95.
- Cordaro, M., Salinaro, A. T., Siracusa, R., et al. 2021. Hidrox® roles in neuroprotection: biochemical links between traumatic brain injury and Alzheimer's disease. *Antioxidants* 10(5):818.
- Cordero, J. G., García-Escudero, R., Avila, J., Gargini, R. and García-Escudero, V. 2018. Benefit of oleuropein aglycone for Alzheimer's disease by promoting autophagy. *Oxidative Medicine and Cellular Longevity* 2018:5010741.
- Correa, J. A., López-Villodres, J. A., Asensi, R., Espartero, J. L., Rodríguez-Gutiérrez, G. and De La Cruz, J. P. 2009. Virgin olive oil polyphenol hydroxytyrosol acetate inhibits *in vitro* platelet aggregation in human whole blood: comparison with hydroxytyrosol and acetylsalicylic acid. *British Journal of Nutrition* 101(8):1157-64.
- Covas, M. I., Konstantinidou, V. and Fitó, M. 2009. Olive oil and cardiovascular health. *Journal of Cardiovascular Pharmacology* 54(6):477-82.
- D'Adamo, S., Cetrullo, S., Guidotti, S., Borzì, R. M. and Flamigni, F. 2017. Hydroxytyrosol modulates the levels of microRNA-9 and its target sirtuin-1 thereby counteracting oxidative stress-induced chondrocyte death. *Osteoarthritis and Cartilage* 25(4):600-10.

- D'Angelo, C., Franceschelli, S., Quiles, J. L. and Speranza, L. 2020. Wide biological role of hydroxytyrosol: possible therapeutic and preventive properties in cardiovascular diseases. *Cells* 9(9):1932.
- de Bock, M., Thorstensen, E. B., Derraik, J. G., Henderson, H. V., Hofman, P. L. and Cutfield, W. S. 2013. Human absorption and metabolism of oleuropein and hydroxytyrosol ingested as olive (*Olea europaea* L.) leaf extract. *Molecular Nutrition and Food Research* 57(11):2079-85.
- Difonzo, G., Troilo, M., Squeo, G., Pasqualone, A. and Caponio, F. 2021. Functional compounds from olive pomace to obtain high-added value foods – a review. *Journal of the Science of Food and Agriculture* 101(1):15-26.
- Dima, C., Assadpour, E., Dima, S. and Jafari, S. M. 2020. Bioavailability and bioaccessibility of food bioactive compounds; overview and assessment by *in vitro* methods. *Comprehensive Reviews in Food Science and Food Safety* 19(6):2862-84.
- Ditano-Vázquez, P., Torres-Peña, J. D., Galeano-Valle, F., et al. 2019. The fluid aspect of the Mediterranean diet in the prevention and management of cardiovascular disease and diabetes: the role of polyphenol content in moderate consumption of wine and olive oil. *Nutrients* 11(11):2833.
- Drira, R., Chen, S. and Sakamoto, K. 2011. Oleuropein and hydroxytyrosol inhibit adipocyte differentiation in 3 T3-L1 cells. *Life Sciences* 89(19):708-16.
- Durazzo, A., Lucarini, M., Souto, E. B., et al. 2019. Polyphenols: a concise overview on the chemistry, occurrence, and human health. *Phytotherapy Research* 33(9):2221-43.
- Emma, M. R., Augello, G., Di Stefano, V., et al. 2021. Potential uses of olive oil secoiridoids for the prevention and treatment of cancer: A narrative review of preclinical studies. *International Journal of Molecular Sciences* 22(3):1-22.
- Fabiani, R., Vella, N. and Rosignoli, P. 2021. Epigenetic modifications induced by olive oil and its phenolic compounds: a systematic review. *Molecules* 26(2):273.
- Facchini, A., Cetrullo, S., D'Adamo, S., et al. 2014. Hydroxytyrosol prevents increase of osteoarthritis markers in human chondrocytes treated with hydrogen peroxide or growth-related oncogene α . *PLoS One* 9(10):e109724.
- Feng, Z., Li, X., Lin, J., et al. 2017. Oleuropein inhibits the IL-1 β -induced expression of inflammatory mediators by suppressing the activation of NF- κ B and MAPKs in human osteoarthritis chondrocytes. *Food and Function* 8(10):3737-44.
- Ferguson, J. F., Allayee, H., Gerszten, R. E., et al. 2016. Nutrigenomics, the microbiome, and gene-environment interactions: new directions in cardiovascular disease

- research, prevention, and treatment: a scientific statement from the American Heart Association. *Circulation: Genomic and Precision Medicine* 9(3):291-313.
- Fraga, C. G., Galleano, M., Verstraeten, S. V. and Oteiza, P. I. 2010. Basic biochemical mechanisms behind the health benefits of polyphenols. *Molecular Aspects of Medicine* 31(6):435-45.
- Ghanbari, R., Anwar, F., Alkharfy, K. M., Gilani, A.-H. and Saari, N. 2012. Valuable nutrients and functional bioactives in different parts of olive (*Olea europaea* L.) - a review. *International Journal of Molecular Sciences* 13(3):3291-3340.
- Giner, E., Recio, M.-C., Ríos, J.-L. and Giner, R.-M. 2013. Oleuropein protects against dextran sodium sulfate-induced chronic colitis in mice. *Journal of Natural Products* 76(6):1113-20.
- Giner, E., Recio, M. C., Ríos, J. L., Cerdá-Nicolás, J. M. and Giner, R. M. 2016. Chemopreventive effect of oleuropein in colitis-associated colorectal cancer in c57bl/6 mice. *Molecular Nutrition and Food Research* 60(2):242-55.
- González-Casanova, J. E., Pertuz-Cruz, S. L., Caicedo-Ortega, N. H. and Rojas-Gomez, D. M. 2020. Adipogenesis regulation and endocrine disruptors: emerging insights in obesity. *BioMed Research International* 2020:7453786.
- González-Santiago, M., Fonollá, J. and Lopez-Huertas, E. 2010. Human absorption of a supplement containing purified hydroxytyrosol, a natural antioxidant from olive oil, and evidence for its transient association with low-density lipoproteins. *Pharmacological Research* 61(4):364-70.
- Gorzynik-Debicka, M., Przychodzen, P., Cappello, F., et al. 2018. Potential health benefits of olive oil and plant polyphenols. *International Journal of Molecular Sciences* 19(3):686.
- Granados-Principal, S., Quiles, J. L., Ramirez-Tortosa, C., et al. 2011. Hydroxytyrosol inhibits growth and cell proliferation and promotes high expression of sfrp4 in rat mammary tumours. *Molecular Nutrition and Food Research* 55(S1):S117-26.
- Grawish, M. E., Zyada, M. M. and Zaher, A. R. 2011. Inhibition of 4-NQO-induced F433 rat tongue carcinogenesis by oleuropein-rich extract. *Medical Oncology* 28(4):1163-68.
- Grewal, R., Reutzel, M., Dilberger, B., et al. 2020. Purified oleocanthal and ligstroside protect against mitochondrial dysfunction in models of early Alzheimer's disease and brain ageing. *Experimental Neurology* 328:113248.
- Hidalgo, M., Prieto, I., Abriouel, H., et al. 2018. Changes in gut microbiota linked to a reduction in systolic blood pressure in spontaneously hypertensive rats fed an extra virgin olive oil-enriched diet. *Plant Foods for Human Nutrition* 73(1):1-6.

- Hormozi, M., Salehi Marzijerani, A. and Baharvand, P. 2020. Effects of hydroxytyrosol on expression of apoptotic genes and activity of antioxidant enzymes in LS180 Cells. *Cancer Management and Research* 12:7913-19.
- Houston, M. 2018. The role of noninvasive cardiovascular testing, applied clinical nutrition and nutritional supplements in the prevention and treatment of coronary heart disease. *Therapeutic Advances in Cardiovascular Disease* 12(3):85-108.
- Hussein, O., Grosovski, M., Lasri, E., Svalb, S., Ravid, U. and Assy, N. 2007. Monounsaturated fat decreases hepatic lipid content in non-alcoholic fatty liver disease in rats. *World Journal of Gastroenterology* 13(3):361-368.
- Irimie, A. I., Braicu, C., Pasca, S., et al. 2019. Role of key micronutrients from nutrigenetic and nutrigenomic perspectives in cancer prevention. *Medicina* 55(6):283.
- Klen, T. J., Wondra, A. G., Vrhovšek, U., Sivilotti, P., and Vodopivec, B. M. 2015. Olive fruit phenols transfer, transformation, and partition trail during laboratory-scale olive oil processing. *Journal of Agricultural and Food Chemistry* 63(18):4570-79.
- Klen, T. J. and Vodopivec, B. M. 2012. The fate of olive fruit phenols during commercial olive oil processing: traditional press versus continuous two- and three-phase centrifuge. *LWT - Food Science and Technology* 49(2):267-74.
- Ji, M., Gong, X., Li, X., Wang, C. and Li, M. 2020. Advanced research on the antioxidant activity and mechanism of polyphenols from *Hippophae* Species - A review. *Molecules* 25(4):917.
- Kerimi, A., Nyambe-Silavwe, H., Pyner, A., et al. 2019. Nutritional implications of olives and sugar: attenuation of post-prandial glucose spikes in healthy volunteers by inhibition of sucrose hydrolysis and glucose transport by oleuropein. *European Journal of Nutrition* 58(3):1315-30.
- Khalili, A., Nekooeian, A. A. and Khosravi, M. B. 2017. Oleuropein improves glucose tolerance and lipid profile in rats with simultaneous renovascular hypertension and type 2 diabetes. *Journal of Asian Natural Products Research* 19(10):1011-21.
- Kimura, Y. and Sumiyoshi, M. 2009. Olive leaf extract and its main component oleuropein prevent chronic ultraviolet B radiation-induced skin damage and carcinogenesis in hairless mice. *The Journal of Nutrition* 139(11):2079-86.
- Kiritsakis, A. K., Kiritsakis, K. A. and Tsitsipas, C. K. 2020. A review of the evolution in the research of antioxidants in olives and olive oil during the last four decades. *Journal of Food Bioactives* 11:31-56.
- Kolluru, G. K., Shen, X., and Kevil, C. G. 2020. Reactive sulfur species. *Arteriosclerosis, Thrombosis, and Vascular Biology* 40(4):874-884.

- Konstantinidou, V., Covas, M.-I., Muñoz-Aguayo, D., et al. 2010. *In vivo* nutrigenomic effects of virgin olive oil polyphenols within the frame of the Mediterranean diet: a randomized controlled trial. *The FASEB Journal* 24(7):2546-57.
- Larussa, T., Imeneo, M., and Luzzza, F. 2019. Olive tree biophenols in inflammatory bowel disease: when bitter is better. *International Journal of Molecular Sciences* 20(6):1390.
- Larussa, T., Oliverio, M., Suraci, E., et al. 2017. Oleuropein decreases cyclooxygenase-2 and interleukin-17 expression and attenuates inflammatory damage in colonic samples from ulcerative colitis patients. *Nutrients* 9(4):391.
- Li, S., Han, Z., Ma, Y., et al. 2014. Hydroxytyrosol inhibits cholangiocarcinoma tumor growth: an *in vivo* and *in vitro* study. *Oncology Reports* 31(1):145-152.
- Liu, Z., Wang, N., Ma, Y. and Wen, D. 2019. Hydroxytyrosol improves obesity and insulin resistance by modulating gut microbiota in high-fat diet-induced obese mice. *Frontiers in Microbiology* 10(390):1-12.
- López de las Hazas, M.-C., Martín-Hernández, R., Crespo, M.-C., et al. 2019. Identification and validation of common molecular targets of hydroxytyrosol. *Food and Function* 10(8):4897-4910.
- Lu, H. Y., Zhu, J. S., Zhang, Z., et al. 2019. Hydroxytyrosol and oleuropein inhibit migration and invasion of MDA-MB-231 triple-negative breast cancer cell via induction of autophagy. *Anti-Cancer Agents in Medicinal Chemistry* 19(16):1983-90.
- Luccarini, I., Grossi, C., Rigacci, S., et al. 2015. Oleuropein aglycone protects against pyroglutamylated- β amyloid- β toxicity: biochemical, epigenetic and functional correlates. *Neurobiology of Aging* 36(2):648-63.
- Marcelino, G., Hiane, P. A., Freitas, K. de C., et al. 2019. Effects of olive oil and its minor components on cardiovascular diseases, inflammation, and gut microbiota. *Nutrients* 11(8):1826.
- Marrano, N., Spagnuolo, R., Biondi, G., et al. 2021. Effects of extra virgin olive oil polyphenols on beta-cell function and survival. *Plants* 10(2):286.
- Martín-Peláez, S., Castañer, O., Konstantinidou, V., et al. 2017. Effect of olive oil phenolic compounds on the expression of blood pressure-related genes in healthy individuals. *European Journal of Nutrition* 56(2):663-70.
- Martínez-Martos, J. M., Mayas, M. D., Carrera, P., et al. 2014. Phenolic compounds oleuropein and hydroxytyrosol exert differential effects on glioma development via antioxidant defense systems. *Journal of Functional Foods* 11:221-34.
- Mateos, R., Sarria, B. and Bravo, L. 2020. Nutritional and other health properties of olive pomace oil. *Critical Reviews in Food Science and Nutrition* 60(20):3506-21.

- Meza-Miranda, E. R., Rangel-Zúñiga, O. A., Marín, C., et al. 2016. Virgin olive oil rich in phenolic compounds modulates the expression of atherosclerosis-related genes in vascular endothelium. *European Journal of Nutrition* 55(2):519-27.
- Višnjevec A. M., Baker, P., Charlton A., et al. 2021. Developing an olive biorefinery in Slovenia: analysis of phenolic compounds found in olive mill pomace and wastewater. *Molecules* 26(1):7.
- Milenkovic, D., Jude, B. and Morand, C. 2013. miRNA as molecular target of polyphenols underlying their biological effects. *Free Radical Biology and Medicine* 64:40-51.
- Miro-Casas, E., Covas, M. I., Farre, M., et al. 2003. Hydroxytyrosol disposition in humans. *Clinical Chemistry* 49(6):945-52.
- Mitra, V. and Metcalf, J. 2012. Metabolic functions of the liver. *Anaesthesia and Intensive Care Medicine* 13(2):54-5.
- Mondragón-Rodríguez, S., Perry, G. and Peña-Ortega, F. 2017. Tau Proteins. In *Drug Discovery Approaches for the Treatment of Neurodegenerative Disorders*, eds. A. Adejare, 145-160. Academic Press.
- Monti, M. C., Margarucci, L., Riccio, R., and Casapullo, A. 2012. Modulation of tau protein fibrillization by oleocanthal. *Journal of Natural Products* 75(9):1584-88.
- Moreno-Luna, R., Muñoz-Hernandez, R., Miranda, M., et al. 2012. Olive oil polyphenols decrease blood pressure and improve endothelial function in young women with mild hypertension. *American Journal of Hypertension* 25(12):1299-1304.
- Nishida, M., Kumagai, Y., Ihara, H., Fujii, S., Motohashi, H. and Akaike, T. 2016. Redox signaling regulated by electrophiles and reactive sulfur species. *Journal of Clinical Biochemistry and Nutrition* 58(2):91-8.
- Nocella, C., Cammisotto, V., Fianchini, L., et al. 2018. Extra virgin olive oil and cardiovascular diseases: benefits for human health. *Endocrine, Metabolic and Immune Disorders* 18(1):4-13.
- Nunes, M. A., Costa, A. S. G., Bessada, S., et al. 2018. Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components. *Science of The Total Environment* 644:229-36.
- Nunes, M. A., Pimentel, F., Costa, A. S. G., Alves, R. C. and Oliveira, M. B. P. P. 2016. Cardioprotective properties of grape seed proanthocyanidins: an update. *Trends in Food Science and Technology* 57:31-9.
- Olmo-García, L., Kessler, N., Neuweger, H., et al. 2018. Unravelling the distribution of secondary metabolites in *Olea europaea* L.: exhaustive characterization of eight olive-tree derived matrices by complementary platforms (LC-ESI/APCI-MS and GC-APCI-MS). *Molecules* 23(10):2419.

- Olson, K. R. 2020. Reactive oxygen species or reactive sulfur species: why we should consider the latter. *The Journal of Experimental Biology* 223(4):jeb196352.
- Ortega, F. J., Mercader, J. M., Catalán, V., et al. 2013. Targeting the circulating microRNA signature of obesity. *Clinical Chemistry* 59(5):781-792.
- Pantano, D., Luccarini, I., Nardiello, P., Servili, M., Stefani, M. and Casamenti, F. 2017. Oleuropein aglycone and polyphenols from olive mill waste water ameliorate cognitive deficits and neuropathology. *British Journal of Clinical Pharmacology* 83(1):54-62.
- Parenti, A., Spugnoli, P., Masella, P., and Calamai, L. 2008. The effect of malaxation temperature on the virgin olive oil phenolic profile under laboratory-scale conditions. *European Journal of Lipid Science and Technology* 110(8):735-41.
- Peña-Romero, A. C., Navas-Carrillo, D., Marín, F., and Orenes-Piñero, E. 2018. The future of nutrition: nutrigenomics and nutrigenetics in obesity and cardiovascular diseases. *Critical Reviews in Food Science and Nutrition* 58(17):3030-41.
- Peralbo-Molina, Á., Priego-Capote, F. and Luque de Castro, M. D. 2012. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography–tandem mass spectrometry with a quadrupole–quadrupole–time-of-flight mass detector. *Journal of Agricultural and Food Chemistry* 60(46):11542-50.
- Peyrol, J., Riva, C. and Amiot, M. J. 2017. Hydroxytyrosol in the prevention of the metabolic syndrome and related disorders. *Nutrients* 9(3):306.
- Piroddi, M., Albini, A., Fabiani, R., et al. 2017. Nutrigenomics of extra-virgin olive oil: a review. *Biofactors* 43(1):17-41.
- Pirozzi, C., Lama, A., Simeoli, R., et al. 2016. Hydroxytyrosol prevents metabolic impairment reducing hepatic inflammation and restoring duodenal integrity in a rat model of NAFLD. *The Journal of Nutritional Biochemistry* 30:108-15.
- Poddar, M., Chetty, Y. and Chetty, V. T. 2017. How does obesity affect the endocrine system? A narrative review. *Clinical Obesity* 7(3):136-44.
- Priore, P., Cavallo, A., Gnoni, A., Damiano, F., Gnoni, G. V. and Siculella, L. 2015. Modulation of hepatic lipid metabolism by olive oil and its phenols in nonalcoholic fatty liver disease. *IUBMB Life* 67(1):9-17.
- Qin, C., Hu, S., Zhang, S., et al. 2021. Hydroxytyrosol acetate improves the cognitive function of APP/PS1 transgenic mice in ER β -dependent manner. *Molecular Nutrition and Food Research* 65(3):e2000797.
- Raubenheimer, D. and Simpson, S. J. 2016. Nutritional ecology and human health. *Annual Review of Nutrition* 36:603-26.

- Robles-Almazan, M., Pulido-Moran, M., Moreno-Fernandez, J., et al. 2018. Hydroxytyrosol: bioavailability, toxicity, and clinical applications. *Food Research International* 105:654-67.
- Rodis, P. S., Karathanos, V. T. and Mantzavinou, A. 2002. Partitioning of olive oil antioxidants between oil and water phases. *Journal of Agricultural and Food Chemistry* 50(3):596-601.
- Rodríguez-Lara, A., Mesa, M. D., Aragón-Vela, J., et al. 2019. Acute/subacute and sub-chronic oral toxicity of a hidroxytyrosol-rich virgin olive oil extract. *Nutrients* 11(9):2133.
- Rodríguez-López, P., Lozano-Sanchez, J., Borrás-Linares, I., Emanuelli, T., Menéndez, J. A. and Segura-Carretero, A. 2020. Structure–biological activity relationships of extra-virgin olive oil phenolic compounds: health properties and bioavailability. *Antioxidants* 9(8):685.
- Rodríguez-Morató, J., Boronat, A., Kotronoulas A., et al. 2016. Metabolic disposition and biological significance of simple phenols of dietary origin: hydroxytyrosol and tyrosol. *Drug Metabolism Reviews* 48(2):218-36.
- Roselló-Soto, E., Koubaa, M., Moubarik, A., et al. 2015. Emerging opportunities for the effective valorization of wastes and by-products generated during olive oil production process: non-conventional methods for the recovery of high-added value compounds. *Trends in Food Science and Technology* 45(2):296-310.
- Saibandith, B., Spencer, J. P. E., Rowland, I. R. and Commane, D. M. 2017. Olive polyphenols and the metabolic syndrome. *Molecules* 22(7):1082.
- Sánchez-Fidalgo, S., Villegas, I., Aparicio-Soto, M., et al. 2015. Effects of dietary virgin olive oil polyphenols: hydroxytyrosyl acetate and 3, 4-dihydroxyphenylglycol on DSS-induced acute colitis in mice. *The Journal of Nutritional Biochemistry* 26(5):513-20.
- Santangelo, C., Filesi, C., Vari, R., et al. 2016. Consumption of extra-virgin olive oil rich in phenolic compounds improves metabolic control in patients with type 2 diabetes mellitus: a possible involvement of reduced levels of circulating visfatin. *Journal of Endocrinological Investigation* 39(11):1295-301.
- Santos-Sánchez, N. F., Salas-Coronado, R., Villanueva-Cañongo, C. and Hernández-Carlos, B. 2019. Antioxidant compounds and their antioxidant mechanism. In *Antioxidants*, eds. E. Shalaby, 1-28. London: IntechOpen.
- Santos, M. M., Piccirillo, C., Castro, P. M. L., Kalogerakis, N. and Pintado, M. E. 2012. Bioconversion of oleuropein to hydroxytyrosol by lactic acid bacteria. *World Journal of Microbiology and Biotechnology* 28(6):2435-40.

- Scoditti, E., Carpi, S., Massaro, M., et al. 2019. Hydroxytyrosol modulates adipocyte gene and miRNA expression under inflammatory condition. *Nutrients* 11(10):2493.
- Scotece, M., Conde, J., Abella, V., et al. 2018. Oleocanthal inhibits catabolic and inflammatory mediators in LPS-activated human primary osteoarthritis (OA) chondrocytes through MAPKs/NF- κ B pathways. *Cellular Physiology and Biochemistry* 49(6):2414-26.
- Shahidi, F. and Peng, H. 2018. Bioaccessibility and bioavailability of phenolic compounds. *Journal of Food Bioactives* 4:11–68.
- Shamshoum, H., Vlatcheski, F. and Tsiani, E. 2017. Anticancer effects of oleuropein. *Biofactors* 43(4):517-28.
- Terzuoli, E., Donnini, S., Giachetti, A., et al. 2010. Inhibition of hypoxia inducible factor-1 α by dihydroxyphenylethanol, a product from olive oil, blocks microsomal prostaglandin-E synthase-1/vascular endothelial growth factor expression and reduces tumor angiogenesis. *Clinical Cancer Research* 16(16):4207-16.
- Torić, J., Marković, A. K., Brala, C. J. and Barbarić, M. 2019. Anticancer effects of olive oil polyphenols and their combinations with anticancer drugs. *Acta Pharmaceutica* 69(4):461-82.
- Veza, T., Rodríguez-Nogales, A., Algieri, F., et al. 2019. The metabolic and vascular protective effects of olive (*Olea europaea* L.) leaf extract in diet-induced obesity in mice are related to the amelioration of gut microbiota dysbiosis and to its immunomodulatory properties. *Pharmacological Research* 150:104487.
- Visioli, F., Davalos, A., López de las Hazas, M.-C., Crespo, M. C. and Tomé-Carneiro, J. 2020. An overview of the pharmacology of olive oil and its active ingredients. *British Journal of Pharmacology* 177(6):1316-30.
- Vissers, M. N., Zock, P. L., and Katan, M. B. 2004. Bioavailability and antioxidant effects of olive oil phenols in humans: a review. *European Journal of Clinical Nutrition* 58(6):955-65.
- Wadey, R. M., Connolly, K. D., Mathew, D., Walters, G., Rees, D. A. and James, P. E. 2019. Inflammatory adipocyte-derived extracellular vesicles promote leukocyte attachment to vascular endothelial cells. *Atherosclerosis* 283:19-27.
- Wang, N., Ma, Y., Liu, Z., et al. 2019. Hydroxytyrosol prevents PM_{2.5}-induced adiposity and insulin resistance by restraining oxidative stress related NF- κ B pathway and modulation of gut microbiota in a murine model. *Free Radical Biology and Medicine* 141:393-407.
- Winterbourn, C. C. 2020. Biological chemistry of superoxide radicals. *ChemTexts* 6(1):7.

- Zhang, X., Cao, J. and Zhong, L. 2009. Hydroxytyrosol inhibits pro-inflammatory cytokines, iNOS, and COX-2 expression in human monocytic cells. *Naunyn-Schmiedeberg's Archives of Pharmacology* 379(6):581-6.
- Zhi, L. Q., Yao, S. X., Liu, H. L., Li, M., Duan, N. and Ma, J. B. 2018. Hydroxytyrosol inhibits the inflammatory response of osteoarthritis chondrocytes via SIRT6-mediated autophagy. *Molecular Medicine Reports* 17(3):4035-42

This page was intentionally left in blank.

Chapter 3 - Olive pomace strategies valorisation

Chapter 3 describes the approaches that were tested and developed to valorise olive pomace. First, olive pomace lipid- and water-soluble fractions were characterized, and bioactive compounds assessed. Bioactive compounds extraction processes to concentrate olive pomace bioactive compounds, namely a membrane-assisted process, were explored. Monocultivar olive pomaces were discriminated by chemical and spectroscopic techniques. Research has been focused on finding green, simple and low-cost solutions for an industrial valorisation of olive pomace and maximizing its antioxidant properties, namely by finding possible synergistic interaction among different olive pomaces.

The balance between environmental sustainability and the high-yield extraction of compounds is challenging, mainly from the industrial point of view. Keeping in mind the insights brought from the olive oil sector in several University-Industry interactions, processes and products with a possible industrial application were also developed, giving them, subsequently, a socio-economic valorisation. This resulted in two patent applications.

Therefore, **Chapter 3** is divided into two subsections - “Science to Science” and “Science to Society” - to aggregate, first, the scientific publications disseminated within the scientific community, and then the research applied to industry. The ultimate and very challenging goal was to find concrete valorisation strategies with a positive impact on outcomes to the olive oil sector, the community around this sector and the national socio-economic activity. However, we recognize that “Science to Science” and “Science to Society” are highly interconnected when open innovation is attained.

“Science to Society” describes two patent applications related with the valorisation of two by-products of the olive oil processing: extracted and wet olive pomace.

This page was intentionally left in blank.

3.1 Science to Science

3.1.1 Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components

M. Antónia Nunes^a, Anabela S.G. Costa^a, Sílvia Bessada^a, Joana Santos^a, Helder Puga^b, Rita C. Alves^a, Vítor Freitas^c, M. Beatriz P.P. Oliveira^a

^aREQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy, University of Porto, R. J. Viterbo, 4050-313 Porto, Portugal

^bCentre for Micro-Electromechanical Systems, University of Minho, 4800-058 Guimarães, Portugal

^cREQUIMTE/LAQV, Department of Chemistry and Biochemistry, Faculty of Sciences, University of Porto, R. Campo Alegre s/n, 4169-007 Porto, Portugal

- Nunes, M.A., Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P. (2018). Olive pomace as a valuable source of bioactive compounds: a study regarding its lipid- and water-soluble components. *Science of the Total Environment*, 644, 229–236.

Abstract

Olive pomace is a major output of olive oil processing. This by-product is a valuable source of bioactive compounds with well-recognized benefits for human health and well-being.

In this work, the proximate composition and the profiles of vitamin E (HPLC-DAD-FLD), fatty acids (GC-FID) and phenolics (HPLC-DAD-FL/MSⁿ) were determined. Additionally, a sustainable process for antioxidants extraction - Multi-frequency Multimode Modulated (MMM) ultrasonic technique – was compared to a conventional solid-liquid extraction. The total phenolics content and antioxidant activity (ferric reducing antioxidant power and DPPH• scavenging ability) of the extracts were analysed to assess the efficacy of both extraction methodologies. The vitamin E profile of the olive pomace comprised the vitamers α -tocopherol, β -tocopherol, α -tocotrienol and γ -tocopherol. α -Tocopherol was the major form (2.63 mg/100 g), while the other vitamers were present in amounts lower than 0.1 mg/100 g. The lipid fraction was especially rich

in oleic acid (75%), followed by palmitic (10%), linoleic (9%), and stearic (3%) acids. Hydroxytyrosol and comsegolosite represented $\approx 79\%$ of the total phenolics present in olive pomace. Hydroxytyrosol content was 83.6 mg/100 g, while tyrosol was present in lower amounts (3.4 mg/100 g).

Concerning the antioxidants extraction, the MMM technique allowed a faster and higher recovery ($p < 0.05$) of the compounds, compared to the conventional solid-liquid extraction. By this way, it seems to be a very promising eco-friendly and effective methodology to extract antioxidants from this and other matrices

Keywords: Olive, pomace bioactive compounds, antioxidants, extraction, ultrasound.

1. Introduction

In 2050, it is expected that the world population (7.6 billion) will reach 9.1 billion (United Nations, 2017). Globally, more 30% of food will be necessary compared to the current days and, as a consequence, several major challenges are coming, especially related to food security and agricultural practices (Wezel et al., 2014). Indeed, over the last decades, the demand for larger amounts of food production has been increasing, which has been raising a key challenge to agriculture: it must be sustainable.

Sustainable agriculture is an interconnected system that involves environment protection, social fairness, technologies application and economic profitability (DeLonge et al., 2016). In addition to the world food supply, other issues emerge that compromises the Earth natural resources, such as water quality; higher demand for water, wood, and energy; increase of the land area for urbanization; soil degradation; loss of biodiversity; climate changes; among others (Lichtfouse et al., 2009). Overall, agriculture sustainability must enhance the food production without compromising the future generations (Wezel et al., 2014).

The olive oil industry is becoming one of the larger agro-food business sectors. In fact, the organoleptic characteristics together with recent advances on health properties of the olive oil have led to an increase of its consumption and production (Bañas et al., 2017). Two different techniques can be used for olive oil production: the two-phase method or the three-phase method. In the first one, no water is added, and a humid semi-solid pomace is obtained as by-product (wet olive pomace). Olive pomace is one of the major outputs of olive oil production and despite resulting from an eco-friendly system, it is considered phytotoxic due to its high phenolics content (Malapert et al., 2018). Simultaneously, those bioactive compounds can have health and well-being applications (e.g., for food and cosmetic industries) (Nunes et al., 2016; Rodrigues et al., 2017). More and more, the study of eco-friendly technologies that maximize the yield of extraction of such bioactive compounds, at low-cost, in a minimum period of time, and using environmentally friendly solvents are mandatory (Galanakis, 2012). By this way, it is possible to obtain natural ingredients to develop new food products, adding economic value to the olive oil processing segment, and simultaneously reducing the environmental burden of wastes, contributing, therefore, to a sustainable agriculture system.

In this work, the nutritional profile and chemical composition (regarding fatty acids, vitamin E and phenolic compounds) of olive pomace was assessed. Moreover, the

efficiency of a new sustainable process of antioxidants extraction (Multi-frequency Multimode Modulated Ultra-sonic technique) was compared with that of a conventional extraction method.

2. Material and methods

2.1 Sample preparation

Olive pomace (about 5 kg) was collected in Alfândega da Fé (Trás-Os-Montes, Portugal), in 2015, from a two-phase extraction olive oil unit. The sample was homogenized, divided in different batches, frozen at -80°C , and freeze-dried (Telstar Cryodos-80 Terrassa, Barcelona) for preservation till analysis.

2.2 Chemical characterization

2.2.1 Proximate analysis

Sample moisture was determined using an infrared balance (Scaltec model SMO01, Scaltec Instruments, Heiligenstadt, Germany). Total fat (AOAC 991.36) and total protein (AOAC 928.08) were determined by Soxhlet and Kjeldahl methods, respectively (AOAC, 2012). The total protein content was calculated using 6.25 as the nitrogen conversion factor (Tontisirin, 2003). Ash content was determined by incineration at 500°C (AOAC 920.153) (AOAC, 2012). Total carbohydrates were calculated by difference (Tontisirin, 2003).

2.2.2 Vitamin E profile by HPLC-DAD-FLD

An aliquot (≈ 20 mg) of the lipid fraction, obtained according to Pimentel et al. (2014), was mixed with 50 μL of internal standard (tocol, 100 $\mu\text{g}/\text{mL}$) and 1 mL of *n*-hexane. The chromatographic analysis was carried out in a HPLC system (Jasco, Tokyo, Japan) equipped with a multiwavelength diode array detector (MD-2015) coupled to a FP-2020 fluorescence detector (Jasco, Tokyo, Japan), programmed for excitation at 290 and emission at 330 nm. The chromatographic separation of the compounds was achieved using a normal phase SupelcosilTM LC-SI column (75 mm $\times$ 3.0 mm, 3.0 μm) (Supelco, Bellefonte, USA) according to Alves et al. (2009).

Vitamin E vitamers were identified based on their UV spectra and by comparing their retention times with those of standards. Quantification was performed based on the fluorescence signals.

2.2.3 Fatty acids profile by GC-FID

The lipid fraction of olive pomace was obtained using the Soxhlet method (4 h). The fatty acid profile was, then, determined according to ISO 12966-2:2011 (ISO 12966-2:2011, 2011). Briefly, about 15 mg of oil were vigorously mixed with 3 mL of *n*-hexane. Then, 200 μ L of 2 M KOH in methanol were added. After addition of anhydrous sodium sulfate, the mixture was centrifuged (850 $\times$ g, 5 min) and the supernatant was transferred to an injection vial.

The compounds separation was achieved in a Gas Chromatograph (Shimadzu GC-2010 Plus, Tokyo, Japan) coupled to a split/splitless AOC-20i auto-injector (Shimadzu, Tokyo, Japan) and a Flame Ionization Detector (Shimadzu, Tokyo, Japan). Helium was the carrier gas. A CP-Sil 88 silica capillary column (50 m $\times$ 0.25 mm i.d., 0.20 μ m film thickness) from Varian (Middelburg, Netherlands) was used. The temperature program was as follows: 80 $^{\circ}$ C for 5 min; increase to 160 $^{\circ}$ C at 5 $^{\circ}$ C/min; 160 $^{\circ}$ C for 5 min; increase to 170 $^{\circ}$ C at 5 $^{\circ}$ C/min; 170 $^{\circ}$ C for 1 min; increase to 220 $^{\circ}$ C at 5 $^{\circ}$ C/min; 220 $^{\circ}$ C for 15 min. The injector and detector temperatures were 250 and 270 $^{\circ}$ C, respectively. A split ratio of 1:25 was used and the injection volume was 1.0 μ L.

The fatty acid methyl esters were identified by comparison with a standard mixture (FAME 37, Supelco, Bellefonte, PA, USA). Data were analyzed based on relative peak areas. The results were expressed in relative percentage of each fatty acid.

2.2.4 Phenolics profile by HPLC-DAD-FLD and HPLC-DAD-MSⁿ

Aqueous extracts were prepared according to Costa et al. (2014) and freeze-dried. The obtained residue was dissolved in 1 mL of acetic acid (1% v/v). The solutions were centrifuged and filtered with syringe filter (0.22 μ m) prior injection in the HPLC-DAD-FLD system (Jasco, Tokyo, Japan). This system was composed of an AS-2057 automated injector, a PU-2089 pump, a MD-2018 multi-wavelength diode array detector (DAD) and a fluorescence detector (FLD) FP-2020 (Jasco, Japan), programmed for excitation at 280 and emission at 330 nm. The gradient elution program used was the following: 0 min, 10% B; 20 min, 30% B; 50 min, 75% B; 63 min, 100% B; 65 min, 10% B; 70 min, 10% B, being solvent A) 1% acetic acid and B) 100% methanol. The chromatographic column used was a Zorbax-SB-C18 (5 μ m; 25 $\times$ 4.6 mm; Agilent Technologies, USA), at 20 $^{\circ}$ C, with a flow rate of 1 mL/min. The DAD recorded from 200 to 600 nm, being each run monitored at 280, 320 and 335 nm. To further identify the compounds, the extract was also analyzed in an HPLC-

DAD-ESI-MSⁿ system (Thermo Finnigan, LCQ Deca XP Max, San Jose, USA). The same elution conditions were applied, being the connection to the ion trap mass spectrometer made with split of half of the initial flow to 0.5 mL/min. An electrospray interface in the negative mode was used in the following conditions: capillary voltage of 15 V, tube lens voltage of 30 V, capillary temperature of 325 °C, sheath gas flow rate of 60 (arbitrary units) and aux/sweep gas flow rate of 23(arbitrary units). The mass scan ranged from m/z 100 to 1000 and MS/ MS automatic mode was used to study the fragmentation patterns of the more abundant ions. The identification was based on the compounds retention time, UV–Vis and mass spectra with those obtained from standard solutions, when available. The other peaks were tentatively identified comparing the obtained information with available data reported in the literature.

The identified compounds were quantified by external calibration curves constructed based on their maximum UV signal and/or fluorescence signals. The results were presented in mg/100 g of olive pomace.

2.3 Antioxidant activity

2.3.1 Extracts preparation

Extracts were prepared using an innovative Multi-frequency Multi- mode Modulated (MMM) ultrasonic technique and a conventional solid-liquid extraction. Table 1 details the extraction conditions for both methods.

Table 1. Extraction conditions used in the present study.

Extracts	MMM technique			Conventional extraction
	A	B	C	D
Frequency (kHz)	20.05 ± 0.03	20.00 ± 0.03	20.01 ± 0.03	n/a
Input electric power (W)	90	160	160	n/a
Time of extraction (min)	10	10	5	60
Solvent	100% water	100% water	100% water	100% water
Temperature (°C)	RT	RT	RT	40
Rpm	n/a	n/a	n/a	600

MMM, multi-frequency multimode modulated; RT, room temperature; Rpm, revolutions per minute; n/a, not applicable.

In the first case (MMM technique), an ultrasound device was used (Fig. 1). Previously, the ultrasonic device was adjusted through Finite Element Model simulation based in pre-established mathematical models (Puga et al., 2017). The sample mass/volume ratio used was 1 g/50 mL. As described in Table 1, different input electric powers (90 and 160 W) and extraction times (5 and 10 min) were used. After centrifugation ($1077 \times g$, 10 min) and filtration (Whatman No. 4), the extracts were stored at $-20\text{ }^{\circ}\text{C}$ for further analyses.

The same sample mass/volume ratio was used in the conventional solid-liquid extraction. In this case, the mixture was continuously stirred at 600 rpm in a heating plate (MS-H-S10 10-Channel Classic Magnetic Hotplate Stirrer) at $40 \pm 1\text{ }^{\circ}\text{C}$ for 60 min. Then, it was centrifuged, filtered, and the final extracts were stored at $-20\text{ }^{\circ}\text{C}$ till analyses.

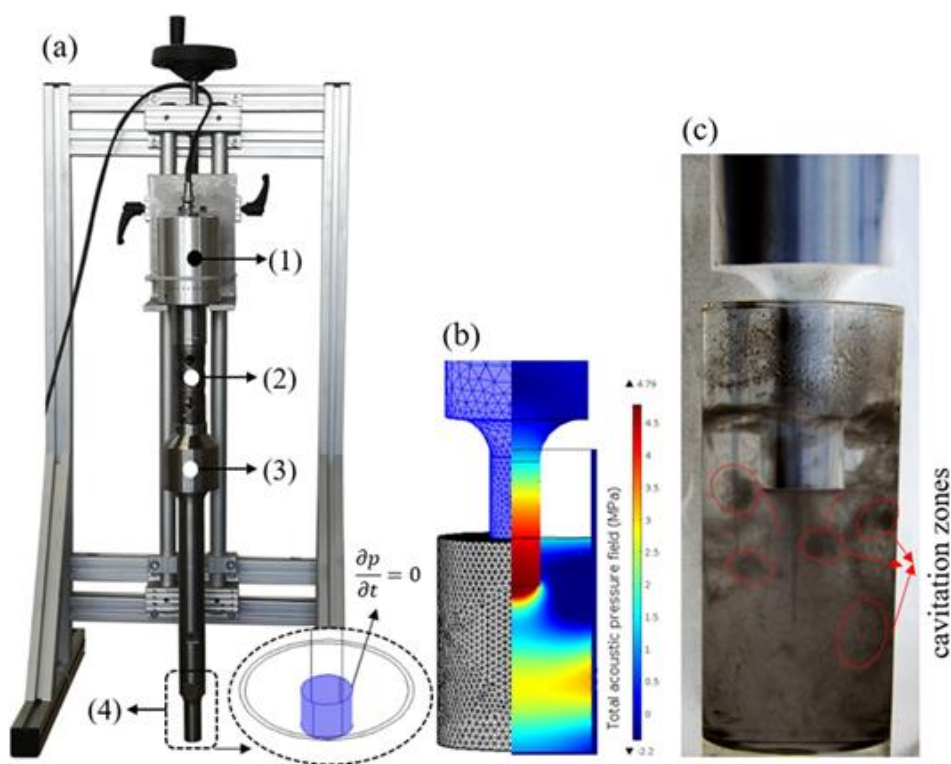


Figure 1. Ultrasonic extraction set-up: (a) experimental apparatus, (b) numerical modelling, (c) experimental acoustic cavitation zones. (1) Transducer, (2) waveguide, (3) booster, (4) special acoustic radiator.

2.3.2 Total phenolics content

Total phenolics content was determined by spectrophotometry (BioTek Instruments, Inc.) according to Costa et al. (2014), with minor modifications. In brief, 30 μL of the extract was mixed with 150 μL of Folin-Ciocalteu reagent (1:10). Then, 120 μL of sodium carbonate (7.5% m/v) were added and the mixture was incubated at $45\text{ }^{\circ}\text{C}$ for 15 min,

protected from light. After 30 min at room temperature, the absorbance was measured at 765 nm. A calibration curve was prepared with gallic acid (5–100 µg/mL; $R^2 = 0.9996$). Total phenolics were expressed as gallic acid equivalents (GAE).

2.3.3 Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out according to Costa et al. (2017). An 35 µL aliquot of extract was mixed with 265 µL of FRAP reagent (0.3 M acetate buffer, 10 mM TPTZ solution, and 20 mM of ferric chloride). The mixture was incubated at 37 °C for 30 min (protected from light).

Absorbance was measured at 595 nm. A calibration curve was prepared with ferrous sulfate (25–500 µmol/L; $R^2=1$), and the ferric reducing antioxidant power was expressed as ferrous sulfate equivalents (FSE).

2.3.4 DPPH• (2, 2-diphenyl-1-picrylhydrazyl radical) scavenging ability

The radical scavenging ability of the different extracts was evaluated according to Costa et al. (2014), with slight modifications. Initially, 270 µL of a DPPH• solution (6.0×10^{-5} mol/L in ethanol) were mixed with 30 µL of a diluted extract. The absorbance decrease was measured every 2 min, at 515 nm, in order to observe the kinetic reaction. The reaction endpoint was attained in 30 min. A calibration curve was prepared with trolox (2.5–175 µg/mL; $R^2=0.9984$) and the scavenging activity was expressed as trolox equivalents (TE).

2.4 Statistical analysis

Statistical analysis was performed using IBM SPSS v. 20 (IBM Corp., Armonk, 241 NY, USA). Data were expressed as mean $\pm$ standard deviation (n=9). The Shapiro-Wilk test was used to evaluate data normality. The one-way ANOVA was used to assess significant differences between samples, followed by Tukey's HSD or Dunnett T3 post-hoc test (selected based on the equality of the variances) to make pairwise comparisons between means. The level of significance for all hypothesis tests (p) was 0.05. Correlations among the different parameters analysed were achieved by Pearson correlation coefficients (r) at a significance level of 99%.

3. Results and discussion

Olive pomace production is strongly affected by seasonality (October to January). Usually, during this period of time, olive oil production units transfer and discharge olive pomace in large open-air evaporation pounds to be stored through long periods until processing of olive pomace oil (Valta et al., 2015).

Olive pomace contains the remaining olive fat, water and a great amount of bioactive compounds (water- and liposoluble), that can have further valuable and novel applications. In particular, the olive pomace derived from the two-phase system, as the one used in this study, is characterized by a high content in water that can reach 70% (Roselló-Soto et al., 2015).

The proximate composition of the studied olive pomace, as well as the vitamin E and fatty acids profile of its lipid fraction, is detailed in Table 2.

Indeed, the sample presented a moisture content of 61.1 g/100 g (fresh weight, fw). The protein and ash contents were 2.6 and 0.7 g/100 g (fw), respectively. Notice that edaphoclimatic factors can influence the proximate composition, as well as the bioactive compounds profile of olive pomace, besides other factors such as the agricultural practices, cultivar or the ripening stage (Portarena et al., 2017).

The total fat content of the olive pomace analysed in this study was of 2 g/100 g (fw). This remaining oil can have applications in either food or cosmetic industries. For example, in cosmetics, several oil applications are described as soap manufacturing, excipients in cosmetic formulations or even as active ingredients (Rodrigues et al., 2017; Rodrigues et al., 2015; Roselló-Soto et al., 2015).

Vitamin E is a liposoluble powerful antioxidant that comprises a group of eight vitamers with a common structure of phenolic- chromanol ring linked to an isoprenoid side chain that is saturated (α , β , δ , γ -tocopherols) or unsaturated (α , β , δ , γ -tocotrienols) (Roselló-Soto et al., 2015). Regarding the vitamin E profile, α -tocopherol is the major vitamin E form found in the olive pomace (2.63 mg/100 g, fw). α -Tocopherol is the most biologically active form of vitamin E, having a major role in the prevention of lipid peroxidation and scavenging of lipid peroxy radicals (Fiedor and Burda, 2014; Montenegro et al., 2015). In turn, α -tocotrienol (0.1), γ -tocopherol (0.04), and β -tocopherol (0.03 mg/100 g, fw) are minor components of olive pomace. The vitamers β -tocotrienol, γ -tocotrienol, δ -tocopherol and δ -tocotrienol were not detected in this sample (Table 2). The limit of detection (LOD) for β -tocotrienol, γ -tocotrienol, δ -tocopherol and δ -tocotrienol is respectively, 0.008, 0.006, 0.016, and 0.006 mg/100 g. LOD was calculated as 3.3 times of the standard deviation of the response/the slope of the calibration curve, as previously described by Alves et al. (2009).

Table 2. Chemical characterization of olive pomace.

Olive pomace composition		
Proximal analysis	g/100 g (dw)	g/100 g (fw)
Moisture	-	61.1 ± 0.7
Total protein	7.4 ± 0.2	2.6 ± 0.1
Nitrogen	1.2 ± 0.0	0.4 ± 0.0
Total fat	5.8 ± 0.3	2.0 ± 0.1
Ash	1.9 ± 0.0	0.7 ± 0.0
Total carbohydrates	84.9 ± 0.4	34.03 ± 0.1
Vitamin E	mg/100 g (dw)	mg/100 g (fw)
α-tocopherol	7.64 ± 0.81	2.63 ± 0.28
α-tocotrienol	0.29 ± 0.04	0.10 ± 0.01
β-tocopherol	0.09 ± 0.02	0.03 ± 0.01
γ-tocopherol	0.13 ± 0.02	0.04 ± 0.01
Fatty acids	Relative %	
C14:0 (myristic)	0.04 ± 0.00	
C16:0 (palmitic)	10.40 ± 0.05	
C17:0 (heptadecanoic)	0.13 ± 0.00	
C18:0 (stearic)	3.27 ± 0.01	
C20:0 (arachidic)	0.42 ± 0.01	
C22:0 (behenic)	0.16 ± 0.01	
C24:0 (lignoceric)	0.07 ± 0.01	
C16:1 (palmitoleic)	0.68 ± 0.03	
C17:1 (<i>cis</i> -10-heptadecenoic)	0.06 ± 0.01	
C18:1n9c (oleic)	75.25 ± 0.19	
C18:2n6c (linoleic)	8.46 ± 0.19	
C18:3n3 (α-linolenic)	0.80 ± 0.01	
C20:1n9 (<i>cis</i> -11-eicosenoic)	0.26 ± 0.01	
Σ SFA	14.49	
Σ MUFA	76.25	
Σ PUFA	9.26	
C18:1n9c/C18:2n6c	8.89	

Data presented as mean ± standard deviation. SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; dw, dry weight; fw, fresh weight.

External oxidative factors, such as light and oxygen, and intrinsic factors as antioxidants/prooxidants content can influence the composition of the olive pomace oil. The exposure to oxidative factors will lead, for example, to modifications in the fatty acids profile and/or vitamin E content of the remaining oil (Yun and Surh, 2012). Moreover, oil degradation can lead to unpleasant features as rancidity and discoloration that

besides decreasing its economic value, reduces the oil performance as functional ingredient. Moreover, the degree of the oil instability is also associated with a high content of polyunsaturated fatty acids (PUFA) due to their susceptibility to oxidation (Li et al., 2013).

A mixture of saturated (SFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA) composes the lipid fraction of the olive pomace. The fatty acid composition (relative %) of the oil extracted from olive pomace is presented in Table 2. Thirteen fatty acids were detected. The oleic acid was the most abundant, representing 75% of the total fatty acids, followed by palmitic, linoleic, and stearic acids (10, 9, and 3%, respectively). The other fatty acids were found in small quantities (Table 2). These results are in agreement with literature for olive products and by-products (Dubois et al., 2007; Orsavova et al., 2015; Uribe et al., 2014).

Considering the oxidative stability based on the fatty acids profile, it is possible to observe that the total PUFA is 9%. When compared to other oils used, for example, in cosmetic formulations, the lipid fraction of the olive pomace has a lower content in PUFA. Almond, apricot, and grapeseed oils have been used in cosmetic formulations. These oils have PUFA contents that can vary between 7.1 and 31% (almond), 25–36% (apricot), or 58–77% (grapeseed) (Rabasco Alvarez and González Rodríguez, 2000). As the olive pomace oil fraction is essentially rich in MUFA, its susceptibility to oxidation is reduced. Additionally, the presence of vitamin E, a potent liposoluble antioxidant, protects long chain fatty acids against oxidative damage (Uribe et al., 2013). The ratio of oleic acid to linoleic acid (C18:1/C18:2) was determined. In this case, olive pomace oil has a ratio of 8.89, which is comparable to virgin olive oil. Important olive components that remain in olive pomace, as MUFA, pigments, vitamin E, and phenolic compounds, may act synergistically, making this oil more stable than others (Allalout et al., 2009; Orsavova et al., 2015).

Phenolic compounds are released during olive oil processing being distributed into water and olive oil, accordingly to their chemical structure. A minimal phenolic fraction passes into the oil phase (2%). The majority of phenolics from olive fruit (98%), namely hydroxytyrosol and its derivatives, remain in olive pomace (Chanioti and Tzia, 2017). Complex chemical transformations occur during olive fruit maturation, in olive oil processing, and in olive pomace storage. For example, free forms of hydroxytyrosol, tyrosol or oleuropein, among other compounds, can be found in olive by-products or in olive oil along with several analogues (Malapert et al., 2018). Moreover, also the

agronomic practices or even the plant varieties have impact in the bioactive compounds profile (Peralbo-Molina et al., 2012).

Table 3 shows the phenolic profile of the olive pomace aqueous extract.

The main phenolic compounds and derivatives identified in the olive pomace of this study and their percent distribution are presented in Fig. 2.

The major compounds identified are distributed as follows:

hydroxytyrosol>comselogoside>elenolic acid derivative>tyrosol>oleoside riboside.

They are mostly distributed as phenolic alcohols (hydroxytyrosol and tyrosol), lignans (D(+)-erythro-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol and pinoresinol), secoiridoids and derivatives (oleuropein derivative, oleoside, oleoside riboside, aldehydic form of decarboxymethyl elenolic acid, elenolic acid derivative, oleoside diglucoside, verbascoside derivative, caffeoyl-6'-secologanoside) groups. These compounds have been previously described in olive pomace (Araújo et al., 2015; DellaGreca et al., 2004; Malapert et al., 2018; Peralbo-Molina et al., 2012; Yakhlef et al., 2018).

Among the major olive pomace phenolic compounds, hydroxytyrosol has gained particular interest over the years. This molecule presents strong antioxidant activity, anti-inflammatory and antimicrobial properties. Therefore, there is a high interest in this compound by cosmetics and pharmaceutical industries. The beneficial role of hydroxytyrosol in cardio and neurovascular diseases, as well as its anti-tumour effect, has also been demonstrated (Robles-Almazan et al., 2018). Moreover, its effect on food quality (chemical, nutritional and sensory properties) has also been explored (Nunes et al., 2016). Fig. 3 depicts the concentrations of hydroxytyrosol in olive pomace (83.59 and 238.42 mg/100 g in fresh and dry weight, respectively).

Table 3. Phenolic compounds identified (or tentatively identified) in olive pomace.

Phenolic compounds	RT (min)	UV-Vis max	MS (m/z)	MS ⁿ (m/z)	Reference
Hydroxytyrosol	10.7	280	153	123	(a)
Tyrosol	16.0	276			(a)
D(+)-Erythro-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol	17.6	276	213	195; 183; 151; 139	Obied et al., 2007
Oleuropein derivative	21.4	288	183	165; 139	Savarese et al., 2007
Oleoside	22.9	271	389	345; 209; 179; 165; 139	Fu et al., 2010
Oleoside riboside	26.8	274	505	389	Peralbo-Molina et al., 2012
Aldehydic form of decarboxymethyl elenolic acid	29.0	276	215	197; 185; 171; 153	Lozano-Sánchez et al., 2011
Elenolic acid derivative	32.0	274	243	211; 167; 123	Rubio-Senent et al., 2012
Oleoside digluside	35.7	276	713	669; 551; 533; 507; 489; 443	Peralbo-Molina et al., 2012
Verbascoside derivative	36.7	276	539	495; 463; 367	Savarese et al., 2007
Caffeoyl-6'-secologanoside	37.7	280/328	551	507; 389; 281; 251; 179; 161	Obied et al., 2007
Pinoresinol	38.2	280			(a)
Comselogoside	40.3	312 sh290	535	491; 389; 345; 265	Klen and Vodopivec, 2011

(a) Comparison with standard; RT, retention time

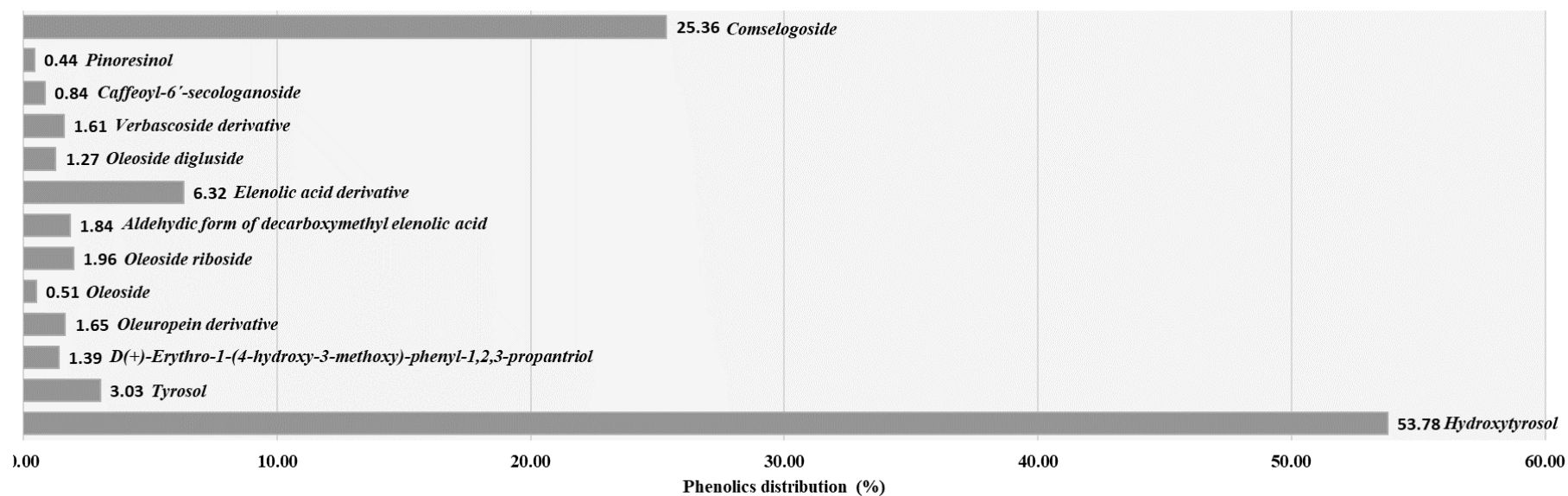


Figure 2. Distribution (in percentage) of the olive pomace phenolic compounds. Hydroxytyrosol, tyrosol, and pinoresinol were identified by comparison with standards. The other compounds were tentatively identified by HPLC-DAD-MSⁿ. Tentative identification based on accurate mass and literature data.

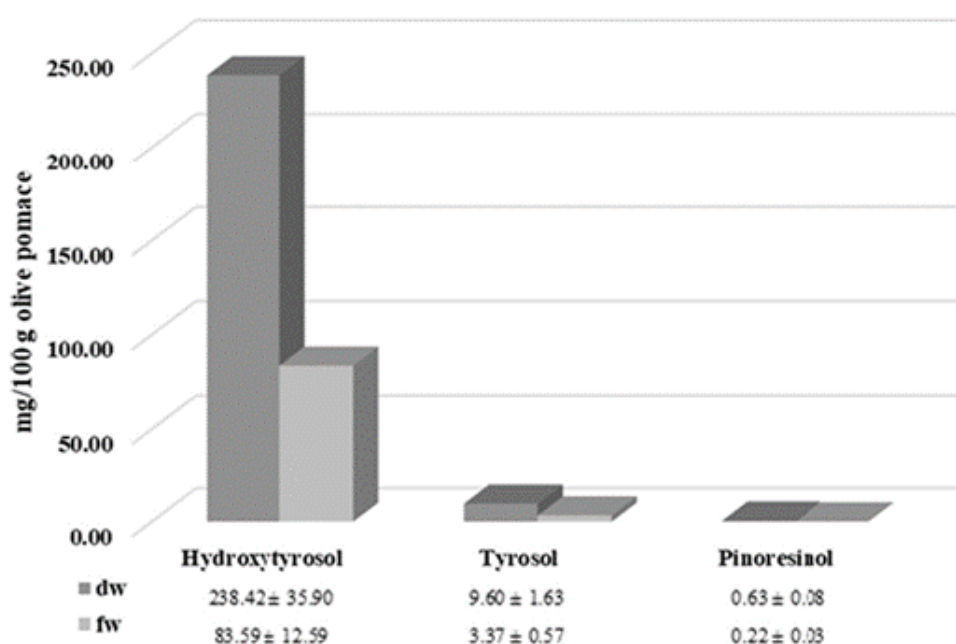


Figure 3. Hydroxytyrosol, tyrosol, and pinoresinol contents in olive pomace. Data presented as mean ± standard deviation. dw, dry weight; fw, fresh weight.

Two minor compounds were also quantified: tyrosol and pinoresinol. Phenolics such as verbascoside, caffeic acid, and tyrosol have been associated with the prevention of cardiovascular diseases due to the capacity to inhibit low-density lipoproteins oxidation or platelet aggregation. Particularly, cardio and neuro protective effects, anti-osteoporosis, anti-inflammatory, anti-diabetic capacities have been associated to tyrosol (Chandramohan et al., 2015; Loru et al., 2009). Promising but still ongoing studies about the benefits of dietary lignans, as pinoresinol, have been reported (Bendini et al., 2007; Brenes et al., 2000; Figueiredo- González et al., 2018; López-Biedma et al., 2016). Also, this compound has been proposed as a discriminant marker of olive varieties, namely Picual (Becerra-Herrera et al., 2018).

A MMM ultrasonic technique and a conventional solid-liquid extraction were used to extract the antioxidants from olive pomace (Table 1). In a conventional solid-liquid extraction, the soluble components are removed from solids using a solvent. For that, the extraction material and the solvent are well mixed during a long period of time, using or not temperature to enhance the extraction yield. This methodology implies fully agitation, long surface contact and stable temperatures. Conventional solid-liquid extraction presents some limitations because it is time and energy consuming and usually requires high amounts of solvents (Costa et al., 2014; Da Porto et al., 2013).

In turn, the MMM technique produces high intensity multimode vibrations which are uniform and repeatable. Therefore, a homogeneous distribution of cavitation and acoustical activities in the system are achieved, with avoidance of stationary and standing waves. The solution is fully agitated, and the sample is fragmented (Puga et al., 2017). The ultrasound parameters used in this study are described in Table 1. In order to assess and compare the efficacy of both extraction methodologies, the total phenolics content and the antioxidant activity of the extracts were evaluated. Figure 4 details the total phenolics content of extracts A, B, C, and D prepared with different conditions (Table 1).

Likewise, antioxidant activity evaluated by FRAP and DPPH[•] scavenging ability assays are also presented. Figure 4 shows that the MMM technique allowed a higher recovery of phenolic compounds when compared to the conventional extraction (60 min, 256 µg GAE/mL). Indeed, with the MMM technique, also using water as solvent and an input electric power of 160 W, it was possible to increase significantly ($p < 0.05$) the yield of antioxidants extraction in only 5 min (extract C, 402 µg GAE/mL). Within the experimental MMM technique conditions, the extract C was that with a higher extraction of phenolic compounds. Indeed, considering that for extract B the extraction time was increased, a higher total phenolic content was expected in this extract. On the contrary, the extended time (10 min) presented a detrimental impact on the compounds maybe due to the increase of the temperature of the medium during the extraction.

Figure 5 shows the increase of temperature with the extension of time in the MMM technique. This study is in agreement with other works that showed that high temperatures of extraction may reduce the antioxidant activity of the olive cake and particular phenolic compounds as flavonoids (Aliakbarian et al., 2011; Uribe et al., 2013).

Also, in other matrices, the impact of the temperature in antioxidants has been described (Costa et al., 2014; Sasidharan and Menon, 2011). Based on these results, in this study, the MMM technique with an input electric power of 160 W, a frequency of 20 KHz, during 5 min was the selected optimal extraction method. These conditions allowed the medium to reach ≈ 75 °C. The FRAP and DPPH[•] inhibition values are in total agreement with the total phenolics content.

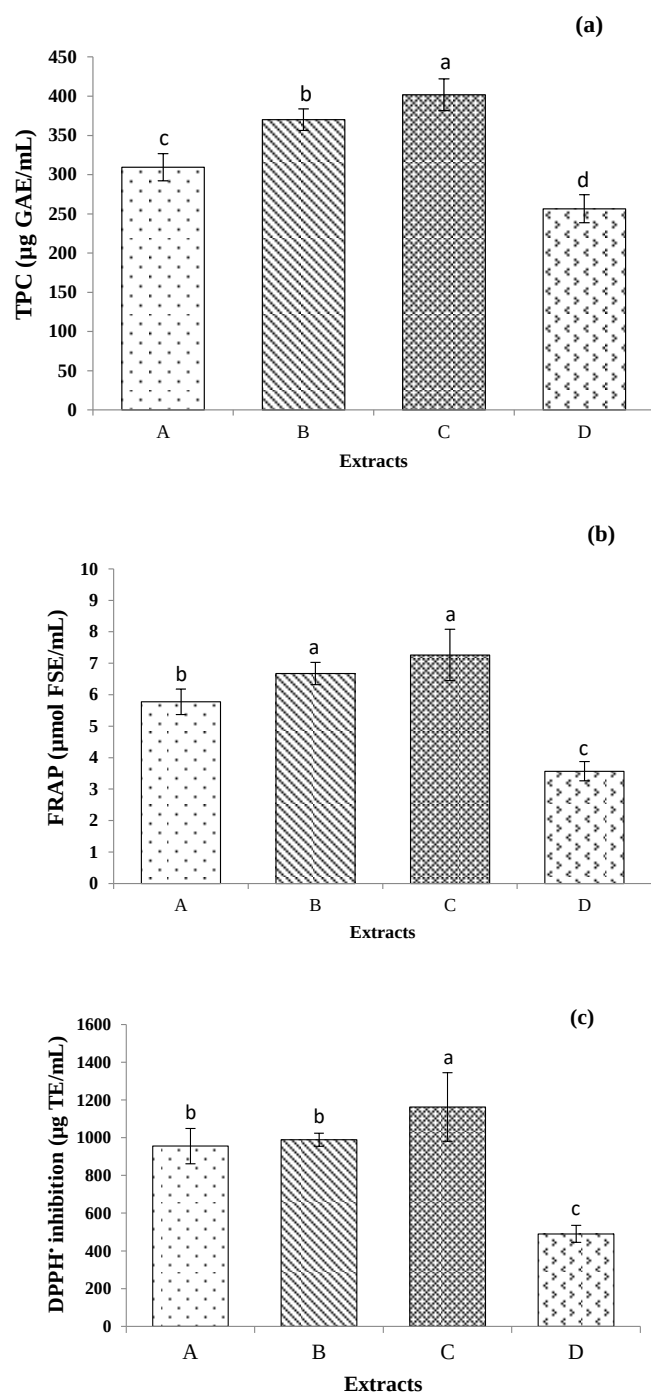


Figure 4. Total phenolics content and antioxidant activity of the different extracts: (a) TPC - total phenolic compounds; (b) FRAP - ferric reducing antioxidant power; (c) DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) scavenging ability. A, B, C, are extracts obtained by MMM technique and D expresses the results of conventional method. Data presented as mean $\pm$ standard deviation. Different letters within each graph represent significant differences ($p < 0.05$) between extracts. GAE, gallic acid equivalents; FSE, ferrous sulfate equivalents; TE, trolox equivalents.

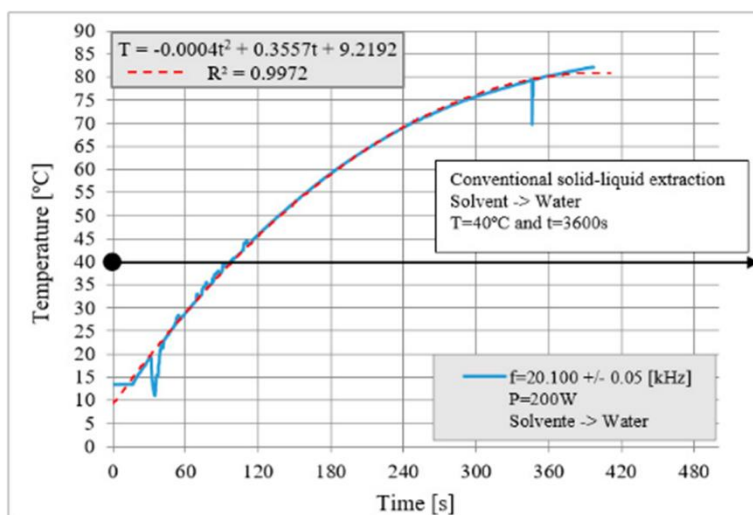


Figure 5. Temperature evolution in the Multi-frequency Multimode Modulated ultrasonic technique.

Significantly high correlations ($r > 0.872$) were obtained between the parameters analyzed, showing that the phenolic compounds present in the extract are responsible for the antioxidant activity presented (Table 4).

Additionally, compared to a conventional solid-liquid method of extraction, the MMM vibration process increased the amount of antioxidant compounds extracted from olive pomace, in a shorter period of time. Therefore, this process seems to be a very effective technique to extract antioxidants from olive pomace.

Attending to the profile and content of the bioactive compounds present either in lipid fraction or in the aqueous fraction of the olive pomace, this residue is an exploitable source to recover bioactive compounds.

Table 4. Pearson correlation coefficients (r) between antioxidant parameters.

	TPC	FRAP	DPPH• inhibition
TPC	1	0.930**	0.872**
FRAP	0.930**	1	0.946**
DPPH• inhibition	0.872**	0.946**	1

**Significant correlation at $p < 0.01$. TPC, total phenolic compounds; FRAP, ferric reducing antioxidant power; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical.

4. Conclusions

Olive oil production is increasing worldwide. A huge amount of by-products is consequently produced, being olive pomace the most representative one. This is an emergent problem to sustainable agriculture due to the olive pomace environmental burden. On the other hand, olive pomace is a rich source of bioactive compounds. By this way, there is a growing concern on finding effective ways to extract, isolate and use the bioactive compounds for added-value applications.

This study presents a comprehensive analysis of the chemical composition of olive pomace. The first part of this research pointed out olive pomace as a great source of lipid bioactive compounds whereas the second one presents an effective method to recover water-soluble antioxidants. Overall, the lipid fraction of the olive pomace is a source of antioxidants such as vitamin E, namely its more biologically active form: α -tocopherol; in addition, the high MUFA content of this oil is favourable to further applications in food and cosmetics formulations due to its higher oxidative stability compared with other PUFA-rich oils. The phenolics analysis showed that the compounds present in the aqueous fraction are mostly secoiridoids and derivatives. Nevertheless, hydroxytyrol is the major phenolic compound present (approximately 54% of the total phenolic compounds). Due to the high antioxidant activity of hydroxytyrosol, it is possible to foresee innovative applications as a natural additive/ingredient in food and cosmetics.

References

- Aliakbarian, B., Casazza, A.A., Perego, P., 2011. Valorization of olive oil solid waste using high pressure–high temperature reactor. *Food Chem.* 128, 704–710.
- Allalout, A., Krichène, D., Methenni, K., Taamalli, A., Oueslati, I., Daoud, D., Zarrouket, M., 2009. Characterization of virgin olive oil from super intensive Spanish and Greek varieties grown in northern Tunisia. *Sci. Hortic.* 120, 77–83.
- Alves, R., Casal, S., Oliveira, M.B.P.P., 2009. Determination of vitamin E in coffee beans by HPLC using a micro-extraction method. *Food Sci. Technol. Int.* 15, 57–63.
- AOAC, 2012. Official Methods of Analysis. Association of Analytical Communities, USA.
- Araújo, M., Pimentel, F.B., Alves, R.C., Oliveira, M.B.P.P., 2015. Phenolic compounds from olive mill wastes: health effects, analytical approach and application as food antioxidants. *Trends Food Sci. Technol.* 45, 200–211.
- Banias, G., Achillas, C., Vlachokostas, C., Moussiopoulos, N., Stefanou, M., 2017. Environmental impacts in the life cycle of olive oil: a literature review. *J. Sci. Food Agric.* 97, 1686–1697.

- Becerra-Herrera, M., Vélez-Martín, A., Ramos-Merchante, A., Richter, P., Beltrán, R., Sayago, A., 2018. Characterization and evaluation of phenolic profiles and color as potential discriminating features among Spanish extra virgin olive oils with protected designation of origin. *Food Chem.* 241, 328–337.
- Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gómez-Caravaca, A., Segura-Carretero, A., Fernández-Gutiérrez, A., Lercker, G., 2007. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. *Molecules* 12, 1679–1719.
- Brenes, M., Hidalgo, F.J., García, A., Rios, J.J., García, P., Zamora, R., Garrido, A., 2000. Pinoresinol and 1-acetoxypinoresinol, two new phenolic compounds identified in olive oil. *J. Am. Oil Chem. Soc.* 77, 715–720.
- Chandramohan, R., Pari, L., Rathinam, A., Sheikh, B.A., 2015. Tyrosol, a phenolic compound, ameliorates hyperglycemia by regulating key enzymes of carbohydrate metabolism in streptozotocin induced diabetic rats. *Chem. Biol. Interact.* 229, 44–54.
- Chanioti, S., Tzia, C., 2017. Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: oil recovery, unsaponifiable matter, total phenol content and antioxidant activity. *LWT Food Sci. Technol.* 79, 178–189.
- Costa, A.S.G., Alves, R.C., Vinha, A.F., Barreira, S.V.P., Nunes, M.A., Cunha, L.M., Oliveira, M.B.P.P., 2014. Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. *Ind. Crop. Prod.* 53, 350–357.
- Costa, A.S.G., Alves, R.C., Vinha, A.F., Costa, E., Costa, C.S.G., Nunes, M.A., Almeida, A.A., Santos-Silva, A., Oliveira, M.B.P.P., 2017. Nutritional, chemical and antioxidant/pro- oxidant profiles of silverskin, a coffee roasting by-product. *Food Chem.* <https://doi.org/10.1016/j.foodchem.2017.03.106>.
- Da Porto, C., Porretto, E., Decorti, D., 2013. Comparison of ultrasound-assisted extraction with conventional extraction methods of oil and polyphenols from grape (*Vitis vinifera* L.) seeds. *Ultrason. Sonochem.* 20, 1076–1080.
- DellaGreca, M., Previtera, L., Temussi, F., Zarrelli, A., 2004. Low-molecular-weight components of olive oil mill waste-waters. *Phytochem. Anal.* 15, 184–188.
- DeLonge, M.S., Miles, A., Carlisle, L., 2016. Investing in the transition to sustainable agriculture. *Environ. Sci. Pol.* 55, 266–273.
- Dubois, V., Breton, S., Linder, M., Fanni, J., Parmentier, M., 2007. Fatty acid profiles of 80 vegetable oils with regard to their nutritional potential. *Eur. J. Lipid Sci. Technol.* 109, 710–732.

- Fiedor, J., Burda, K., 2014. Potential role of carotenoids as antioxidants in human health and disease. *Nutrients* 6, 466–488.
- Figueiredo-González, M., Reboredo-Rodríguez, P., González-Barreiro, C., Carrasco-Pancorbo, A., Simal-Gándara, J., Cancho-Grande, B., 2018. Nutraceutical potential of phenolics from ‘Brava’ and ‘Mansa’ extra-virgin olive oils on the inhibition of enzymes associated to neurodegenerative disorders in comparison with those of ‘Picual’ and ‘Cornicabra’. *Molecules* 23:722.
- Fu, S., Arráez-Roman, D., Segura-Carretero, A., Menéndez, J.A., Menéndez-Gutiérrez, M.P., Micol, V., Fernández-Gutiérrez, A., 2010. Qualitative screening of phenolic compounds in olive leaf extracts by hyphenated liquid chromatography and preliminary evaluation of cytotoxic activity against human breast cancer cells. *Anal. Bioanal. Chem.* 397, 643–654.
- Galanakis, C.M., 2012. Recovery of high added-value components from food wastes: conventional, emerging technologies and commercialized applications. *Trends Food Sci. Technol.* 26, 68–87.
- ISO 12966-2:2011 - Animal and Vegetable Fats and Oils - Gas Chromatography of Fatty Acid Methyl Esters - Part 2: Preparation of Methyl Esters of Fatty Acids.
- Klen, T.J., Vodopivec, B.M., 2011. Ultrasonic extraction of phenols from olive mill wastewater: comparison with conventional methods. *J. Agric. Food Chem.* 59, 12725–12731.
- Li, H., Fan, Y.W., Li, J., Tang, L., Hu, J.N., Deng, Z.Y., 2013. Evaluating and predicting the oxidative stability of vegetable oils with different fatty acid compositions. *J. Food Sci.* 78, 633–641.
- Lichtfouse, E., Navarrete, M., Debaeke, P., Souchère, V., Alberola, C., Ménassieu, J., 2009. Agronomy for sustainable agriculture: a review. In: Lichtfouse, E., Navarrete, M., Debaeke, P., Véronique, S., Alberola, C. (Eds.), *Sustainable Agriculture*. Springer Netherlands, Dordrecht, pp. 1–7.
- López-Biedma, A., Sánchez-Quesada, C., Beltrán, G., Delgado-Rodríguez, M., Gaforio, J.J., 2016. Phytoestrogen (+)-pinoresinol exerts antitumor activity in breast cancer cells with different oestrogen receptor statuses. *BMC Complement. Altern. Med.* 16, 350.
- Loru, D., Incani, A., Deiana, M., Corona, G., Atzeri, A., Melis, M., Rosa, A., Dessì, M.A., 2009. Protective effect of hydroxytyrosol and tyrosol against oxidative stress in kidney cells. *Toxicol. Ind. Health* 25, 301–310.
- Lozano-Sánchez, J., Giambanelli, E., Quirantes-Piné, R., Cerretani, L., Bendini, A., Segura-Carretero, A., Fernández-Gutiérrez, A., 2011. Wastes generated during the

- storage of extra virgin olive oil as a natural source of phenolic compounds. *J. Agric. Food Chem.* 59, 11491–11500.
- Malapert, A., Reboul, E., Loonis, M., Dangles, O., Tomao, V., 2018. Direct and rapid profiling of biophenols in olive pomace by UHPLC-DAD-MS. *Food Anal. Methods* 11, 1001–1010.
- Montenegro, L., Rapisarda, L., Ministeri, C., Puglisi, G., 2015. Effects of lipids and emulsifiers on the physicochemical and sensory properties of cosmetic emulsions containing vitamin E. *Cosmetics* 2, 35–47.
- Nunes, M.A., Pimentel, F.B., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P., 2016. Olive by-products for functional and food applications: challenging opportunities to face environmental constraints. *Innovative Food Sci. Emerg. Technol.* 35, 139–148.
- Obied, H.K., Bedgood, D.R., Prenzler, P.D., Robards, K., 2007. Chemical screening of olive biophenol extracts by hyphenated liquid chromatography. *Anal. Chim. Acta* 603, 176–189.
- Orsavova, J., Misurcova, L., Ambrozova, J., Vicha, R., Mlcek, J., 2015. Fatty acids composition of vegetable oils and its contribution to dietary energy intake and dependence of cardiovascular mortality on dietary intake of fatty acids. *Int. J. Mol. Sci.* 16, 12871–12890.
- Peralbo-Molina, Á., Priego-Capote, F., Luque de Castro, M.D., 2012. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography–tandem mass spectrometry with a quadrupole–quadrupole–time-of-flight mass detector. *J. Agric. Food Chem.* 60, 11542–11550.
- Pimentel, F.B., Alves, R.C., Costa, A.S.G., Fernandes, T.J.R., Torres, D., Almeida, M.F., Oliveira, M.B.P.P., 2014. Nutritional composition of low protein and phenylalanine-restricted dishes prepared for phenylketonuric patients. *LWT Food Sci. Technol.* 57, 283–289.
- Portarena, S., Baldacchini, C., Brugnoli, E., 2017. Geographical discrimination of extra-virgin olive oils from the Italian coasts by combining stable isotope data and carotenoid content within a multivariate analysis. *Food Chem.* 215, 1–6.
- Puga, H., Alves, R.C., Costa, A.S., Vinha, A.F., Oliveira, M.B.P.P., 2017. Multi-frequency multimode modulated technology as a clean, fast, and sustainable process to recover antioxidants from a coffee by-product. *J. Clean. Prod.* 168, 14–21.
- Rabasco Alvarez, A.M., González Rodríguez, M.L., 2000. Lipids in pharmaceutical and cosmetic preparations. *Grasas Aceites* 51, 1–23.
- Robles-Almazan, M., Pulido-Moran, M., Moreno-Fernandez, J., Ramirez-Tortosa, C., Rodriguez-Garcia, C., Quiles, J.L., Ramirez-Tortosa, M.C., 2018. Hydroxytyrosol: bio-availability, toxicity, and clinical applications. *Food Res. Int.* 105, 654–667.

- Rodrigues, F., Pimentel, F.B., Oliveira, M.B.P.P., 2015. Olive by-products: challenge application in cosmetic industry. *Ind. Crop. Prod.* 70, 116–124.
- Rodrigues, F., Nunes, M.A., Oliveira, M.B.P.P., 2017. Chapter 12 - applications of recovered bioactive compounds in cosmetics and health care products. In: Galanakis, C.M. (Ed.), *Olive Mill Waste - Recent Advances for Sustainable Management*. Academic Press, pp. 255–274.
- Roselló-Soto, E., Koubaa, M., Moubarik, A., Lopes, R.P., Saraiva, J.A., Boussetta, N., Grimi, N., Barba, F.J., 2015. Emerging opportunities for the effective valorization of wastes and by-products generated during olive oil production process: non-conventional methods for the recovery of high-added value compounds. *Trends Food Sci. Technol.* 45, 296–310.
- Rubio-Senent, F., Rodríguez-Gutiérrez, G., Lama-Muñoz, A., Fernández-Bolaños, J., 2012. New phenolic compounds hydrothermally extracted from the olive oil byproduct Alperujo and their antioxidative activities. *J. Agric. Food Chem.* 60, 1175–1186.
- Sasidharan, I., Menon, A.N., 2011. Effects of temperature and solvent on antioxidant properties of curry leaf (*Murraya koenigii* L.). *J. Food Sci. Technol.* 48, 366–370.
- Savarese, M., De Marco, E., Sacchi, R., 2007. Characterization of phenolic extracts from olives (*Olea europaea* cv. Pisciotana) by electrospray ionization mass spectrometry. *Food Chem.* 105, 761–770.
- Tontisirin, K., 2003. Chapter 2: methods of food analysis. *Food Energy: Methods of Analysis and Conversion Factors: Report of a Technical Workshop*, Rome, 3–6 December 2002. Food and Agriculture Organization of the United Nations, pp. 7–17.
- United Nations, 2017. World population prospects: the 2017 revision, key findings and advance tables. Working Paper No. ESA/P/WP/248. Department of Economic and Social Affairs, Population Division.
- Uribe, E., Lemus-Mondaca, R., Vega-Gálvez, A., López, L.A., Pereira, K., López, J., Ah-Hen, K., Di Scala, K., 2013. Quality characterization of waste olive cake during hot air drying: nutritional aspects and antioxidant activity. *Food Bioprocess Technol.* 6, 1207–1217.
- Uribe, E., Lemus-Mondaca, R., Vega-Gálvez, A., Zamorano, M., Quispe-Fuentes, I., Pasten, A., Di Scala, K., 2014. Influence of process temperature on drying kinetics, physico-chemical properties and antioxidant capacity of the olive-waste cake. *Food Chem.* 147, 170–176.
- Valta, K., Aggeli, E., Papadaskalopoulou, C., Panaretou, V., Sotiropoulos, A., Malamis, D., Moustakas, K., Haralambous, K.-J., 2015. Adding value to olive oil production

- through waste and wastewater treatment and valorisation: the case of Greece. *Waste Biomass Valoriz.* 6, 913–925.
- Wezel, A., Casagrande, M., Celette, F., Vian, J.-F., Ferrer, A., Peigné, J., 2014. Agroecological practices for sustainable agriculture. A review. *Agron. Sustain. Dev.* 34, 1–20.
- Yakhlef, W., Arhab, R., Romero, C., Brenes, M., de Castro, A., Medina, E., 2018. Phenolic composition and antimicrobial activity of Algerian olive products and by-products. *LWT Food Sci. Technol.* 93, 323–328.
- Yun, J.-M., Surh, J., 2012. Fatty acid composition as a predictor for the oxidation stability of Korean vegetable oils with or without induced oxidative stress. *Prev. Nutr. Food Sci.* 17, 158–165.

3.1.2 Olive pomace phenolics extraction: conventional vs emergent methodologies

^aM.A. Nunes, ^aR.C. Alves, ^aA.S.G. Costa, ^bH. Puga, ^aM.B.P.P. Oliveira

^aREQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy,
University of Porto, R. J. Viterbo, 4050-313 Porto, Portugal

^bCentre for Micro-Electromechanical Systems, University of Minho, 4800-058
Guimarães, Portugal

- Nunes, M.A., Alves, R.C., Costa, A.S.G., Puga, H., Oliveira, M.B.P.P. (2018). Olive pomace phenolics extraction: conventional vs emergent methodologies, in *Wastes solutions, treatments and opportunities II*. Editores: Cândida Vilarinho, Fernando Castro e Maria de Lurdes Lopes, CRC Press, Taylors & Francis. pp. 395–401. ISBN: 978-1-138-19669-8.

Abstract

Olive oil production has been steadily increasing. Along with the growth of this sector, a high rate of wastes is produced. Olive pomace presents high levels of phenolic compounds with potential applications in food, cosmetic and pharmaceutical industries. Sustainable and cost-effective technologies for bioactive compounds recovery are required. In this work, conventional extraction methodologies were compared with an ultrasound technique using a probe and only water as solvent. Total phenolics and antioxidant activity of the extracts were assessed.

A conventional solid-liquid extraction (hydroethanolic solvent, 40°C, 60 min) was the best procedure to recover phenolics compounds from pomace (487 µg GAE/ml). However, in only 5 min, the sonication probe allowed significantly higher yields of recovery compared to aqueous conventional extraction (60 min) (402 and 257 µg GAE/ml, respectively). Therefore, it seems to be a very promising technique to obtain antioxidants from olive pomace in an organic solvent-free medium.

1. Introduction

In Europe, for waste management, the Waste Framework Directive (2008/98/EC) is the core legislative act. Accordingly, it is emergent to avoid waste generation and to improve the use of wastes as resources (Directive 2008/98/EC, 2008). Green practices are mandatory to allow a sustainable growth of the agro-industry sector. The conversion of wastes into added-value products is a challenging task that can have a positive socio-economic impact and simultaneously reduce the environmental burden.

Olive oil production has been increasing worldwide over the last years. European Union leads the international market, producing ≈ 80 – 84% of the total olive oil. Olive oil has a great economic importance in Mediterranean countries and in new producing countries of America, Africa, and Australia. Nevertheless, as other agro-industries, olive oil production generates a large quantity of wastes, namely olive pomace (Nunes et al., 2016). The resulting wastes depend on the technique applied for extracting olive oil. The common systems used are pressing mills, two-phase and three-phase processes. In the two-phase process, unlike the three-phase system, no water is used. This means that both olive oil and a humid semi-solid pomace are obtained. Despite resulting from an eco-friendly system, olive pomace has a high-water content, which can difficult a proper storage. This semi-solid residue also includes skins, pulp and stones. After olive oil processing, olive pomace is discharged in large open-air containers (evaporation ponds) where it is stored through long periods until further transformation in olive pomace oil and a last final residue – extracted olive pomace (Fernández-Hernández et al., 2014). As the production of olive oil is increasing, the discharge of olive pomace is also escalating. Olive pomace oil industry partially solves this problem. Still, a final residue exists at the end of the processing that usually is used as fuel with a minimal added-value (Roselló-Soto et al., 2015).

In olive pomace, a huge amount of phenolic compounds and fatty acids still remains. These bioactive compounds are phytotoxic. Despite some countries allow land spreading, it must be done under a tight control (Di Bene et al., 2013). Although those pomace compounds are a hazard for the environment, they present benefic effects for human health (Nunes et al., 2016). This feature anticipates new opportunities to recover functional ingredients for further applications, namely for cosmetics and food industry.

The recovery of bioactive compounds from olive by-products, intending innovative applications and enhanced technological functions have been strongly explored over the last years. The added-value of these agro-industry by-products has been recognized, as well as the benefits of their bioactive compounds (Galanakis, 2012). The extraordinary

content in phenolic compounds, such as phenolic acids and alcohols, secoiridoids, lignans and flavones, stands out olive pomace as a promissory valuable by-product (Mirabella et al., 2014).

Several conventional (e.g., solvents, heat and grinding) and non-conventional methods (e.g., supercritical fluid extraction, pulsed electric fields and microwaves) have been studied in order to achieve maximum recoveries of olive pomace bioactive compounds (Roselló-Soto et al., 2015). Nevertheless, an emergent question always rises: how to obtain, simultaneously, maximum yields of extraction, at low-cost, using eco-friendly technologies and in a minimum period of time? The development of sustainable methodologies for the recovery of bioactive compounds from agro-industrial by-products is emergent. Indeed, it is important to find simple, fast, cost-effective and efficient processes to achieve that aim. Extraction methodologies using solvents, microwave assisted, or supercritical fluids have been used consistently for bioactive compounds extraction (Galanakis, 2012).

The useful effects of ultrasonic liquid processing are related to efficient transformation of electric energy (using an ultrasonic power supply or generator) into mechanical vibration by means of an ultrasonic transducer (operating in resonance), which is in the last step radiated to a medium in form of acoustic intensity. During this process, it is necessary to minimize thermal losses (in the ultrasonic generator, piezoelectric transducer and acoustic radiator) and maximize the real power load of acoustic energy in the water medium. Thus, with the application of the acoustic intensity, it is created kinetic energy in the liquid, which promotes the homogenization of the mixture. Moreover, the increasing of the medium temperature (related to bubbles collapse) promotes and accelerates the extraction.

This work aimed to explore and compare conventional methods of extraction (solid-liquid extraction with different solvents) with an emerging processing (an ultrasonic vibration technique using a probe and only water as extractor) in order to achieve an optimal extraction of olive pomace antioxidants.

2. Materials and methods

2.1 Reagents and standards

Gallic acid, heptahydrate ferrous sulfate, trolox, Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ferric chloride, 2,4,6-tripyridyl-striazine (TPTZ), and sodium acetate were all acquired from Sigma-Aldrich (St. Louis, USA). Anhydrous

sodium carbonate and absolute ethanol were purchased from Merck (Darmstadt, Germany). All reagents were of analytical grade.

2.2 Sample and sample preparation

Olive pomace was obtained from an olive oil extraction unit in the Protected Designation of Origin Trás-Os-Montes (Portugal). In order to preserve olive pomace, samples were frozen (-80 °C) and lyophilized (Telstar Cryodos-80 Terrassa, Barcelona).

2.3 Nutritional analysis

Moisture was determined using an infrared balance (Scaltec model SMO01, Scaltec Instruments, Heiligenstadt, Germany). All the analysis (protein, ash and fat contents) were performed according to AOAC (AOAC, 2012). Total carbohydrates were calculated by difference.

2.4 Extracts preparation

2.4.1 Conventional extraction

A classic solid-liquid extraction was performed using three different solvents: i) water, ii) water: ethanol (1:1, v/v), and iii) ethanol. Extracts were prepared using a rigorously weighted amount (1 g) of lyophilized sample and 50 ml of solvent. The bioactive compounds were extracted with magnetic stirring at 40 °C, for 1 h. Then, they were centrifuged (3000 rpm; 10 min) and filtered (Whatman No. 4).

2.4.2 Ultrasonic extraction

An ultrasound device (Figure 1(a)) was used for extraction, comprising a piezoelectric transducer, an acoustic radiator, a generator, a CompactDAQ for temperature acquisition, a type S thermocouple and a Windows compatible software to control processing. The whole ultrasonic device was previously optimized through Finite Element Model simulation (Fig. 1(b)) based in pre-established mathematical models. Parameters of extraction are described in Table 1.

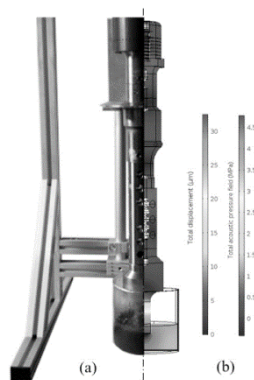


Figure 1. Ultrasonic extraction. (a) Experimental apparatus; (b) Numerical results of acoustic pressure in the sonicated medium (input electrical power of 160 W and $f_0=20.01 \pm 0.03$ kHz).

2.5 Total phenolics content

The total amount of phenolic compounds was determined by spectrophotometry (BioTek Instruments, Inc) based on Costa et al. (2014), with some modifications. Briefly, 30 μ l of each extract were mixed with 150 μ l of Folin-Ciocalteu reagent (1:10) and 120 μ l of a sodium carbonate solution (7.5 g/100 ml). The mixture was incubated at 45 °C, protected from light, during 15 min. After 30 min at room temperature, the absorbance was measured at 765 nm. A calibration curve prepared with gallic acid was used (5–100 μ g/ml; $R^2=0.9996$). Total phenolics were expressed as μ g of gallic acid equivalents (GAE)/ml of extract. The assay was performed in triplicate.

2.6 Ferric reducing antioxidant power (FRAP)

The FRAP assay was carried out according to Benzie and Strain (Benzie and Strain, 1996) with minor modifications (Costa et al., *in press*). In short, an aliquot of 35 μ l of extract were mixed with 265 μ l of the FRAP solution (containing 0.3 M acetate buffer, 10 mM TPTZ solution and 20 mM of ferric chloride). The mixture was kept for 30 min at 37 °C protected from light. Absorbance was measured at 595 nm. A calibration curve was prepared with ferrous sulfate (25–500 μ mol/l; $R^2=1$), and the ferric reducing antioxidant power was expressed as μ mol of ferrous sulfate equivalents (FSE)/ml of extract. The assay was performed in triplicate.

2.7 DPPH• (2,2-diphenyl-1-picrylhydrazyl) scavenging activity

The radical scavenging ability of the different extracts was evaluated according to Costa et al. (2014), with slight modifications. Briefly, the reaction was initiated by transferring 30 μ l of a diluted extract to 270 μ l of a freshly prepared DPPH• solution (6.0×10^{-5} mol/l

in ethanol). The decrease of the absorbance was measured in equal time intervals (2 min) at 515 nm, in order to observe the kinetic reaction. The reaction endpoint was attained in 30 min. A calibration curve was prepared with trolox (2.5–175 µg/ml; $R^2=0.9984$) and the scavenging activity was expressed as µg of trolox equivalents (TE)/ml of extract. The assay was performed in triplicate.

2.8 Statistical analysis

Statistical analysis was performed using IBM SPSS v. 20 (IBM Corp., Armonk, 241 NY, USA). Data were expressed as mean $\pm$ standard deviation ($n=9$). The Shapiro-Wilk test was used to evaluate data normality. The one-way ANOVA was used to assess significant differences between samples, followed by Tukey's HSD or Dunnett T3 post-hoc test (selected based on the equality of the variances) to make pairwise comparisons between means. The level of significance for all hypothesis tests (p) was 0.05. Correlations among the different parameters analyzed were achieved by Pearson correlation coefficients (r) at a significance level of 99%.

3. Results and discussion

The olive pomace analyzed in this study was composed, in a dry weight basis, by 3% of moisture, 7% of protein, 1% of ash, 6% of lipids and 83% of total carbohydrates (in which total fiber is included). Conventional solid-liquid extractions using different solvents were performed and compared with aqueous extraction using a sonication probe (Table 1).

The efficacy of extraction was assessed by analyzing the total phenolics content of the extracts, as well as their antioxidant activity. This was determined using two complementary methods. In the DPPH• scavenging assay, the radical can be neutralized by both direct reduction (via electron transfer) or by radical quenching (via H atom transfer). In the FRAP assay, the reduction of Fe^{3+} -TPTZ to a colored product occurs, detecting natural compounds with redox potentials lower than 0.7 V (that of Fe^{3+} -TPTZ), but not compounds that act by radical quenching (H transfer) since only an electron transfer mechanism occurs. Nevertheless, both assays are reasonable screening methods to evaluate the potential ability of natural compounds to maintain the redox status in cells or tissues (Costa et al., in press). Table 1 presents the antioxidant profile of the olive pomace extracts obtained with the different experimental conditions.

As it can be clearly observed in Table 1, the conventional solid-liquid extraction using a hydroethanolic solvent (1:1), at 40 °C, for 60 min, was the best procedure to recover antioxidants from olive pomace. Indeed, significantly higher values of total phenolics

and antioxidant activity ($p<0.05$) were achieved, compared to all the remaining tested procedures. This is certainly due to the type of phenolic compounds present in olive pomace (such as hydroxytyrosol, tyrosol, oleuropein, apigenin, luteolin, among others) (Araújo et al., 2015), which together are more extractable with an intermediate polarity solvent. Nevertheless, comparing the aqueous extracts, in only 5 min (using an input electric power of 160 W), the sonication probe allowed a significantly higher ($p<0.05$) yield of extraction ($\approx$ more 60% of total phenolics) than that obtained with the aqueous conventional method (60 min). An extension of this extraction to 10 min showed to be detrimental probably due to the temperature increase of the medium (Fig. 2), which can lead to the degradation of the compounds. In fact, Uribe et al. (2013) also reported a loss of total phenolic compounds, especially above 70 °C, in olive pomace submitted at air-drying temperature. These authors concluded that air drying temperatures affected antioxidant activity (Uribe et al., 2013) and our results are in agreement to those conclusions. Based on that, less extraction time and a higher input electric power showed to be more adequate to extract the natural antioxidants of this matrix.

The composition of the extracts varied widely according to extraction procedure used. Nevertheless, significantly high correlations ($p<0.01$) were obtained between the different parameters analyzed (Table 2), namely between 0.828 and 0.963, showing clearly that phenolic compounds of olive pomace are the chemical constituents responsible for the sample antioxidant activity.

Several factors have impact on the olive pomace phenolic compounds. Primarily, olive pomace depends on the phenolic profile of olives, which, in turn, varies greatly with their variety, stage of ripeness, storage time, geographical origin and edaphoclimatic conditions; secondly, the olive oil processing technique will also influence the pomace composition. In a last instance, sample preparation and extraction methodologies (e.g., time, temperature, solvent) can lead to the great range of results found in scientific literature (Uribe et al., 2013). Nonetheless, a review from Dermeche et al. (2013) showed that total phenolics varied between 0.4 and 2.43%, which is in total agreement with the results of this work. In addition, an optimized methodology for phenolics recovery which studied the effect of a multistep extraction, including the time-temperature binomial, and a solvents sequence is also in line with the results here presented (Alu'datt et al., 2010).

Table 1. Total phenolics content and antioxidant activity of differently prepared extracts.

	Extract	Solvent	Frequency (kHz)	Input electric power (W)	Time (min)	Total phenolics $\mu\text{g GAE/ml}$	FRAP $\mu\text{mol/ml FSE}$	DPPH• inhibition $\mu\text{g TE/ml}$
Sonication	1	Water	20.05 ± 0.03	90	10	$309.3 \pm 17.3^{\text{d}}$	$5.78 \pm 0.41^{\text{c}}$	$955.4 \pm 93.2^{\text{c}}$
	2	Water	20.00 ± 0.03	160	10	$370.2 \pm 13.7^{\text{c}}$	$6.68 \pm 0.36^{\text{b}}$	$989.1 \pm 34.3^{\text{c}}$
	3	Water	20.01 ± 0.03	160	5	$401.9 \pm 20.2^{\text{b}}$	$7.26 \pm 0.82^{\text{b}}$	$1162.8 \pm 181.5^{\text{b}}$
Conventional	4	Water	n/a	n/a	60	$256.4 \pm 17.8^{\text{e}}$	$3.57 \pm 0.31^{\text{d}}$	$490.0 \pm 44.8^{\text{d}}$
	5	Water:Ethanol (1:1)	n/a	n/a	60	$487.2 \pm 22.8^{\text{a}}$	$9.40 \pm 0.38^{\text{a}}$	$1403.1 \pm 110.7^{\text{a}}$
	6	Ethanol	n/a	n/a	60	$307.4 \pm 13.2^{\text{d}}$	$5.57 \pm 0.35^{\text{c}}$	$1070.6 \pm 102.6^{\text{bc}}$

Different letters within each column represent significant differences at $p < 0.05$.

n/a, not applicable; GAE, gallic acid equivalents; FRAP, ferric reducing antioxidant power; FSE, ferrous sulfate equivalents; DPPH•, (2,2-diphenyl-1-picrylhydrazyl); TE, trolox equivalents.

Table 2. Pearson correlations coefficients (r) between the analyzed parameters.

	Total phenolics	FRAP	DPPH• inhibition
Total phenolics	1	0.963**	0.828**
FRAP	0.963**	1	0.892**
DPPH• inhibition	0.828**	0.892**	1

**Significant correlation at $p < 0.01$. FRAP, ferric reducing antioxidant power; DPPH•, 2,2-diphenyl-1-picrylhydrazyl.

Ultrasonic extraction seems, thus, to be a very promising technique to extract antioxidants in an organic solvent-free medium although, in this case, optimization should be further attempted in order to achieve the extraction yields obtained with the hydroalcoholic solvent.

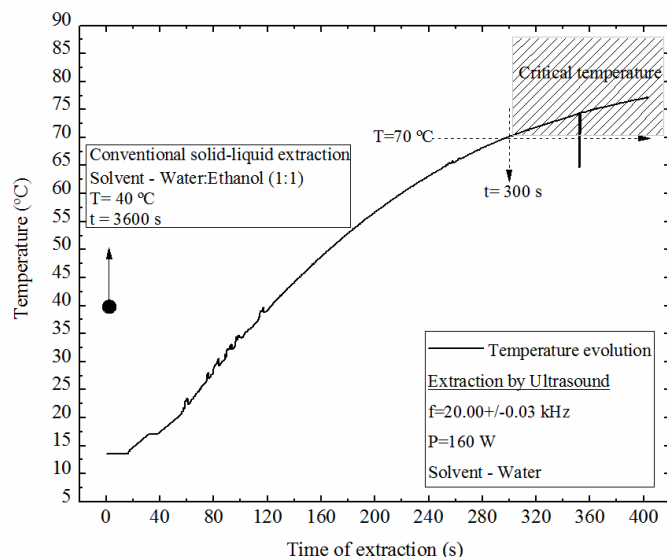


Figure 2. Temperature increase during ultrasonic olive pomace antioxidants extraction.

4. Conclusions

Due to the olive pomace extracts richness in bioactive compounds, its valorization should be promoted. This study was conducted in order to evaluate the efficacy of different procedures to extract phenolic compounds from this by-product, contributing to its valorization and recycling (in a circular economy perspective), foreseeing health benefits since its extracts can be further used in food or cosmetic industries. New approaches to recover and stabilize bioactive compounds from extracts as well as new industrial applications are also promising.

High amounts of phenolic compounds were quantified in olive pomace, namely between 1.3 and 2.4 g GAE/100 g of pomace, depending on the extraction method used. Moreover, this work highlights the potential of ultrasound as a green extraction technique that requires less energy and time, without using organic solvents. The results of this work show that this type of technology can be of high interest to recover antioxidants from natural products, in a clean and efficient way. Nevertheless, optimization can still be performed in order to achieve the highest extraction yield obtained with the hydroalcoholic solvent.

References

- AOAC, 2012. Official methods of analysis. Association of Official Analytical Chemists, Arlington VA, USA.
- Alu'datt, M.H., Alli, I., Ereifej, K., Alhamad, M., Al-Tawaha, A.R., & Rababah, T., 2010. Optimisation, characterisation and quantification of phenolic compounds in olive cake. *Food Chemistry* 123, 117-122.
- Araújo, M., Pimentel, F. B., Alves, R. C. & Oliveira, M. B. P. P. 2015. Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. *Trends in Food Science & Technology* 45: 200-211.
- Benzie, I. F. F. & Strain, J. J. 1996. The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry* 239: 70-76.
- Costa, A. S. G., Alves, R. C., Vinha, A. F., Barreira, S. V. P., Nunes, M. A., Cunha, L. M. & Oliveira, M. B. P. P. 2014. Optimization of antioxidants extraction from coffee silverskin, a roasting by-product, having in view a sustainable process. *Industrial Crops and Products* 53: 350-357.
- Costa, A. S. G., Alves, R. C., Vinha, A. F., Costa, E., Costa, C. S. G., Nunes, M. A., Almeida, A. A., Santos-Silva, A. & Oliveira, M. B. P. P. Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chemistry*. doi: 10.1016/j.foodchem.2017.03.106
- Dermeche, S., Nadour, M., Larroche, C., Moulti-Mati, F., & Michaud, P. 2013. Olive mill wastes: Biochemical characterizations and valorization strategies. *Process Biochemistry* 48: 1532-1552.
- Di Bene, C., Pellegrino, E., Debolini, M., Silvestri, N. & Bonari, E. 2013. Short- and long-term effects of olive mill wastewater land spreading on soil chemical and biological properties. *Soil Biology and Biochemistry* 56: 21-30.
- Directive 2008/98/EC of the European Parliament and of the Council of 19 November 2008 on waste and repealing certain Directives. *Official Journal of the European Union* 312: 1-9.
- Fernández-Hernández, A., Roig, A., Serramiá, N., Civantos, C. G.-O. & Sánchez-Monedero, M. A. 2014. Application of compost of two-phase olive mill waste on olive grove: Effects on soil, olive fruit and olive oil quality. *Waste Management* 34: 1139-1147.
- Galanakis, C. M. 2012. Recovery of high added-value components from food wastes: Conventional, emerging technologies and commercialized applications. *Trends in Food Science & Technology* 26: 68-87.

- Mirabella, N., Catellani, V. & Sala, S. 2014. Current options for the valorization of food manufacturing waste: a review. *Journal of Cleaner Production* 65: 28-41.
- Nunes, M. A., Pimentel, F. B., Costa, A. S. G., Alves, R. C. & Oliveira, M. B. P. P. 2016. Olive by-products for functional and food applications: Challenging opportunities to face environmental constraints. *Innovative Food Science & Emerging Technologies* 35: 139-148.
- Roselló-Soto, E., Koubaa, M., Moubarik, A., Lopes, R. P., Saraiva, J. A., Bousseta, N., Grimi, N. & Barba, F. J. 2015. Emerging opportunities for the effective valorization of wastes and by-products generated during olive oil production process: Non-conventional methods for the recovery of high-added value compounds. *Trends in Food Science & Technology* 45: 296-310.
- Uribe, E., Lemus-Mondaca, R., Vega-Gálvez, A., López, L. A., Pereira, K., López, J., Ah-Hen, K. & Di Scala, K. 2013. Quality Characterization of Waste Olive Cake During Hot Air Drying: Nutritional Aspects and Antioxidant Activity. *Food and Bioprocess Technology* 6: 1207-1217.

This page was intentionally left in blank.

3.1.3 Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration

^aM. Antónia Nunes, ^bSylwin Pawlowski, ^aAnabela S.G. Costa, ^aRita C. Alves,

^aM. Beatriz P.P. Oliveira, ^bSvetlozar Velizarov

^aLAQV/REQUIMTE, Department of Chemical Sciences, Faculty of Pharmacy,
Universidade do Porto, 4050-313 Porto, Portugal

^bLAQV/REQUIMTE, Department of Chemistry, Faculty of Science and Technology,
Universidade NOVA de Lisboa, 2829-516 Caparica, Portugal

- Nunes, M.A., Pawlowski, S., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P., Velizarov, S. (2019). Valorization of olive pomace by a green integrated approach applying sustainable extraction and membrane-assisted concentration. *Science of the Total Environment*, 652, 40-47.

Abstract

Olive pomace is a semi-solid paste resulting from the two-phase olive oil production, being the most significant waste generated by this agro-industry. Olive pomace is reported as an environmental hazard due to its high content in phenolic compounds (phytotoxic). Nevertheless, these compounds, when recovered, can have impactful actions in different human physiological conditions, namely, skin protection, dysfunction treatment or diseases prevention. Therefore, their recovery from olive pomace is crucial for environmental and economical sustainability, without forgetting the functional challenge.

In a previous work, lipid and aqueous fractions of olive pomace were studied regarding its major bioactive compounds. The present research aims to describe an environmentally friendly integrated approach to extract and concentrate (by a pressure-driven membrane processing) the phytotoxic compounds of olive pomace.

Three types of polymeric composite membranes (NF90, NF270 and BW30) were tested. The composition of the resulting streams (permeates and concentrates) were compared and the process efficiency assessed based on: (1) antioxidant activity and total phenolic

and flavonoid contents; (2) inorganic elemental composition (by Inductively Coupled Plasma Atomic Emission Spectroscopy); (3) pH, conductivity and total organic carbon; and (4) permeate flux, membranes' apparent target solutes rejection and fouling index. The BW30 membrane presented the lowest fouling index and was the most effective for extracts concentration, with no phenolic compounds in the permeates, preventing completely the loss of such compounds.

1. Introduction

Over the last years, olive oil consumption increased worldwide, and its global production doubled between 1990 and 2014, surpassing 3 million tons (Mattas and Tsakiridou, 2017). The majority of producers use the two-phase olive oil system, leading to less wastewater production and considered more eco-friendly than other processing options (Nunes et al., 2016). The main waste obtained in this processing method is olive pomace (OP). OP is a semi-solid paste, mainly composed by fragments of olive skin, pulp and stone (solid phase), water and oil (Artajo et al., 2007; Nunes et al., 2016; Peralbo-Molina et al., 2012). As only a minimal phenolic fraction passes into the oil phase, the majority of phenolics ($\approx 98\%$), namely hydroxytyrosol and its derivatives (hydrophilic compounds) remain in OP (Reboredo-Rodríguez et al., 2017; Rodis et al., 2002). Due to the great amount of phenolics, OP can have a high polluting organic load and a phytotoxic effect becoming a significant environmental hazard (Nunes et al., 2016). On the other hand, OP may be a natural and low-cost source of phenolic compounds, with beneficial human physiological actions (Aliakbarian et al., 2017). This is the case of hydroxytyrosol, oleuropein and oleocanthal related to the prevention of cardiovascular and neurodegenerative diseases and reported as anti-tumoral, antimicrobial and antioxidant compounds (Parkinson and Cicerale, 2016). Their use as active ingredients has been challenging for food and cosmetic industries (Nunes et al., 2016; Rodrigues et al., 2015).

New technologies have been explored, such as ultrasound-assisted extraction or pulsed electric field, in order to attain maximum extraction yields of bioactive compounds. Nonetheless, in an extraction process selection, the economic and environmental cost-benefit analysis of each method ought to be considered (Galanakis, 2013).

The “bioeconomy” concept entails the recovery of bioactive compounds from natural renewable resources in order to develop added-value products (Ravindran and Jaiswal, 2016). The most common by-products used for bioactive compounds extraction are generated from fruits and vegetables processing (Galanakis, 2012). Generally, five fundamental steps are carried out in order to recover the high added-value compounds present in agro-industrial by-products (Galanakis, 2012). The recovery process often initiates with a macroscopic pre-treatment that can include, for instance, a mechanical pressing step and/or centrifugation. Then, it is followed by a macro and micro-molecules separation, extraction, and isolation and purification. Finally, the fifth stage is the product preparation (e.g., encapsulation or drying). Although this step is not truly comprised in the recovery process, it is essential and should be taken into account, since

it allows the preservation of compounds functional properties (Galanakis, 2012). Considering the 5-stages of the recover process, the membrane technologies might be included in the first, second and fourth stages. Indeed, the removal of suspended solids, the concentration of macromolecules, as well as clarification processes, can be achieved using a proper membranes system (Galanakis et al., 2014). In fact, membrane separation processes are considered clean and economically feasible for concentration, purification and valorisation of valuable products in food (Syed et al., 2017), beverages (Pan et al., 2013) and biotechnological industries (Monte et al., 2018; Sosa et al., 2016; Velizarov and Crespo, 2010). However, there are a limited number of studies regarding the use of membrane technologies in olive oil industry. Besides, they are mostly focused on olive mill wastewater purification and not on the OP bioactive compounds recovery (Ochando-Pulido and Martinez-Ferez, 2015). Depending on the process used - ultrafiltration (UF) (Galanakis et al., 2010; Cassano et al., 2013), nanofiltration (NF) (Cassano et al., 2013; Coskun et al., 2010; Galanakis et al., 2010) or reverse osmosis (RO) (Coskun et al., 2010) - valuable antioxidant compounds can be kept in the retentate (RO, NF) or distributed between the permeate and the retentate streams (UF).

In a recent study, the distribution of OP bioactive compounds between the oil and aqueous fractions was assessed (Nunes et al., 2018). As a sequence, and considering the promising results obtained, the aim of the present work was to develop an integrated approach to extract (by an environmentally friendly process) and concentrate (by a pressure-driven membrane processing) the hydrophilic bioactive compounds of OP. Indeed, the obtained extracts were processed using different NF and RO membranes in order to evaluate and compare their potential to obtain high added-value concentrated extracts. Taking into account their composition, several applications for food and cosmetic industries can be highlighted. The recapture and concentration of OP valuable compounds is, by this way, presented to obtain functional compounds focusing the R&D of new products. Additionally, a possible use of the obtained permeate streams as irrigation water is proposed.

2. Material and methods

2.1 Reagents and standards

Gallic acid, epicatechin, heptahydrate ferrous sulfate, trolox, Folin- Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl radical, ferric chloride, 2,4,6-tripyridyl-triazine, sodium nitrite, aluminium chloride, sodium acetate, petroleum ether were all acquired from Sigma-Aldrich (St. Louis, USA). Absolute ethanol, sodium carbonate decahydrate, sodium hydroxide, Kjeldahl tablets catalyst, sulfuric acid concentrated, n-hexane, and

sodium sulfate anhydrous were purchased from Merck (Darmstadt, Germany). Boric acid 4% was purchased from Panreac (Barcelona, Spain). All other reagents were of analytical grade.

2.2 Samples characterization and extracts preparation

Raw OP (about 5 kg) was collected in Alfândega da Fé, Portugal, in an olive mill from the region of the Protected Designation of Origin of Trás-Os-Montes olive oil (41°19'53.9"N 6°58'19.4"W). The sample was obtained from an olive oil two-phase extraction system in the season 2015/2016 and composed by the following olive cultivars: Cobrançosa, Madural, Verdeal Transmontana, and Cordovil. After collection, it was homogenized and grouped in batches. Some of them were frozen at $-80\text{ }^{\circ}\text{C}$ and freeze-dried for further analysis. Simultaneously, three batches of 100 g each of raw OP (fresh OP) were separated and subjected to a hydraulic press (Patent WO/2017/212450, 2017). Two different products were obtained: an olive fat-based mixture (water and oil) (Patent WO/2017/212450, 2017) and a pressed OP solid phase. The olive fat-based mixture was removed, conditioned, and used for further studies. The pressed OP was preserved at $-20\text{ }^{\circ}\text{C}$ until extraction of the remaining fraction of the bioactive compounds (Fig. 1).

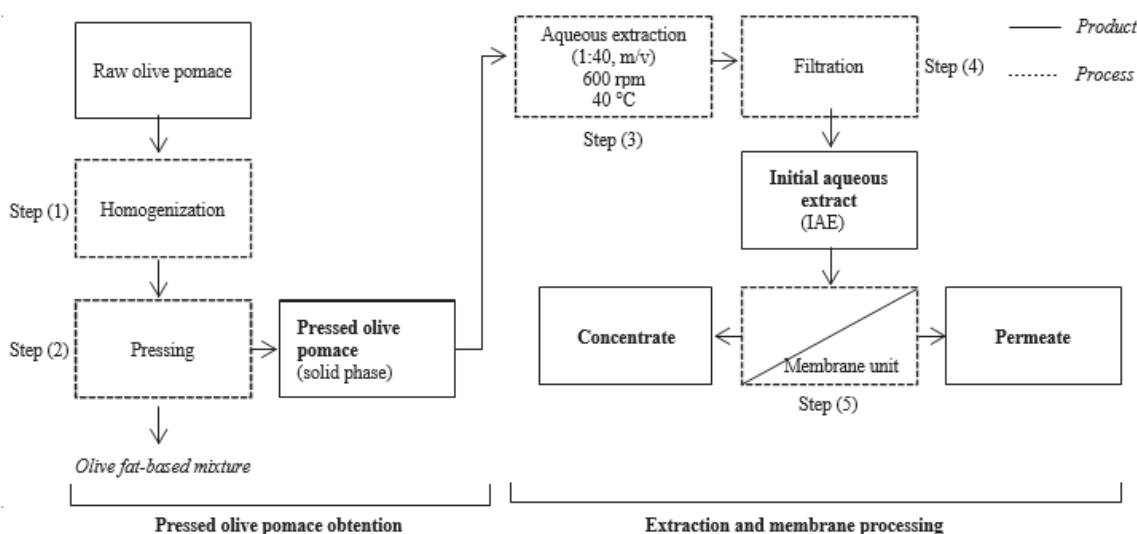


Figure 1. A flow chart of the steps from the olive pomace collection to the membrane processing.

For an initial sample characterization, a proximate analysis of raw and pressed OP was performed. Moisture was determined using an infrared balance (Scaltec model SMO01, Scaltec Instruments, Heiligenstadt, Germany). Total protein (AOAC 928.08), ash (quantified after incineration at $500\text{ }^{\circ}\text{C}$) (AOAC 920.153) and total fat (AOAC 991.36) contents were determined according to AOAC methods (AOAC, 2012). For the protein

content, a 6.25 nitrogen conversion factor was used (Tontisirin, 2003). Total carbohydrates (%) were calculated as follows (Tontisirin, 2003):

$$\text{Total carbohydrates} = 100 - (\% \text{moisture} + \% \text{protein} + \% \text{fat} + \% \text{ash}) \quad (1)$$

A previously patented process (WO/2017/212450, 2017) was applied to extract the bioactive compounds from the pressed OP. Briefly, the sample was subjected to an aqueous extraction (1:40; m/v) during 2 h at 40 ± 5 °C on a stir plate (MS-H-S10 10-Channel Classic Magnetic Hotplate Stirrer) at 600 rpm. The extracts were prepared with deionized water (pH = 6.4; conductivity = 0.01 dS/m). Afterwards, the solution was filtered through Whatman n. °4 filter paper, and the obtained extracts (initial aqueous extracts, IAE) were stored at -20 °C (Fig. 1).

2.3 Extracts treatment via membrane processing

The aqueous extracts (IAE) obtained in Section 2.2 were subjected to batch filtration in a dead-end stainless steel membrane unit (METCell, Membrane Extraction Technology, UK) at a controlled pressure of 20 bar. The feed vessel solution was stirred at 200 rpm and the permeate weighted online with an electronic balance (Kern 572, Kern, Germany).

Three types of flat sheet polymeric membranes: NF90, NF270 and BW30 (Dow Water & Process Solutions, USA), all with an effective membrane area of 51.4 cm², were tested. NF270 and NF90 are nanofiltration membranes, while BW30 is a brackish water reverse osmosis membrane. Each membrane was used twice: first to process 200 mL and afterwards 150 mL of OP extract. A volume concentration factor (CF) of 10 was reached in each filtration. The membranes were compacted with deionized water at pressure of 25 bar, until constant volumetric water flux, before use.

The instantaneous permeate volumetric flux, J_v (L/(m²·h)) was calculated according to the following Eq. (2):

$$J_v = \frac{\Delta m_{perm}}{\rho \cdot A \cdot \Delta t}, \quad (2)$$

where Δm_{perm} (kg) is the permeate mass recovered during a defined interval of time, Δt (s), ρ (kg/m³) is the density of the permeate and A (m²) is the membrane area. The concentration factor, CF, was calculated along the filtration process as:

$$CF = \frac{m_o}{(m_o - m_{perm})}, \quad (3)$$

where m_o (kg) is the solution mass in the feed vessel at the beginning of the experiment and m_{perm} (kg) is the mass of permeate recovered up to a given instant. Before, between,

and after OP extract filtrations, deionized water was flushed through the system to determine the membrane fouling index I_f (%), expressed as a percentage drop in permeate flux as follows:

$$I_f = \left(1 - \frac{J_{v1}}{J_{v0}}\right) \cdot 100, \quad (4)$$

where J_{v1} (L/(m²·h)) and J_{v0} (L/(m²·h)) are the pure water fluxes measured after and before filtration of the OP extracts (IAE), respectively.

The apparent membrane rejection, R_i (%) of a target compound i (e.g., phenolic compounds, flavonoids and elements such as K, Na, etc.) was calculated as:

$$R_i = \left(1 - \frac{c_{i,perm}}{c_{i,conc}}\right) \cdot 100, \quad (5)$$

where $c_{i,perm}$ (mg/L) and $c_{i,conc}$ (mg/L) are the concentrations of compound i in the permeate and concentrate solutions, respectively. Fig. 1 shows a flow chart of the steps from OP collection, subsequent extracts preparation (Section 2.2) and, finally, extracts treatment via membrane processing (Section 2.3).

2.4 Extracts characterization

Total phenolics, total flavonoids, antioxidant activity, conductivity, total organic carbon, pH and elemental composition were measured in the extracts (IAE), concentrates, and permeates using the methodologies described below. The sodium adsorption ratio (SAR) was also calculated.

2.4.1 Total phenolics content

Total phenolics content (expressed as mg of gallic acid equivalents (GAE)/L of extract) was determined by the Folin-Ciocalteu method as previously described in Nunes et al. (2018). A calibration curve (5–100 mg/L; $R^2=0.9996$) was prepared and absorbance measured at 765 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.4.2 Total flavonoids content

Total flavonoids (expressed as milligrams of epicatechin equivalents (EE)/L of extract) were quantified by a colorimetric assay based on Costa et al. (2018). Epicatechin was used to plot a standard curve (5–300 mg/L; $R^2=0.9998$) and absorbance read at 595 nm with a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.4.3 Antioxidant activity

Two spectrophotometric methods were used in this determination, namely Ferric Reducing Antioxidant Power (FRAP) and DPPH• scavenging ability assays. Both methods present complementary mechanisms of action and are worthwhile to be used together (Huang et al., 2005).

2.4.3.1 FRAP assay

The FRAP assay (expressed as μmol of ferrous sulfate equivalents (FSE)/L of extract) was carried out according to Nunes et al. (2018). A calibration curve was prepared with ferrous sulfate (25–500 $\mu\text{mol/L}$; $R^2=1$) and absorbance measured at 595 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

2.4.3.2 DPPH• (2,2-diphenyl-1-picrylhydrazyl) scavenging ability

The radical inhibition was evaluated according to Nunes et al. (2018). The absorbance decrease, in order to observe the kinetic reaction, was monitored in 2 min intervals at 525 nm (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA) until the endpoint was attained (20 min). A calibration curve was prepared with trolox (2.5–175 mg/L; $R^2=0.9946$) and the results expressed as mg of trolox equivalents (TE)/L of extract.

2.4.4 Conductivity, total organic carbon, pH and elemental composition

Electrical conductivity (EC) was measured using an ORION electrical conductivity meter (Model 120) and total organic carbon (TOC) contents analysed using a total organic carbon analyser (Shimadzu Corporation, Kyoto, Japan). The pH was measured with an ORION pH meter (Model 720 A). Elemental composition was analysed with Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) (Ultima model, Horiba Jobin-Yvon, France) equipped with a radiofrequency (RF) generator of 40.68 MHz, a Czerny-Turner type monochromator with 1.00 m (sequential), AS500 auto-sampler and data acquisition software.

2.4.5 Sodium adsorption ratio

The sodium adsorption ratio (SAR) is an indicator of the suitability of water for agricultural irrigation (FAO, 1985; Quist-Jensen et al., 2015) (Table 1) and was calculated as follows:

$$\text{SAR} = \frac{\text{Na}^+ (\text{meq/L})}{\sqrt{\frac{\text{Ca}^{2+} (\text{meq/L}) + \text{Mg}^{2+} (\text{meq/L})}{2}}}, \quad (6)$$

Table 1. Water quality for irrigation considering only the sodium adsorption ratio (SAR) and electrical conductivity (EC) taking together (FAO, 1985).

SAR	Degree of restriction on the use of irrigation water		
	None	Slight to moderate	Severe
	Electrical conductivity (dS/m)		
0 - 3	>0.7	0.7-0.2	<0.2
3 - 6	>1.2	1.2-0.3	<0.3
6 - 12	>1.9	1.9-0.5	<0.5
12 - 20	>2.9	2.9-1.3	<1.3
20 - 40	>5.0	5.0-2.9	<2.9

2.5 Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v.25 (IBM Corp., Armonk, 241 NY, USA). Data are expressed as mean $\pm$ standard deviation (n = 3). The one-way ANOVA was used to evaluate differences between samples, followed by Tukey's HSD or Dunett T3 post hoc test (selected based on the equality of variances) to make pairwise comparisons between means. Paired Student's t-test was used to compare OP proximate composition before and after pressing processing. The level of significance for all hypothesis tests was 0.05.

3. Results and discussion

3.1 Olive pomace characterization

A proximate analysis was performed in order to characterize OP before and after the pressing procedure (Fig. 2).

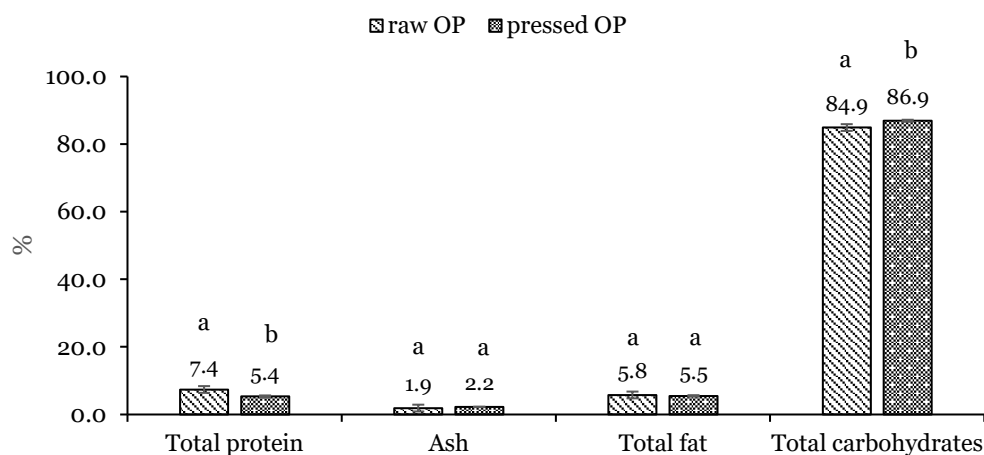


Figure 2. OP proximate composition (g/100 g dry weight) before (raw OP) and after pressing process (pressed OP). Different letters indicate significant differences between raw and pressed OP at $p < 0.05$.

In general, no significant differences ($p > 0.05$) were found regarding total fat and ash. Notwithstanding, a significant ($p < 0.05$) loss of protein was observed, probably due to the solubility of proteins in water and losses during pressing. The moisture content changed from 61.1 (raw OP) to 49.3% (pressed OP).

The extracts prepared from pressed OP comprise mostly hydrophilic compounds because the used solvent was 100% water. Although other solvents could be used (e.g., ethanol, methanol and hydro-alcoholic solutions) (Alu'datt et al., 2010), this study aims to answer to the emergent concern of the bioactive compounds recovery, from agro wastes, by green methodologies.

The results concerning the composition of the extracts before and after membrane processing will be discussed further below.

3.2 Characterization of membrane processing

Concerning the olive oil processing by-products, the membranes separation technology has been used mostly for olive mill wastewaters rather than for OP extracts treatment. For instance, Galanakis et al. (2010) used a pilot UF module for processing high-added value products derived from olive mill wastewater (pectin and phenolics containing solutions). The current tendency to produce olive oil using the two-phase systems (more eco-friendly) points out to the importance of the development of new solutions regarding the recover and valorisation of the bioactive compounds present in OP. Besides olive oil processing by-products, promising results have been obtained in various agro-industrial by-products (winery sludge, oat mill waste, cheese whey, apple pomace, etc.) using

emerging technologies as membranes-assisted processes (Kammerer et al., 2014; Patsioura et al., 2011; Galanakis et al., 2014). In this study, the potential of NF and RO for separation and concentration of the bioactive compounds present in the aqueous extracts (IAE) was explored and compared. Three membranes, NF270, NF90 and BW30, from the same manufacturer (Dow Water & Process Solutions, USA) were tested. NF270 is a piperazine based semi-aromatic polyamide thin film composite membrane, while NF90 and BW30 are fully aromatic polyamide thin film composite membranes (Tang et al., 2007). According to the pure water fluxes, salt rejection, and rejection layer thickness, the membranes can be further classified as “loose” NF (NF270), standard (NF90) and “loose” RO (BW30) membranes. The most important operating parameters, namely permeate flux, fouling influence on process performance, and quality/composition of the permeate and concentrate streams, were evaluated. Fig. 3 shows the permeate fluxes in function of the volume concentration factor for the investigated membranes.

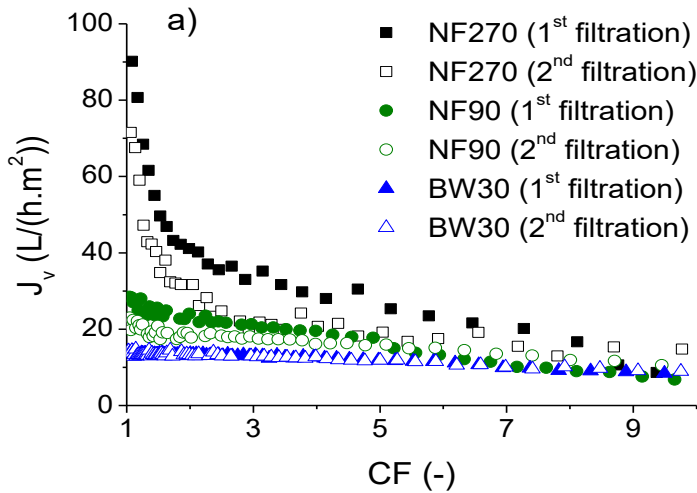


Figure 3. Permeate fluxes (J_v) in function of the concentration factor (CF) obtained during filtration of aqueous extracts of olive pomace (IAE) at 20 bar, with the investigated membranes.

As previously described (Section 2.3), each membrane was used twice, and thoroughly washed with deionized water between the two filtrations.

As can be seen in Fig. 3, the volumetric permeate flux profiles are different for the three membranes, which can be attributed to the “tightness” of their rejection layers. For NF270, the permeate flux was the highest one at the beginning of the 1st filtration (until a CF of 2), but quickly dropped to values similar to those obtained with the other membranes (NF90 and BW30). The permeate fluxes obtained during the 2nd filtration were about 10 $\text{L}/(\text{h}\cdot\text{m}^2)$ lower in comparison to those obtained in the 1st filtration. For

the case of NF90, only a small permeate flux drop was observed during, and between the two filtrations. In the BW30 membrane almost no differences between the filtrations and no permeate flux drop was observed. The relatively loose semi-aromatic skin rejection layer of the NF270 membrane can explain the highest initial permeate fluxes obtained due to its lower hydraulic resistance to water transport. The reverse osmosis BW30 membrane offers the highest hydraulic resistance, leading to the smallest permeate flux. During filtration of IAE, the present compounds rapidly fouled the NF270 and NF90 membranes. It can be argued that due to the relatively looser polymeric structure of the rejection skin layers of such membranes, these foulants had penetrated into them more easily, increasing hydraulic resistances and corresponding reduction of their permeate fluxes. Contrary, for the BW30 membrane, due to its tighter rejection layer, these foulants could not easily penetrate, thus the permeate flux obtained remains relatively constant.

In practice, it will be important to guarantee a long-term stability and possibility for repeated use of the selected membrane. Therefore, the membranes fouling index was investigated. The reversibility of the observed fouling and disclosure of the process robustness is shown in Fig. 4. The fouling index is a membrane-related parameter, which compares the permeate fluxes of a membrane before and after filtration, thus showing how the membrane performance is affected (not necessarily only by fouling) during processing. The lower the fouling index, the better is the long-term operational stability of a membrane. As it can be seen in Fig. 4, BW30 presents the lowest fouling indices, meaning that this membrane was almost not affected during operation. In addition, even if some foulants accumulated on its surface, they were easily removed by washing out with water.

In the case of NF270 and NF90, their performance strongly changed during operation, although via distinct process dynamics. NF90 gets fouled at once, probably because the polymeric structure sufficiently loose (open) allows penetration of foulants into its voids. At the same time, they are sufficiently small to be closed/blocked in a single step.

On the other hand, NF270 is getting fouled progressively. Its rejection layer intra-polymeric voids are too large to be blocked at once, but it is also impossible to remove the foulants from the membrane surface by washing with water, since the foulants penetrate irreversibly into its structure.

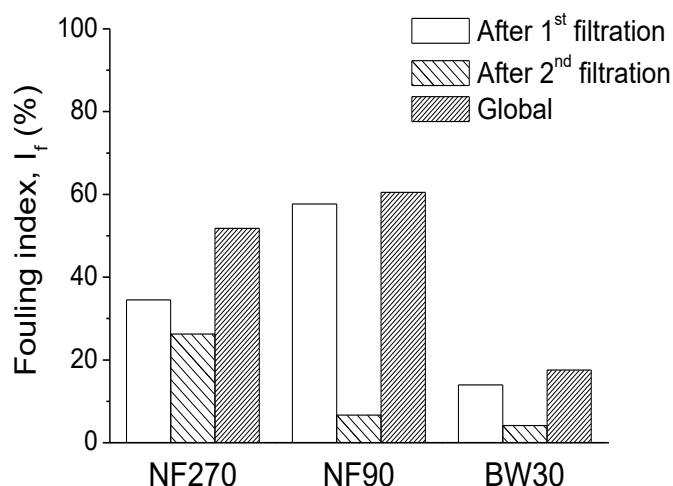


Figure 4. Fouling index (I_f) expressing the drop in permeate fluxes, obtained during filtration of aqueous extracts of olive pomace (IAE) at 20 bar, with the investigated membranes.

3.3 Characterization of IAE (feed), permeate and concentrate streams

Chemical analyses of the IAE (feed), permeate and concentrate streams (Table 2) were carried out regarding their total phenolics and flavonoids contents and antioxidant activity.

The initial extracts (IAE) showed a ferric reducing power of 845 $\mu\text{mol FSE/L}$ and a DPPH $\cdot$ scavenging ability of 42 mg TE/L. The total phenolics and total flavonoids attained 110 mg GAE/L and 50 mg EE/L in IAE, respectively. Several antioxidant compounds are described in OP that contribute to these antioxidant properties. Hydroxytyrosol (2-(3,4-dihydroxyphenyl)-ethanol) is the major phenolic present in OP ($\approx 54\%$), followed by comsologoside ($\approx 25\%$) (Nunes et al., 2018). Besides, tyrosol, secoiridoids and derivatives (e.g., oleuropein, oleuropein aglycone, verbascoside, 10-hydroxy-oleuropein), iridoids precursors (e.g., loganin, loganin glucoside, loganic acid, loganic acid glucoside, eleanolic acid), lignans (e.g., pinoreosin, hydroxypinoreosin, acetoxypinoreosin), phenolic acids (cinnamic, *p*-coumaric, caffeic, protocatechuic, vanillic, ferulic and gallic acids) and flavonoids (e.g., rutin, apigenin, luteolin, apigenin glucoside, luteolin glucoside, quercetin) have also been described in OP (Nunes et al., 2018; Peralbo-Molina et al., 2012).

Table 2. Physicochemical characteristics of the IAE (feed), permeate and concentrate streams obtained using the selected membranes (NF90, NF270, BW30) and their apparent rejection (Ri) values toward total organic carbon (TOC), phenolics and flavonoids at CF = 10.

Type of extracts		TOC (mg/L)	Phytochemicals		Antioxidant activity		Conductivity (μ S/cm)	pH
			Total phenolics (mg GAE/L)	Total flavonoids (mg EE/L)	FRAP (μ mol FSE/L)	DPPH $\cdot$ inhibition (mg ET/L)		
IAE		850.5	109.8 $\pm$ 1.3 ^c	50.2 $\pm$ 0.7 ^d	844.6 $\pm$ 15.2 ^c	41.8 $\pm$ 1.5 ^b	320	5.6
Concentrates	NF270	7477	1063.9 $\pm$ 30.9 ^b	448.8 $\pm$ 3.1 ^b	8227.0 $\pm$ 186.1 ^b	406.3 $\pm$ 55.0 ^a	1400	5.1
	NF90	7434	1069.4 $\pm$ 11.1 ^b	403.7 $\pm$ 4.8 ^c	8024.6 $\pm$ 393.7 ^b	425.1 $\pm$ 9.6 ^a	2840	5.2
	BW30	14754	1234.3 $\pm$ 54.0 ^a	464.2 $\pm$ 3.1 ^a	9183.3 $\pm$ 275.9 ^a	405.9 $\pm$ 14.0 ^a	3310	5.3
Permeates	NF270	56.38	4.4 $\pm$ 0.2 ^d	1.5 $\pm$ 0.2 ^e	42.0 $\pm$ 0.5 ^d	nd	85.4	6.2
	NF90	10.21	0.2 $\pm$ 0.1 ^e	nd	7.2 $\pm$ 0.4 ^e	nd	17.4	6.1
	BW30	9.89	nd	nd	2.6 $\pm$ 0.4 ^f	nd	9.4	5.6
Ri (%)	NF270	99.25	99.59	99.66	99.49	100.00	93.90	
	NF90	99.86	99.98	100.00	99.91	100.00	99.39	
	BW30	99.93	100.00	100.00	99.97	100.00	99.72	

FRAP, ferric-reducing antioxidant power; DPPH $\cdot$, 2,2-diphenyl-1-picrylhydrazyl radical; GAE, gallic acid equivalents; EE, epicatechin equivalents; TE, trolox equivalents; FSE, ferrous sulfate equivalents; IAE, initial aqueous extract (extract obtained after the pressed OP extraction and before membrane processing); nd, not detected. Different letters indicate significant differences between the IAE, concentrates and permeates at $p < 0.05$. Mean values with the same superscript letters (a, b, c, d or e) were similar and no significant differences were observed between these samples.

Significant differences ($p < 0.05$) between the concentrates from the different membranes can be observed (Table 2). Total phenolics and flavonoids contents of the concentrate from BW30 was significantly higher (around 15%) than those achieved with NF270 and NF90. 100% rejection (R_i) of these compounds was achieved with BW30, avoiding completely the loss of these compounds into the permeate. No significant differences between NF270 and NF90 concentrates were observed, regarding their phenolic contents ($p > 0.05$). As expected, the antioxidant activity of the concentrate streams was significantly higher (around 10 times, which corresponds to the defined concentration factor) than that of the IAE. The concentrate using the BW30 membrane showed the highest FRAP value, while no significant differences ($p > 0.05$) were observed among the DPPH $\cdot$ scavenging ability of the three concentrates. Accordingly, the permeate from the BW30 membrane showed the lowest FRAP value and no DPPH $\cdot$ inhibition was observed in all permeates. In the BW30 permeate, phenolics and flavonoids were not detected, which is consistent with the negligible FRAP values observed (eventually resulting from other chemical compounds with electron transfer ability) (Table 2).

3.4 Challenging applications of concentrates and permeates

Bioactive compounds from OP (e.g., phenolic compounds, polysaccharides and fibres) can be used i) to enhance antioxidant properties of food products; ii) to act as stabilizing agents; and iii) as source of oligo-, di-, and mono-saccharides, for example. Novel applications of olive pomace extracts in food products as edible oils, fortification and preservation of meat and meat products and probiotics have also been reported (Galanakis, 2018; Nunes et al., 2016). Also, food products preservation has been tested using phenolics recovered from olive oil processing by-products. Recently, Galanakis et al. (2018a) studied the microbial growth in bakery products (bread and rusks) fortified with polyphenols recovered from olive mill wastewater. In this case, ascorbic acid, a tocopherols mixture and α -tocopherol were also used. The results showed that the antioxidants (including olive polyphenols) presented antimicrobial properties in these kind of products, increasing their shelf life (Galanakis et al., 2018a).

Likewise, in the cosmetic field, some bioactive compounds of OP (antioxidants and minerals) have particular interest as potential ingredients in formulations (anti-aging and moisturizing, for example) (Rodrigues et al., 2017; Rodrigues et al., 2015). Moreover, phenolics obtained from olive mill wastewater, can have an effective protection against ultra-violet radiation and thus be used as ingredient in cosmetic formulations (Galanakis et al., 2018b, 2018c). Hence, the concentrate obtained in this study can be used as functional ingredient in food and cosmetic industries due to its richness in particular

bioactive compounds and its absence of solvent residues. Globally, the agriculture sector is the major consumer of freshwater. Irrigation practices are responsible by about 70% of the total water consumed. Climate changes and the scarce water resources intensify the need to implement agricultural sustainable solutions (Gobin et al., 2017; Knox et al., 2012). Thus, a hypothetical subsequent application of the permeates as irrigation water was envisaged. Despite their health properties, phenolic compounds have also been described as phytotoxic when discarded into soil at high amounts (Caporaso et al., 2017). Therefore, only “phenolics free” waters, such as the BW30 permeate, are suitable for irrigation.

Table 3 shows a detailed elemental composition of BW30 permeate stream.

Table 3. Elemental composition of the permeate obtained with the BW30 membrane.

Element	Concentration (mg/L)
B	0.57
Ca	1.01
K	2.15
Mg	0.45
Na	0.48
S	1.30
Si	0.10
Zn	0.03

Note: Silver (Ag), Aluminium (Al), Arsenic (As), Barium (Ba), Bismuth (Bi), Cadmium (Cd), Cobalt (Co), Chromium (Cr), Copper (Cu), Iron (Fe), Mercury (Hg), Lithium (Li), Manganese (Mn), Molybdenum (Mo), Nickel (Ni), Phosphorus (P), Lead (Pb), Antimony (Sb), Selenium (Se), Tin (Sn), Strontium (Sr), Titanium (Ti), Thallium (Tl), Vanadium (V), Tungsten (W), and Zirconium (Zr) were not detected.

Potassium (K) is the major element (2.15 mg/L) present in the BW30 permeate. K is a key factor in plants physiology, namely in the sugars translocation and root growth. Moreover, its presence is associated with crop resistance against pest and diseases. Thus, its availability in irrigation water has an added value against biotic and abiotic stresses affecting plants metabolism (Wang et al., 2013). Concerning its individual value and the maximum recommended in water for human consumption (10 mg/L), according to Decree-Law n.º 236/98, of 1 August (Portugal), it is possible to anticipate that the permeate can be suitable for watering purposes (Decree-Law n.º 236/98, of 1 August). Nevertheless, FAO (Food and Agriculture Organization of the United Nations) water quality classification is the most used and reported guideline concerning water quality for agriculture (Bortolini et al., 2018). Taking into account FAO classification, the K value determined in this work (2.15 mg/L) is slightly higher than the maximum established by FAO (0–2 mg/L) (FAO, 1985). Boron (B) is an essential element for plants growth but in

high amounts can be toxic. Levels of 0.57 mg/L of B were detected in the BW30 permeate which is consistent with no restriction of use as irrigation water (0.7 mg/L) (FAO, 1985). Crops as pepper, pea, carrot, radish, potato, and cucumber are moderately sensitive to B (1–2 mg/L), lettuce, cabbage, oats, and corn moderately tolerant (2–4 mg/L), and tomato, alfalfa, vetch, sugar beet, and table beet tolerant (4–6 mg/L) (Hopkins et al., 2007). Furthermore, FAO proposes a wide list of crops that are very sensitive to B (<0.5 mg/L); sensitive (0.5–0.75 and 0.5–1 mg/L); moderately sensitive (1.0–2.0 mg/L); moderately tolerant (2.0–4.0 mg/L); tolerant (4.0–6.0 mg/L); and very tolerant (6.0–15.0 mg/L). Therefore, the crop selection can be a strategic solution when regarding irrigation waters with potentially toxic elements (FAO, 1985). Also, as phytotoxicity is cumulative, the type of the crop selected - orchards and perennial crops versus seasonal crops - determine the sensitivity to certain elements.

The BW30 permeate contains 0.48 mg/L of Sodium (Na), 1.01 mg/L of Calcium (Ca) and 0.45 mg/L of Magnesium (Mg) (Table 3). Ca and Mg are important elements to counteract Na-related effects. Excessive salt amounts supplied by irrigation have impact in the soil quality and crop production (e.g., reduces the water uptake by plant roots) and accumulate in soil. Ca and Mg are essential plant nutrients besides their key role in soil aggregation and friability. Also, high contents in both elements lead to a pH increase which reduces soil phosphorus (P) availability (Al-Shammiri et al., 2005). In these circumstances, taking into account the reduced amounts of Ca and Mg (FAO, 1985) and that P was not detected in the permeate, a water blending strategy would be advisable to avoid detrimental effects in the soil and/or enhance the crop nutrient and consequently the productivity. The BW30 permeate presented a conductivity of just 0.0094 dS/m and a SAR ratio of 0.1. Taking together EC and SAR values, the permeate use is restricted (Table 1) (FAO, 1985). Notably, some Mediterranean areas, namely olive oil producers regions, are arid and semi-arid and chronically water-stressed. The water in these regions can have a very high EC (Aissa and Boutoutaou, 2017; Bortolini et al., 2018). Therefore, blending the permeate with the irrigation waters of these areas can have a positive effect on lowering its EC.

Regarding pH, the common range for irrigation water is 6.5–8.4 (FAO, 1985). A pH of 5.6 was determined for the BW30 permeate. However, pH levels of 5.0–7.0 can be advantageous for some plants that require acidic conditions (Seufert et al., 2012). It is noteworthy that the pH of the deionized water used in this study was 6.4. Hereafter, in order to obtain permeates with chemical features consistent with those obtained in this study, the solvent (water) properties must be established and maintained. In addition,

other studies can be developed to obtain a comprehensive analysis of the influence of the water chemical characteristics on the permeates composition.

Another irrigation water quality indicator is TOC, which represents the organic molecules in water, such as fatty acids, sugars, and amino acids. The recommended TOC values in irrigation water range from 5 to 10 mg/L. In the BW30 permeate, the TOC value is 9.89 mg/L which is therefore adequate for agricultural applications (Ingram et al., 2011). Due to water-saving concerns, the BW30 permeate is foreseen to be used for re-extraction of other samples of pressed OP bioactive compounds and then reused for watering purposes. By this way, it is possible to achieve a more sustainable process to extract the OP bioactive compounds.

Several factors are considered for the evaluation of water quality and application for irrigation purposes. Major factors as crop type, edaphoclimatic conditions, and irrigation method (Bortolini et al., 2018) will determine the utilization of the BW30 permeate as irrigation water. The agronomic quality indicators evaluated in this work should be monitored frequently to avoid detrimental effects on crops and soil fertility (Bortolini et al., 2018). Also, strategic solutions (e.g., water fortification with selected elements or crops selection) ought to be considered as well as blending waters practices. Overall, taking into account the presented results, it is necessary to consider some issues: i) water quality is defined by physical, chemical and biological characteristics, which means that further analysis to permeate should be performed; ii) as water is a scarce resource, despite the low availability of some nutrients, BW30 permeate can be considered for irrigation with amendments; and iii) to develop the process presented, it is important bearing in mind the seasonality variation of olives and edaphoclimatic conditions of the different region/countries.

4. Conclusions

Bioactive compounds with health and economic added value were recovered and concentrated from OP using an integrated process: extraction by an eco-friendly patented methodology and a membrane-assisted processing. Two nanofiltration (NF270 and NF90) and one reverse osmosis (BW30) membranes were evaluated in terms of permeate fluxes, fouling influence on process performance and physicochemical composition of the obtained permeate and concentrate streams. For the future scale-up of the process, the BW30 membrane was found to be the most appropriate to be used since:

- 1) it was less affected by fouling – the fouling index was lower than 20%, while for NF270 and NF90 it was ≈ 50 and 60%, respectively;
- 2) its permeate flux was almost constant during the whole operation, ≈ 15 L/(h·m²) (at 20 bar);
- 3) the rejection of BW30 toward total organic carbon (TOC), salts, phenolic and flavonoids compounds was of 99.93, 99.72, 100 and 100%, respectively, which are the highest rejection values obtained;
- 4) the BW30 concentrate presented the highest antioxidant activity, concentrations of phenolic and flavonoids compounds by at least 12, 15 and 4%, respectively;
- 5) no phenolic and flavonoids compounds were detected in the permeate from BW30, and its total organic carbon (TOC) content was below 10 mg/L (9.89 mg/L).

Overall, the antioxidant concentrate obtained by BW30 has an added value for pharmaceutical, cosmetic and food industries. Further validation studies are still needed to identify the most suitable crops to be irrigated considering the permeate chemical features. Additionally, when scaling up the process some parameters should be re-evaluated as the extraction water pH and EC.

Usually, raw OP is processed into OP oil. A final raw material (extracted OP) remains after obtaining the OP oil, which is accumulated in the industries. Indeed, OP has low economic value and is essentially used as biomass for heat and energy production. However, the increase of competition, especially in what concerns to the biomass substitute products, has led to a decrease in its profitability. Thus, it is important to find alternative ways to valorise OP. This work anticipated a clean and low-cost method to extract the bioactive compounds from OP, using only water and low temperatures. The market is demanding by natural ingredients/additives (with high economic value) and the OP process presented can respond to this request at low cost. Moreover, the studied process aims to accomplish the “zero wastes” strategy looked-for in the Patent WO/2017/212450 (2017). In what concerns to the OP solid phase, free of phytotoxic compounds, it is a promising agricultural substrate for crops production.

References

- Aissa, R.B., Boutoutaou, D., 2017. Characterization of groundwater in arid zones (case of Ouargla basin). *Energy Procedia* 119, 556–564.
- Aliakbarian, B., Paini, M., Adami, R., Perego, P., Reverchon, E., 2017. Use of supercritical assisted atomization to produce nanoparticles from olive pomace extract. *Innovative Food Sci. Emerg. Technol.* 40, 2–9.
- Al-Shammiri, M., Al-Saffar, A., Bohamad, S., Ahmed, M., 2005. Waste water quality and reuse in irrigation in Kuwait using microfiltration technology in treatment. *Desalination* 185, 213–225.
- Alu'datt, M.H., Alli, I., Ereifej, K., Alhamad, M., Al-Tawaha, A.R., Rababah, T., 2010. Optimisation, characterisation and quantification of phenolic compounds in olive cake. *Food Chem.* 123, 117–122.
- AOAC, 2012. Official Methods of Analysis of AOAC. seventeenth ed. Association of Analytical Communities, USA.
- Artajo, L.-S., Romero, M.-P., Suárez, M., Motilva, M.-J., 2007. Partition of phenolic compounds during the virgin olive oil industrial extraction process. *Eur. Food Res. Technol.* 225, 617–625.
- Bortolini, L., Maucieri, C., Borin, M., 2018. A tool for the evaluation of irrigation water quality in the arid and semi-arid regions. *Agronomy* 8, 1–15.
- Caporaso, N., Formisano, D., Genovese, A., 2017. Use of phenolic compounds from olive mill wastewater as valuable ingredients for functional foods. *Crit. Rev. Food Sci. Nutr.* 1–13.
- Cassano, A., Conidi, C., Giorno, L., Drioli, E., 2013. Fractionation of olive mill wastewaters by membrane separation techniques. *J. Hazard. Mater.* 248–249, 185–193.
- Coskun, T., Debik, E., Demir, N.M., 2010. Treatment of olive mill wastewaters by nanofiltration and reverse osmosis membranes. *Desalination* 259, 65–70.
- Costa, A.S.G., Alves, R.C., Vinha, A.F., Costa, E., Costa, C.S.G., Nunes, M.A., Almeida, A.A., Santos-Silva, A., Oliveira, M.B.P.P., 2018. Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chem.* 267, 28–35.
- FAO-Food and Agriculture Organization of the United Nations, 1985. Water quality for agriculture. <http://www.fao.org/docrep/003/T0234E/T0234E00.htm#TOC>, Accessed date: 9 October 2018.

- Galanakis, C.M., 2012. Recovery of high added-value components from food wastes: conventional, emerging technologies and commercialized applications. *Trends Food Sci. Technol.* 26, 68–87.
- Galanakis, C.M., 2013. Emerging technologies for the production of nutraceuticals from agricultural by-products: a viewpoint of opportunities and challenges. *Food Bioprod. Process.* 91, 575–579.
- Galanakis, C.M., 2018. Phenols recovered from olive mill wastewater as additives in meat products. *Trends Food Sci. Technol.* 79, 98–105.
- Galanakis, C.M., Tornberg, E., Gekas, V., 2010. Clarification of high-added value products from olive mill wastewater. *J. Food Eng.* 99, 190–197.
- Galanakis, C.M., Chasiotis, S., Botsaris, G., Gekas, V., 2014. Separation and recovery of proteins and sugars from Halloumi cheese whey. *Food Res. Int.* 65, 477–483.
- Galanakis, C.M., Tsatalas, P., Charalambous, Z., Galanakis, J.M., 2018a. Control of microbial growth in bakery products fortified with polyphenols recovered from olive mill wastewater. *Environ. Technol. Innov.* 10, 1–15.
- Galanakis, C.M., Tsatalas, P., Galanakis, I.M., 2018b. Phenols from olive mill wastewater and other natural antioxidants as UV filters in sunscreens. *Environ. Technol. Innov.* 9, 160–168.
- Galanakis, C.M., Tsatalas, P., Galanakis, I.M., 2018c. Implementation of phenols recovered from olive mill wastewater as UV booster in cosmetics. *Ind. Crop. Prod.* 111, 30–37. Gobin, A., Kersebaum, K., Eitzinger, J., Trnka, M., Hlavinka, P., Takáč, J., Kroes, J., Ventrella, D., Marta, A., Deelstra, J., Lalić, B., Nejedlik, P., Orlandini, S., Peltonen-Sainio, P., Rajala, A., Saue, T., Şaylan, L., Stričević, R., Vučetić, V., Zoumides, C., 2017. Variability in the water footprint of arable crop production across European regions. *Water* 9, 93.
- Hopkins, B.G., Horneck, D.A., Stevens, R.G., Ellsworth, J.W., Sullivan, D.M., 2007. Managing Irrigation Water Quality for Crop Production in the Pacific Northwest. A Pacific North- west Extension publication, USA.
- Huang, D., Ou, B., Prior, R.L., 2005. The chemistry behind antioxidant capacity assays. *J. Agric. Food Chem.* 53, 1841–1856.
- Ingram, D.T., Patel, J., Sharma, M., 2011. Effect of repeated irrigation with water containing varying levels of total organic carbon on the persistence of *Escherichia coli* O157:H7 on baby spinach. *J. Food Prot.* 74, 709–717.

- Kammerer, D.R., Kammerer, J., Valet, R., Carle, R., 2014. Recovery of polyphenols from the by-products of plant food processing and application as valuable food ingredients. *Food Res. Int.* 65, 2–12.
- Knox, J.W., Kay, M.G., Weatherhead, E.K., 2012. Water regulation, crop production, and agricultural water management-understanding farmer perspectives on irrigation efficiency. *Agric. Water Manag.* 108 (3–8).
- Mattas, K., Tsakiridou, E., 2017. The functional olive oil market: marketing prospects and opportunities. In: Shahidi, F., Kiritsakis, A. (Eds.), *Olives and Olive Oil as Functional Foods*. John Wiley & Sons Ltd., pp. 647–658.
- Monte, J., Sá, M., Galinha, C.F., Costa, L., Hoekstrad, H., Brazinha, C., Crespo, J.G., 2018. Harvesting of *Dunaliella salina* by membrane filtration at pilot scale. *Sep. Purif. Technol.* 190, 252–260.
- Nunes, M.A., Pimentel, F.B., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P., 2016. Olive by-products for functional and food applications: challenging opportunities to face environmental constraints. *Innovative Food Sci. Emerg. Technol.* 35, 139–148.
- Nunes, M.A., Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P., 2018. Olive pomace as a valuable source of bioactive compounds: a study regarding its lipid- and water-soluble components. *Sci. Total Environ.* 644, 229–236.
- Ochando-Pulido, J.M., Martinez-Ferez, A., 2015. On the recent use of membrane technology for olive mill wastewater purification. *Membranes* 5, 513–531.
- Pan, B., Yan, P., Zhu, L., Li, X., 2013. Concentration of coffee extract using nanofiltration membranes. *Desalination* 317, 127–131.
- Parkinson, L., Cicerale, S., 2016. The health benefiting mechanisms of virgin olive oil phenolic compounds. *Molecules* 21, 1734.
- Patent WO/2017/212450. 2017. Olive pomace products, method of production and their uses. University of Porto.
- Patsioura, A., Galanakis, C.H., Gekas, V., 2011. Ultrafiltration optimization for the recovery of β -glucan from oat mill waste. *J. Membr. Sci.* 373, 53–63.
- Peralbo-Molina, Á., Priego-Capote, F., Luque De Castro, M.D., 2012. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography–tandem mass spectrometry with a quadrupole–quadrupole-time-of-flight mass detector. *J. Agric. Food Chem.* 60, 11542–11550.
- Quist-Jensen, C.A., Macedonio, F., Drioli, E., 2015. Membrane technology for water production in agriculture: desalination and wastewater reuse. *Desalination* 364, 17–32.

- Ravindran, R., Jaiswal, A.K., 2016. Exploitation of food industry waste for high-value products. *Trends Biotechnol.* 34, 58–69.
- Reboredo-Rodríguez, P., Figueiredo-González, M., González-Barreiro, C., Simal-Gándara, J., Salvador, M.D., Cancho-Grande, B., Fregapane, G., 2017. State of the art on functional virgin olive oils enriched with bioactive compounds and their properties. *Int. J. Mol. Sci.* 18, 668.
- Rodis, P.S., Karathanos, V.T., Mantzavinou, A., 2002. Partitioning of olive oil antioxidants between oil and water phases. *J. Agric. Food Chem.* 50, 596–601.
- Rodrigues, F., Pimentel, F.B., Oliveira, M.B.P.P., 2015. Olive by-products: challenge application in cosmetic industry. *Ind. Crop. Prod.* 70, 116–124.
- Rodrigues, F., Nunes, M.A., Oliveira, M.B.P.P., 2017. Chapter 12 - applications of recovered bioactive compounds in cosmetics and health care products. In: Galanakis, C. (Ed.), *Olive Mill Waste*. Academic Press. Elsevier, pp. 255–274.
- Seufert, V., Ramankutty, N., Foley, J.A., 2012. Comparing the yields of organic and conventional agriculture. *Nature* 485, 229.
- Sosa, A.P., Roca, C., Velizarov, S., 2016. Membrane assisted recovery and purification of bio-based succinic acid for improved process sustainability. *J. Membr. Sci.* 501, 236–247.
- Syed, U.T., Brazinha, C., Crespo, J.G., Ricardo-Da-Silva, J.M., 2017. Valorisation of grape pomace: fractionation of bioactive flavan-3-ols by membrane processing. *Sep. Purif. Technol.* 172, 404–414.
- Tang, C.Y., Kwon, Y.-N., Leckie, J.O., 2007. Fouling of reverse osmosis and nanofiltration membranes by humic acid—effects of solution composition and hydrodynamic conditions. *J. Membr. Sci.* 290, 86–94.
- Tontisirin, K., 2003. Chapter 2 - methods of Food Analysis. Food and Agriculture Organization of the United Nations, *Food Energy: Methods of Analysis and Conversion Factors: Report of a Technical Workshop*, pp. 7–17.
- Velizarov, S., Crespo, J.G., 2010. Chapter 5 - membrane processing for the recovery of bio- active compounds in agro-industries. In: Ribeiro, C.P., Passos, M.L. (Eds.), *Innovation in Food Engineering: New Techniques and Products*. CRC Press, Taylor & Francis Group, pp. 137–160.
- Wang, M., Zheng, Q., Shen, Q., Guo, S., 2013. The critical role of potassium in plant stress response. *Int. J. Mol. Sci.* 14, 7370.

This page was intentionally left in blank.

3.1.4 Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: The case of monocultivar pomaces

M. Antónia Nunes, Ricardo N.M.J. Páscoa, Rita C. Alves, Anabela S.G. Costa, Sílvia Bessada, M. Beatriz P.P. Oliveira

REQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy of the University of Porto, Porto, Portugal

- ¹Nunes, M.A., ¹Páscoa, R.N.M.J., Alves, R.C., Costa, A.S.G., Bessada, S., Oliveira, M.B.P.P. (2020). Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: the case of monocultivar pomaces. *Waste Management*, 103, 378-387. ¹Both authors contributed equally to this work.

Abstract

Olive pomace, a wet semi-solid paste that remains after olive oil extraction, is a major waste of the process and its recovery is mandatory due to its phytotoxicity when rejected directly into the soil. Innovative applications have been studied, but simple and reliable methods that fulfil the gap between the recovery of compounds and their use by industries (contributing to the sustainability and circular economy of the chain) still need to be explored. In this work, four monocultivar olive pomaces (Arbequina, Arbosana, Oliana, and Koroneiki) were studied regarding their nutritional composition, fatty acids and vitamin E profiles, total phenolic and flavonoid contents, antioxidant activity, and Fourier Transform Near Infrared and Mid Infrared spectra. Principal Component Analysis and Partial Least Square Discriminant Analysis were used to discriminate samples. Arbosana pomace presented the highest total fat (15%, dw) and vitamin E contents (1.4 mg/g of oil), being α -tocopherol the main vitamer in all samples. Koroneiki pomace was the richest in phenolic compounds (9 g gallic acid eq./kg). The major fatty acid was oleic acid. Stearic acid, linoleic acid, and FRAP levels differed significantly among cultivars. NIR spectra showed differences in all spectral regions (best separation from 6504 to 5389 cm^{-1} and 4961 to 4035 cm^{-1}), while MIR spectra presented differences only in some spectral regions. The results showed that Near Infrared spectroscopy together with Principal Component Analysis is a powerful tool to discriminate olive pomace cultivars, with ability to be used in an industrial context.

1. Introduction

Olea europaea L. is one of the most ancient domestic fruit trees. Its cultivation covering area (7.5 million ha) makes it one of the most extensively cultivated fruit crops (Gökdoğan and Erdoğan, 2018). Over 1,000 olive cultivars grow worldwide, each one presenting a natural unique flavour and specific chemical features (Rugini et al., 2016).

Olive oil consumption has been increasing worldwide due to its well-recognized human health benefits and organoleptic properties. The major olive oil production is concentrated in Europe (76%), being Spain the major producer (1787.7 thousands of tonnes), followed by Greece (185.0 thousands of tonnes), Italy (175.0 thousands of tonnes), and Portugal (101.1 thousands of tonnes) (2018/2019 campaign) (European Commission, 2019). According to European Commission, it is estimated that Portugal, in the 2019/2020 olive campaign, will produced 125.4 thousands of tonnes of olive oil, a record for the country (European Commission, 2019). Other countries such as Tunisia, Turkey, Syria, Morocco and China have also been increasing significantly its olive oil production (Rugini et al., 2016; Foscolou et al., 2018).

The olive oil processing is mostly performed using the three- or the two-phase systems. In the first, water is added to the process. The two-phase procedure is considered more ecological because no water is used and consequently, the production of olive mill wastewaters does not occur. In this case, the major output is a wet semi-solid paste (olive pomace) (Nunes et al., 2016, 2018). The use of these two-phase systems to produce olive oil has been increasing in Spain, Italy, Greece, and Portugal (Sygouni et al., 2019).

Olive pomace is composed by olive pulp (21–33%), skin (10–11%), and pit fragments (42–54%). It also contains the remaining oil which can vary between 8 and 12% and water. Depending on the extraction system used (two-phase *versus* three phase system), moisture can vary: 70% for olive pomace derived from the two-phase system; 45% for the olive pomace obtained from the three-phase system (Chanioti and Tzia, 2019). Olive pomace lipid fraction (the remaining olive oil) is rich in vitamin E, namely in α -tocopherol, its most biologically active form. Also, the oil presents a high amount of monounsaturated fatty acids (MUFA), mainly due to the presence of oleic acid (C18:1n9). Furthermore, pomace is a rich source of phenolic compounds as secoiridoids and derivatives, being hydroxytyrosol its major phenolic compound (Peralbo-Molina et al., 2012; Nunes et al., 2018).

In this agro-industry, waste management is still a challenge, but strongly necessary (Bernardi et al., 2018). Infrared spectroscopy techniques can be useful to a fast characterization of industrial olive pomaces (Barros et al., 2009; Lee et al., 2018), providing information about several chemical and physical properties of the samples in a single measurement. Moreover, they are green analytical techniques due to the absence of reagents, no effluents production, no need of sample preparation, and also present a low cost per analysis.

Near infrared (NIR) spectroscopy arises from overtones and combination of fundamental vibrations from C-H, N-H, O-H and S-H bands within the spectral range of 14,000 to 4,000 cm^{-1} . Mid infrared spectroscopy (MIR) is located within 4,000 to 400 cm^{-1} and is originated from fundamental vibrations (stretching, bending, rotating) of some covalent bonds, originating more well-defined absorption bands than NIR spectroscopy. Both techniques need the application of chemometric tools to extract useful information (Dos Santos et al., 2017). The use of NIR and/or MIR spectroscopic techniques for discrimination purposes has already been explored with successful results (Machado et al., 2018; Páscoa et al., 2018). MIR spectroscopy has been already used to discriminate monocultivar olive oils at different maturation stages (Gouvinhas et al., 2015), olives from different cultivars (Vergara- Barberán et al., 2015), and extra virgin olive oils from different geographic origins (Tapp et al., 2003). Gouvinhas et al. (2015) pointed out a higher discrimination of the maturation stages than the different cultivars, while Tapp et al. (2003) revealed around 95% of correct predictions. NIR spectroscopy has already been used to discriminate extra virgin olive oil samples of three different monovarietal cultivars. The results obtained, in terms of prediction capacity (around 85%) after variable selection, were very interesting (Sinelli et al., 2010). The determination of different parameters in olive paste and olive oil, namely fat content, moisture and free acidity was already accomplished using NIR spectroscopy with coefficients of determination higher than 0.9 for all the parameters analysed (Bendini et al., 2007).

Concerning the application of these techniques in olive pomace, just a few works are known (Muik et al., 2004; Pizzi et al., 2018). In one of them, NIR spectroscopy was used to determine the oil and water content and the results obtained regarding coefficients of determination were very positive (higher than 0.9 for both parameters). In another work, NIR spectroscopy was used to characterize olive pomace based on gross calorific value and moisture and ash contents. The results obtained from the developed PLS models demonstrated that this technique can be used to predict accurately moisture and ash

contents (RPD values higher than 3 for both) but can only be used as a screening procedure for gross calorific value (RPD value lower than 2) (Pizzi et al., 2018).

From the above mentioned researches, none aimed to discriminate monocultivar olive pomaces. The aim of this work was to study the chemical composition of different monocultivar olive pomaces (Arbequina, Arbosana, Oliana, and Koroneiki) and try their discrimination through principal component analysis (PCA), as well as by means of NIR and MIR spectroscopies. These cultivars were selected since they are widely produced in intensive orchards in Portugal (due to their fast growth and high yield of olive oil production), and huge amounts of the respective olive pomaces are discarded every year. In this way, this work also aims to contribute to olive pomace valorization developing tools that can rapidly (and in a clean way) discriminate the most interesting samples to be used in new industrial contexts, answering to the principles of sustainability and circular economy.

2. Material and methods

2.1 Reagents and standards

Absolute ethanol, sodium carbonate decahydrate, sodium hydroxide, Kjeldahl tablets catalyst, concentrated sulfuric acid, *n*-hexane, and anhydrous sodium sulfate were purchased from Merck (Darmstadt, Germany). Boric acid (4%) was purchased from Panreac (Barcelona, Spain). Gallic acid, epicatechin, heptahydrate ferrous sulfate, trolox, Folin-Ciocalteu's reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH), ferric chloride, 2,4,6-triphenyl-1,3,5-triazine (TPTZ), sodium nitrite, aluminium chloride, sodium acetate, petroleum ether, and Supelco 37 Component FAME Mix were all acquired from Sigma-Aldrich (St. Louis, USA). Tocopherols and tocotrienols standards were purchased from Calbiochem (La Jolla, CA, USA) and tocol from Matreya Inc. (Pennsylvania, USA). HPLC-grade solvents were obtained from Chem-Lab (Zedelgem, Belgium) or Merck (Darmstadt, Germany). Ultrapure water was treated in a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

2.2 Samples

Olive pomaces were obtained from a two-phase olive mill allocated in Alentejo (38°05'17.600 N 8°10'028.800 W), which is the major area of olive oil production in Portugal. The samples were collected after olive oil processing and correspond to the olive cultivars Arbequina, Arbosana, Koroneiki, and Oliana.

2.3 Sample preparation

The individual samples were divided into batches, frozen at 80 °C, and freeze-dried (Telstar Cryodos-80 Terrassa, Barcelona). Before analyses, samples were ground (GM200 Grindomix, Retsh, Germany) into a fine powder and sieved using 0.2 mm mesh.

2.4 Proximate analysis

Soxhlet and Kjeldahl methods were used for total fat and total protein assessment, respectively (AOAC, 2012). Ash content was determined by incineration at 500 °C (AOAC, 2012). Samples moisture was determined using an infrared balance (Scaltec model SMO01, Scaltec Instruments, Heiligenstadt, Germany). The total carbohydrates were calculated by difference as follows (Tontisirin, 2003): Total carbohydrates = 100% – (% moisture + % protein + % fat + % ash). The results are presented in g/100 g of dry weight (dw).

2.5 Analysis of lipid fraction: Fatty acid and vitamin E profiles

The lipid fraction of the samples was extracted by Soxhlet, using *n*-hexane, during 4 h. After elimination of the remaining solvent with a N₂ stream, the obtained oil was used for the subsequent analyses.

2.5.1 Fatty acid profile

The fatty acid profile was determined, accordingly to Nunes et al. (2018). Briefly, KOH in methanol (2 M) was used for transesterification of fatty acids and analysis was performed by gas chromatography (Shimadzu GC-2010 Plus with a flame ionization detector and a Shimadzu AOC-20i automatic injector, Tokyo, Japan). A CP-Sil 88 silica capillary column (50 m x 0.25 mm i.d.; 0.20 µm of film thickness) from Varian (Middelburg, Netherlands) was used to separate the fatty acids methyl esters (FAME) (Nunes et al., 2018). FAME were identified comparing the retention times with those of the standard mixture Supelco 37 Component FAME Mix (Bellefonte, PA, USA). The results were expressed as relative percentage of fatty acid.

2.5.2 Vitamin E profile

The vitamin E profile was analysed according to Pimentel et al. (2014). Very briefly, an aliquot of olive pomace oil was mixed with tocol (internal standard) and *n*-hexane, followed by chromatographic analysis using a Jasco HPLC system (Tokyo, Japan) with a MD-2015 diode array and a FP-2020 fluorescence detectors (excitation wavelength at 290 and emission at 330 nm). A Supelcosil™ LC-SI column (75 mm 3.0 mm, 3.0 µm) (Supelco, Bellefonte, USA) was used for compounds separation according to Alves et al.

(2009). The respective UV spectra were used to confirm the identity of vitamins. Quantification was carried out using the fluorescence signals.

2.6 Phytochemical analysis and antioxidant activity

2.6.1 Extracts preparation

The extracts were prepared according to a patented process (WO/2017/212450, 2017). Very briefly, extraction was performed with deionised water, during 2 h (magnetic stirring) at 40 ± 5 °C. The extracts were filtered with a Whatman n.º 4 filter paper and prepared in triplicate.

2.6.2 Total phenolics content

Total phenolics content (TPC) was determined according to Costa et al. (2018), with minor modifications. Briefly, 30 µL of extract was mixed with 150 µL of Folin-Ciocalteu reagent (1:10) and 120 µL of 7.5% sodium carbonate, followed by incubation at 45 °C (15 min). The absorbance was measured at 765 nm after 30 min. TPC were expressed as g of gallic acid equivalents (GAE) per kg of olive pomace (dw).

2.6.3 Total flavonoids content

Total flavonoids (TFC) were quantified as described by Costa et al. (2018). In short, an extract aliquot was mixed with deionised water and 25% sodium nitrite. After 5 min, 10% AlCl_3 was added, and after 1 min, 2 mL 1 M NaOH were also added, followed by deionised water. The absorbance was read at 510 nm. TFC was expressed as g of epicatechin equivalents (EC) per kg of olive pomace (dw).

2.6.4 Ferric reducing antioxidant power

The ferric reducing antioxidant power (FRAP) was assessed according to Costa et al. (2018). The FRAP reagent was prepared immediately before analysis (Costa et al., 2018) and added to an extract aliquot. After incubation at 37 °C (30 min) protected from light, the absorbance was measured at 595 nm. The reducing power was expressed as g of ferrous sulfate equivalents (FSE) per kg of olive pomace (dw).

2.6.5 DPPH• scavenging ability

The anti-radical activity of extracts was studied by mixing 270 µL of a DPPH• solution (6.0×10^{-5} mol/L in ethanol) with an extract aliquot (Costa et al., 2018). The absorbance kinetics of the reaction was monitored in equal intervals of time (2 min), at 515 nm, till a plateau was achieved. DPPH• inhibition was expressed as g of trolox equivalents (TE) per kg of olive pomace (dw).

2.7 Fourier transform NIR and Fourier transform MIR spectroscopies

Samples were transferred into borosilicate flasks for spectra acquisition. For each sample, spectra of five different portions were acquired at both instruments (NIR and MIR). NIR spectra were acquired within the wavenumber interval of 10,000 to 4,000 cm^{-1} from the average of 64 scans with an 8 cm^{-1} resolution, on a Fourier transform near infrared spectrometer (FTLA 2000, ABB, Canada) equipped with an indium-gallium-arsenide (InGaAs) detector in diffuse reflectance mode. The instrument control was made through Bomen-Grams software (version 7, ABB, Canada). A Teflon reference material was used to perform the background. MIR spectra were acquired within the wavenumber interval of 4,000 cm^{-1} to 600 cm^{-1} from the average of 32 scans with a resolution of 4 cm^{-1} , on a Fourier transform PerkinElmer Spectrum BX FTIR System spectrophotometer with a DTGS detector (USA). The spectra were collected in diffuse reflectance mode through a PIKE Technologies Gladi ATR accessory with a pressure of 150 N cm^{-2} . Between spectra acquisition of different samples, the ATR crystal was cleaned and a background on air performed.

2.8 Data analysis

The one-way ANOVA was used to assess significant differences between samples, followed by Duncan post-hoc test to make pairwise comparisons between means, at $p < 0.05$ (IBM SPSS v. 25, IBM Corp., NY, USA).

Data analysis was also performed using two different chemometric methods, namely principal component analysis (PCA) (Naes et al., 2004) and partial least square discriminant analysis (PLSDA) (Barker and Rayens, 2003). The detection of outliers and common patterns was made through PCA for the chemical parameters determined and NIR and MIR spectra while PLSDA was used to develop discrimination models for NIR and MIR spectra. The infrared spectra were mean centered before PCA or PLSDA.

For PLSDA, samples were classified according to the respective olive pomace cultivar and the available data were divided into two data sets, one part for calibration (70%) and the rest for validation (30%). The division was performed randomly but maintaining the same proportion between each olive pomace cultivar to avoid unbalanced categories (Machado et al., 2018). The selection of the optimal number of latent variables (LV) to prevent model over-fitting was made through leave-one-sample-out cross-validation procedure using only the calibration set. Five spectral regions were set for both NIR and MIR data to understand which regions were more important for olive pomace cultivars differentiation and in this way to select the best spectral region. The MIR regions were:

R1 from 3982 to 2602 cm^{-1} , R2 from 2600 to 1802 cm^{-1} , R3 from 1800 to 1482 cm^{-1} , R4 from 1480 to 902 cm^{-1} and R5 from 900 to 608 cm^{-1} . In the case of NIR, the spectral regions were: R1 from 9960 to 7279 cm^{-1} , R2 from 7275 to 6507 cm^{-1} , R3 from 6504 to 5389 cm^{-1} , R4 from 5385 to 4964 cm^{-1} and R5 from 4961 to 4035 cm^{-1} . All these regions were tested individually and in all possible combinations, which makes a total of 31 possible combinations, for each data set (NIR and MIR data sets). Different pre-processing techniques including standard normal variate (SNV) and Savitzky-Golay filter (with first and second derivatives, different filter widths and polynomial orders) were also tested individually and in combination. After finding the best pre-processing technique and spectral region, the validation set was used to obtain the percentage of correct predictions in terms of olive pomace cultivars. The results were expressed as confusion matrices (Páscoa et al., 2018) where the total percentage of correct predictions is given by adding the diagonal elements of the respective confusion matrix. This was performed for each spectroscopic technique (MIR and NIR). The loadings of the first principal components and regression coefficient vectors of the best PCA and PLSDA models developed, respectively, were analysed to understand which chemical vibrations were more important for the respective models. Data analyses were performed in Matlab version 8.6 (MathWorks, Natick, USA) and PLS Toolbox version 8.2.1 (Eigen- vector Research Inc., Wenatchee, USA).

3. Results and discussion

3.1 Chemical characterization of olive pomaces

The chemical features of olive fruits, and consequently olive oils and pomaces, are dependent on environmental factors such as soil and climate, production system (traditional, intensive or super intensive), agronomic practices (e.g., irrigation and fertilization), harvesting time and system, and post-harvest storage conditions (Uncu and Ozen, 2016). These settings, as well as the olive cultivar, influence the secondary metabolites profile of olive fruits that are responsible for organoleptic properties. Also, they have effect on the antioxidant activity and in the fatty acid composition, particularly in the oleic acid (C18:1n9) amount (Rugini et al., 2016) of the fruits. Thus, the same olive cultivar can have different chemical profiles depending on the place where it grows (Kandylis et al., 2011). Currently, olive orchards comprise genotypes chosen among traditional and novel cultivars as those approached in this work (Arbequina, Arbosana, Oliana, and Koroneiki). However, many others are used, according to the region of olive oil production. Koroneiki is the most representative Greek olive cultivar that has been disseminated to other countries such as Spain, Australia, Tunisia, and Portugal (Kandylis

et al., 2011). This cultivar produces a very small fruit and has an oil production yield of 24–28% (Vossen, 2007). Arbequina fruits are similar to those of Arbosana cultivar, and Arbequina oil production yield ranges from 22 to 27% (Vossen, 2007). These three olive varieties have been selected for super-high production systems because, when compared with other olive varieties, they produce a good crop and the olive oil has good characteristics (Vossen, 2007; Tous et al., 2008). Moreover, they have a few large lateral branches, and even so they are compact which facilitates harvesting (Vossen, 2007). The Oliana cultivar is recent and results from crossing two of the most common varieties in superintensive production: Arbequina and Arbosana. Arbequina, Arbosana, and Koroneiki are the dominant varieties in super-high density orchards (Connor et al., 2014).

In this work, Arbosana pomace presented the highest total fat content (15 g/100 g, dw), followed by Oliana (13 g), Koroneiki (10 g), and Arbequina (8 g) samples (Table 1). Considering the chemical analysis performed in the lipid fraction, also Arbosana shows the major content of vitamin E (1.4 mg/g of oil), being α -tocopherol the main vitamer (1.22 mg/g of oil) (Table 1).

The fatty acids that compose the lipid fraction of the four samples are predominantly monounsaturated (Table 1). The major fatty acid present in the samples was oleic acid: 75% in Koroneiki; 73% in Oliana; 72% in Arbosana, and 70% in Arbequina. Thus, the olive pomace oil presents a high oxidative stability, due to the few double bonds of its fatty acids, that make it an ingredient suitable for being used in the food and cosmetic fields.

Phenolics are secondary plant metabolites that are characterized by the presence of an aromatic ring structure with one or more hydroxyl groups. The phenolics with two or more hydroxyl groups present *in vitro* antioxidant capacity, whereas phenolics with one hydroxyl group have low or even none antioxidant activity, as is the case of tyrosol (Tripoli et al., 2005; Peralbo-Molina et al., 2012). During the olive oil processing, several factors affect the total amount of antioxidants in olive pomace as the operation conditions and the phenolics solubility in the water and aqueous phases. Considering that the olive antioxidants are amphiphilic in nature and are more soluble in the water than in the oily phase, a significant part remains in the olive pomaces derived from the two-phases system due to its amount of water (Rodis et al., 2002; Chanioti and Tzia, 2017).

Table 1. Chemical composition of olive pomaces from Arbequina, Arbosana, Oliana, and Koroneiki cultivars.

	Olive pomaces			
	Arbequina	Arbosana	Oliana	Koroneiki
Proximate analysis (g/100 g dw)				
Total fat	8.0 ± 0.9 ^b	14.5 ± 0.8 ^a	12.9 ± 0.4 ^a	9.7 ± 0.3 ^b
Total protein	8.0 ± 0.0 ^a	7.4 ± 0.6 ^a	7.9 ± 1.3 ^a	7.1 ± 0.1 ^a
Ash	4.3 ± 0.1 ^{bc}	4.7 ± 0.1 ^a	4.0 ± 0.1 ^c	4.5 ± 0.0 ^{ab}
Total carbohydrates	79.7 ± 0.9 ^a	73.4 ± 0.1 ^c	75.2 ± 1.6 ^{bc}	78.7 ± 0.5 ^{ab}
Fatty acids (relative %)				
C14:0 (Myristic)	0.08 ± 0.03 ^a	0.03 ± 0.01 ^b	0.02 ± 0.00 ^b	0.05 ± 0.01 ^{ab}
C16:0 (Palmitic)	14.06 ± 0.10 ^a	13.95 ± 0.19 ^a	13.40 ± 0.04 ^a	11.90 ± 0.86 ^b
C16:1 (Palmitoleic)	1.16 ± 0.05 ^b	1.31 ± 0.07 ^b	1.62 ± 0.01 ^a	0.92 ± 0.13 ^c
C17:0 (Heptadecanoic)	0.09 ± 0.01 ^a	0.08 ± 0.01 ^a	0.04 ± 0.00 ^b	0.05 ± 0.01 ^b
C18:0 (Stearic)	2.00 ± 0.03 ^b	1.84 ± 0.03 ^c	1.61 ± 0.01 ^d	2.03 ± 0.50 ^a
C18:1n9c (Oleic)	70.38 ± 0.08 ^c	71.48 ± 0.05 ^{bc}	72.76 ± 0.06 ^b	75.25 ± 1.24 ^a
C18:2n6c (Linoleic)	10.66 ± 0.02 ^a	9.56 ± 0.37 ^b	8.97 ± 0.01 ^c	8.14 ± 0.24 ^d
C20:0 (Arachidic)	0.39 ± 0.01 ^b	0.39 ± 0.01 ^b	0.34 ± 0.00 ^c	0.43 ± 0.01 ^a
C18:3n3 (α-Linolenic)	0.63 ± 0.04 ^b	0.75 ± 0.02 ^a	0.69 ± 0.01 ^{ab}	0.70 ± 0.08 ^{ab}
C20:1n9 (cis-11-Eicosenoic)	0.31 ± 0.01 ^a	0.32 ± 0.01 ^a	0.32 ± 0.00 ^a	0.29 ± 0.01 ^b
C22:0 (Behenic)	0.17 ± 0.01 ^a	0.18 ± 0.00 ^a	0.15 ± 0.00 ^a	0.18 ± 0.03 ^a
C24:0 (Lignoceric)	0.08 ± 0.01 ^a	0.09 ± 0.01 ^a	0.07 ± 0.00 ^a	0.08 ± 0.01 ^a
MUFA	71.85 ± 0.05 ^d	73.11 ± 0.12 ^c	74.70 ± 0.05 ^b	76.45 ± 1.10 ^a
PUFA	11.29 ± 0.05 ^a	10.31 ± 0.36 ^b	9.67 ± 0.01 ^c	8.83 ± 0.32 ^d
SFA	16.86 ± 0.08 ^a	16.57 ± 0.24 ^a	15.64 ± 0.05 ^{ab}	14.71 ± 1.40 ^b
C18:1/C18:2	6.60 ± 0.01 ^d	7.49 ± 0.30 ^c	8.11 ± 0.01 ^b	9.25 ± 0.15 ^a
MUFA/PUFA	6.36 ± 0.03 ^d	7.09 ± 0.26 ^c	7.73 ± 0.01 ^b	8.66 ± 0.20 ^a
Vitamin E profile (mg/g oil)				
α-tocopherol	1.02 ± 0.03 ^a	1.22 ± 0.15 ^a	1.18 ± 0.21 ^a	0.35 ± 0.04 ^b
α-tocotrienol	0.10 ± 0.01 ^a	0.14 ± 0.00 ^a	0.14 ± 0.02 ^a	0.04 ± 0.00 ^b
β-tocopherol	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a	0.02 ± 0.00 ^a
γ-tocopherol	0.01 ± 0.00 ^b	0.02 ± 0.00 ^b	0.01 ± 0.00 ^b	0.11 ± 0.01 ^a
Total vitamin E	1.15 ± 0.05 ^a	1.40 ± 0.23 ^a	1.35 ± 0.32 ^a	0.52 ± 0.07 ^b
Phytochemicals				
Total phenolics (g GAE/kg dw)	5.37 ± 0.41 ^c	7.51 ± 0.50 ^b	5.91 ± 0.62 ^c	9.26 ± 0.32 ^a
Total flavonoids (g EE/kg dw)	0.15 ± 0.00 ^b	0.17 ± 0.00 ^a	0.15 ± 0.00 ^b	0.15 ± 0.00 ^b
Antioxidant activity				
FRAP (g FSE/kg dw)	66.38 ± 4.61 ^d	87.65 ± 4.66 ^b	72.02 ± 3.35 ^c	101.51 ± 2.30 ^a
DPPH• inhibition (g TE/kg dw)	3.60 ± 0.28 ^b	4.61 ± 0.53 ^a	4.14 ± 0.46 ^{ab}	4.63 ± 0.19 ^a

Data expressed as mean ± standard deviation. Different letters within each line represent significant differences between samples, at $p < 0.05$. MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; GAE, gallic acid equivalents; EE, epicatechin equivalents; FRAP, ferric reducing antioxidant power; FSE, ferrous sulfate equivalents; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; TE, trolox equivalents; dw, dry weight.

Therefore, phenolics are responsible for the great antioxidant activity of olive pomace. Previously, hydroxytyrosol was identified as the most representative phenolic compound in olive pomace, ranging from 10 to 238 mg/100 g (Peralbo-Molina et al., 2012; Nunes et al., 2018). Hydroxytyrosol results from the hydrolysis of oleuropein that occurs during olive maturation, storage and when the olives are prepared as table olives (Granados-Principal et al., 2010). The great antioxidant and chemotherapeutic efficacy of this compound (higher than tyrosol) is due to the presence of the OH group at the orthoposition (Bonechi et al., 2019). Koroneiki pomace presented 9.26 g GAE/kg of total phenolics, whereas Arbosana, Oliana, and Arbequina samples presented 7.51, 5.91, and 5.37 g, respectively (Table 1). In addition, the antioxidant activity of the samples is positively correlated with the phenolics content, meaning that this is the group of compounds responsible for the antioxidant activity of olive pomace. Nevertheless, also other compounds can contribute to the antioxidant activity of the samples, as carotenoids and vitamin E (Tripoli et al., 2005). Despite the similarities of the fruits of Arbequina and Arbosana cultivars (Vossen, 2007), in this work, Arbequina pomace presented a lower total phenolic content. Moreover, considering the overall chemical profile, it was possible to discriminate Koroneiki and Arbosana olive pomaces from the other two samples.

3.2 Spectral analyses

The average raw spectra obtained for each cultivar with both instruments (MIR and NIR) is shown in Figs. 1 and 2. Analysing both, it is possible to visualize some differences between MIR and NIR spectra according to each cultivar, but the distinction among samples through raw MIR spectra (Fig. 1) was less clear than that obtained using raw NIR spectra (Fig. 2). In fact, raw NIR spectra showed differences in all spectral regions while raw MIR spectra present differences only in some spectral regions. These results suggest a better capacity of NIR spectra to differentiate olive pomace cultivars.

3.3 Principal component analysis

Initially, the PCA was used to screen possible outliers using all the spectra obtained at NIR instrument. This same procedure was repeated considering only all the spectra obtained at MIR instrument. The analysis of Hotelling's T² and Q residuals statistics did not reveal the presence of outliers (data do not shown) for the spectra obtained at both instruments. Therefore, all the spectra were used in the subsequent analyses.

After outlier screening, PCA was applied to verify the formation of clusters according to the cultivars. This was performed considering NIR and MIR spectra separately.

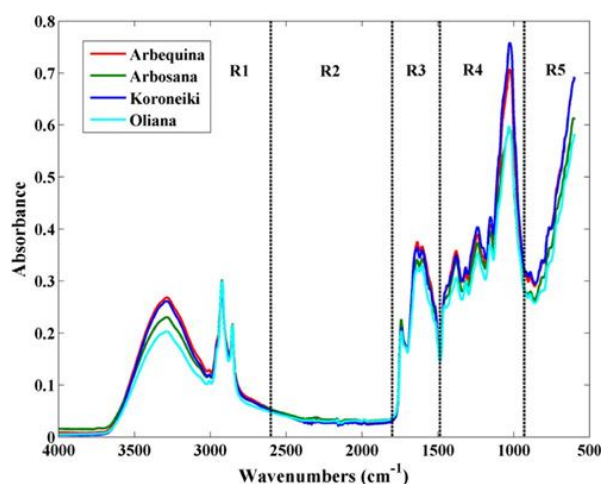


Figure 1. Average raw MIR spectra of each olive pomace cultivar.

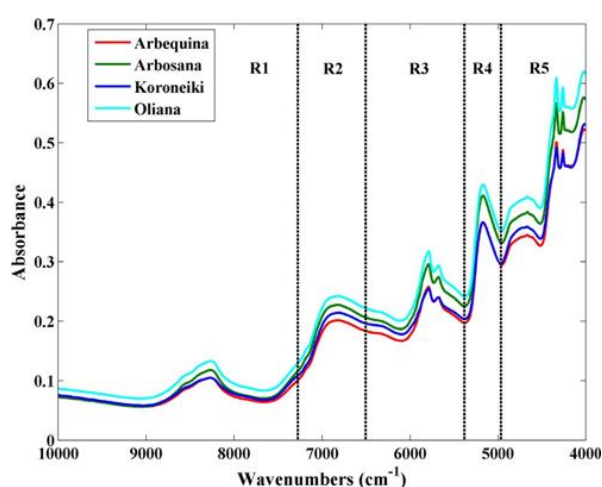


Figure 2. Average raw NIR spectra of each olive pomace cultivar.

Several combinations of spectral regions and pre-processing techniques were tested, as above mentioned, aiming to obtain the best separation among each olive pomace cultivar using only the first four principal components (Figs. 3 and 4). The best separation of olive pomace cultivars considering MIR data was obtained using the spectral region R4 individually and with the spectra pre-processed with Savitzky-Golay filter (first derivative, 15 points of filter width and second polynomial order), followed by SNV with the scores at PC1 and PC3 (Fig. 3).

The separation obtained using MIR spectra for all cultivars is not clear and it seems that the Arbosana pomace is similar to Oliana pomace. Indeed, chemical composition, especially total fat, total carbohydrates and total vitamin E contents (Table 1), also showed that these two samples are similar and distinguishable from Arbequina and Koroneiki. Therefore, these results suggest that the MIR absorptions of Arbosana

cultivar are alike to the ones obtained with Oliana cultivar. In this sense, the PCA using MIR spectra was not able to cluster according to each olive pomace cultivar.

Regarding NIR data, the best separation was obtained using the spectral regions R3 and R5 in combination and with the spectra pre-processed with Savitzky-Golay filter (first derivative, 15 points of filter width and second polynomial order) considering the scores obtained at principal component (PC) 1 and PC2 (Fig. 4).

As it can be observed, there is a clear separation between the samples because each olive pomace cultivar appeared in a different quadrant of the plot, except one sample of Arbequina cultivar. This indicates that NIR spectra contain chemical information that is related with each olive pomace cultivar. Moreover, this result suggests that NIR spectroscopy is better to differentiate olive pomace cultivars than MIR spectroscopy. This is somehow in agreement with the differences found between NIR and MIR spectra of olive pomace cultivars (Figs. 1 and 2).

It is important to analyse the loadings of PC1 and PC2 (Fig. 5) of the best PCA model obtained with NIR spectra to understand which chemical bonds were responsible for this differentiation and in this way relate them with chemical compounds present in the samples. This analysis was only performed for the PCA using NIR spectra because it was the one that showed a clear separation between each olive pomace cultivar.

The more important wavenumber regions for PC1 and PC2 were around 4070 cm^{-1} , from 4467 to 4359 cm^{-1} , around 4910 cm^{-1} and from 5890 to 5760 cm^{-1} . The wavenumbers around 4070 cm^{-1} can be related to C-H plus C-C absorption bands (combination band region) while from 4456 to 4359 cm^{-1} can be related with C-H plus C-H absorption bands including also CH_2 and CH_3 absorption bands (combination band region). The former absorption bands can be associated possibly with fatty acids and carbohydrates present in olive pomace while the latter with tocopherols.

The wavenumbers around 4910 cm^{-1} belong to the N-H combination region, namely CONH_2 absorption bands (combination bands region) which can be associated with vitamin B₃ (Xiao et al., 2012). The wavenumbers from 5890 to 5760 cm^{-1} can be related with C-H (namely CH , CH_2 and CH_3) and S-H first overtone absorption bands (first overtone region) which can be connected with the amino acid cysteine.

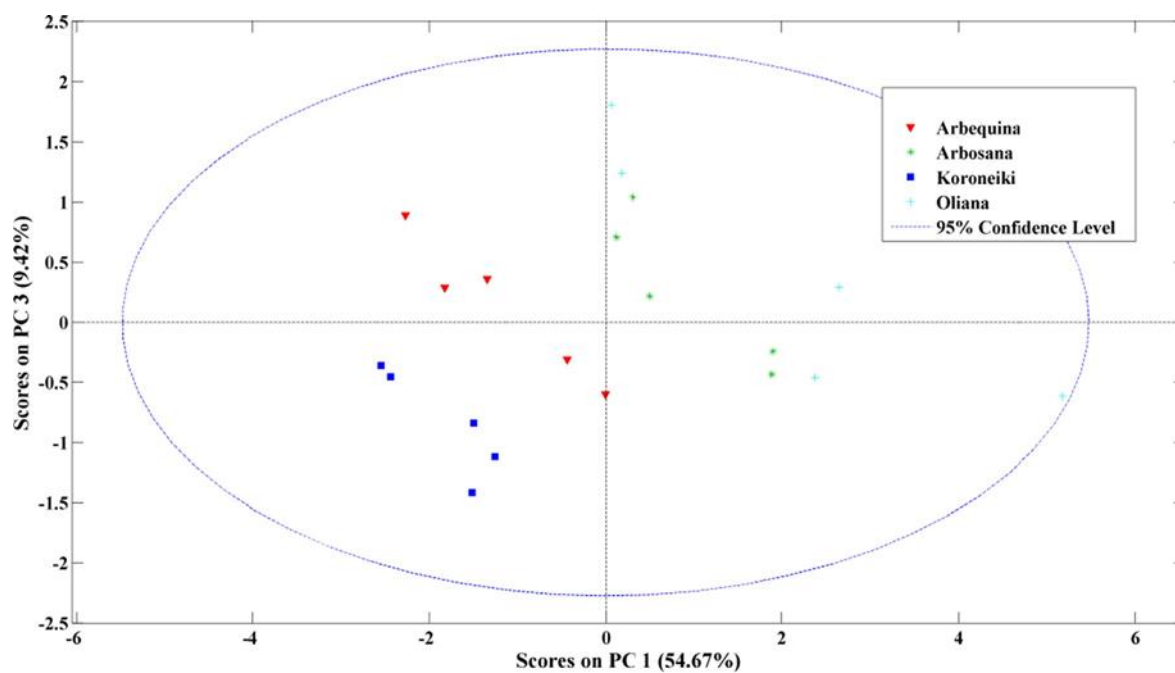


Figure 3. Scores obtained of PC1 versus scores obtained of PC3 considering MIR spectra.

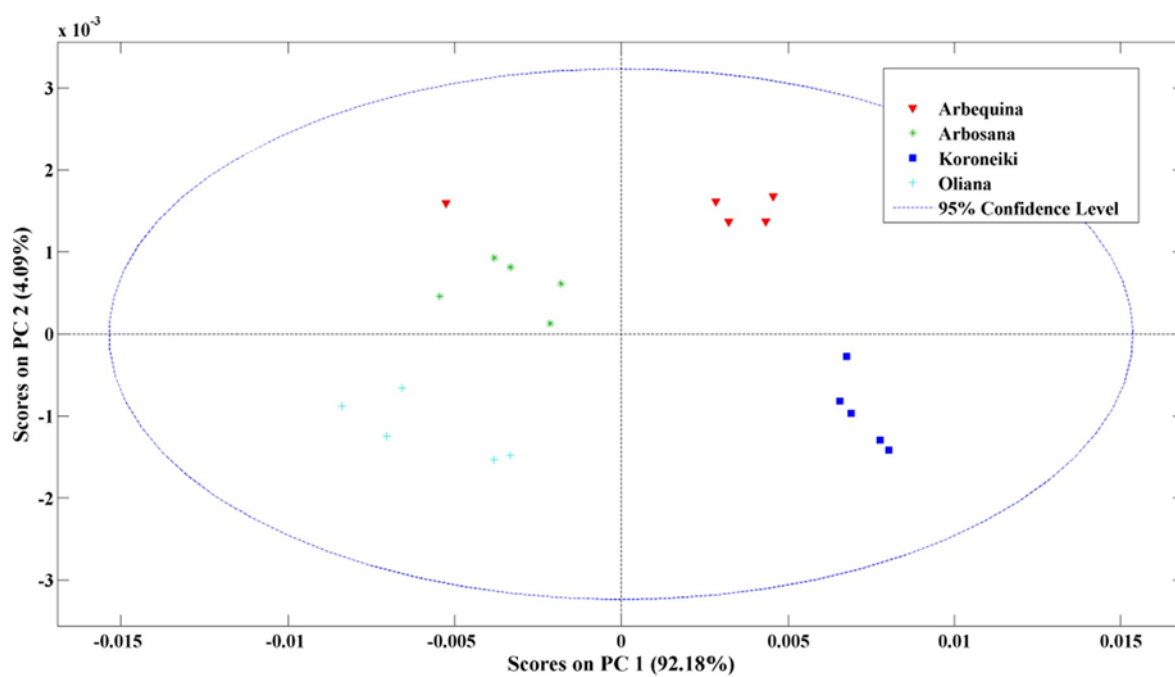


Figure 4. Scores obtained of PC1 versus scores obtained of PC2 considering NIR spectra.

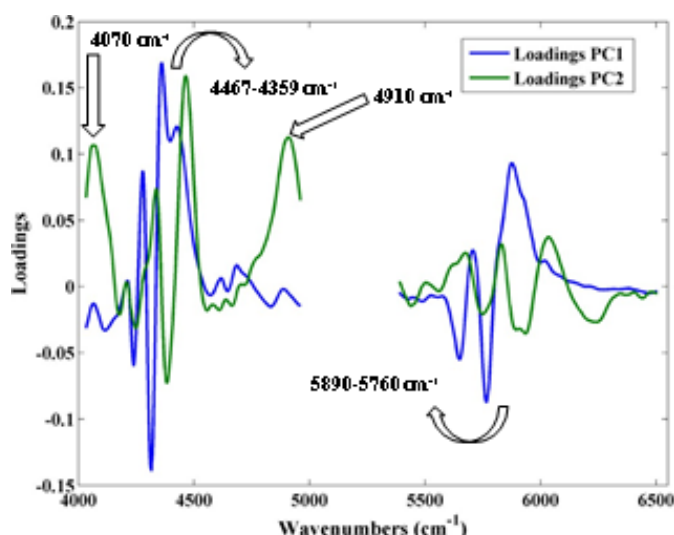


Figure 5. Loadings of PC1 and PC2 obtained with the PCA that showed the best separation between each olive pomace cultivar considering NIR data.

A PCA of data obtained by chemical analysis was also performed. The mean values of all chemical parameters determined were considered and the data were auto-scaled. The score bi-plot map generated is shown in Fig. 6. The map generated revealed that the chemical parameters/properties determined contained differences between each olive pomace cultivar and that it is possible to differentiate each olive pomace cultivar. However, the chemical profile of Arbosana and Oliana olive pomace cultivars are similar because their scores clustered close to each other. This similarity is in agreement with the results obtained through MIR spectroscopy where the scores of PC1 and PC3 of these same cultivars were also very similar. Moreover, it is possible to refer that the most important chemical parameters responsible for this differentiation are total fats amount, carbohydrates amount and the amount of C14:0 and C18:0.

It should be mentioned that the separation obtained through PCA using NIR spectra (Fig. 4) was clearer than the one obtained with the chemical parameters (Fig. 6). This suggests that NIR spectroscopy is able to better differentiate the olive pomace cultivars than all these chemical parameters/properties analysed.

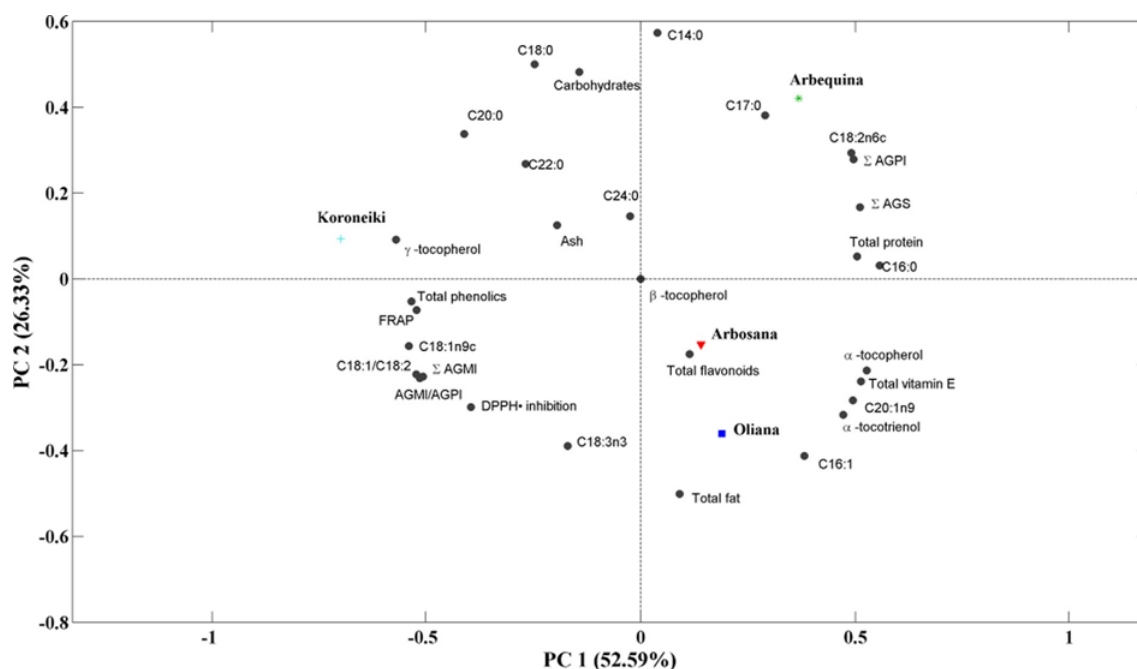


Figure 6. Biplot score map generated with the mean values of all chemical parameters.

3.4 Partial least square discriminant analysis

PCA is not a supervised method, it only reduces data dimension and maximizes the variance without forcing the model to find any relations. PLSDA is an example of a supervised classification method, that forces the model to find a relation between X and Y variables and in this case between olive pomace cultivars spectra (X) and each cultivar (Y). The results obtained with PCA (where some differentiation between each olive pomace cultivar was seen) suggest that both NIR and MIR spectra contain relevant information for the discrimination of each olive pomace cultivar. In this sense, several PLSDA models were developed considering NIR and MIR spectra. The spectral regions and pre-processing techniques that showed the best separation between each olive pomace cultivar in PCA (for both NIR and MIR spectra) are not necessarily the best options for PLSDA because the mathematical procedures within each chemometric method are different (Barker and Rayens, 2003; Naes et al., 2004). Therefore, several PLSDA models were developed to find the best spectral regions and pre-processing techniques.

For MIR spectra, the spectral region R4 and pre-processing technique Savitzky-Golay filter (second derivative, 15 points filter width and second polynomial order) using 4 LV presented the best PLSDA model: around 95% of correct predictions were obtained for the validation set. The respective confusion matrices obtained using only the calibration

set and the projection of the validation set can be consulted in the Supplementary Material 1.

The results obtained in the confusion matrix revealed that all olive pomace cultivars were well classified and therefore attested the suitability of MIR spectroscopy to classify olive pomace cultivars. About NIR spectra, the best PLSDA model was obtained when using spectral region R5, pre-processed with Savitzky-Golay filter (second derivative, 15 points filter width and second polynomial order) followed by SNV and using 4 LV. Around 98% of correct predictions for the validation set were obtained demonstrating the accuracy of NIR spectroscopy for the discrimination of olive pomace cultivars. The respective confusion matrices obtained using only the calibration set and the projection of the validation set (Supplementary Material 2) show that all olive pomace cultivars were well predicted. The PLSDA results obtained with NIR and MIR spectroscopy confirmed the ability of these methods to discriminate olive pomace cultivars. These results confirm the good separation obtained in PCA analysis with both techniques.

In this context, the suitability of NIR and MIR spectroscopy to discriminate olive pomace cultivars was demonstrated and NIR spectroscopy revealed the best results. The results obtained with MIR spectroscopy were lower than those obtained with NIR spectroscopy but also accurate.

Once again, it is important to understand which chemical compounds present in olive pomace samples could be responsible for the discriminations obtained. This can be done through the analysis of regression coefficient vectors of the developed PLSDA models (Supplementary Materials 3 and 4). These vectors reveal the more important wavenumbers for the developed PLSDA models which can be further related with specific chemical bonds absorptions.

The regression coefficient vectors obtained with MIR spectra revealed that wavenumbers within 1400–1300 cm^{-1} and 1200–1000 cm^{-1} were the more important for the developed PLSDA model. The wavenumbers within 1400–1300 cm^{-1} can be attributed to C-H bending of alkanes, =C-H bending of alkenes, C-N stretching of aromatic amines, NO_2 symmetric stretching of aliphatic and aromatic amines and P = O stretching of aromatic compounds. Volatile aromatic compounds are formed through biogenic pathways of the olive fruit, mainly by the lipoxygenase pathways and fatty acids and amino acids metabolism. In olive oil, several volatile compounds had been identified (Angerosa et al., 2004). For instance, octenol is produced during the oxidative breakdown of the linoleic acid. Many other products are derived by possible fermentation conversion of some amino acids or oxidative processes during olive oil production that could be associated

to the identified structures (Peres et al., 2017). The wavenumbers within 1200–1000 cm^{-1} can be associated with C-H bending in aromatic compounds, C-O stretching in alcohols, phenols and cyclic ethers, probably associated with polyols, such as hydroxyflavonoids (Silva et al., 2014). In addition, the oxidation of olive pomace along storage time results in the formation of hydroperoxides. Then, compounds such as alcohols, aldehydes, acids and hydrocarbons can be produced.

Olives also present alkane series of saturated hydrocarbons. In olive oil, the major hydrocarbon is squalene, a natural 30-carbon triterpene ($\text{C}_{30}\text{H}_{50}$), that represents 85–90% of the hydrocarbon fraction. Other hydrocarbons are present, in this case in the lipid fraction of the olive pomace, as C14-C30 n-alkanes and other n-alkenes (Gómez-Coca et al., 2016; Guinda et al., 1996).

The type and amount of phenolics present in olive pomace are wide and include compounds with a polyphenol structure (hydroxyl groups on aromatic rings) and compounds with a phenyl ring (e.g., phenolic acids and phenolic alcohols, such as hydroxytyrosol, tyrosol, or catechol). The major compounds identified in olive pomace belong to groups as phenolic alcohols (e.g., hydroxytyrosol and tyrosol), lignans (e.g., D(+)-*erythro*-1-(4-hydroxy-3-methoxy)-phenyl-1,2,3-propantriol and pinoresinol), secoiridoids and derivatives as oleuropein derivative, oleoside, oleoside riboside, aldehydic form of decarboxymethyl elenolic acid, elenolic acid derivative, oleoside diglucoside, verbascoside derivative, caffeoyl-6' secologanoside (Tripoli et al., 2005; Nunes et al., 2018). Regarding the PSLDA model obtained with NIR spectra, the wavenumbers that showed the highest contribution were located within 4500–4050 cm^{-1} . These wavenumbers can be related to C-H plus C-C absorption bands (combination band region) and to C-H plus C-H absorption bands including also CH_2 and CH_3 absorption bands (combination band region), possibly related to the presence of fatty acids and tocopherols.

4. Conclusions

Currently, the amount of olive pomace generated every year throughout the world, as well as its bioactive properties, demand the use of this residue. In this context, several questions arise:

- (i) as olive oil production increases, which is the solution to give to the high amounts of olive pomace generated?
- (ii) it is possible to transform lab scale projects in new viable and profitable industrial processes?

(iii) and taking into account industrial applications, how to obtain immediate and precise analysis of the raw material that arrives in the industries?

Based on these issues, analytical methods able to characterize olive oil industrial residues in a quick, non-expensive and accurate way are mandatory. The development of such methods will enhance companies interested in using olive pomace to decide which by-product is richer in terms of bioactive compounds and, at the same time, attribute a fair price to them. NIR and MIR spectroscopies demonstrated to be interesting “greener” alternatives to replace the laborious, toxic and time-consuming reference procedures used in this work. Moreover, these are multi-parametric, non-expensive, rapid and can be easily applied *in-situ* at industrial facilities. Among all the procedures studied in this work, NIR spectroscopy together with chemometric analysis were able to better differentiate the olive pomace cultivars, even when compared to the reference analytical methodologies applied.

References

- Alves, R., Casal, S., Oliveira, M.B.P.P., 2009. Determination of vitamin E in coffee beans by HPLC using a micro-extraction method. *Food Sci. Technol. Int.* 15, 57– 63.
- Angerosa, F., Servili, M., Selvaggini, R., Taticchi, A., Esposto, S., Montedoro, G., 2004. Volatile compounds in virgin olive oil: Occurrence and their relationship with the quality. *J. Chromatogr. A* 1054, 17–31.
- AOAC, 2012. Official Methods of Analysis. Association of Analytical Communities, USA.
- Barker, M., Rayens, W., 2003. Partial least squares for discrimination. *J. Chemom.* 17, 66–173.
- Barros, A.S., Nunes, A., Martins, J., Delgadillo, I., 2009. Determination of oil and water in olive and olive pomace by NIR and multivariate analysis. *Sens. Instrum. Food Qual. Saf.* 3, 180–186.
- Bendini, A., Cerretani, L., Di Virgilio, F., Belloni, P., Lercker, G., Toschi, T.G., 2007. In-process monitoring in industrial olive mill by means of FT-NIR. *Eur. J. Lipid Sci. Technol.* 109, 498–504.
- Bernardi, B., Falcone, G., Stillitano, T., Benalia, S., Strano, A., Bacenetti, J., De Luca, A.I., 2018. Harvesting system sustainability in Mediterranean olive cultivation. *Sci. Total Environ.* 625, 1446–1458.
- Bonechi, C., Donati, A., Tamasi, G., Pardini, A., Rostom, H., Leone, G., Lamponi, S., Consumi, M., Magnani, A., Rossi, C., 2019. Chemical characterization of liposomes containing nutraceutical compounds: Tyrosol, hydroxytyrosol and oleuropein. *Biophys. Chem.* 246, 25–34.

- Chanioti, S., Tzia, C., 2017. Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: oil recovery, unsaponifiable matter, total phenol content and antioxidant activity. *LWT- Food Sci. Technol.* 79, 178–189.
- Chanioti, S., Tzia, C., 2019. Evaluation of ultrasound assisted and conventional methods for production of olive pomace oil enriched in sterols and squalene. *LWT- Food Sci. Technol.* 99, 209–216.
- Connor, D.J., Gómez-del-Campo, M., Rousseaux, M.C., Searles, P.S., 2014. Structure, management and productivity of hedgerow olive orchards: A review. *Sci. Hortic.* 169, 71–93.
- Costa, A.S.G., Alves, R.C., Vinha, A.F., Costa, E., Costa, C.S.G., Nunes, M.A., Almeida, A. A., Santos-Silva, A., Oliveira, M.B.P.P., 2018. Nutritional, chemical and antioxidant/prooxidant profiles of silverskin, a coffee roasting by-product. *Food Chem.* 267, 28–35.
- Dos Santos, C.A.T., Páscoa, R.N.M.J., Lopes, J.A., 2017. A review on the application of vibrational spectroscopy in the wine industry: From soil to bottle. *TrAC Trends Anal. Chem.* 88, 100–118.
- European Commission, 2019. Olive oil: detailed information on the market situation, price developments, balance sheets, production and trade. Balance sheet. https://ec.europa.eu/info/food-farming-fisheries/plants-and-plant-products/plant-products/olive-oil_en (accessed 19 November 2019).
- Foscolou, A., Critselis, E., Panagiotakos, D., 2018. Olive oil consumption and human health: A narrative review. *Maturitas* 118, 60–66.
- Gökdoğan, O., Erdoğan, O., 2018. Evaluation of energy balance in organic olive (*Olea Europaea* L.) production in Turkey. *Erwerbs-Obstbau* 60, 47–52.
- Gómez-Coca, R.B., Pérez-Camino, M. del C., Moreda, W., 2016. Saturated hydrocarbon content in olive fruits and crude olive pomace oils. *Food Addit. Contam., Part A* 33, 391–402.
- Gouvínhas, I., de Almeida, J.M.M.M., Carvalho, T., Machado, N., Barros, A.I.R.N.A., 2015. Discrimination and characterisation of extra virgin olive oils from three cultivars in different maturation stages using Fourier transform infrared spectroscopy in tandem with chemometrics. *Food Chem.* 174, 226–232.
- Granados-Principal, S., Ramirez-Tortosa, M.C., Quiles, J.L., Ramirez-Tortosa, C.L., Sanchez-Rovira, P., 2010. Hydroxytyrosol: From laboratory investigations to future clinical trials. *Nutr. Rev.* 68, 191–206.
- Guinda, A., Lanzón, A., Albi, T., 1996. Differences in hydrocarbons of virgin olive oils obtained from several olive varieties. *J. Agric. Food Chem.* 44, 1723–1726.

- Kandyli, P., Vekiari, A.S., Kanellaki, M., Grati Kamoun, N., Msallem, M., Kourkoutas, Y., 2011. Comparative study of extra virgin olive oil flavor profile of Koroneiki variety (*Olea europaea* var. *Microcarpa alba*) cultivated in Greece and Tunisia during one period of harvesting. *LWT-Food Sci. Technol.* 44, 1333–1341.
- Lee, C., Polari, J.J., Kramer, K.E., Wang, S.C., 2018. Near-Infrared (NIR) Spectrometry as a fast and reliable tool for fat and moisture analyses in olives. *ACS Omega* 3, 16081–16088.
- Machado, J.C., Faria, M.A., Ferreira, I.M.P.L.V.O., Páscoa, R.N.M.J., Lopes, J.A., 2018. Varietal discrimination of hop pellets by near and mid infrared spectroscopy. *Talanta* 180, 69–75.
- Montenegro, L., Rapisarda, L., Ministeri, C., Puglisi, G., 2015. Effects of lipids and emulsifiers on the physicochemical and sensory properties of cosmetic emulsions containing vitamin E. *Cosmetics* 2, 35–47.
- Muik, B., Lendl, B., Molina-Díaz, A., Pérez-Villarejo, L., Ayora-Cañada, M.J., 2004. Determination of oil and water content in olive pomace using near infrared and Raman spectrometry. A comparative study. *Anal. Bioanal. Chem.* 379, 35–41.
- Naes, T., Isaksson, T., Fearn, T., Davies, T., 2004. *A User-friendly Guide to Multivariate Calibration and Classification*. IM Publications, United Kingdom.
- Nunes, M.A., Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P., 2018. Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components. *Sci. Total Environ.* 644, 229–236.
- Nunes, M.A., Pimentel, F.B., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P., 2016. Olive by-products for functional and food applications: Challenging opportunities to face environmental constraints. *Innovative Food Sci. Emerging Technol.* 35, 139–148.
- Páscoa, R.N., Moreira, S., Lopes, J.A., Sousa, C., 2018. Citrus species and hybrids depicted by near- and mid-infrared spectroscopy. *J. Sci. Food Agric.* 98, 3953–3961.
- Patent WO/2017/212450, 2017. Olive pomace products, method of production and their uses. University of Porto.
- Peralbo-Molina, Á., Priego-Capote, F., Luque de Castro, M.D., 2012. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography–tandem mass spectrometry with a quadrupole–quadrupole- time-of-flight mass detector. *J. Agric. Food Chem.* 60, 11542–11550.
- Peres, F., Martins, L.L., Ferreira-Dias, S., 2017. Influence of enzymes and technology on virgin olive oil composition. *Crit. Rev. Food Sci. Nutr.* 57, 3104–3126.

- Pimentel, F.B., Alves, R.C., Costa, A.S.G., Fernandes, T.J.R., Torres, D., Almeida, M.F., Oliveira, M.B.P.P., 2014. Nutritional composition of low protein and phenylalanine-restricted dishes prepared for phenylketonuric patients. *LWT- Food Sci. Technol.* 57, 283–289.
- Pizzi, A., Toscano, G., Foppa Pedretti, E., Duca, D., Rossini, G., Mengarelli, C., Ilari, A., Renzi, A., Mancini, M., 2018. Energy characteristics assessment of olive pomace by means of FT-NIR spectroscopy. *Energy* 147, 51–58.
- Rodis, P.S., Karathanos, V.T., Mantzavinou, A., 2002. Partitioning of olive oil antioxidants between oil and water phases. *J. Agric. Food Chem.* 50, 596–601.
- Rugini, E., Cristofori, V., Silvestri, C., 2016. Genetic improvement of olive (*Olea europaea* L.) by conventional and *in vitro* biotechnology methods. *Biotechnol. Adv.* 34, 687–696.
- Shahidi, F., de Camargo, A.C., 2016. Tocopherols and tocotrienols in common and emerging dietary sources: Occurrence, applications, and health benefits. *Int. J. Mol. Sci.* 17, 1–29.
- Silva, A.J., Silva, J.R., de Souza, N.C., Souto, P.C.S., 2014. Membranes from latex with propolis for biomedical applications. *Mater. Lett.* 116, 235–238.
- Sinelli, N., Casale, M., Di Egidio, V., Oliveri, P., Bassi, D., Tura, D., Casiraghi, E., 2010. Varietal discrimination of extra virgin olive oils by near and mid infrared spectroscopy. *Food Res. Int.* 43, 2126–2131.
- Sygouni, V., Pantziaros, A.G., Iakovides, I.C., Sfetsa, E., Bogdou, P.I., Christoforou, E.A., Paraskeva, C.A., 2019. Treatment of two-phase olive mill wastewater and recovery of phenolic compounds using membrane technology. *Membranes* 27, 1–16.
- Tapp, H.S., Defernez, M., Kemsley, E.K., 2003. FTIR Spectroscopy and multivariate analysis can distinguish the geographic origin of extra virgin olive oils. *J. Agric. Food Chem.* 51, 6110–6115.
- Thiele, J.J., Hsieh, S.N., Ekanayake-Mudiyanselage, S., 2005. Vitamin E: Critical review of its current use in cosmetic and clinical dermatology. *Dermatol. Surg.* 31, 805–813.
- Tous, J., Romero, A., Plana, J., Hermoso, J.F., 2008. Olive oil cultivars suitable for very-high density planting conditions. *Acta Hortic.* 791, 403–408.
- Tripoli, E., Giammanco, M., Tabacchi, G., Di Majo, D., Giammanco, S., La Guardia, M., 2005. The phenolic compounds of olive oil: structure, biological activity and beneficial effects on human health. *Nutr. Res. Rev.* 18, 98–112.
- Uncu, O., Ozen, B., 2016. Geographical differentiation of a monovarietal olive oil using various chemical parameters and mid-infrared spectroscopy. *Anal. Methods* 8, 4872–4880.

- Vergara-Barberán, M., Lerma-García, M.J., Herrero-Martínez, J.M., Simó-Alfonso, E.F., 2015. Cultivar discrimination of Spanish olives by using direct FTIR data combined with linear discriminant analysis. *Eur. J. Lipid Sci. Technol.* 117, 1473–1479.
- Vossen, P., 2007. Olive oil: History, production, and characteristics of the world's classic oils. *HortScience* 42, 1093–1100.
- Xiao, X., Hou, Y., Du, J., Sun, D., Bai, G., Luo, G., 2012. Determination of vitamins B2, B3, B6 and B7 in corn steep liquor by NIR and PLSR. *Trans. Tianjin Univ.* 18, 372–377.

Appendix A. Supplementary material

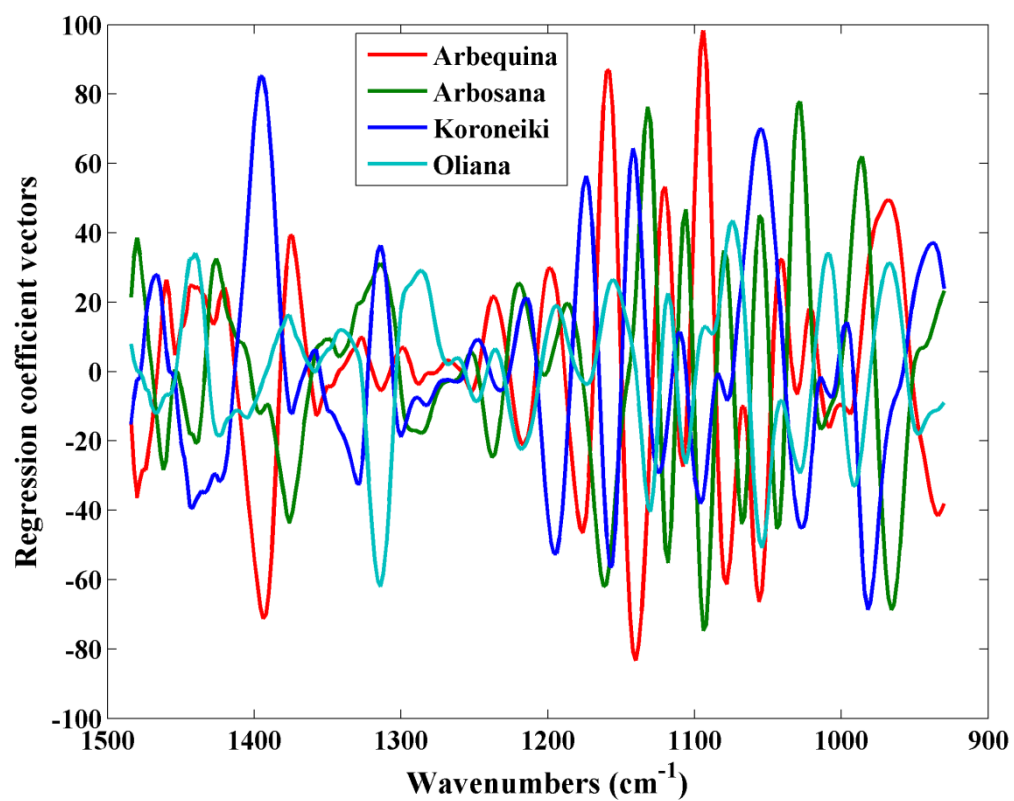
Supplementary material 1. Composition percentage of olive pomace samples.

Sample number	Olive pomace cultivars (%)			
	Arbosana	Koroneiki	Oliana	Arbequina
1	100	0	0	0
2	0	100	0	0
3	0	0	100	0
4	0	0	0	100
5	10	12.5	32.5	45
6	20	7.5	17.5	55
7	30	17.5	45	7.5
8	40	32.5	25	2.5
9	50	22	22.5	5.5
10	60	5.5	12	22.5
11	70	13.75	4	12.25
12	80	2	13.5	4.5
13	90	2.5	2.5	5
14	67.5	10	5.5	17
15	55	19.75	18	7.25
16	35	30	14.25	20.75
17	15	40	31	14
18	5	50	3.75	41.25
19	12.5	60	16.5	11
20	2.5	70	8.75	18.75
21	7.5	80	6	6.5
22	1.5	90	4.75	3.75
23	45	24.5	10	20.5
24	19	28	20	33
25	2	42	30	26
26	9	20.5	40	30.5
27	33.5	4	50	12.5
28	4	9	60	27
29	23	3.5	70	3.5
30	7	11	80	2
31	3	6	90	1
32	13	33	44	10
33	25	3	52	20
34	6.5	55	8.5	30
35	12	16	32	40
36	23.5	15	11.5	50
37	16.5	10.5	13	60
38	4.5	21	4.5	70
39	6	5	9	80
40	1	8	1	90
41	2.75	65	29	3.25
43	5.5	4.5	27.5	62.5
44	85	6.5	3.25	5.25
45	3.5	85	2.75	8.75
46	5.75	1.25	85	8
47	7.25	0.75	7	85
48	21	23	27	29
49	18	36	21	25
50	1.25	26.75	35	37
51	27	11.75	15	46.25
52	16.75	16.75	37	29.5
53	48	26	11	15

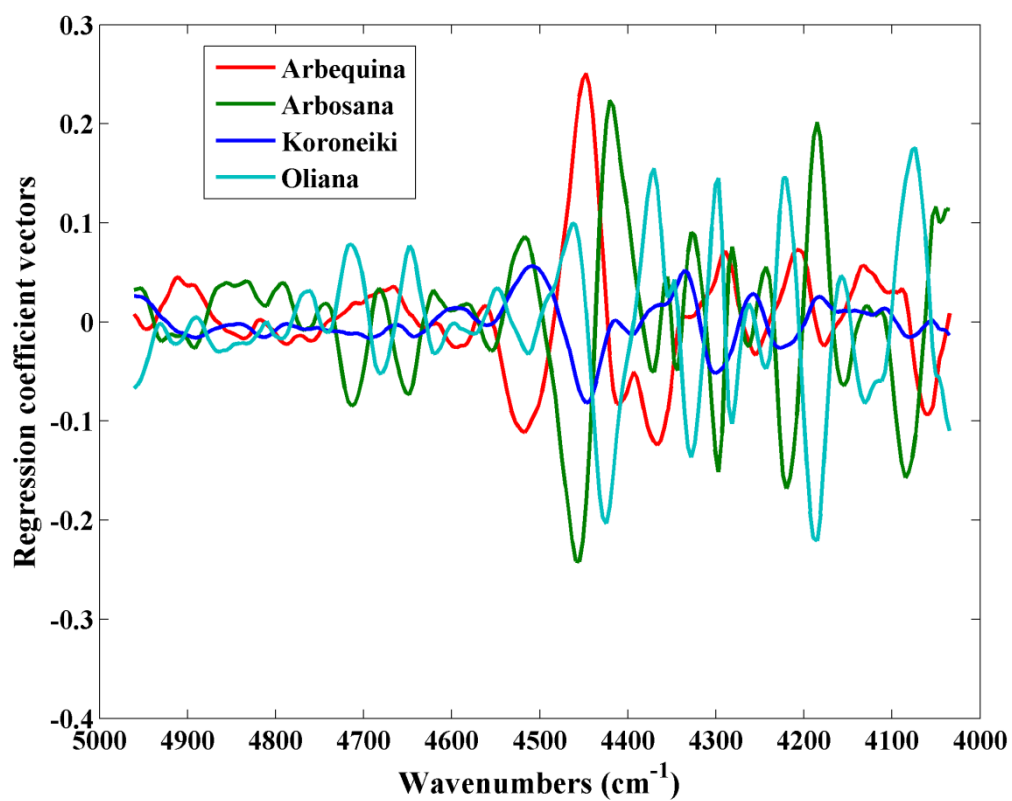
Supplementary material 2. Confusion matrices for the best PLSDA olive pomace cultivar discrimination model based on NIR spectra considering only the calibration set (100% overall correct predictions with 4 LV) and the projection of the validation set (97.8% overall correct predictions with 4 LV). Values are expressed in %.

Real olive pomace cultivars	Predicted olive pomace cultivars							
	Calibration set				Validation set			
	Arbequina	Arbosana	Koroneiki	Oliana	Arbequina	Arbosana	Koroneiki	Oliana
Arbequina	25	0	0	0	24.6	0	0.4	0
Arbosana	0	25	0	0	0.1	24.6	0	0.3
Koroneiki	0	0	25	0	1.0	0	23.8	0.3
Oliana	0	0	0	25	0	0	0.3	24.8

Supplementary material 3. Regression coefficient vectors of the PLSDA model using MIR spectra and considering the spectral region R4.



Supplementary material 4. Regression coefficient vectors of the PLSDA model using NIR spectra and considering the spectral region R5.



This page was intentionally left in blank.

3.1.5 Near infrared (NIR) spectroscopy as a tool to assess blends composition and discriminate antioxidant activity of olive pomace cultivars

¹M. Antónia Nunes, ¹Ricardo N.M.J. Páscoa, Filip Reszczyński, Anabela S.G. Costa, M. Beatriz P.P. Oliveira, Rita C. Alves

REQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy of the University of Porto, Porto, Portugal

- ¹Nunes, M.A., ¹Páscoa, R.N.M.J., Reszczyński, P., Costa, A.S.G., Oliveira, M.B.P.P., Alves, R.C. (2021). Near Infrared (NIR) spectroscopy as a tool to assess blends composition and discriminate antioxidant activity of olive pomace cultivars. *Waste and Biomass Valorization*, 12, 4901-4313. ¹Both authors contributed equally to this work.

Abstract

Purpose

Currently, monovarietal olive oils are increasingly present on the market and generate large amounts of monovarietal olive pomaces. Considering the high production of this by-product and its bioactive content, it is essential to explore green, reliable, easy-to-go, and low-cost techniques that allow their easy analysis and discrimination. In this sense, the goal of this work was to use near infrared (NIR) spectroscopy to assess the composition and to estimate the antioxidant activity of olive pomace.

Methods

A total of 53 samples including four monovarietal cultivars (Arbequina, Arbosana, Koroneiki and Oliana) and 49 different blends were used for this study. The antioxidant activity was evaluated through ferric reducing antioxidant power (FRAP), total phenolic content (TPC) and the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH[•]) scavenging ability. The NIR spectra of olive samples were calibrated against these chemical parameters and the composition of each sample using PLS modelling. The discrimination of the olive pomace samples with a high amount of bioactive compounds in terms of TPC, FRAP and DPPH scavenging ability was performed through PLSDA modelling.

Results

The PLS models results regarding olive pomace samples composition yielded coefficients of determination of prediction (R^2_p) and range error ratio (RER) values higher than 0.9 and 12, respectively, while the PLS models results for the determination of the antioxidant activity yielded unsatisfactory results. For the PLS-DA models, the percentage of correct predictions obtained using the validation set was 82, 90, 61% for DPPH scavenging ability, TPC and FRAP, respectively.

Conclusions

Globally, the obtained results attest that NIR spectroscopy is an interesting green analytical technique for the valorization of olive industry by-products, namely olive pomace, through the determination of their composition and the discrimination between samples with a higher amount of bioactive compounds.

Statement of novelty

Olive oil processing generates a major by-product known as olive pomace, which represents $\approx 80\%$ of the process. It contains high amounts of bioactive compounds, namely antioxidants, that can be used on the R&D of new products. Currently, monovarietal olive oils are increasingly present on the market and generate large amounts of monovarietal olive pomaces. The developed method proved to be rapid, accurate, cost-effective and environmentally friendly which fits the standards of the green analytical concept. While the reference procedures for the same purpose are laborious, expensive, time-consuming and toxic waste producers. In this way, the companies interested in the recovery of these valuable compounds can use this green analytical tool for attributing a value to this residue.

1. Introduction

At the present, there is a significant demand for environmentally friendly analytical techniques due to the undeniable pollution problems throughout the world caused by human beings' way of living. In this sense, near infrared (NIR) spectroscopy fulfills the requests of a green analytical technique because it is reagents free and consequently does not generate effluents. Moreover, it presents a low cost per analysis, is rapid and multi-parametric, can be applied in on-, in- and at-line measurements and does not need sample preparation. NIR spectra are characterized by overtones and combinations of fundamental vibrations of hydrogen atoms bonds, namely, C–H, O–H, N–H and S–H, on the wavenumber interval of 14,000 to 4000 cm^{-1} . For this reason, it is considered a fingerprinting technique because it reflects the properties (physical and chemical) of the samples. However, the resulting spectrum is quite complex and as so, chemometric tools need to be used to obtain useful data [1].

The application of NIR spectroscopy for the characterization of agro-food by-products (composition and antioxidant properties), namely in coffee [2] and wine [3] residues has already been performed. In the former study [2], the antioxidant properties (antioxidant activity, total phenolics and flavonoids content) of spent coffee grounds were analysed by the common reference procedures and NIR spectroscopy. PLS models were developed for each parameter analysed and the determination coefficients of prediction (R^2_P) obtained were higher than 0.9 for all the parameters. In the latter study [3], the composition (in terms of seed and skin percentages) and antioxidant properties (total phenolics and antioxidant activity) of raw and milled grape pomace samples were assessed. The best results were obtained for milled grape pomace with R^2_P higher than 0.9 for all the parameters. Both these works demonstrated the suitability of this technique for the characterization and valorization of agro-food by-products. Regarding the use of NIR spectroscopy in olive pomace samples there are few works published [4–8]. Most of them are focused on the determination of water and oil content [4, 5, 7] and only two explore the valorization of olive pomace samples [6, 8]. The obtained results on the works aiming the determination of oil and water content revealed R^2_P higher than 0.9 [4, 7] except in Giovenzana et al. 2018 [5] where the R^2_P were slightly lower. In relation to the works employing NIR spectroscopy as a tool for olive pomace valorization [6, 8], there are some interesting results. One of these works used NIR spectroscopy to assess the content of several elements namely, potassium, chlorine, sulphur, phosphorous, sodium, calcium, magnesium, iron and silicon because these parameters may provide an undesirable effect on the final quality of the produced biodiesel [6]. The PLS models obtained for chlorine, potassium and sulphur were the best, with R^2_P greater than 0.9

and residual predictive determinations (RPD) higher than 3. In the other work [8], NIR spectroscopy was tested for the characterization of olive pomace samples through gross calorific value, ash and moisture content. A RPD higher than 3 was obtained for the quantification of moisture and ash contents while for gross calorific values a RPD lower than 2 was obtained [8]. Both these works reflect the suitability of this technology for the valorization of olive pomace samples.

Olive pomace is the major waste generated during olive oil processing [9]. Its hydrophilic compounds, such as phenolics, have been focus of several research. Different phenolic profiles and amounts have been reported, varying according to distinct factors, namely the methodology used for compounds extraction [10, 11]. Recently, a combination of enzymatic and microwave procedures allowed to attain 341 mg gallic acid equivalents (GAE) of phenolics/g of sample and a 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) inhibition higher than 60% [12]. Other authors optimized extraction procedures by exploring the type of solvents, sample-to-solvent ratio and time of extraction to obtain a maximum concentration of phenolics (0.6–2 mg GAE/g) and a DPPH-inhibition between 60 and 80%, depending on the monovarietal olive pomaces [13]. Albahari et al. used ultrasound-assisted processing reaching 8–16 mg GAE/g [14] and Goldsmith and colleagues obtained 20 mg GAE/g and 31 mg trolox equivalents/g of sample [15].

Considering the olive pomace richness in phenolics and its relevance for human health, this natural available resource should be valorized. This mostly follows three axes: environment, health, and olive oil sector economic growth. Indeed, olive pomace is considered an environmental burden precisely due to the presence of phenolics and fatty acids, which can be phytotoxic. Hence, it is mandatory to reduce the amount of olive pomace discarded to the environment and, simultaneously, recover the compounds that, on one hand, are harmful to the environment but, on the other hand, are beneficial for human health.

Circular and green economies are key policies to add value to this waste. By this way, the bioactive compounds of olive pomace can be used for R&D of new food products and/or cosmetics [9]. Moreover, the global market of such products (e.g., nutraceuticals) is in clear expansion, offering great advantageous opportunities [16].

Since olive pomace is produced in high amounts in a very short time [9], a fast characterization of this raw material can allow an effective selection of the olive pomaces based on their bioactive contents, and support the development of high-yield mixtures, foreseeing their use in other industries. Hence, it will be interesting to develop a rapid, low-cost, and online analytical technique able to assess olive pomace antioxidant

properties in industries. Moreover, this analytical technique should also be capable of determining the composition of olive pomace samples in terms of its cultivars since they can be quite distinct. As so, this work aims to assess the composition of olive pomace samples in terms of its cultivars and to ascertain their respective antioxidant activity (through ferric reducing antioxidant power, total phenolics and the 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability) using NIR spectroscopy. Therefore, the valorization of bio-residues using a green analytical procedure is accomplished.

2. Material and methods

2.1 Samples

The samples were collected in a two-phase olive mill from Alentejo (38°05'17.6"N 8°10'28.8"W), the Southern region of Portugal and the main olive oil production area.

After olive oil processing, four olive pomace cultivars (Arbosana, Arbequina, Oliana and Koroneiki) were collected. The samples were freeze-dried and then ground (GM200 Grindomix, Retch, Germany) to obtain a fine powder and sieved using a 0.25 mm mesh. After this, samples of the four olive pomace cultivars were mixed according to a D-optimal mixture experimental design to obtain the samples set used in this work (Supplementary material, Table S1). A total of 53 samples were obtained.

2.2 Extracts Preparation

A hydroalcoholic solution (80% ethanol/20% water; v/v) was used for phenolics extraction. The samples were submitted to agitation (Horizontal Shaker KS 15 B, Edmund Bühler GmbH, Germany) at room temperature for 3 h, followed by filtration. Then, the extracts were stored (– 20 °C) for analysis.

2.2.1 Total phenolics compounds (TPC)

For the determination of the TPC, 30 µL of sample extract were mixed with 150 µL of a 10% Folin-Ciocalteu solution and 120 µL of Na₂CO₃ solution (7.5%). Then, the mixture was incubated at 45 °C for 15 min (kept in the dark). The absorbance was measured at 765 nm (microplate reader Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). Data were expressed in dry weight as mg of gallic acid equivalents (GAE)/g of olive pomace [17].

2.2.2 Ferric reducing antioxidant power (FRAP)

FRAP was assessed according to Costa et al. [17]. For that, 35 µL of sample extract were mixed with 265 µL of FRAP reagent. The solution was incubated (30 min; 37 °C) and

absorbance was measured at 595 nm (microplate reader Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA). Results were presented in dry weight as mg ferrous sulfate equivalents (FSE)/g of olive pomace.

2.2.3 DPPH• scavenging ability

The DPPH• scavenging ability was determined by mixing 30 µL of sample extract with 270 µL of an ethanolic DPPH• solution (6.0×10^{-5} mol/L). The kinetic of the reaction was monitored at 525 nm, with 2 min of interval, until attain the endpoint (20 min) (microplate reader Synergy HT, BioTek Instruments, Inc., Winooski, VT, USA) [17]. Data were presented in dry weight as mg of trolox equivalents (TE)/g of olive pomace.

2.3 NIR Spectra Acquisition

Before spectral acquisition, the olive pomace samples were placed into borosilicate flasks. The spectral acquisition was performed on a Fourier transform near infrared spectrometer (FTLA 2000, ABB, Canada) using a detector of indium- gallium-arsenide (InGaAs) in diffuse reflectance mode. The Bomen-Grams software (version 7, ABB, Canada) was used for spectral acquisition within 10,000 and 4000 cm^{-1} , with a 8 cm^{-1} resolution and from the average of 64 scans. The average of three spectra from each sample was considered for further analysis. The background was corrected using a Teflon reference material. Data Modelling and Statistical Analysis.

Different multivariate data analysis tools were used. The detection of outliers using squared residual statistics and Hotelling's T^2 was performed through principal component analysis (PCA) [18]. The discrimination models between NIR spectra of olive pomace samples and the chemical parameters obtained through TPC, FRAP, and DPPH• scavenging ability assays were performed through partial least square discriminant analysis (PLSDA) [19]. Partial least squares (PLS) [20] regression was selected to establish quantitative models between NIR spectra of olive pomace samples and its respective physical (composition of the samples) and chemical parameters (TPC, FRAP, and DPPH• scavenging ability). NIR spectra were previously mean centered before PCA, PLSDA and PLS.

Before diving the samples into two sets, the following procedure was adopted only for PLSDA aiming to classify the olive pomace samples into two different groups: group 1- samples with chemical parameters values $\leq 75\%$ of the maximum value; group 2- sample with chemical parameters values $> 75\%$ of the maximum value. This value was selected based on a compromise of having samples with a high amount of bioactive compounds (as companies interested in extracting bioactive compounds from olive pomace residues

will prefer samples with a high amount of bioactive compounds) and an acceptable number of samples in samples set group 2. This was done for each of the analysed chemical parameter. Thus, for each analysed chemical parameter the groups composition was different. Then, for both PLS and PLSDA, the data set was separated into two sets, one for calibration (70% of the total spectra) and the other for validation (the remaining 30% of the total spectra). A random division was made. For PLSDA, both groups of olive pomace samples maintained the same proportion of samples to prevent unbalanced categories [21]. The leave-one-out cross validation method considering only the calibration set was selected to estimate the optimal number of latent variables (LV) for both PLS and PLSDA [21]. In this way, model over-fitting was prevented. Both PLS and PLSDA models were optimized according to the best pre-processing technique and best spectral region using only the calibration set. The best pre-processing techniques were selected through testing individually and in all possible combinations Savitzky-Golay filter (with polynomial orders, using the first and second derivatives and different filter widths) and standard normal variate (SNV). The olive pomace NIR spectra was divided into five different spectral regions to understand which region was more important for each developed model (PLS or PLSDA) and in this way select the best spectral region. Then, the spectral regions were modelled individually and in all possible combinations (which gives a total of 31 possible combinations). The five spectral regions were set as follows: R1 from 4961 to 4035 cm⁻¹, R2 from 5385 to 4964 cm⁻¹, R3 from 6504 to 5389 cm⁻¹, R4 from 7622 to 6507 cm⁻¹ and 9960 to 7626 cm⁻¹. Subsequently, the projection of the validation set onto the calibration set was performed to obtain the correct predictions percentage for the PLSDA models and to obtain the prediction results for the PLS models. The PLSDA results were compiled in the format of confusion matrices [21] which gives the total percentage of correct predictions through the addition of the diagonal elements of each respective confusion matrix. The PLS results were evaluated in terms of different parameters, namely, root mean square error of calibration (RMSEC), root mean square error of cross-validation (RMSECV), root means square error of prediction (RMSEP), coefficient of determination of prediction (R²_p) and range error ratio (RER). RMSEC, RMSECV and RMSEP were calculated according to the following equation:

$$RMSE = \sqrt{\frac{\sum_{i=1}^N (\hat{\gamma}_i - \gamma_i)^2}{N}}, \quad (1)$$

In Eq. (1), N is the number of samples, γ_i is the experimental result for sample i , and $\hat{\gamma}_i$ is the value obtained with the calibration (RMSEC) using the calibration set, cross-validation (RMSECV) using the calibration set and prediction (RMSEP) using the

validation set. The Pearson's correlation coefficient was also calculated to compare experimental values with the estimations using the validation set. The RER parameter was calculated according to the following equation:

$$RER = \frac{(\gamma_{max} - \gamma_{min})}{RMSEP}, \quad (2)$$

In Eq. (2), γ_{max} and γ_{min} are the maximum and minimum values of the validation set, respectively, of each model parameter.

The more significant chemical vibrations for the best PLS and PLSDA models were visualized through the analysis of the respective regression coefficient vectors. All data modelling was carried out in Matlab version 8.6 (MathWorks, Natick, USA) and PLS Toolbox version 8.2.1 (Eigenvector Research Inc., Wenatchee, USA).

3. Results and Discussion

3.1 Spectral analysis

Figure 1 shows the raw spectra of olive pomace samples. The obtained spectra are very similar with others obtained on previous works [4, 7]. The most intense bands can be visualized in the spectral region, R1, within 4400 and 4200 cm^{-1} and can be attributed to the vibration of $-\text{CH}$, $-\text{CH}_2$ and $-\text{CH}_3$ bonds in the combination band region. The band between 5000 and 4500 cm^{-1} can be ascribed to several vibrations, namely, $-\text{NH}$ bonds, $-\text{CHO}$ bonds and combinations between $-\text{NH}$ and $-\text{CH}$ bonds in the combination band region. Regarding the spectral region R2 there is only one band present, between 5350 and 5000 cm^{-1} , that can be connected with the vibration of $-\text{OH}$ bonds present in the first overtone region and combination band region of water molecules. In the spectral region R3, the most intense bands are found between 6000 and 5600 cm^{-1} and can be attributed to the vibration of $-\text{CH}$, $-\text{CH}_2$ and $-\text{CH}_3$ bonds in the first overtone region. The most intense band visualized in the spectral region R4, can be ascribed with the vibration of $-\text{OH}$ bonds present in the second overtone region of water molecules. At last, the bands present in the spectral region R5, within 9000 to 8000 cm^{-1} , may be connected to the vibration of $-\text{CH}$, $-\text{CH}_2$ and $-\text{CH}_3$ bonds in the second overtone region.

All the spectra were analysed for the outlier detection through squared residual statistics and Hotelling's T^2 . No outliers were detected, therefore, all the spectra were considered for further modelling.

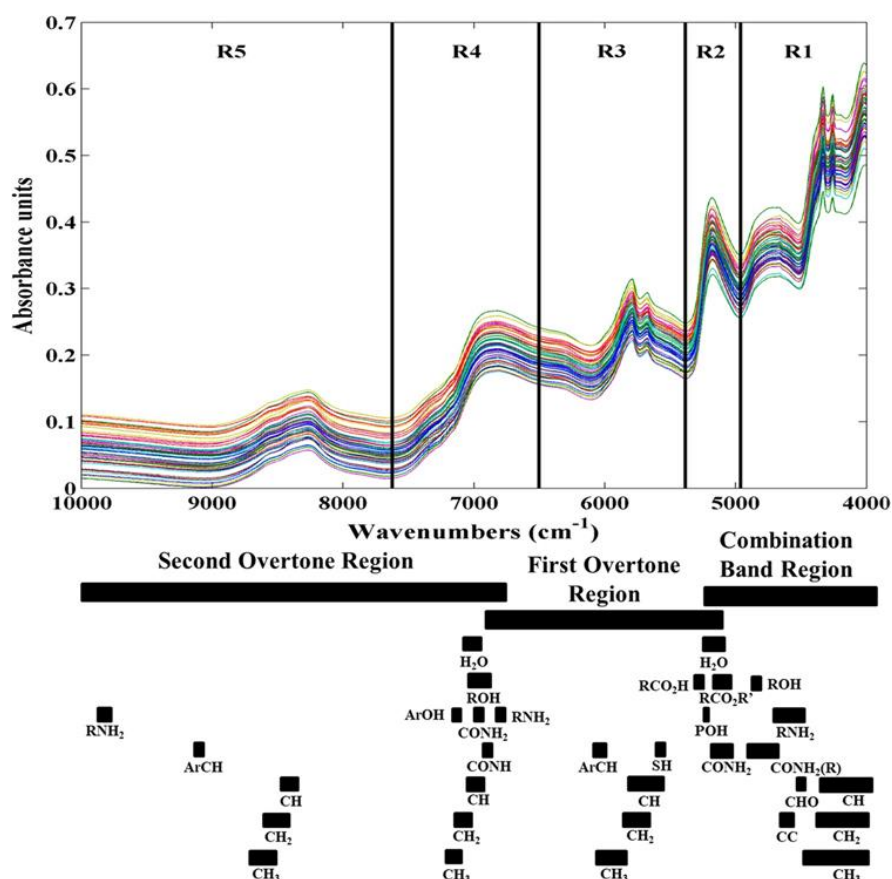


Figure 1. Raw NIR spectra of olive pomace samples and respective characteristic NIR absorption bands.

3.2 Composition determination

The chemical composition of grapes is known to be affected by several variables among which the “terroir” and cultivar [22]. In this sense, the olive pomace samples chemical’s composition as well as any other crop is influenced by the same factors. Olive cultivars are selected accordingly to yield, organoleptic characteristics, resistance to pests, among other features. The four varieties selected for this work (Arbequina, Arbosana, Koroneiki and Oliana) are predominant in super-high-density orchards. Arbosana and Arbequina are similar regarding their fruit characteristics and yield and Oliana derives from a genetic crossing between those two cultivars. This is a highly productive variety adapted to the recent harvesting techniques. Koroneiki produces a small fruit but its oil shows high stability [23–25]. Hence, different oils can be obtained and used to blend regarding the production of olive oils with specified organoleptic and chemical characteristics [26]. The edaphoclimatic conditions and the degree of fruit ripeness have also a strong influence on olive oil composition [27]. The cultivars explored in this study were processed in the same olive mill, were subjected to similar agronomic procedures, and

are from the same geographic zone, which allows to foresee that the cultivar is the major factor that could determine chemical differences in the pomaces.

Therefore, through the determination of olive pomace composition (in terms of their cultivars), it is possible to indirectly relate their composition to the chemical compounds present, namely bioactive compounds, or other valuable compounds. As above mentioned, the NIR spectra of olive pomace samples were divided into five different spectral regions and several pre-processing techniques were tested to optimize the PLS models. The best spectral region and pre-processing technique for the determination of olive pomace samples composition was obtained using spectral region R1 and through the application of Savitzky-Golay (15 points filter width, first derivate and second polynomial order) followed by SNV. The best PLS models' results for the determination olive pomace samples composition are presented in Fig. 2.

The obtained results demonstrated that it is possible to accurately determine the composition of olive pomace samples through their NIR spectra. The obtained R^2_p and RER parameters through PLS for all the cultivars were above 0.9 and 12, respectively. Arbequina cultivar determination presented the worst results when compared to the other cultivars. As so, the figures of merit of these PSL models are very acceptable and point to the fact that this technology may be used to estimate olive pomace samples composition with the advantage of no sample preparation. In fact, PLS models with RER values ≥ 10 and ≥ 15 are considered to be acceptable for quality control and very good for research quantification, respectively [28].

In this way, the method hereby described using NIR spectroscopy may be applied to characterize olive pomace samples for companies interested in recovering target compounds that exist in a higher amount at a specific cultivar and as so contribute to the valorization and reuse of this by-product.

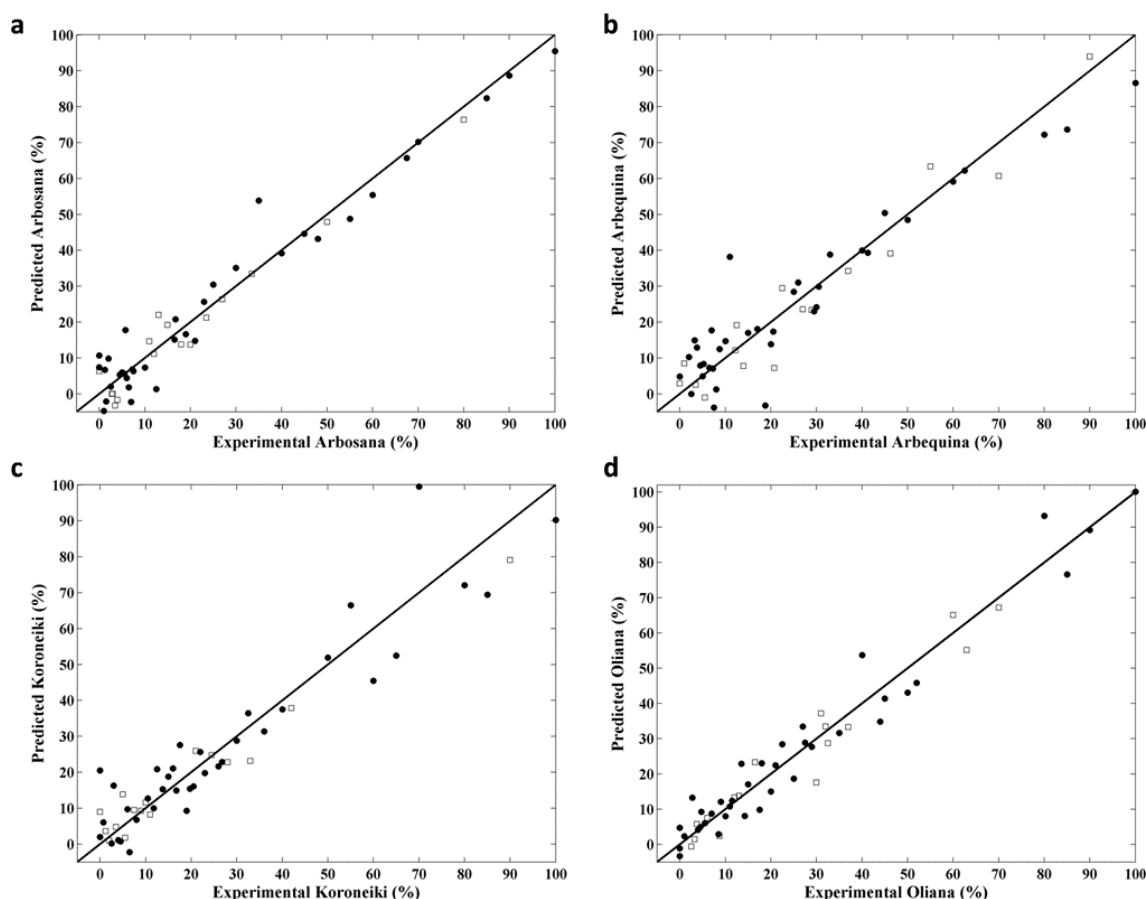


Figure 2. Experimental values against PLS cross-validation predictions (filled circle) and independent test set predictions (open square) for the composition determination of olive pomace samples (**a** Arbosana cultivar, **b** Arbequina cultivar, **c** Koroneiki cultivar, **d** Oliana cultivar) including also the corresponding parameters values of the PLS models.

The wavenumbers that showed the highest contribution in the PLS models developed can be visualized in the respective regression coefficient vectors (Fig. 3).

From the analysis of regression coefficient vectors it is possible to conclude that the more important wavenumbers were positioned within 4600 and 4300 cm^{-1} . The main NIR absorption bands present in this region are C-H and N-H vibrations that could be assigned to the oil fraction [29, 30] and proteins [30] of olive pomace samples. These absorption bands can also be related with carbohydrates [31]. This suggests that the main differences detected by NIR spectroscopy for the characterization of olive pomace samples in terms of its composition may be related with the oil fraction, proteins and carbohydrates content.

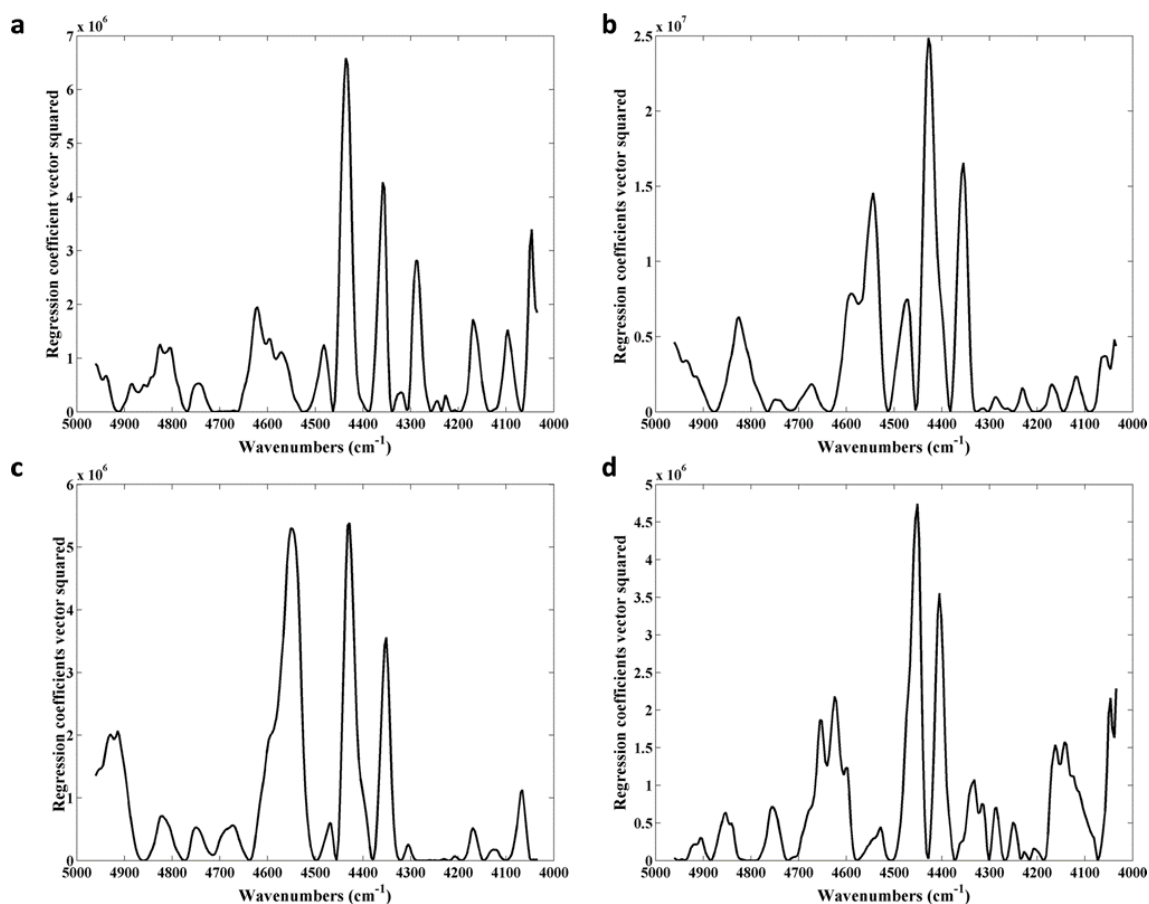


Figure 3. Regression coefficients vectors squared of the PLS models built for the composition determination of olive pomace samples with spectral region R1, from 4961 to 4035 cm^{-1} (**a** Arbosana cultivar, **b** Arbequina cultivar, **c** Koroneiki cultivar, **d** Oliana cultivar).

3.3 Chemical parameters determination

Another goal of the hereby described method was to apply NIR spectroscopy for the determination of total phenolics and the antioxidant activity (through FRAP and DPPH· scavenging ability assays) of olive pomace samples. The range and mean of the chemical evaluated parameters on olive pomace samples are given in Table 1.

Considering the 53 samples, total phenolics ranged between 8.7 and 21.2 mg GAE/g, with significant differences between monovarietal pomaces (Arbosana, 15.7; Koroneiki, 15.4; Oliana, 8.7; Arbequina, 10.7 mg GAE/g). In addition, samples blending can cause antagonistic or synergistic effects due to, for example, a stronger antioxidant that regenerates a weaker one or regeneration of stronger antioxidants, respectively [32].

Table 1. Total antioxidant activity through FRAP and DPPH[•] scavenging ability assays and total phenolics values obtained for olive pomace samples.

	Assay		
	TPC	FRAP	DPPH [•] scavenging ability
	(mg GAE/g of sample)	(mg FSE/g of sample)	(mg TE/g of sample)
Range	8.7 – 21.2	27.9 – 59.7	12.4 – 22.8
Mean + S.D.	13.9 ± 2.7	45.5 ± 6.7	18.2 ± 2.6

S.D. – standard deviation; *TPC* - total phenolics content; *FRAP* - ferric reducing antioxidant power; *DPPH[•]* - 2,2-diphenyl-1-picrylhydrazyl radical; *GAE* - gallic acid equivalents; *FSE* - ferrous sulfate equivalents; *TE* - trolox equivalents.

De Bruno et al. [13] determined the phenolics amount of the monovarietal olive pomaces Carolea and Ottobratica and phenolics ranged from 0.6 to 2 mg GAE/g [13]. The reported values were distinct from the results obtained in the present study, probably due to the olive variety, edaphoclimatic conditions, agricultural practices, the way the olive oil was processed, or even due to the different conditions used to extract the compounds [27]. Indeed, as different extraction procedures can have various degrees of efficiency and complexity, Chanioti and Tzia [11] explored combinations of green solvents and extraction-assisted methods. The extraction of phenolics was enhanced using those techniques when compared to a conventional mixture of solvents [11]. Nevertheless, solid–liquid extraction is the most used extraction method owing to its simplicity and convenience [13]. Despite that, over the last years, advanced techniques such as microwave-assisted extraction, pressurized liquid extraction, solid-phase extraction, and ultrasound-assisted extraction emerged. Ultrasound-assisted extraction is regarded as a cost-effective and simple method to be used in industrial processes. With this green process, it was possible to attain a phenolics concentration of 20 mg GAE/g using only water as a solvent [15]. Nunes et al. [9] also explored a sustainable process for phenolics extraction, a multi-frequency multimode modulated ultrasonic technique. A faster and higher recovery of phenolics (5 min, 402 µg GAE/mL) was achieved in comparison to a conventional solid–liquid extraction (60 min, 256 µg GAE/mL) [9]. Also, applying chemical-free processing to two-phase olive pomaces, it was possible to obtain juice rich in hydroxytyrosol, a major phenolic of olive pomace [33, 34].

Increasingly, solvent-free and ecological methods have been proposed for agro-wastes valorization bearing in mind efficiency versus low-cost [35]. From an industrial point of view, it is important to develop extraction techniques that present higher yields and are sustainable alternatives to the laborious extraction procedures.

Nevertheless, before the application of methodologies to recover bioactive compounds from the olive pomace, the use of a screening step as the one described in this work, can be advantageous. This can be a way of ascertaining which olive pomace or blend is richer in phenolics. Thus, it is aimed that the valorization of pomaces begins with NIR spectroscopy, despite the extraction methodology applied to recover the bioactive compounds.

Again, the development of the PLS models regarding the chemical parameters followed the same procedure adopted in the previous section, namely pre-processing technique and spectral region optimization. However, the results achieved for all the developed PLS models were unsatisfactory independently of the spectral region or pre-processing technique used. In fact, there were no significant differences between all the pre-processing techniques and spectral regions tested. The R^2_P and RER parameters were in the range between 0.5–0.6 and 4–6, respectively, for all the analysed parameters. This was not expected since other previous published works exploring NIR spectroscopy for the evaluation of the same parameters (FRAP, total phenolics and DPPH $\cdot$ scavenging ability) revealed better results [2, 3, 36–38].

It is known that chemical compounds such as bioactive compounds can interact with each other, and synergistic effects were found in olive oil [39]. Therefore, these synergistic effects or other effects (such as additive and antagonist) between the bioactive compounds that occur naturally in samples of olive pomace could explain the poor PLS results attained. Further studies regarding the effects between the bioactive compounds present in samples of olive pomace samples are needed. Another possible explanation for the poor PLS obtained results could be connected to the extraction procedure and the range studied. In fact, the chemical parameters range studied was lower than most of the other previously works cited and the extraction procedure used a solution of ethanol and water of (80/20, v/v) while the other works used a solution of water and ethanol (50/50, v/v) [2, 3], only methanol [36] and only deionized water [37, 38]. Additional studies are required to clarify these hypotheses.

3.4 Chemical parameters discrimination

The PLS models of the analysed chemical parameters generated unsatisfactory results. Thus, a different approach based on the discrimination of olive pomace samples through PLSDA was tested. The samples were classified as above-mentioned in 2.5 Data modelling section. The optimization of the pre-processing techniques and spectral regions revealed that pre-processing the entire spectra with Savitzky- Golay (15 points filter width, second polynomial order, first derivate) followed by SNV yielded the best

results. This was consistent for all the analysed chemical parameters. The percentage of correct predictions was 82, 90, 61% for DPPH[•] scavenging ability, TPC and FRAP, respectively, using 2 LV in all the models and only the validation samples. The respective confusion matrices are shown in Table 2.

Table 2. Confusion matrices obtained through PLSDA for the discrimination of olive pomace samples with high values of DPPH[•] scavenging ability, TPC and FRAP with an overall percentage of correct predictions of 82, 90 and 61%. Values are expressed in %.

Real olive pomace samples	Predicted olive pomace samples					
	DPPH [•] scavenging ability assay		TPC assay		FRAP assay	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Group 1	17	13	70	7	17	24
Group 2	6	65	4	20	15	44

DPPH[•] - 2,2-diphenyl-1-picrylhydrazyl radical; TPC - total phenolics content; FRAP - ferric reducing antioxidant power.

The obtained results for olive pomace samples discrimination based on DPPH[•] scavenging ability and TPC assays indicate that this technology may be used as a rapid, non-expensive, screening method for the valorization of these by-products. Nonetheless, the obtained results based on DPPH[•] scavenging ability assay revealed that the respective PLSDA model found some problems to discriminate group 1 samples. In fact, almost 45% of the samples of group 1 were misclassified as belonging to group 2 while the samples belonging to group 2 were well classified (around 90% of correct predictions were obtained). A possible explanation for these results can be connected with the fact that half of the samples included in group 1 (8 samples in a total of 16) presented DPPH[•] scavenging ability values very close to the limit of group division (75% of the maximum value). Another explanation could be related with the synergistic/additive/antagonist effects between bioactive compounds which are not seen in the NIR spectra. Nonetheless, from all the analysed samples, 82% of them were correctly discriminated which indicates that somehow the PLSDA model developed is quite effective. The obtained results for olive pomace samples discrimination based on FRAP assay were not satisfactory. The differences obtained on the PLSDA results can derive from the fact that different chemical compounds respond to each assay. Consequently, the chemical vibrations of some of these compounds are better captured in NIR spectra than others.

From the regression coefficient vectors analysis' it is possible to visualize the spectral regions with more impact in each PLSDA model (Fig. 4).

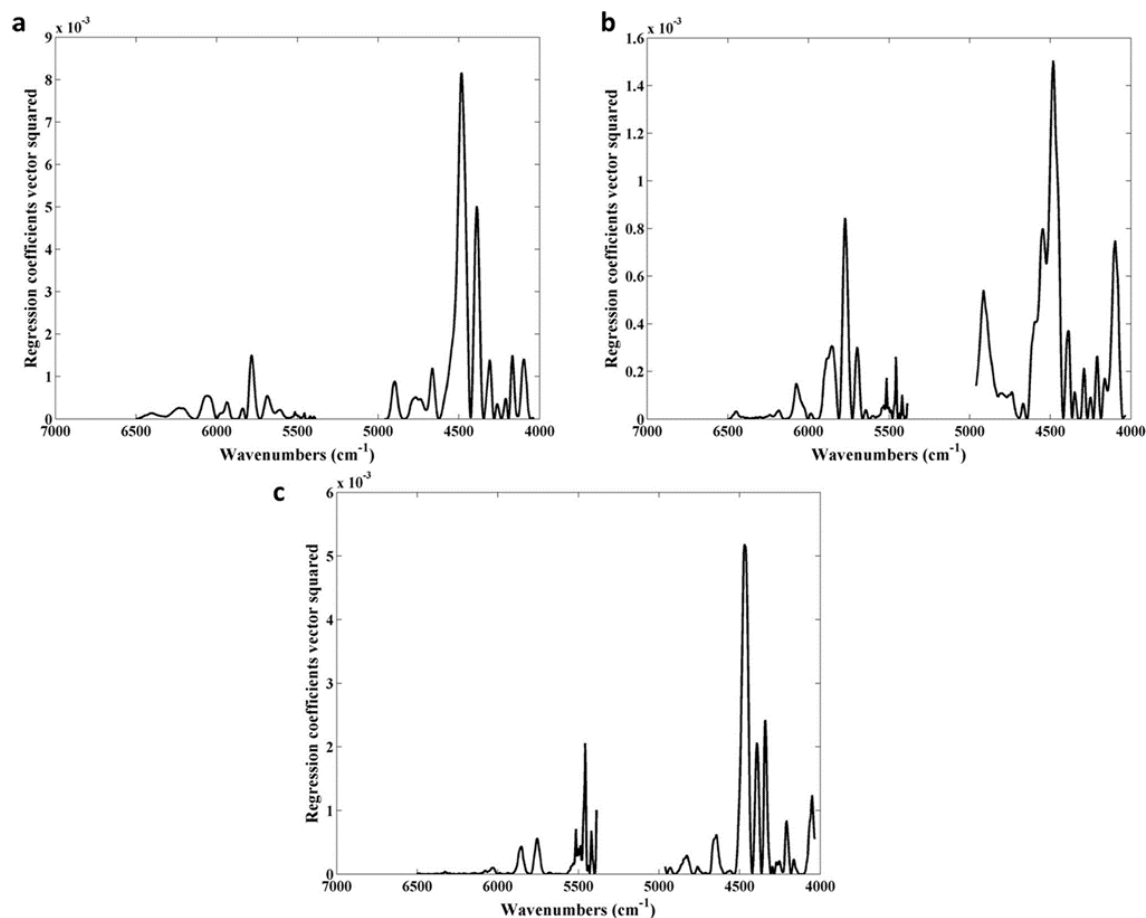


Figure 4. Regression coefficients vectors squared of the PLSDA models built for the discrimination of olive pomace samples based on DPPH $\cdot$, TPC and FRAP assays results' using the entire spectra (**a** DPPH $\cdot$ assay, **b** TPC assay, **c** FRAP assay).

Regarding DPPH $\cdot$ scavenging ability assay, the wavenumbers that showed to be more important for the respective model were present within 4550 to 4350 cm^{-1} which can be connected with several bonds -CH, -CH $_2$, -CH $_3$, -NH, -CHO in the combination band region. Several compounds contain these bonds, namely antioxidant compounds, lipids, proteins and carbohydrates as above mentioned.

The main wavenumbers concerning the PLSDA model based on TPC results were found between 5800 and 5750 cm^{-1} , 4600 and 4450 and around 4100 cm^{-1} . Phenolic compounds can be ascribed to the first interval [40]. Several vibrations bonds are located within the second interval, namely -CH, -CH $_2$, -CH $_3$, -NH, -CHO in the combination band region. In relation to 4100 cm^{-1} , the vibrations present are -CH and -CH $_2$. Several phenolic compounds possess these chemical bonds, thus it is possible that some of these compounds are being responsible for the discrimination of TPC in olive pomace samples.

About the PLSDA model based on FRAP assay, the wavenumbers with the highest importance were located around 5450 cm^{-1} and within 4500 and 4300 cm^{-1} . Around 5450 cm^{-1} , the vibrations present may be associated with the -C=O bond vibration. The last interval can be associated with the following vibrations -CH , -CH_2 , -CH_3 , -NH , -CHO in the combination band region as abovementioned. Again, several compounds as antioxidant compounds contain these bonds.

Further studies are needed to enlighten the chemical compounds that can be ascribed to these bands.

Overall, the results herein described prove that NIR spectroscopy can be an appropriate green analytical technique towards the valorization of olive industry by-products, namely olive pomace, through the determination of their composition and the discrimination between samples with a higher amount of bioactive compounds. In this context, companies interested in the extraction of chemical compounds present in olive pomace can use this method to search for a specific cultivar and/or residue with a high amount of bioactive compounds. In this way, the olive pomace can be valued (being some more valuable than others) through an environmentally and cost-effective technique when compared to the classical analysis. We believe this method will enhance the reuse of this by-product.

4. Conclusions

The quantity of olive pomace produced all over the world and their rich composition in terms of bioactive properties support the development of new approaches aiming its reuse, for example the extraction of bioactive compounds. The herein described pioneer application yielded satisfactory results regarding the determination of olive pomace cultivars composition and its bioactive properties using NIR spectroscopy. The assessment of the composition of olive pomace samples in terms of their cultivars through PLS provided satisfactory results, with R^2_P and RER values above 0.9 and 12, respectively. The obtained results attest the possibility of using NIR spectroscopy as a quality control tool. Concerning the antioxidant activity assessment (DPPH $\cdot$ scavenging ability, TPC and FRAP assays) of olive pomace samples through PLS, unsatisfactory results were obtained. This can be related with the interactions between the bioactive compounds present in olive pomace samples which can lead to synergistic/additive/antagonist effects, the extraction procedure and the range studied.

The application of PLSDA for the discrimination of olive pomace samples with a high amount of bioactive compounds presented good results for DPPH $\cdot$ scavenging ability and

TPC assays with 82 and 90%, respectively, of total correct predictions. For FRAP assay, the percentage of correct predictions obtained with PLSDA was 61%.

Globally, the results presented in this manuscript were satisfactory and enhance the reuse and valorization of olive pomace samples aiming the extraction of valuable compounds. Moreover, the method herein described based on NIR spectroscopy is rapid, accurate, multi-parametric, cost-effective and environmentally friendly which fits the standards of the green analytical concept.

References

- dos Santos, C.A.T., Pascoa, R., Lopes, J.A.: A review on the application of vibrational spectroscopy in the wine industry: from soil to bottle. *Trac-Trends Anal. Chem.* 88, 100–118 (2017).
- Pascoa, R., Magalhaes, L.M., Lopes, J.A.: FT-NIR spectroscopy as a tool for valorization of spent coffee grounds: Application to assessment of antioxidant properties. *Food Res. Int.* 51(2), 579–586 (2013).
- Pascoa, R., Machado, S., Magalhaes, L.M., Lopes, J.A.: Value adding to red grape pomace exploiting eco-friendly FT-NIR spectroscopy technique. *Food Bioprocess Technol.* 8(4), 865–874 (2015).
- Barros, A.S., Nunes, A., Martins, J., Delgadillo, I.: Determination of oil and water in olive and olive pomace by NIR and multivariate analysis. *Sens. Instrum. Food Qual. Saf.* 3(3), 180–186 (2009).
- Giovenzana, V., Beghi, R., Romaniello, R., Tamborrino, A., Guidetti, R., Leone, A.: Use of visible and near infrared spectroscopy with a view to on-line evaluation of oil content during olive processing. *Biosyst. Eng.* 172, 102–109 (2018).
- Mata-Sanchez, J., Perez-Jimenez, J.A., Diaz-Villanueva, M.J., Serrano, A., Nunez-Sanchez, N., Lopez-Gimenez, F.J.: Corrosive properties prediction from olive byproducts solid biofuel by near infrared spectroscopy. *Energy Fuels* 28(8), 5136–5143 (2014).
- Muik, B., Lendl, B., Molina-Diaz, A., Perez-Villarejo, L., Ayora-Canada, M.J.: Determination of oil and water content in olive pomace using near infrared and Raman spectrometry A comparative study. *Anal. Bioanal. Chem.* 379(1), 35–41 (2004).
- Pizzi, A., Toscano, G., Pedretti, E.F., Duca, D., Rossini, G., Mengarelli, C., Ilari, A., Renzi, A., Mancini, M.: Energy characteristics assessment of olive pomace by means of FT-NIR spectroscopy. *Energy* 147, 51–58 (2018).

- Nunes, M.A., Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., Oliveira, M.B.P.P.: Olive pomace as a valuable source of bioactive compounds: a study regarding its lipid- and water-soluble components. *Sci. Total Environ.* 644, 229–236 (2018).
- Araújo, M., Pimentel, F.B., Alves, R.C., Oliveira, M.B.P.P.: Phenolic compounds from olive mill wastes: Health effects, analytical approach and application as food antioxidants. *Trends Food Sci. Technol.* 45(2), 200–211 (2015).
- Chanioti, S., Tzia, C.: Extraction of phenolic compounds from olive pomace by using natural deep eutectic solvents and innovative extraction techniques. *Innov. Food Sci. Emerg. Technol.* 48, 228–239 (2018).
- Macedo, G.A., Santana, A.L., Crawford, L.M., Wang, S.C., Dias, F.F.G., Bell, J.M.L.N.M.: Integrated microwave-and enzyme- assisted extraction of phenolic compounds from olive pom- ace. *LWT-Food Sci. Technol.* 138, 110621 (2021).
- De Bruno, A., Romeo, R., Fedele, F.L., Sicari, A., Piscopo, A., Poiana, M.: Antioxidant activity shown by olive pomace extracts. *J. Environ. Sci. Health B* 53(8), 526–533 (2018).
- Albahari, P., Jug, M., Radić, K., Jurmanović, S., Brnčić, M., Brnčić, S.R., Vitali Čepo, D.: Characterization of olive pomace extract obtained by cyclodextrin-enhanced pulsed ultrasound assisted extraction. *LWT-Food Sci. Technol.* 92, 22–31 (2018).
- Goldsmith, C.D., Vuong, Q.V., Stathopoulos, C.E., Roach, P.D., Scarlett, C.J.: Ultrasound increases the aqueous extraction of phenolic compounds with high antioxidant activity from olive pomace. *LWT-Food Sci. Technol.* 89, 284–290 (2018).
- Bansal, R., Dhiman, A.: Nutraceuticals: a comparative analysis of regulatory framework in different countries of the World. *Endocr. Metab. Immune Disord. Drug Targets* 20(10), 1654–1663 (2020).
- Costa, A.S.G., Alves, R.C., Vinha, A.F., Costa, E., Costa, C.S.G., Nunes, M.A., Almeida, A.A., Santos-Silva, A., Oliveira, M.: Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chem.* 267, 28–35 (2018).
- Naes, T., Isaksson, T., Fearn, T., Davies, T.: Interpreting PCR and PLS solutions. A user-friendly guide to multivariate calibration and classification 1, 39–54 (2004).
- Barker, M., Rayens, W.: Partial least squares for discrimination. *J. Chemometr.* 17(3), 166–173 (2003).
- Geladi, P., Kowalski, B.R.: Partial least-squares regression - a tutorial. *Anal. Chim. Acta* 185, 1–17 (1986).

- Pascoa, R., Moreira, S., Lopes, J.A., Sousa, C.: Citrus species and hybrids depicted by near- and mid-infrared spectroscopy. *J. Sci. Food Agric.* 98(10), 3953–3961 (2018).
- Pascoa, R., Lopo, M., dos Santos, C.A.T., Graca, A.R., Lopes, J.A.: Exploratory study on vineyards soil mapping by visible/ near-infrared spectroscopy of grapevine leaves. *Comput. Electron. Agric.* 127, 15–25 (2016).
- Connor, D.J., Gomez-del-Campo, M., Rousseaux, M.C., Searles, P.S.: Structure, management and productivity of hedgerow olive orchards: A review. *Sci. Hortic.* 169, 71–93 (2014).
- Dabbou, S., Dabbou, S., Chehab, H., Brahmi, F., Taticchi, A., Servili, M., Hammami, M.: Chemical composition of virgin olive oils from Koroneiki cultivar grown in Tunisia with regard to fruit ripening and irrigation regimes. *Int. J. Food Sci. Technol.* 46(3), 577–585 (2011).
- Vossen, P.: Olive oil: history, production, and characteristics of the world's classic oils. *HortScience* 42(5), 1093–1100 (2007).
- Pereira, C., Freitas, A.M.C., Cabrita, M.J., Garcia, R.: Assessing tyrosol and hydroxytyrosol in Portuguese monovarietal olive oils: Revealing the nutraceutical potential by a combined spectroscopic and chromatographic techniques - based approach. *LWT-Food Sci. Technol.* 118, 7 (2020).
- Talhaoui, N., Gomez-Caravaca, A.M., Leon, L., De la Rosa, R., Fernandez-Gutierrez, A., Segura-Carretero, A.: From olive fruits to olive oil: phenolic compound transfer in six different olive cultivars grown under the same agronomical conditions. *Int. J. Mol. Sci.* 17(3), 14 (2016).
- Rambo, M.K.D., Amorim, E.P., Ferreira, M.M.C.: Potential of visible-near infrared spectroscopy combined with chemometrics for analysis of some constituents of coffee and banana residues. *Anal. Chim. Acta* 775, 41–49 (2013).
- Ayora-Canada, M.J., Muik, B., Garcia-Mesa, J.A., Ortega-Calderon, D., Molina-Diaz, A.: Fourier-transform near-infrared spectroscopy as a tool for olive fruit classification and quantitative analysis. *Spectr. Lett.* 38(6), 769–785 (2005).
- Saha, U., Jackson, D.: Analysis of moisture, oil, and fatty acid composition of olives by near-infrared spectroscopy: development and validation calibration models. *J. Sci. Food Agric.* 98(5), 1821–1831 (2018).
- Rodriguez-Saona, L.E., Fry, F.S., McLaughlin, M.A., Calvey, E.M.: Rapid analysis of sugars in fruit juices by FT-NIR spectroscopy. *Carbohydr. Res.* 336(1), 63–74 (2001).
- Olszowy-Tomczyk, M.: Synergistic, antagonistic and additive antioxidant effects in the binary mixtures. *Phytochem. Rev.* 19(1), 63–103 (2020).

- Malapert, A., Reboul, E., Loonis, M., Dangles, O., Tomao, V.: Direct and rapid profiling of biophenols in olive pomace by UHPLC-DAD-MS. *Food Anal. Methods* 11(4), 1001–1010 (2018).
- Ribeiro, T.B., Costa, C.M., Bonifácio - Lopes, T., Silva, S., Veiga, M., Monforte, A.R., Nunes, J., Vicente, A.A., Pintado, M.: Prebiotic effects of olive pomace powders in the gut: *In vitro* evaluation of the inhibition of adhesion of pathogens, prebiotic and antioxidant effects. *Food Hydrocoll.* 112, 106312 (2021).
- Panzella, L., Moccia, F., Nasti, R., Marzorati, S., Verotta, L., Napolitano, A.: Bioactive phenolic compounds from agri-food wastes: An update on green and sustainable extraction methodologies. *Nutr. Front.* (2020).
- Revilla, I., Vivar-Quintana, A.M., Gonzalez-Martin, I., Escuredo, O., Seijo, C.: The potential of near infrared spectroscopy for determining the phenolic, antioxidant, color and bactericide characteristics of raw propolis. *Microchem J.* 134, 211–217 (2017).
- Wu, D., Chen, J.Y., Lu, B.Y., Xiong, L.N., He, Y., Zhang, Y.: Application of near infrared spectroscopy for the rapid determination of antioxidant activity of bamboo leaf extract. *Food Chem.* 135(4), 2147–2156 (2012).
- Xia, F.B., Li, C.C., Zhao, N., Li, H., Chang, Q., Liu, X.M., Liao, Y.H., Pan, R.L.: Rapid determination of active compounds and antioxidant activity of Okra seeds using Fourier Transform Near Infrared (FT-NIR) spectroscopy. *Molecules* 23(3), 10 (2018).
- Tresserra-Rimbau, A., Lamuela-Raventos, R.M., Moreno, J.J.: Polyphenols, food and pharma. Current knowledge and directions for future research. *Biochem. Pharmacol.* 156, 186–195 (2018).
- Cozzolino, D., Kwiatkowski, M.J., Parker, M., Cynkar, W.U., Damberg, R.G., Gishen, M., Herderich, M.J.: Prediction of phenolic compounds in red wine fermentations by visible and near infrared spectroscopy. *Anal. Chim. Acta* 513(1), 73–80 (2004).

This page was intentionally left in blank.

3.1.6 Influence of olive pomace blending on antioxidant activity: additive, synergistic, and antagonistic effects

M. Antónia Nunes, Filip Reszczyński, Ricardo N.M.J. Páscoa,
Anabela S.G. Costa, Rita C. Alves, Maria Beatriz P.P. Oliveira

REQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy of the
University of Porto, Porto, Portugal

- Nunes, M.A., Reszczyński, P., Páscoa, R.N.M.J., Costa, A.S.G., Alves, R.C., Oliveira, M.B.P.P. (2021). *Influence of olive pomace blending on antioxidant activity: additive, synergistic, and antagonistic effects. Molecules*, 26, 169.

Abstract

Food innovation is moving rapidly and comprises new categories of food products and/or ingredients with a natural and ecological origin. Monocultivar olive pomaces, individually or combined, can be a source of natural bioactive compounds suitable for food or cosmetic applications. This work aimed to assess the phenolics content and antioxidant activity of four monocultivar olive pomaces (Arbosana, Koroneiki, Oliana, and Arbequina) and forty-nine blends prepared with different proportions of each. Additive, synergistic, and antagonistic effects were studied. Among the monocultivar pomaces, Koroneiki and Arbosana were the richest in total phenolics (~15 mg gallic acid eq./g). Most of the interactions found in the blends were additive or synergistic, while very few antagonistic effects were observed. The best results were obtained for those blends where the Koroneiki variety predominated: (i) 90% Koroneiki, 4.75% Oliana, 3.75% Arbequina, 1.5% Arbosana; (ii) 65% Koroneiki, 29% Oliana, 3.25% Arbequina, 2.75% Arbosana; and (iii) 85% Koroneiki, 8.75% Arbequina, 3.5% Arbosana, 2.75% Oliana. In sum, these combinations can be advantageous in comparison to the individual use of monocultivar pomaces, presenting a higher potential to be used as functional ingredients or for bioactive compounds extraction, having in view the obtention of natural preservatives or food/cosmetic formula enhancers.

1. Introduction

In recent years, science and socio-economic players have been exploring sustainable solutions for agriculture. Ag-tech systems have been emerging to increase the production of several crops, in line with the belief that the current agro-food revolution must be complemented by ecological practices, such as the management of by-products [1]. The development of new products using natural compounds recovered from food by-products is a challenge and an attempt to meet the consumers' current demand for natural and ecological products/ingredients. At the same time, it reduces the environmental and economic burden related to by-products management [2–4].

Considering the olive oil sector, the European Union produces 91% (14 million tonnes) of the world's olive oil, and the Mediterranean region contributes to 70% of that amount [5]. In recent decades, there was a global intensification of olive oil production, particularly in some countries like Portugal, due to the innovation of the sector, new plantations, and national and European investments [6]. Along with the increasing production of olive oil, the disposal of raised amounts of several by-products (such as leaves, olive mill wastewaters, and olive pomace) is also a reality.

Nowadays, monocultivar olive oils are increasingly present on the market, but some producers also develop olive oil blends based on the chemical and organoleptic features of individual olive varieties [7]. For example, Arbequina, Arbosana, and Koroneiki are predominant cultivars in super-high-density orchards [8]. The Spanish Arbosana and Arbequina cultivars are mostly known for their precocity and high yield of fruits, and their olives have similar characteristics [9]. With a Greek origin, Koroneiki is very resistant to environmental conditions, as water stress and wind, but sensitive to infestation. The fruit is very hard to harvest due to its small size. However, its oil is very stable [10]. The Oliana cultivar was introduced very recently in olive orchards as a result of genetic crossing of Arbequina and Arbosana. Its most important feature is the minor size when compared with the parental trees and the larger productivity that facilitates the modern harvest techniques [11].

Olive pomace is a major output of olive oil production. The oil itself constitutes, depending on the selected cultivar and the extraction procedure, about 15–20% of the fruit [12,13]. The remaining part constitutes the olive pomace. This by-product contains, among other components, water, polysaccharides, minerals, fatty acids, and phenolic compounds [14], retaining around 98% of the polyphenols originally present in the fruit [15]. The total amount and profile of these compounds change according to the periods of growth, maturation, and ripening of the olive. Moreover, the intensity of phenolics

biosynthesis is inversely correlated with the water availability in the soil where the tree grows. Indeed, the total content of phenolics in irrigated olive trees can be lower than in rain fed trees, and the profile can also differ [16,17]. Additionally, the olive oil processing techniques such as crushing, malaxation, and centrifugation can lead to a modification of the phenolics profile of olive oil and pomace [17].

Olive pomace antioxidants are regarded as cost-effective and a green alternative to currently used food preservatives. They can also be applied as enhancers of the nutritional profile of foodstuffs [18,19]. The main aim of this work was to ascertain the effect of blending monocultivar olive pomaces on the antioxidant properties of the samples. For that, the phenolics content and antioxidant activity of four monocultivar olive pomaces (Arbosana, Koroneiki, Oliana, and Arbequina) were explored for the first time, as well as the additive synergistic, and antagonistic effects occurring when different olive pomaces are blended in different proportions.

2. Results and discussion

The phenolics found in olive pomace are a complex combination of compounds from different groups but connected by similarities such as chemical structure and biological properties. Some of the major compounds are (i) hydroxytyrosol, tyrosol and derivatives, (ii) iridoid precursors, (iii) secoiridoids (oleuropein, oleuropein aglycone, ligstroside, verbascoside) their derivatives), (iv) flavonoids (luteolin, apigenin, rutin and derivatives), (v) lignans (pinorexinol and derivatives), and (vi) phenolic acids (gallic acid, caffeic acid, cinnamic acid, p-coumaric acid, ferulic acid, vanillic acid, and shikimic acid) [19,20].

Hydroxytyrosol is one of the most relevant antioxidants of olive pomace. Its antioxidant activity is related to its ortho-diphenol moiety which acts as chain breaker by donating a hydrogen atom to peroxy-radicals [21]. In this way, it contributes to the prevention of oxidative stress and related physiological and pathological processes (e.g., ageing, cancer, neurodegenerative and cardiovascular diseases). Hydroxytyrosol is also involved in the modulation of inflammatory response and insulin resistance [22], as well as in the activation of different cellular signaling pathways, increasing the endogenous defense systems against an oxidative stress state [23]. In general, the above-mentioned phenolics are multifunctional, which means that they can exert several antioxidant actions such as: (i) scavenging free radicals; (ii) inactivating metal catalysts; (iii) reducing hydroperoxides into stable hydroxyl derivatives; (iv) interacting with other reducing compounds in a synergistic way [24]. Table 1 shows the total phenolic content and the antioxidant activity of both monocultivar olive pomaces and different blends prepared

with variable proportions of each monocultivar. Considering the monocultivar samples, Koroneiki showed the highest total phenolics content (15.4 mg GAE/g), whereas Arbosana, Arbequina, and Oliana presented lower values, by this order: 14.7, 10.7, and 8.7 mg GAE/g. Concerning the antioxidant activity, Koroneiki achieved the highest values assessed by Ferric Reducing Antioxidant Power (FRAP) and DPPH[•] inhibition assays (58.4 mg FSE/g and 20.0 mg TE/g, respectively), although no significant differences ($p>0.05$) were observed in this last assay between Koroneiki and Arbosana (19.4 mg TE/g), the two varieties with the highest phenolics contents. Regarding the FRAP assay, no significant differences ($p>0.05$) were observed between Oliana (27.9 mg FSE/g) and Arbequina (32.0 mg FSE/g) samples (Table 1).

It is known that the phenolic content of olives differs not only according to the agronomic practices (cultivation, irrigation management, and olive maturation), soil, and edaphoclimatic conditions, but also according to genetics. These factors are reflected in the phenolics amount and profiles of olive fruits and olive oils obtained from different cultivars [25,26]. Consequently, it was also expected that olive pomaces had different compositions. For instance, Talhaoui and colleagues [25] studied the phenolic compounds of olives and extra-virgin olive oil of six different olive cultivars: Arbequina, Picual, Sikitita, Arbosana, Changlot Real, and Koroneiki. Additionally, these authors also investigated the phenolics transfer to the oil. The olives and the respective oils presented a total of 33 and 20 phenolic compounds, respectively. Regarding the fruits, Changlot Real presented the highest values in total phenolics, followed by Arbosana. In turn, the olive oil with a major phenolics content was Picual, followed by Koroneiki. Significant differences were observed among all cultivars and it was concluded that, in general, a low amount of phenolics are transferred from the fruits to the oils [25]. According to this, it is possible to infer that phenolics remain mainly in the olive oil processing by-products, such as olive pomace [27]. Moreover, the chemical compounds present in olives undergo through a cascade of transformations during processing due to, for example, the enzymatic activity of polyphenol oxidase and β -glucosidase during pressing and malaxation. These changes can be both qualitative and quantitative since they are reflected either in the phenolic profile or in the total amount of the compounds of olive fruits and oils. Thus, similar variations in olive pomace phenolics are also expected [25].

Table 1. Total phenolics and antioxidant activity (FRAP and DDPH• scavenging ability) of the monovarietal olive pomaces and mixtures. Experimental, predicted results, and type of interaction.

						Total phenolics (mg GAE/g)				Ferric reducing antioxidant power (mg FSE/g)				DPPH• scavenging ability (mg TE/g)			
Samples		Ar	K	Ol	Aq	Experimental	Predicted	p-value	Interaction	Experimental	Predicted	p-value	Interaction	Experimental	Predicted	p-value	Interaction
1	Mono1	100	0	0	0	14.7±1.1 ^{efghijkl}				48±3.8 ^{defghijk}				19.4±1.2 ^{abcdefg}			
2	Mono2	0	100	0	0	15.4±1.3 ^{cdefghijk}				58.4±10.1 ^{ab}				20±1.9 ^{abcdef}			
3	Mono3	0	0	100	0	8.7±0.7 ^p				27.9±1.5 ^q				12.5±0.4 ^q			
4	Mono4	0	0	0	100	10.7±0.8 ^o				32±0.3 ^{pq}				15.1±0.2 ^{ijklmnopq}			
5	Mix1	10	12.5	32.5	45	10.8±0.1 ^o	11±0.5	0.409	Ad	30.5±0.3 ^{pq}	35.6±1.2	0.000	At	14.8±0 ^{klmnopq}	15.3±0.3	0.124	Ad
6	Mix2	20	7.5	17.5	55	10.7±0.8 ^o	11.5±0.5	0.100	Ad	29±3.8 ^q	36.5±1.3	0.000	At	14.1±2 ^{opq}	15.9±0.5	0.024	At
7	Mix3	30	17.5	45	7.5	12.6±0.9 ^{ijklmno}	11.8±0.5	0.134	Ad	38.3±3.3 ^{nop}	39.6±1.9	0.365	Ad	17.6±1 ^{cdefghijklmno}	16.1±0.7	0.009	Sy
8	Mix4	40	32.5	25	2.5	13.1±0.4 ^{hijklmno}	13.3±0.8	0.613	Ad	41.5±4.3 ^{klmno}	46±3.5	0.051	Ad	20.5±1.8 ^{abcd}	17.8±1	0.007	Sy
9	Mix5	50	22	22.5	5.5	14.9±1.1 ^{defghijkl}	13.3±0.9	0.047	Sy	44.5±3.9 ^{ijklmno}	44.9±3.1	0.820	Ad	18.8±0.2 ^{abcdeghij}	17.8±1.3	0.194	Ad
10	Mix6	60	5.5	12	22.5	15.4±1 ^{cdefghijk}	13.1±0.9	0.002	Sy	46.4±3.1 ^{ghijklm}	42.6±2.3	0.030	Sy	18.3±2.1 ^{abcdeghijk}	17.7±1.7	0.563	Ad
11	Mix7	70	13.75	4	12.25	15.6±1.2 ^{cdeghij}	14±1.1	0.045	Sy	43.5±3.3 ^{ijklmno}	46.7±3.3	0.088	Ad	17.8±1.1 ^{bdeghijklmn}	18.7±2	0.435	Ad
12	Mix8	80	2	13.5	4.5	10.6±0.9 ^o	13.7±1.2	0.001	At	49.9±7.5 ^{cdeghij}	44.8±2.6	0.083	Ad	16.7±1.9 ^{efghijklmnop}	18.3±2.4	0.195	Ad
13	Mix9	90	2.5	2.5	5	10.9±0.2 ^o	14.3±1.4	0.000	At	46.8±2.6 ^{fghijklm}	47±3.1	0.904	Ad	18±1.3 ^{abcdeghijkl}	19.1±2.7	0.469	Ad
14	Mix10	67.5	10	5.5	17	10.6±0.7 ^o	13.7±1	0.000	At	50.6±3.7 ^{bdeghij}	45.2±2.9	0.016	Sy	19.1±2.4 ^{abcdeghij}	18.4±1.9	0.509	Ad
15	Mix11	55	19.75	18	7.25	12.7±0.9 ^{ijklmno}	13.4±0.9	0.250	Ad	41.9±2.3 ^{ijklmno}	45.3±3.1	0.050	Ad	18.1±1.7 ^{abcdeghijkl}	18±1.5	0.907	Ad
16	Mix12	35	30	14.25	20.75	13.1±0.2 ^{hijklmno}	13.2±0.7	0.707	Ad	46±5.3 ^{hijklmno}	44.9±3.4	0.659	Ad	20.1±1.4 ^{abcdef}	17.7±0.9	0.005	Sy
17	Mix13	15	40	31	14	16.2±0.1 ^{cdegh}	12.5±0.5	0.000	Sy	47.6±0.2 ^{efghijk}	43.7±3.4	0.043	Sy	18.8±0.4 ^{abcdeghij}	16.9±0.7	0.001	Sy
18	Mix14	5	50	3.75	41.25	18.1±0.4 ^{abcd}	13.2±0.6	0.002	Sy	46.7±0.7 ^{fghijklm}	45.9±4.3	0.680	Ad	17.9±0.2 ^{bdeghijklm}	17.7±1	0.839	Ad
19	Mix15	12.5	60	16.5	11	17.8±1.8 ^{bcd}	13.7±0.7	0.000	Sy	50.5±0 ^{bdeghij}	49.2±5.1	0.631	Ad	17.5±1.7 ^{cdeghijklmno}	18.2±1	0.644	Ad
20	Mix16	2.5	70	8.75	18.75	17.5±0.1 ^{bcd}	13.9±0.8	0.000	Sy	43.4±5.4 ^{ijklmno}	50.6±5.7	0.043	At	18.3±1.5 ^{abcdeghijk}	18.4±1.4	0.946	Ad
21	Mix17	7.5	80	6	6.5	17.3±1.8 ^{bcd}	14.6±1	0.007	Sy	41.8±0.3 ^{klmno}	54.1±6.6	0.001	At	17.3±0.9 ^{cdeghijklmno}	19.2±1.5	0.946	Ad
22	Mix18	1.5	90	4.75	3.75	20.1±0.8 ^{ab}	14.9±1	0.001	Sy	56.9±6.3 ^{abc}	55.8±7.3	0.776	Ad	20.9±1.3 ^{abc}	19.4±1.7	0.065	Ad
23	Mix19	45	24.5	10	20.5	15.1±1.3 ^{defghijkl}	13.4±0.8	0.026	Sy	41.8±5.4 ^{klmno}	45.3±3.3	0.150	Ad	15.9±0.5 ^{ghijklmnopq}	18±1.1	0.093	Ad

Table 1. Total phenolics and antioxidant activity (FRAP and DDPH• scavenging ability) of the monovarietal olive pomaces and mixtures. Experimental, predicted results, and type of interaction (*cont.*).

Samples		Total phenolics (mg GAE/g)								Ferric reducing antioxidant power (mg FSE/g)				DDPH• scavenging ability (mg TE/g)			
		Ar	K	Ol	Aq	Experimental	Predicted	p-value	Interaction	Experimental	Predicted	p-value	Interaction	Experimental	Predicted	p-value	Interaction
24	Mix20	19	28	20	33	14.8±2.2 ^{efghijkl}	12.4±0.5	0.030	Sy	43.4±3.8 ^{ijklmno}	41.6±2.7	0.312	Ad	20.5±1 ^{abcd}	16.8±0.5	0.000	Sy
25	Mix21	2	42	30	26	16.2±0.7 ^{cdefgh}	12.1±0.5	0.000	Sy	53.5±0.6 ^{abcde fgh}	42.2±3.3	0.000	Sy	18.2±0.1 ^{abcde fghijk}	16.5±0.9	0.008	Sy
26	Mix22	9	20.5	40	30.5	13.5±0.1 ^{hijklmno}	11.2±0.4	0.006	Sy	48.2±2 ^{defghijk}	37.2±1.7	0.000	Sy	15.3±0.2 ^{ijklmnopq}	15.5±0.4	0.463	Ad
27	Mix23	33.5	4	50	12.5	12.2±0.4 ^{klmno}	11.2±0.5	0.025	Sy	47.5±1 ^{efghijkl}	36.4±1	0.000	Sy	14.7±0.3 ^{lmnopq}	15.5±0.9	0.106	Ad
28	Mix24	4	9	60	27	10.9±0.3 ^o	10.1±0.5	0.081	Ad	39.5±2.5 ^{lmno}	32.6±0.6	0.000	Sy	12.7±0.1 ^q	14.2±0.4	0.080	Ad
29	Mix25	23	3.5	70	3.5	10.8±0.5 ^o	10.4±0.5	0.371	Ad	44.9±1.7 ^{ijklmno}	33.7±0.6	0.000	Sy	13.2±0.4 ^{pq}	14.5±0.6	0.008	At
30	Mix26	7	11	80	2	12.4±0.3 ^{ijklmno}	9.9±0.4	0.000	Sy	45.6±1.5 ^{ijklmno}	32.8±0.6	0.000	Sy	14.3±0.4 ^{mnopq}	13.9±0.4	0.492	Ad
31	Mix27	3	6	90	1	11±0.1 ^{no}	9.3±0.5	0.001	Sy	43.3±1.8 ^{ijklmno}	30.4±0.7	0.000	Sy	12.4±0.3 ^q	13.2±0.5	0.235	Ad
32	Mix28	13	33	44	10	12.3±0.4 ^{ijklmno}	11.9±0.4	0.327	Ad	42.7±4.5 ^{ijklmno}	41±2.6	0.410	Ad	15.7±0.3 ^{ijklmnopq}	16.2±0.6	0.314	Ad
33	Mix29	25	3	52	20	13.1±0.8 ^{hijklmno}	10.8±0.5	0.000	Sy	45.3±1.7 ^{ijklmno}	34.7±0.7	0.000	Sy	17.1±0.4 ^{defghijklmno}	15±0.6	0.005	Sy
34	Mix30	6.5	55	8.5	30	17.1±1.2 ^{bcdefg}	13.4±0.6	0.000	Sy	56.1±6 ^{abcd}	47.2±4.7	0.010	Sy	21.5±0.8 ^a	17.9±1.1	0.000	Sy
35	Mix31	12	16	32	40	11.9±1 ^{lmno}	11.3±0.4	0.285	Ad	43.7±2.9 ^{ijklmno}	36.8±1.5	0.001	Sy	16.6±1.2 ^{fghijklmnopq}	15.6±0.4	0.084	Ad
36	Mix32	23.5	15	11.5	50	14.2±0.1 ^{ghijklmn}	12.1±0.5	0.005	Sy	48.4±1.1 ^{defghijk}	39.3±2	0.000	Sy	18.4±0.8 ^{abcde fghijk}	16.6±0.5	0.002	Sy
37	Mix33	16.5	10.5	13	60	15.2±1.1 ^{defghijk}	11.6±0.5	0.000	Sy	48.4±0.7 ^{defghijk}	36.9±1.5	0.000	Sy	17.9±0.9 ^{abcde fghijkl}	16±0.4	0.045	Sy
38	Mix34	4.5	21	4.5	70	13.6±0.4 ^{hijklmno}	11.8±0.6	0.000	Sy	46.3±0.4 ^{ghijklmn}	38.1±2.2	0.000	Sy	18.2±0.5 ^{abcde fghijk}	16.2±0.6	0.008	Sy
39	Mix35	6	5	9	80	14.6±0.5 ^{efghijkl}	11±0.7	0.000	Sy	48.5±0.5 ^{defghijk}	33.9±1.1	0.000	Sy	18.5±0.1 ^{abcde fghij}	15.4±0.4	0.000	Sy
40	Mix36	1	8	1	90	13.4±0.2 ^{hijklmno}	11.1±0.8	0.004	Sy	45.3±2.4 ^{ijklmno}	34.2±1.4	0.000	Sy	18.9±1.5 ^{abcde fghi}	15.5±0.5	0.000	Sy
41	Mix37	2.75	65	29	3.25	21.2±0.9 ^a	13.3±0.7	0.000	Sy	54.9±4.7 ^{abede}	48.4±5	0.070	Ad	21.4±1 ^{ab}	17.7±1.2	0.000	Sy
42	Mix38	11	19	63	7	13.6±1 ^{hijklmno}	10.8±0.3	0.000	Sy	41.5±0.6 ^{klmno}	36.2±1.3	0.000	Sy	15.1±0.6 ^{ijklmnopq}	14.9±0.4	0.652	Ad
43	Mix39	5.5	4.5	27.5	62.5	11.1±0.2 ^{mno}	10.6±0.6	0.321	Ad	39±1.6 ^{mno}	33±0.9	0.000	Sy	15±0.3 ^{ijklmnopq}	14.9±0.3	0.707	Ad
44	Mix40	85	6.5	3.25	5.25	18.5±0.6 ^{abc}	14.3±1.3	0.001	Sy	54.3±0.3 ^{abcde fgh}	47.2±3.2	0.001	Sy	19.4±1 ^{abcde fgh}	19±2.5	0.784	Ad
45	Mix41	3.5	85	2.75	8.75	18.6±0.9 ^{abc}	14.8±1	0.002	Sy	59.7±5.7 ^a	54.9±7	0.189	Ad	19.9±1.3 ^{abcde f}	19.3±1.6	0.534	Ad
46	Mix42	5.75	1.25	85	8	10.9±0.5 ^{no}	9.3±0.5	0.004	Sy	38.2±3.1 ^{op}	29.8±0.9	0.000	Sy	14.2±1 ^{mnopq}	13.2±0.4	0.072	Ad

Table 1. Total phenolics and antioxidant activity (FRAP and DDPH• scavenging ability) of the monovarietal olive pomaces and mixtures. Experimental, predicted results, and type of interaction (*cont.*).

						Total phenolics (mg GAE/g)			Ferric reducing antioxidant power (mg FSE/g)				DPPH• scavenging ability (mg TE/g)				
Samples		Ar	K	Ol	Aq	Experimental	Predicted	<i>p</i> -value	Interaction	Experimental	Predicted	<i>p</i> -value	Interaction	Experimental	Predicted	<i>p</i> -value	Interaction
47	Mix43	7.25	0.75	7	85	13.8±0.1 ^{hijklmno}	10.9±0.7	0.001	Sy	46.4±1.8 ^{ghijklm}	33.1±1	0.000	Sy	19±1.4 ^{abcdefghi}	15.3±0.3	0.000	Sy
48	Mix44	21	23	27	29	15.5±1.2 ^{cdefghij}	12.1±0.5	0.001	Sy	47.5±0.1 ^{efghijkl}	40.3±2.3	0.001	Sy	20.2±0.3 ^{abcde}	16.5±0.5	0.000	Sy
49	Mix45	18	36	21	25	14.3±0.3 ^{fghijklm}	12.7±0.5	0.025	Sy	46.9±1.2 ^{efghijklm}	43.5±3.3	0.083	Ad	17.9±0.9 ^{abcdefghijkl}	17.1±0.6	0.264	Ad
50	Mix46	1.25	26.75	35	37	12.7±1 ^{ijklmno}	11.3±0.5	0.052	Ad	41.6±2.6 ^{klmno}	37.8±2	0.016	Sy	15.8±0.9 ^{hijklmnopq}	15.6±0.7	0.755	Ad
51	Mix47	27	11.75	15	46.25	12.4±1 ^{ijklmno}	12±0.5	0.335	Ad	47.7±3 ^{efghijk}	38.8±1.8	0.000	Sy	17.5±0 ^{cdefghijklmno}	16.5±0.6	0.006	Sy
52	Mix48	16.7 5	16.75	37	29.5	13.6±0.1 ^{hijklmno}	11.4±0.4	0.008	Sy	46.3±2.8 ^{ghijklmn}	37.6±1.6	0.001	Sy	17.1±1.5 ^{defghijklmno}	15.7±0.4	0.04	Sy
53	Mix49	48	26	11	15	14.4±2.2 ^{fghijkl}	13.6±0.8	0.420	Ad	54.7±4.6 ^{abcdef}	46.1±3.5	0.004	Sy	19.9±0.8 ^{abcdef}	18.2±1.2	0.035	Sy

In relation to the blends prepared in this study (Table 1), those containing a higher Koroneiki percentage (40, 50, and 60%) demonstrated greater antioxidant properties. Also, the blends with higher proportions of Koroneiki and Arbosana presented higher amounts of total phenolics. Indeed, the samples 18 (50% Koroneiki, 41.25% Arbequina, 5% Arbosana, 3.75% Oliana), 22 (90% Koroneiki, 4.75% Oliana, 3.75% Arbequina, 1.5% Arbosana), 41 (65% Koroneiki, 29% Oliana, 3.25% Arbequina, 2.75% Arbosana), 44 (85% Arbosana, 6.5% Koroneiki, 5.25% Arbequina, 3.25% Oliana), and 45 (85% Koroneiki, 8.75% Arbequina, 3.5% Arbosana, 2.75% Oliana) presented 18.1, 20.1, 21.2, 18.5, and 18.6 mg GAE/g of olive pomace, respectively, significantly higher values compared to the remaining blends. However, the antioxidant activity evaluated through the FRAP assay showed no significant differences between those samples and samples 25 (42% Koroneiki, 30% Oliana, 26% Arbequina, 2% Arbosana), 34 (55% Koroneiki, 30% Arbequina, 8.5% Oliana, 6.5% Arbosana), and 53 (48% Arbosana, 26% Koroneiki, 15% Arbequina, 11% Oliana). In fact, among all the mentioned samples, sample 45 presented the highest FRAP value (59.7 mg FSE/g) and sample 18 the lowest one (46.7 mg FSE/g). Regarding DPPH[•] inhibition, the samples 34 (55% Koroneiki, 30% Arbequina, 8.5% Oliana, 6.5% Arbosana) and 41 (65% Koroneiki, 29% Oliana, 3.25% Arbequina, 2.75% Arbosana) presented the highest value: 21.5 and 21.4 mg TE/g, respectively. The obtained results lead to questioning if there were *in vitro* additive, synergistic, or antagonistic interactions within the olive pomace blends, since all phytochemicals may interact with each other, influencing the total phenolics content of the samples as well as their antioxidant capacity [28]. Indeed, the combination of different compounds or distinct amounts of those compounds can lead to additive and synergistic effects or, in turn, to an antagonistic behavior. In the case of olive oil, synergistic effects between tyrosol, hydrocarbons (squalene) and phytosterols (β -sitosterol) have already been reported [29,30]. The complexity related to the type, amount, and bioactive action of phenolics in each monovarietal olive pomace leads to a multiplicity of interactions that are not limited to phenolic–phenolic ones but also includes other chemical components such as proteins, carbohydrates, fiber, fat and carotenoids [30]. Indeed, it is important to notice that each monovarietal pomace, individually, already presents a multitude of bioactive compounds trapped in a complex net of interactions, which can globally grow when interacting with other pomaces. By this way, in a broad sense, the function “interaction”, in this work, indicates a dynamic relationship among the bioactive compounds, particularly of phenolics, among the four olive pomaces.

Table 1 describes the type of interaction observed for each blend based on the predicted results calculated using the experimental values obtained for each monocultivar pomace.

Although several factors can affect the antioxidant properties of a single compound, namely its concentration and the interaction with other constituents, the specific interactions that are known to occur are still not completely understood [31,32]. The complexity of reactions increases as many compounds are present in a sample and with the number of matrices combined, since this raises the amplitude of interactions [32]. Furthermore, two compounds that interact in one way in a reaction environment can exhibit other behaviors when exposed to another mixture with different constituents [33]. An antagonism occurs when two or more compounds react, and the result is lower than the mathematical sum of the individual effects [32]. Some of the studied blends showed antagonistic interactions, as described in Table 1.

The different interactions observed when mixing the blends can be related to several compounds. Despite the blends being composed of four different olive varieties, the amount of phenolics is alike in Arbosana and Koroneiki pomaces. Additionally, their profile in the monocultivar samples is expected to be similar: hydroxytyrosol is the major phenolic described in olive pomaces, followed mostly by tyrosol, which has a lower antioxidant activity in comparison to the former [34]. However, besides phenolics, several other compounds are present in olive pomaces that can also contribute to their antioxidant activity. The lipid fraction, for example, contains α -tocopherol that has strong antioxidant properties, known as the most potent radical-scavenging lipophilic antioxidant. In a previous study, we found that Arbequina, Arbosana, and Oliana pomaces contained higher amounts of α -tocopherol compared to the Koroneiki one [35].

In complex mixtures, the antagonistic effects can be observed when: (i) an efficient compound regenerates another one with lower antioxidant activity; (ii) complexes and adducts are formed between antioxidant compounds; (iii) antioxidant polymerization (mainly polyphenols) occurs during oxidation, which leads to the reduction in the antioxidant capacity [32,36]. In turn, the regeneration of powerful antioxidants by weaker ones results in synergism. Other mechanisms can be related to synergism, namely (i) the development of stable complexes among compounds with great antioxidant power; (ii) the formation of dimers and adducts and/or new phenolic products with a higher antioxidant capacity than the primary compounds. The additive effect may indicate lack of interactions during the radical neutralization. This means that each antioxidant is acting independently of the others present in a complex mixture [32].

When comparing the results obtained for the different blends (Table 1), few significant differences were observed among the samples regarding not only the total phenolics content, but also the antioxidant activities evaluated by both assays. This can be related

to the low percentage variation between some blends that could be insufficient to cause significant differences in those parameters. However, the broad range of percentages used in this study allowed to foresee, at a large scale, the antioxidant yield of the mixtures.

The higher synergistic effect in phenolics content corresponded to the pomace blends containing Koroneiki (samples 18, 22, 41, and 45) and Arbosana (sample 44) in percentages above 50 and 85%, respectively. However, this was not directly correlated with the FRAP interactions (in which higher values were related to higher Arbosana percentages) or even with DPPH• inhibition. This can be associated with the presence of several compounds that are contributing to the antioxidant activity and the different mechanisms of reaction of each method. While in the FRAP assay, the detected antioxidants are able to transfer an electron and reduce the 2,4,6-tripyridyl-s-triazine/ferric ion complex (antioxidants with a redox potential lower than 0.7 V), the DPPH• inhibition method evaluates the ability of antioxidants to scavenge this radical, which can occur via both electron or H atom transfer (reduction and radical quenching, respectively). These two methods were selected to perform this study, since they have complementary mechanisms of action, which is advisable to better understand the antioxidant properties of the samples [37].

Most of the interactions among the four olive pomaces were synergistic regarding total phenolics (32 samples) and FRAP values (28 samples), and additive for DPPH• inhibition (28 samples). In general, very few antagonistic interactions were observed (samples 12, 13, and 14, for total phenolics; 5 and 6, for FRAP; and 7 and 29, for DPPH• inhibition). Sometimes, the same blend showed a synergism in phenolics content and an antagonism in the FRAP assay (e.g., samples 20 and 21); or an additive effect in phenolics, together with synergism in FRAP and antagonism in DPPH• inhibition (sample 29). In this case, the major olive pomace present was Oliana (70%). This olive variety is not extensively characterized since it is recent in orchards, but it will be relevant to investigate its individual compounds and/or amounts that can differentiate its behavior from the other pomaces. Sample 6 presented antagonistic effects in FRAP and DPPH• assays and an additive interaction in phenolics although, in this case, Arbequina (55%) and Arbosana (20%) were the major pomaces present, followed by Oliana (17.5%).

From all the blends, samples 22, 41, and 45 can be highlighted as they presented higher amounts of total phenolics and higher antioxidant activity. These samples are mostly composed of the Koroneiki olive pomace, the monocultivar with a higher amount of total phenolics, followed by Oliana (samples 22 and 41) and Arbequina (sample 45). All three

blends presented synergistic (total phenolics) and additive (FRAP assay) effects. Concerning DPPH• inhibition, samples 22 and 45 presented an additive effect, whereas in sample 41 a synergistic effect occurred.

In sum, the following combinations of pomace mixtures: (i) 90% Koroneiki, 4.75% Oliana, 3.75% Arbequina, 1.5% Arbosana; (ii) 65% Koroneiki, 29% Oliana, 3.25% Arbequina, 2.75% Arbosana; and (iii) 85% Koroneiki, 8.75% Arbequina, 3.5% Arbosana, 2.75% Oliana, presented major potential to be used as functional ingredients or for bioactive compounds extraction since their antioxidant properties are increased. Those can be applied, for example, as natural preservatives and/or food and cosmetics formula enhancers.

3. Materials and methods

3.1 Reagents and standards

Absolute ethanol and sodium carbonate decahydrate were purchased from Merck (Darmstadt, Germany). Gallic acid, heptahydrate ferrous sulfate, trolox, Folin-Ciocalteu reagent, 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•), ferric chloride, 2,4,6-tripyridyl-triazine, sodium acetate were all acquired from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

3.2 Samples

Olive pomace samples (10 kg) obtained from a two-phase extraction olive mill (in Alentejo, Portugal) were kindly provided by a national company (SOVENA) in the season 2017/2018 (January 2018). Four monocultivar olive pomaces were collected after individual olive oil processing: Arbosana, Koroneiki, Oliana, and Arbequina. As soon as samples arrived at the laboratory, they were freeze-dried and ground (GM200 Grindomix, Retch, Germany). Then, the four olive pomaces were blended to obtain the 49 mixtures described in Table 1. The mixtures were prepared following a D-optimal mixture experimental design with extra samples carried out in the Design Expert software (Stat-Ease Inc., MN, USA).

3.3 Antioxidants extraction procedure

The extraction procedure was performed using an ethanol:water solution (80/20; v/v) with magnetic stirring (Horizontal Shaker KS 15 B, Edmund Bühler GmbH, Germany) at room temperature for 3 h. Then, samples were filtered (Whatman n.º 4 filter) and the extracts analysed. Extractions were performed in triplicate.

3.4 Total phenolics content

The determination of total phenolics content was carried out, in triplicate, as described by Costa et al. [37] with some modifications. Briefly, 30 μL of extract was mixed with 150 μL of Folin–Ciocalteu reagent (10%) and 120 μL of Na_2CO_3 solution (7.5%) in a 96-well microplate. The solution was incubated at 45 °C, for 15 min, in the dark. The absorbance was measured at 765 nm using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The results were expressed as mg of gallic acid equivalents (GAE)/g of olive pomace (dry weight).

3.5 Antioxidant activity

The antioxidant activity was evaluated by two complementary methods: ferric reducing antioxidant power (FRAP) and the DPPH $\cdot$ scavenging ability. Each assay was performed in triplicate.

3.5.1 FRAP

For the FRAP assay, an aliquot (35 μL) of each sample was mixed with 265 μL of FRAP reagent prepared according to Costa et al. [37]. Then, the mixture was incubated for 30 min at 37 °C (protected from light). Measurement of absorbance was performed at 595 nm in a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA). The results were presented as mg ferrous sulfate equivalents (FSE)/g of olive pomace (dry weight).

3.5.2 DPPH $\cdot$ Scavenging ability

The anti-radical ability of the extracts was assessed according to Costa et al. [37]. Briefly, an aliquot (30 μL) was mixed with 270 μL of an ethanolic DPPH $\cdot$ solution (6.0×10^{-5} mol/L). The reaction kinetic was monitored (with 2 min intervals) at 525 nm (Synergy HT Microplate Reader, BioTek Instruments, Inc., Winooski, VT, USA) until a plateau was attained. The results were expressed as mg of trolox equivalents (TE)/g of olive pomace (dry weight).

3.6 Synergistic, additive, and antagonistic interactions

The *in vitro* synergistic, additive, and antagonistic interactions obtained with olive pomaces blending were studied.

Predicted values were calculated through the mathematical sum of the total phenolic contents and antioxidant capacity values obtained for the monocultivar olive pomaces.

Experimental values determined for the blended samples were compared with the respective predicted ones. When significant differences ($p < 0.05$) were found between experimental and predicted data, the effects were classified as synergistic or antagonistic (for positive or negative differences, respectively). When no significant differences ($p > 0.05$) were observed between experimental and predicted data, the effect was classified as additive.

3.7 Statistical analysis

Statistical analysis was performed using IBM SPSS v. 25 (IBM Corp., Armonk, NY, USA). Data are expressed as mean $\pm$ standard deviation ($n=9$). The Shapiro–Wilk test was used to evaluate data normality. The one-way ANOVA was used to assess significant differences between samples, followed by Tukey’s HSD or Dunnett T3 post hoc test (selected based on the equality of the variances) to make pairwise comparisons between means. Student’s t-test was used to define the type of effect (synergistic, antagonistic, or additive) by comparing the expected and the experimental values. The level of significance for all hypothesis tests (p) was 0.05.

4. Conclusions

Olive oil is mostly obtained from different mixed olive cultivars, usually present in the same orchard, but the market demand for monocultivar olive oils has been increasing. Huge amounts of olive pomace are being produced and suggestions on how it can be valued and recovered are needed.

The present work, besides anticipating a valuable application of bioactive compounds derived from Arbosana, Koroneiki, Oliana, and Arbequina cultivars as natural antioxidants, also shows that it can be gainful to mix the monocultivar pomaces rather than use them individually. The key is to select the most promising varieties and the adequate proportions to obtain the greatest synergistic effect among the samples. In this work, the best results were obtained using a mixture where the Koroneiki variety predominates.

Nevertheless, further studies are needed to explore the association of the described interactions and the phenolics profile. Characteristic individual olive pomace compounds and resultant interactions should also be investigated.

References

1. Rotz, S.; Duncan, E.; Small, M.; Botschner, J.; Dara, R.; Mosby, I.; Reed, M.; Fraser, E.D.G. The politics of digital agricultural technologies: A preliminary review. *Sociol. Rural.* 2019, 59, 203–229.
2. Verain, M.C.D.; Sijtsma, S.J.; Antonides, G. Consumer segmentation based on food-category attribute importance: The relation with healthiness and sustainability perceptions. *Food Qual. Prefer.* 2016, 48, 99–106.
3. Aschemann-Witzel, J.; Varela, P.; Peschel, A.O. Consumers' categorization of food ingredients: Do consumers perceive them as 'clean label' producers expect? An exploration with projective mapping. *Food Qual. Prefer.* 2019, 71, 117–128.
4. Angus, A.; Westbrook, G. Top 10 Global Consumer Trends 2020; Euromonitor International: Hong Kong, China, 2020.
5. Sánchez de la Campa, A.M.; Salvador, P.; Fernández-Camacho, R.; Artiñano, B.; Coz, E.; Márquez, G.; Sánchez-Rodas, D.; de la Rosa, J. Characterization of biomass burning from olive grove areas: A major source of organic aerosol in PM₁₀ of Southwest Europe. *Atmos. Res.* 2018, 199, 1–13.
6. Neves, B.; Pires, I.M. The Mediterranean diet and the increasing demand of the olive oil sector: Shifts and environmental consequences. *REGION* 2018, 5, 101–112.
7. Pereira, C.; Costa Freitas, A.M.; Cabrita, M.J.; Garcia, R. Assessing tyrosol and hydroxytyrosol in Portuguese monovarietal olive oils: Revealing the nutraceutical potential by a combined spectroscopic and chromatographic techniques-based approach. *LWT Food Sci. Technol.* 2020, 118, 108797.
8. Connor, D.J.; Gómez-del-Campo, M.; Rousseaux, M.C.; Searles, P.S. Structure, management and productivity of hedgerow olive orchards: A review. *Sci. Hortic.* 2014, 169, 71–93.
9. Vossen, P. Olive oil: History, production, and characteristics of the world's classic oils. *HortScience* 2007, 42, 1093–1100.
10. Dabbou, S.; Dabbou, S.; Chehab, H.; Brahmi, F.; Taticchi, A.; Servili, M.; Hammami, M. Chemical composition of virgin olive oils from Koroneiki cultivar grown in Tunisia with regard to fruit ripening and irrigation regimes. *Int. J. Food Sci. Technol.* 2011, 46, 577–585.
11. Cunill, M.; Salvador, D. Oliana: Una nueva variedad de olivo adaptada al sistema superintensivo. *Olint Plantas Olivo Mag.* 2014, 25, 21–29.
12. Chanioti, S.; Tzia, C. Evaluation of ultrasound assisted and conventional methods for production of olive pomace oil enriched in sterols and squalene. *LWT Food Sci. Technol.* 2019, 99, 209–216.

13. Di Giovacchino, L.; Preziuso, S.M.; Di Serio, M.G.; Mucciarella, M.R.; Di Loreto, G.; Lanza, B. Double extraction of olive oil in large oil mills of Southern Italy: Effects on extraction efficiency, oil quality, and economy of the process. *Eur. J. Lipid Sci. Technol.* 2017, 119, 1600161.
14. Rodrigues, F.; Nunes, M.A.; Oliveira, M.B.P.P. Chapter 12—Applications of recovered bioactive compounds in cosmetics and health care products. In *Olive Mill Waste*; Galanakis, C.M., Ed.; Academic Press: Cambridge, MA, USA, 2017; pp. 255–274.
15. Chanioti, S.; Tzia, C. Optimization of ultrasound-assisted extraction of oil from olive pomace using response surface technology: Oil recovery, unsaponifiable matter, total phenol content and antioxidant activity. *LWT Food Sci. Technol.* 2017, 79, 178–189.
16. Jiménez-Herrera, R.; Pacheco-López, B.; Peragón, J. Water stress, irrigation and concentrations of pentacyclic triterpenes and phenols in *Olea europaea* L. cv. Picual Olive Trees. *Antioxidants* 2019, 8, 294.
17. Servili, M.; Esposto, S.; Fabiani, R.; Urbani, S.; Taticchi, A.; Mariucci, F.; Selvaggini, R.; Montedoro, G.F. Phenolic compounds in olive oil: Antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology* 2009, 17, 76–84.
18. Nunes, M.A.; Pimentel, F.B.; Costa, A.S.G.; Alves, R.C.; Oliveira, M.B.P.P. Olive by-products for functional and food applications: Challenging opportunities to face environmental constraints. *Innov. Food Sci. Emerg. Technol.* 2016, 35, 139–148.
19. Peralbo-Molina, Á.; Priego-Capote, F.; Luque de Castro, M.D. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography–tandem mass spectrometry with a quadrupole–quadrupole-time-of-flight mass detector. *J. Agric. Food Chem.* 2012, 60, 11542–11550.
20. Paze, I.C.; Lozano-Sánchez, J.; Borrás-Linares, I.; Nuñez, H.; Robert, P.; Segura-Carretero, A. Obtaining an extract rich in phenolic compounds from olive pomace by pressurized liquid extraction. *Molecules* 2019, 24, 3108.
21. Boronat, A.; Mateus, J.; Soldevila-Domenech, N.; Guerra, M.; Rodríguez-Morató, J.; Varon, C.; Muñoz, D.; Barbosa, F.; Morales, J.C.; Gaedigk, A.; et al. Cardiovascular benefits of tyrosol and its endogenous conversion into hydroxytyrosol in humans. A randomized, controlled trial. *Free Radic. Biol. Med.* 2019, 143, 471–481.
22. Čepo, D.V.; Radic', K.; Jurmanovic', S.; Jug, M.; Grdic' Rajkovic', M.; Pedisic', S.; Moslavac, T.; Albahari, P. Valorization of olive pomace-based nutraceuticals as antioxidants in chemical, food, and biological models. *Molecules* 2018, 23, 2070.

23. Markovic', A.K.; Toric', J.; Barbaric', M.; Jakobušić' Brala, C. Hydroxytyrosol, tyrosol and derivatives and their potential effects on human health. *Molecules* 2019, 24, 2001.
24. Frankel, E.N.; Finley, J.W. How to standardize the multiplicity of methods to evaluate natural antioxidants. *J. Agric. Food Chem.* 2008, 56, 4901–4908.
25. Talhaoui, N.; Gómez-Caravaca, A.M.; León, L.; De la Rosa, R.; Fernández-Gutiérrez, A.; Segura-Carretero, A. From olive fruits to olive oil: Phenolic compound transfer in six different olive cultivars grown under the same agronomical conditions. *Int. J. Mol. Sci.* 2016, 17, 337.
26. Vidal, A.M.; Alcalá, S.; De Torres, A.; Moya, M.; Espínola, F. Characterization of olive oils from superintensive crops with different ripening degree, irrigation management, and cultivar: (Arbequina, Koroneiki, and Arbosana). *Eur. J. Lipid Sci. Technol.* 2019, 121, 1800360.
27. Klen, T.J.; Vodopivec, B.M. The fate of olive fruit phenols during commercial olive oil processing: Traditional press versus continuous two- and three-phase centrifuge. *LWT Food Sci. Technol.* 2012, 49, 267–274.
28. Wang, S.; Meckling, K.A.; Marccone, M.F.; Kakuda, Y.; Tsao, R. Synergistic, additive, and antagonistic effects of food mixtures on total antioxidant capacities. *J. Agric. Food Chem.* 2011, 59, 960–968.
29. Moreno, J.J. Effect of olive oil minor components on oxidative stress and arachidonic acid mobilization and metabolism by macrophages RAW 264.7. *Free Radic. Biol. Med.* 2003, 35, 1073–1081.
30. Tresserra-Rimbau, A.; Lamuela-Raventos, R.M.; Moreno, J.J. Polyphenols, food and pharma. Current knowledge and directions for future research. *Biochem. Pharm.* 2018, 156, 186–195.
31. Giovagnoli-Vicuña, C.; Pizarro, S.; Briones-Labarca, V.; Delgadillo, A. A square wave voltammetry study on the antioxidant interaction and effect of extraction method for binary fruit mixture extracts. *J. Chem.* 2019, 3, 8706061.
32. Olszowy-Tomczyk, M. Synergistic, antagonistic, and additive antioxidant effects in the binary mixtures. *Phytochem. Rev.* 2020, 19, 63–103.
33. Palma, A.; Ruiz Montoya, M.; Díaz, M.J.; Arteaga, J.F.; Brito, R.E.; Mellado, J.M.R. Evaluation of synergistic and antagonistic effects between some selected antioxidants by means of an electrochemical technique. *Int. J. Food Sci. Technol.* 2017, 52, 1639–1644.
34. Nunes, M.A.; Costa, A.S.G.; Bessada, S.; Santos, J.; Puga, H.; Alves, R.C.; Freitas, V.; Oliveira, M.B.P.P. Olive pomace as a valuable source of bioactive compounds: A study

- regarding its lipid- and water-soluble components. *Sci. Total Env.* 2018, 644, 229–236.
35. Nunes, M.A.; Páscoa, R.N.M.J.; Alves, R.C.; Costa, A.S.G.; Bessada, S.; Oliveira, M.B.P.P. Fourier transform near infrared spectroscopy as a tool to discriminate olive wastes: The case of monocultivar pomaces. *Waste Manag.* 2020, 103, 378–387.
36. Hotta, H.; Sakamoto, H.; Nagano, S.; Osakai, T.; Tsujino, Y. Unusually large numbers of electrons for the oxidation of polyphenolic antioxidants. *Biochim. Biophys. Acta* 2001, 1526, 159–167.
37. Costa, A.S.G.; Alves, R.C.; Vinha, A.F.; Costa, E.; Costa, C.S.G.; Nunes, M.A.; Almeida, A.A.; Santos-Silva, A.; Oliveira, M.B.P.P. Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chem.* 2018, 267, 28–35.

This page was intentionally left in blank.

3.2 Science to Society

3.2.1 Olive pomace products, method of production and their uses

International Application No.: PCT/IB2017/053422

[Publication number: WO/2017/212450](#) (link)

Applicant: University of Porto

Inventors: Da Mota Nunes, Maria Antónia; Botelho Pimentel, Filipa Alexandra; Sílvia Guedes da Costa, Anabela; Prior Pinto Oliveira, Maria Beatriz

Abstract

The present disclosure relates to olive pomace and a green methodology to obtain derivative products for its valorization. The obtained products may be used as a solid substrate, nutraceuticals, cosmetics or food supplements.

Context and scope

The project behind this patent application was denominated as Soilife and aimed to transform a major by-product of the olive oil processing – the extracted olive pomace (EOP) - into a substrate for agricultural production. This biomass is an output from the extraction of the olive pomace oil and still has a high content in phenolics that are toxic for soil. Hence, EOP is phytotoxic, impairs the germination and growth of plants, and is an environmental burden. Moreover, it increasingly has low economic value since it is essentially used as biomass for heat and energy production, and other alternatives have emerged in the market. Figure 1 shows the main by-products resulting from olive oil processing, namely the EOP.

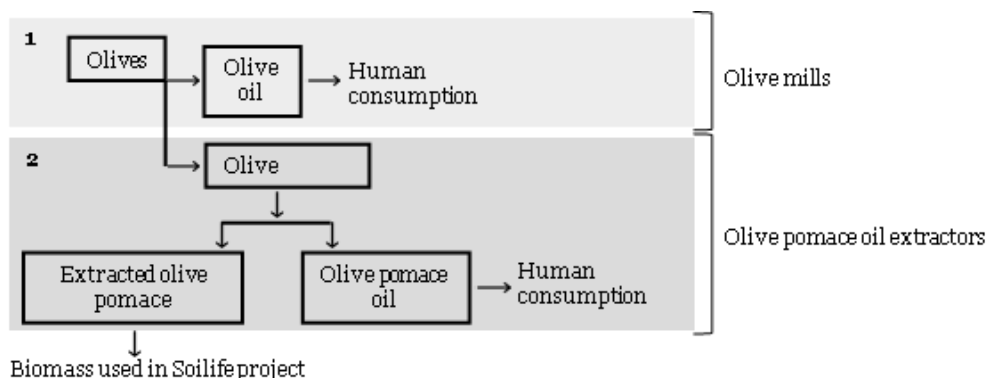


Figure 1. Major by-products of olive oil processing.

Olive pomace oil extractors have massive amounts of EOP stored, which is no longer competitive and an environmental problem. Also, as olive oil production increases, EOP will rise, and the difficulties of these industries will be more significant.

Soilife intended to transform this agro-waste into an agricultural substrate available to produce food suitable for human consumption and, ultimately, to close the cycle, meaning that it was expected to attain a product with no environmental risk, from an environment hazard by-product, being a fertile substrate to grow plants. This contributes to food security by producing nutritionally viable and safe foods and was also in line with the European political measures and circular economy principles that advocate the agricultural sector's sustainable development by promoting the rehabilitation of natural resources.

Adding value to this final biomass, new incomes are generated, and new industrial players emerge. Simultaneously, these olive oil companies will be able to reduce the actual olive oil production ecological footprint.

Soilife was the subject of a business ignition program (2018) in which the market, the target clients, the project differentiation, and the business model were identified. It was awarded in categories related to sustainability, innovation, and agro-business. Then, in 2019, the patent was exclusively licenced to a University of Porto spin-off company that is scaling up the process.

3.2.2 Foodstuff composition comprising a derivate of olive pomace

International Application No.: PCT/IB2018/060111

[Publication number: WO/2019/116343](#) (link)

Applicant: University of Porto

Inventors: Da Mota Nunes, Maria Antónia; Sílvia Guedes da Costa, Anabela; Prior Pinto Oliveira, Maria Beatriz

Abstract

The present disclosure relates to a foodstuff composition, in particular a spreadable food composition, more in particular an olive oil-based functional spread fat composed by olive oil fortified with a functional ingredient – the olive pomace active ingredient. This functional ingredient is a mixture of bioactive compounds, in particular hydroxytyrosol, tyrosol, sterols, tocopherols, triterpenes, coenzyme Q10, K, Mg and Ca, among others, obtained from olive pomace by mechanical pressing.

Context and scope

The raw material used in this project, designated as Sprelive, is wet olive pomace (WOP), obtained from two-phase system olive mills after olive oil processing. This system has been replacing the three-phase system for olive oil processing, mainly in Spain and Portugal, due to its more ecological approach.

Beneficial compounds as phenolics, sterols, tocopherols, triterpenes, coenzyme Q10 and squalene are present in WOP. Also, the remaining lipid phase is constituted by a high amount of monounsaturated fatty acids, particularly oleic acid. Hence, WOP is a rich source of bioactive compounds with benefits for human health. On the other hand, due to the high content of phenolics, WOP can have a phytotoxic effect representing an environmental hazard.

The balance between environmental sustainability, low-cost procedures and high-yield extraction of compounds is challenging. Sprelive attempt to respond to that purpose. For that, the hypothesis that was identified was if the water that naturally exists in WOP, that can reach 70%, can be a natural container or even an extractor of the bioactive compounds. This assumption was based on the fact that a minimal phenolic fraction passes into the oil phase (2%) during olive oil processing. The majority of phenolics from olive fruit (98%), namely hydroxytyrosol and its derivatives, remain in WOP, meaning that its aqueous phase comprises the hydrophilic compounds.

A mechanic engine was designed and then materialized in a prototype. This allows using only physical pressure to separate the water from the biomass WOP.

The sequential physical extraction procedure is the following:

- 1) Compression of WOP.
- 2) Separation of the liquid phase (water and oil) from solid phase (olive skin, pulp, stone and kernel).
- 3) Removal of the solid phase.
- 4) Centrifugation of liquid phase.
- 5) Lyophilization of the superior layer.

The liquid phase obtained in 5) was designated as olive pomace functional ingredient (OFI) and was used to develop an olive oil-based spread (Chapter 5).

At the end of the pressing procedure, a compressed biomass composed mostly of the olive skin, pulp, stone, and stone will remain. This biomass still contains some residual olive oil and, with less volume, can be transferred to the olive oil pomace extractors.

Figure 1 shows the non-confidential procedures that include the OFI obtention and the incorporation in an olive oil-based spread.

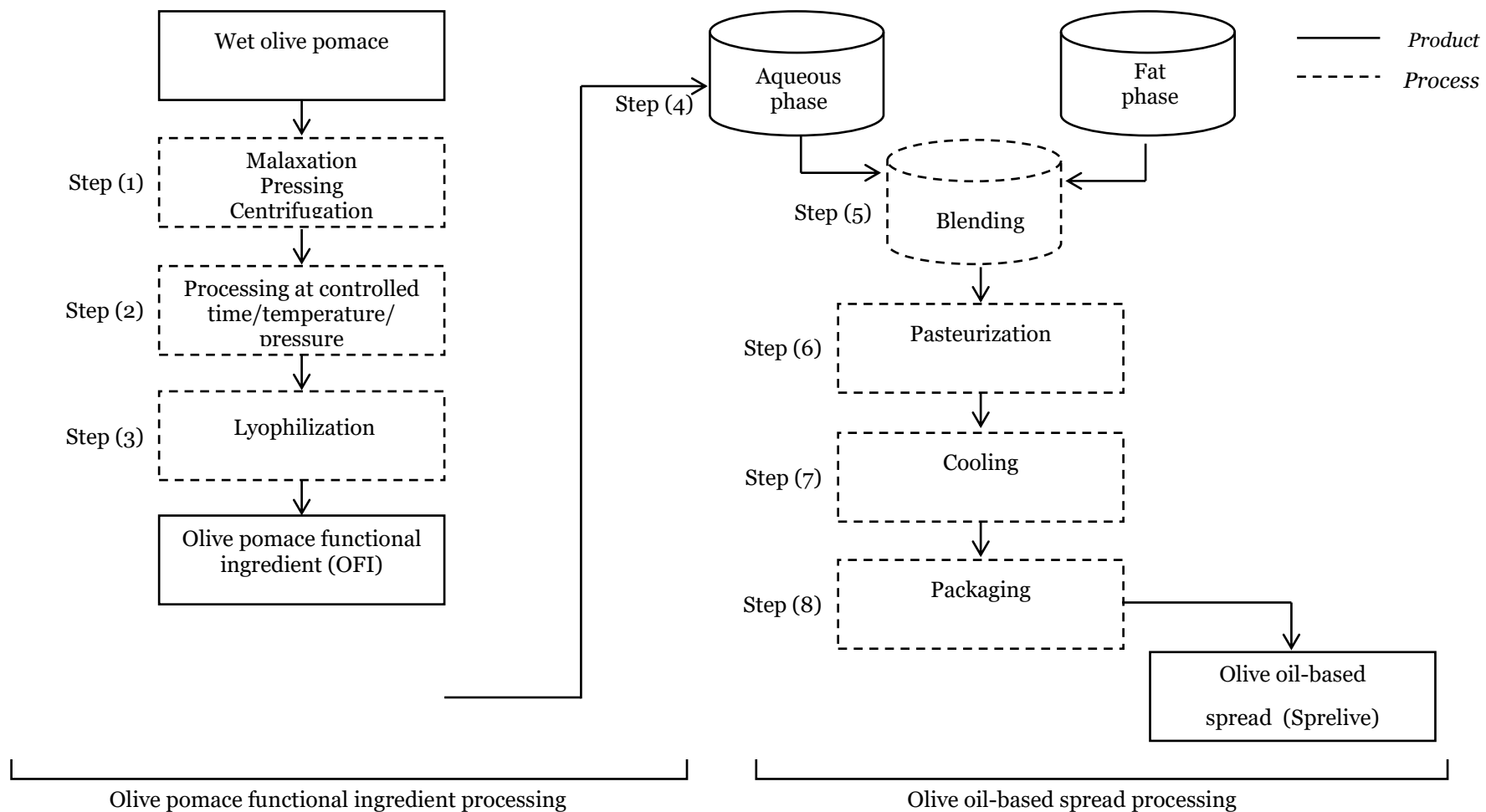


Figure 2. Flow diagram of the olive pomace functional ingredient obtention and olive oil-based spread processing.

3.2.3 Science and technology transfer process

According to the Competence Centre on Technology Transfer of the European Commission, the technology transfer *“refers to the process of conveying results stemming from scientific and technological research to the marketplace and to wider society, along with associated skills and procedures, and is as such an intrinsic part of the technological innovation process.”*

Technology transfer is a linked chain process that begins with research, invention patentability, protection, marketing, and licencing agreements. Ultimately, this process is a cycle as the financial returns of the products generated with the innovative technology can be used in further scientific research and the technology protection supports the investigation, which can make project applications more successful.

The processes associated with both protected technologies went equally through the following steps: communication of invention to the technology transfer office of the University of Porto, technical analysis to assess the fulfilment of the patentability requirements, intellectual property protection/management and technology promotion. Soilife went forward to negotiation and commercialization and was licenced, whereas Sprelive is, at this point, being presented to the olive oil stakeholders and market.

The commercialization processes started after the technology being protected by filing a provisional patent application. The patent applications were submitted to INPI (Instituto Nacional da Propriedade Industrial), the Portuguese intellectual property institute. The patent protects the invention from being used, produced or commercialised by others without the owner's authorisation.

The market analysis identifies the leading companies and/or partners that can engage with the research results, the commercial applications and the principal competitors. Notwithstanding, during the process of commercialization, several actions can be developed. The primary step is to contact companies and present the technology, usually using the Technology Offer as a support document. It comprises the non-confidential information to be shared. During this process, several meetings took place to present both technologies, most of them in Portugal and Spain. Also, meetings with stakeholders from Chile, Italy and Greece occurred.

The partnerships with companies in collaborative projects allowed to, on the one hand, assure financial support to continue developing the project and, on the other hand, attain results that could be of interest to the company. It was developed partnerships with

Acesur (Spain) and SIRO (Portugal), which led to the successful development of the Soilife project.

Scientific meetings and other events are also a way to promote the technology in the market. The Sprelive project was presented in the “The Global Gastronomy Summit in Portugal”, focused on food management and new agri-food and tourism businesses. In this case, the networking was accomplished, and the spread was presented to consumers for the first time.

This page was intentionally left in blank.

Chapter 4 - Olive pomace functional ingredient as a potential food ingredient: chemical and antimicrobial assessment

It is important to present solutions that address the olive pomace valorisation under the eco-nutrition framework. The development of sustainable applications of an olive pomace ingredient is approached in **Chapter 4**, namely exploring the potential utilization of the ingredient as a natural additive. Moreover, a study developed during the prototyping phase of the olive oil-based vegetable cream functionalized with the olive pomace extract is presented, which allowed ascertaining the product attributes, reinforced the consumer preference by the “natural”, and supported the use of by-products to develop new food products.

This page was intentionally left in blank.

4.1 Chemical composition and antimicrobial activity of a new olive pomace functional ingredient

¹Maria Antónia Nunes, ^{2,3}Josman Dantas Palmeira, ¹Diana Melo, ¹Susana Machado,
¹Joana C. Lobo, ¹Anabela S.G. Costa, ¹Rita C. Alves,
²Helena Ferreira, ¹Maria Beatriz P.P. Oliveira

¹REQUIMTE/LAQV, Department of Chemical Sciences, Faculty of Pharmacy,
University of Porto, R. Jorge de Viterbo Ferreira, 228,
4050-313 Porto, Portugal

²REQUIMTE/UCIBIO, Laboratory of Microbiology, Department of Biological
Sciences, Faculty of Pharmacy, University of Porto, R. Jorge de Viterbo Ferreira, 228,
4050-313 Porto, Portugal

³Department of Biology & CESAM, University of Aveiro, Campus de Santiago,
3810-193, Aveiro, Portugal

- Nunes, M.A., Palmeira, J.D., Melo, D., Machado, S., Lobo, J.C., Costa, A.S.G., Alves, R.C., Ferreira, H., Oliveira, M.B.P.P. (2021). Chemical composition and antimicrobial activity of a new olive pomace functional ingredient. *Pharmaceuticals*, 14, 913.

Abstract

Olive pomace, an olive oil processing byproduct, can be upcycled and meet the current demand for natural and sustainable food ingredients. In this work, a patented process was used to obtain a functional ingredient from different olive pomaces. The nutritional, chemical and antioxidant profiles, as well as the antimicrobial activity against *S. aureus*, *E. coli* and *C. albicans*, were investigated for the first time. The amount of phenolics ranged between 3.1 and 3.8 g gallic acid eq./100 g in all samples and flavonoids between 2.0 and 3.2 g catechin eq/100 g. No significant differences were found regarding the antioxidant activity. The total fat varied between 5 and 11%, α -tocopherol being the major vitamer and oleic acid the main fatty acid. The protein and ash contents were 1–4% and 10–17%, respectively. The functional ingredient with a higher hydroxytyrosol content (220 mg/100 g) also presented the best minimal inhibitory concentration against the

tested bacteria. No activity against *C. albicans* was verified. This new functional ingredient presents the potential to be used as a natural preservative or as a nutritional profile enhancer. Moreover, it can be an advantageous ingredient in food products since it comprises specific lipid and hydrophilic bioactive compounds usually not present in other plant extracts.

Keywords: olive pomace; sustainability; antimicrobial activity; natural additive.

1. Introduction

Industrial players and research institutions have been firmly committed to the circular economy principles by exploring efficient and eco-friendly procedures to upcycle agro-industrial byproducts [1]. All phases of the food life cycle (agricultural production, processing and distribution) generate byproducts that are natural resources available in high amounts at low costs and, noticeably, can be sources of high-value nutritional components [2].

In 2020, the EU produced more than 1900 thousand tons of olive oil. Spain was the top producer, followed by Italy, Greece and Portugal [3]. Olive oil production is increasing due to its organoleptic properties and significant benefits in health. Furthermore, the olive oil yield is 15–20%, meaning that the remaining material is olive pomace (80–85%) [4]. Therefore, the generation of this byproduct is currently high and tends to increase. Olive pomace can be a great source of components with bioactivity, such as antioxidants and fatty acids, that can be explored to develop new ingredients or products [5,6]. It was reported that olive products (olive and olive pomace oils) and byproducts exert antimicrobial activity against pathogenic bacteria and toxigenic fungi strains [6]. This effect is mainly related to the presence of phenolics, probably in a synergistic way, and the amount and type of phenolics present can influence the bioaction efficiency [6].

The use of synthetic additives in the food industry is a recurring topic in public health discussions due to the hazard risk related to the concomitant consumers and long-term exposure [7]. This movement aligns with the current health concerns and environmental awareness, which has been guiding consumer behaviors over the last years [8]. Currently, consumers prefer products additive-free or with natural ingredients. Thus, the utilization of natural additives derived from olive oil processing byproducts can address the consumer trend, which is also a challenge for food and beverage companies [9].

This study assessed the chemical composition and antimicrobial activity against the bacteria (*Staphylococcus aureus* and *Escherichia coli*) and yeast (*Candida albicans*) of four olive pomace-based ingredients in order to evaluate their potential to be used in food products. The olive pomace extracts were obtained using an ecological and innovative process (patent international application PCT/IB2018/060111, 2019) and are natural, plant-based and chemical-free. The functional ingredient presented antimicrobial activity against *S. aureus* and *E. coli*, foreseeing its application in food products as a natural and sustainable preservative. Additionally, it can also be valuable to improve the nutritional profile of food products due to the bioactive compounds' health effects, especially hydroxytyrosol.

2. Results and discussion

In this work, a green, sustainable and cost-effective method was used to add value to olive pomace, currently generated in high amounts in the Mediterranean basin, by recovering its bioactive compounds in a functional ingredient. Pomaces from the two major Portuguese olive oil production regions — Trás-Os-Montes (O1 and O2) and Alentejo (O3 and O4) — were analyzed. Table 1 shows the geographical origin and the predominant varieties in each pomace.

Table 1. Predominant olive varieties in the olive pomaces from the northeast region (Trás-Os-Montes) and south (Alentejo) of Portugal.

Olive pomace geographical origin		Predominant olive varieties
Northeast region (Trás-Os-Montes)	Alfândega da Fé	Cobrançosa Madural Verdeal Transmontana Cordovil
	Valpaços	Madural Cordovil
South (Alentejo)	Beja	Cobrançosa
	Ferreira do Alentejo	Arbosana

The phytochemicals (total phenolics and flavonoids) and antioxidant activity results are summarized in Table 2. Different methods were also applied to evaluate the antimicrobial activity of the olive pomace extracts. The incorporation, surface spreading, and disk diffusion methods were used for screening purposes, whereas the microdilution method assessed the MIC. A Gram-positive and a Gram-negative bacteria and yeast (*S. aureus*, *E. coli* and *C. albicans*, respectively) were selected as representatives of the principal types of food contamination and human infection (Tables 3 and 4). Table 3 summarizes the preliminary results for the extracts' antimicrobial activity, being clear that the extracts had an antimicrobial activity for *E. coli* and *S. aureus* but not for *C. albicans*. Table 4 shows the results of the antimicrobial activity against *E. coli* and *S. aureus*.

Table 2. Total phenolics; flavonoids; hydroxytyrosol content and antioxidant activity (FRAP, DPPH and ABTS) of the olive pomace extracts (dry weight).

Samples	Phytochemicals				Antioxidant activity		
	TPC		TF	Hydroxytyrosol	FRAP	DPPH	ABTS
	g GAE/100 g	g HE/100 g	g CE/100 g	mg/100 g	g FSE/100 g	g TE/100 g	g TE/100 g
O1	3.49 ± 0.28 ^a	2.63 ± 0.29 ^a	2.47 ± 0.29 ^b	220 ± 20.57 ^a	2.76 ± 0.52 ^a	0.6 ± 0.07 ^b	1.13 ± 0.07 ^a
O2	3.05 ± 0.07 ^b	2.34 ± 0.12 ^b	2.29 ± 0.12 ^b	122.39 ± 1.92 ^c	2.46 ± 0.13 ^a	0.96 ± 0.21 ^a	0.85 ± 0.03 ^b
O3	3.83 ± 0.2 ^a	2.8 ± 0.11 ^a	3.17 ± 0.11 ^a	172.7 ± 2.09 ^b	2.76 ± 0.16 ^a	0.91 ± 0.05 ^a	1.1 ± 0.1 ^a
O4	3.06 ± 0.23 ^b	2.23 ± 0.2 ^b	1.96 ± 0.09 ^c	63.33 ± 3.59 ^d	2.65 ± 0.51 ^a	0.68 ± 0.07 ^b	0.8 ± 0.04 ^b

O1, Alfândega da Fé; O2, Valpaços; O3, Beja; O4, Ferreira do Alentejo. TPC, total phenolics content; TF, total flavonoids; FRAP, ferric reducing antioxidant power; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability, ABTS, 2,2'-azino-bis(3-ethylenbenzothiazoline-6-sulfonic acid) radical scavenging ability; GAE, gallic acid equivalents; HE, hydroxytyrosol equivalents; CE, catechin equivalents; FSE, ferrous sulfate equivalents; TE, trolox equivalents. Results expressed as mean ± standard deviation. Within each column, different letters represent significant differences between samples at $p < 0.05$.

Table 3. General results of antimicrobial activity of the olive pomace extracts.

Microorganism	Strain	Type	Olive pomace extracts			
			O1	O2	O3	O4
<i>Escherichia coli</i>	ATCC® 25922	Gram-negative	+	+	+	+
<i>Staphylococcus aureus</i>	ATCC® 25923	Gram-positive	+	+	+	+
<i>Candida albicans</i>	ATCC® 10231	Yeast	-	-	-	-

O1, Alfândega da Fé; O2, Valpaços; O3, Beja; O4, Ferreira do Alentejo.

Table 4. Results of antimicrobial activity against *E. coli* and *S. aureus* of the olive pomace extracts.

Method	O1		O2		O3		O4	
	<i>E. coli</i> <i>S. aureus</i>		<i>E. coli</i> <i>S. aureus</i>		<i>E. coli</i> <i>S. aureus</i>		<i>E. coli</i> <i>S. aureus</i>	
	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>S. aureus</i>
Incorporation (IZ-mm)	10	15	10	13	12	14	10	13
Surface spreading (IZ-mm)	10	15	10	14	9	14	0	12
Disk diffusion (IZ-mm)	0	8	0	8	0	8	0	7
MIC (mg/mL)	62.5	31.25	125.0	62.5	125.0	31.25	125	125

O1, Alfândega-da-Fé; O2, Valpaços; O3, Beja; O4, Ferreira do Alentejo. MIC, minimal inhibitory concentration; IZ, in-hibition zone.

Table 5. Chemical analysis of the olive pomace ingredients (total fat, fatty acid and vitamin E profiles, total protein, ash and pH) (dry weight).

	O1	O2	O3	O4
Total fat (g/100 g)	5.55 ± 0.02 ^b	10.54 ± 1.99 ^a	4.62 ± 0.24 ^b	5.86 ± 0.06 ^b
Fatty acids (relative %)				
C16:0 (<i>Palmitic</i>)	11.77 ± 0.0 ^c	11.59 ± 0.04 ^d	13.22 ± 0.03 ^b	14.35 ± 0.02 ^a
C16:1 (<i>Palmitoleic</i>)	0.74 ± 0.01 ^c	0.62 ± 0.02 ^d	0.84 ± 0.05 ^b	1.28 ± 0.01 ^a
C17:0 (<i>Heptadecanoic</i>)	0.19 ± 0.00 ^a	0.16 ± 0.00 ^b	0.12 ± 0.01 ^d	0.14 ± 0.00 ^c
C18:0 (<i>Stearic</i>)	3.16 ± 0.01 ^c	3.65 ± 0.01 ^a	3.44 ± 0.02 ^b	2.28 ± 0.01 ^d
C18:1n9cis (<i>Oleic</i>)	72.94 ± 0.03 ^a	71.7 ± 0.05 ^c	71.82 ± 0.05 ^b	71.78 ± 0.03 ^b
C18:2n6cis (<i>Linoleic</i>)	9.44 ± 0.03 ^b	10.48 ± 0.02 ^a	8.75 ± 0.02 ^c	8.4 ± 0.02 ^d
C20:0 (<i>Arachidic</i>)	0.43 ± 0.01 ^b	0.44 ± 0.02 ^{ab}	0.45 ± 0.02 ^{ab}	0.47 ± 0.01 ^a
C18:3n3 (<i>α-Linolenic</i>)	0.83 ± 0.01 ^b	0.9 ± 0.02 ^a	0.9 ± 0.01 ^a	0.72 ± 0.00 ^c
C20:1n9 (<i>cis-11-Eicosenoic</i>)	0.28 ± 0.01 ^a	0.25 ± 0.01 ^b	0.22 ± 0.01 ^c	0.3 ± 0.01 ^a
C22:0 (<i>Behenic</i>)	0.16 ± 0.00 ^b	0.15 ± 0.00 ^b	0.16 ± 0.01 ^b	0.21 ± 0.01 ^a
C24:0 (<i>Lignoceric</i>)	0.06 ± 0.01 ^b	0.06 ± 0.00 ^b	0.07 ± 0.01 ^a	0.08 ± 0.00 ^a
Total vitamin E (mg/100 g)	1.83 ± 0.01 ^a	1.99 ± 0.44 ^a	0.87 ± 0.06 ^b	2.25 ± 0.17 ^a
α-Tocopherol	1.69 ± 0.01 ^a	1.78 ± 0.42 ^a	0.77 ± 0.06 ^b	1.96 ± 0.15 ^a
α-Tocotrienol	0.04 ± 0.00 ^c	0.07 ± 0.00 ^b	0.04 ± 0.00 ^c	0.21 ± 0.01 ^a
β-Tocopherol	0.03 ± 0.00 ^c	0.05 ± 0.00 ^a	0.02 ± 0.00 ^d	0.04 ± 0.00 ^b
γ-Tocopherol	0.07 ± 0.00 ^b	0.09 ± 0.01 ^a	0.04 ± 0.00 ^c	0.04 ± 0.00 ^c
Total protein (g/100 g)	0.88 ± 0.01 ^c	0.88 ± 0.03 ^c	3.16 ± 0.03 ^b	4.44 ± 0.01 ^a
Ash (g/100 g)	11.25 ± 0.65 ^b	10.63 ± 1.16 ^b	9.93 ± 0.30 ^b	16.68 ± 0.18 ^a
pH	5.23 ± 0.02 ^c	5.36 ± 0.04 ^b	5.26 ± 0.01 ^c	5.63 ± 0.01 ^a

O1, Alfândega da Fé; O2, Valpaços; O3, Beja; O4, Ferreira do Alentejo. Results expressed as mean ± standard deviation. Within each line, different letters represent significant differences between samples at $p < 0.05$.

Regarding the antimicrobial action, the most studied natural compounds are phenolics, although the action of oligopeptides, terpenoids or macrolides have also been reported [10–13].

Phenolics are secondary plant metabolites, able to act as antioxidants by neutralizing reactive oxygen and nitrogen species due to hydroxyl groups, making them good hydrogen donors. Furthermore, they can moderate free radical generation by chelating metal ions. Its antimicrobial action depends on the respective concentration and chemical structure [11]. These compounds can cause the disruption of the membrane structure and leakage of the intracellular components caused by phenolics hydroxyl (-

OH) groups interacting with bacteria membranes [14]. The antibacterial (both Gram-positive and Gram-negative) effect that has been most assessed, and attributed to phenolics, is membrane disruption. The –OH group in phenolics leads to electron delocalization, making them act as proton exchangers. This will promote a gradient reduction across the cytoplasmic membrane of bacterial cells, leading, ultimately, to the cell's death [11].

O1 and O3 presented the highest amounts of phenolics (3.5 and 3.8 g GAE/100 g, respectively) and O3 the highest flavonoids content (3.2 g CE/100 g) (Table 5).

Simultaneously, O1 presented the highest content of hydroxytyrosol (220 mg/100 g) and the best minimal inhibitory concentration for *E. coli* (62.5 mg/mL) (Tables 2 and 4). This value was half the value presented by the other extracts (125 mg/mL). In the case of *S. aureus*, both O1 and O3 presented a similar MIC (31.25 mg/mL) and the lowest value compared to the other samples (Table 4). The O4 MIC value (125 mg/mL) was the highest related to the lowest content in hydroxytyrosol, and O3 presented intermediate values for these two parameters in discussion (Tables 2 and 4).

O3 presented the highest amount in total flavonoids (3.2 g CE/100 g), a phenolics class. This sample also showed the best MIC result, along with O1, against *S. aureus*. The flavonoids described in olive pomace include quercetin, apigenin, rutin, luteolin glucoside, luteolin, dismetin, taxifolin and apigenin glucoside [15]. The flavonoids' molecular actions are mainly due to their capacity to form complexes with proteins that compromise microbial adhesion, microbial enzymes and cell transport proteins, among other mechanisms. Overall, fundamental mechanisms have been proposed for flavonoids: damage of the cytoplasmic membrane, topoisomerase and NADH-cytochrome c reductase inhibitions [16]. Table 6 lists the proposed antimicrobial mechanisms of the phenolics and flavonoids.

Regarding the antioxidant activity evaluated by the DPPH assay, O1 showed the lowest value (0.6 g TE/100 g), although with no significant differences when compared to O4 (0.7 g TE/100 g). The same sample presented the highest value in the ABTS assay, along with O3 (1.13 and 1.1 g ET/100 g, respectively), parallel to the hydroxytyrosol values of these samples.

These data can indicate that other compounds present in the extracts beyond the ones analyzed can have antioxidant actions.

Table 6. Olive pomace bioactive compounds and proposed mechanisms of action.

Compounds	Proposed mechanism	References
Phenolics	Disruption of the membranes structure and leakage of the cellular components	[14,17,18]
	Hydroxyl groups promote the delocalization of electrons, leading to the reduction of the gradient across membranes	
	Reduction of the redox potential of the growth medium, leading to microbial growth constraints	
Flavonoids	Inactivation of the microbial adhesion, enzymes, and cell envelope transport proteins	[16,19]
	Disruption of microbial membranes (lipophilic flavonoids)	
	Perforation and/or a reduction of the membrane fluidity	
	Inhibition of nucleic acid synthesis, energy metabolism and cell membrane synthesis	
Hydroxytyrosol	Capability of chelating transition metals, reducing the reactivity of iron and copper by forming an inert metal–ligand complex, which decreases the bioavailability for bacterial growth	[20,21]
	Reduction of intracellular ATP concentrations	
	Cell membrane depolarization	
	Reduction of the bacterial protein content	
Vitamin E (<i>α-tocopherol</i>)	Damage in the cell membrane affecting the essential components for the integrity of the membrane (reduction in membrane potential and loss of ions, cytochrome C, proteins and radicals, followed by the collapse of proton pumps and decrease in ATP), increasing the membrane permeability	[22]
	Interaction with the lipid bilayer of the bacteria cell membrane modifying the respiratory chain and energy production	
	Capacity of acting in the cell envelope resulting in an imbalance in the fluid mosaic nature of the bacterial membrane	
Fatty acids	Disruption of the electron transport chain by binding to electron carriers	[23,24]
	Leakage of cell metabolites via cell lysis	
	Inhibition of nutrient uptake	
	Formation of peroxidation/auto-oxidation products resulting in cell deactivation	

The selected olive pomace samples are from distinct geographical origins and have different predominant olive cultivars (Table 1). Furthermore, olive processing can influence their phytochemicals amount and profile: modifications not only occur in the amount of phenolics of olive paste, oil and byproducts (due to transference among the fractions) but, also, in the phenolics profile, indicating transformations at the compound level. The olive secoiridoids, such as oleuropein, demethyloleuropein and ligstroside, are degraded during crushing and malaxation, the olive processing initial stages, forming several secoiridoid aglycone derivatives. Despite not being detected in the fruits, tyrosol and hydroxytyrosol and its glucosides occur in all the products of olive oil processing.

Only the hydroxytyrosol glycosidic form has been identified in olives [25]. The crushing process is indeed a critical phase in olive oil processing, since a decrease of about half of the total phenolics can occur. Several modifications happen during this operation related to mechanical mixing, enzymatic and nonenzymatic hydrolyses and oxidations. The oleuropein content, a secoiridoid glucoside, decreases 94%, and the amount of other secoiridoid glucosides (demethyloleuropein and ligstroside) also decline. Glucosides can be biotransformed into their corresponding aglycones. First, the conversion to oleuropein or ligstroside aglycones occurs, followed by their decarboxymethylated forms. The crushing phase allowed an increase of hydroxytyrosol due to the degradation of compounds that contain hydroxytyrosol, which is also verified in tyrosol due to tyrosol-containing phenols hydrolysis [26].

Amongst all the samples, significant differences ($p < 0.05$) were only observed in the hydroxytyrosol content (Table 2). O1 (Alfândega da Fé) presented the highest amount (220 mg/100 g), followed by O3 (173 mg/100 g), O2 (122 mg/100 g) and O4 (63 mg/100 g). Table 5 presents the chemical compositions regarding the total fat, fatty acids profile, total vitamin E and vitamers, protein and ash. The residual oil present in olive pomace depends on the olive cultivar, yield of extraction and the applied olive oil processing. Additionally, in this work, for sample preparation, a physical process was applied to remove the liquid phase, constituted from water and oil. Therefore, the initial characteristics of each pomace could also affect the oil amount of the extracts.

The total fat contents are identical in three of the four samples (5 to 6% and 11% for O2). Likewise, the fatty acid present in a higher amount but with a similar value in the four samples (72 to 73%) was oleic acid (C18:1n9cis).

According to the scientific literature, fatty acids can exhibit antimicrobial activity depending on their structure, namely the chain length, double bonds and action site. This bioaction is more relevant in Gram-positive than in Gram-negative bacteria, probably because of the Gram-positive outer membrane nonexistence, since fatty acids target the internal membrane. The antibacterial effect suggested to fatty acids is the inhibition of the cell's nutrients uptake, peroxidation or auto-oxidation product formation (deleterious to cells), the disruption of the electron transport chain and leakage of the cell metabolites due to cell lysis [24].

The residual oil present in olive pomace that is passed to the extracts has a high content in oleic (C18:1n9cis) acid, followed by palmitic (C16:0) and linoleic (C18:2n6cis) acids, similar to the olive oil profile. The monounsaturated fatty acid oleic acid is, in fact, characteristic of olive oil. It is present in 73% in O1 and 72% in the other extracts in the

study. The activity of membrane-associated enzymes can be inhibited by the oleic and linoleic acids. Specifically, unsaturated fatty acids, such as linoleic, can interfere in the bacteria fatty acid elongation process necessary to form the cellular materials [23]. O1 and O2 present the major contents in linoleic acid (9% and 11%, respectively). Overall, the samples had similar fatty acid profiles. In O1 and O3, which showed the best antimicrobial activity results, besides oleic and linoleic acid, heptadecanoic acid (C17:0) and cis-11-eicosenoic acid (C20:1n9) stand out in sample O1 and linolenic acid (C18:3n3) in O3. Different specific mechanisms for each fatty acid were explored, but they can be summed up in two key actions: the disruption of cell membranes and subsequent loss of cytoplasmic contents [23].

The vitamin E profile was determined in all the extracts (Table 5). Vitamin E is a blend of different liposoluble vitamers present in oils from vegetable sources such as olive oil. α -Tocopherol is the vitamer in higher amounts in all samples and the isoform biologically active with a potent antioxidant action [27]. Liposoluble compounds, such as α -tocopherol, can modify bacterial cell membranes, changing their fluidity and decreasing the barrier effect, allowing the passage of several compounds [22]. Table 6 resumes some mechanisms related to the α -tocopherol antimicrobial mechanism of action. Regarding the α -tocopherol contents in the samples, there are no significant differences among O1, O2 and O4, ranging from 1.7 to 2.0 mg/100 g of extract. O3 presented 0.8 mg/100 g.

Considering the total fat amount (Table 5), O2 presented the highest amount of fat (10.5%) and a high content of α -tocopherol (1.8 mg/100 g). However, this sample had a weak antimicrobial activity (Table 4), suggesting once more that phenolics may be the main contributors to this biological action.

In general, regarding the incorporation, surface spreading and disk diffusion, the olive pomace functional ingredient acted against the studied bacteria (*S. aureus* and *E. coli*) at different levels, as shown in Table 4. The extracts' MIC values ranged from 31.25 to 125 mg/mL (*S. aureus*) and from 62.5 to 125 mg/mL (*E. coli*).

S. aureus and *E. coli* are bacteria associated with foodborne diseases when the ingested food contains the pathogen or a toxin from their production [28]. *S. aureus* is widespread in the environment and is commensal on the human skin and mucosal membranes. It has been detected in several different foodstuffs, such as salads, milk, pork sausage, salmon steaks and shrimp, among others. *S. aureus* is one of the causes of infections most frequent. Hence, its presence in food is a public health concern [28,29]. On the other hand, *E. coli* is the main anaerobic facultative bacteria of the intestinal tract of humans and animals [30,31]. Some strains are more pathogenic to humans than others

due to, for example, the ability to produce toxins. The foodborne diseases associated with *E. coli* are related to contaminated foodstuffs or water ingestion [28].

The cell wall structure and composition of Gram-positive and Gram-negative bacteria are different, which can explain the susceptibility variation towards plant extracts. Structurally, Gram-negative bacteria walls, such as *E. coli*, are more differentiated and complex than Gram-positive. Gram-negative bacteria have a stratified structure functioning as a wall. Its outer membrane lipopolysaccharide component makes them more resistant to antibacterial agents [20]. Gram-positive (*S. aureus*) bacteria have a homogeneous wall that does not contain phospholipids. Gram-positive bacteria are sensitive to several plant extracts. On the other hand, extracts with activity against Gram-negative bacteria are desired and beneficial for different applications [20].

In this study, none of the samples exhibited antimicrobial activity against *C. albicans*, an opportunistic and common fungal pathogen. It is usually present in the human gastrointestinal tract or is ingested with food. The disorders caused by this microorganism are more severe, as the host is compromised. It can be a health concern, for example, in antibacterial treatments with an unbalanced microbiota [32]. *C. albicans* is a morphological and physiologically highly variable and adaptable fungus [33].

Flavonoids, phenolic acids, polyenes, oligopeptides, terpenoids, macrolides and alkaloids are examples of compounds with described fungicide activity. Nevertheless, for olive leaf extracts, moderate antifungal activity was reported, in particular, for oleuropein [10,13,34]. Zoric' et al. tested the effect of oleuropein on *C. albicans* cell membranes, showing that this compound affected the cell membrane [34]. Oleuropein is mainly found in olive leaves and fruits but scarcely in extra virgin olive oil and pomace owing to its hydrophilicity and susceptibility to degradation [35].

Nevertheless, hydroxytyrosol can represent ~50% of the total phenolics in extracts of olive pomace [5]. Medina-Martínez et al. reported that pure hydroxytyrosol solutions at different concentrations showed limited antimicrobial activity. Hence, the antimicrobial results observed can be due not just to the hydroxytyrosol content but, most likely, to a synergistic effect among all the phenolics and other compounds, such as the lipophilic ones present in the olive pomace extract [36].

The total protein and ash contents of the samples showed differences. The protein content was higher in O4 (4%) and O3 (3%), whereas the other samples presented 1%. Regarding the total minerals (ash), O4 was the richest sample (17%), while the other samples ranged between 10 and 11% (Table 5).

Considering the chemical profile of the samples and the variability and differences in the parameters analyzed, it seems that phenolics, particularly hydroxytyrosol, are the key compounds responsible for the antimicrobial activity described. Nevertheless, other components are possibly operating synergistically with phenolics against bacteria rather than individually.

3. Materials and methods

3.1 Chemicals and reagents

Chemicals and reagents were of analytical grade and acquired from Merck (Darmstadt, Germany) (Kjeldahl tablets, sulfuric acid, absolute ethanol, sodium carbonate decahydrate, sodium hydroxide, n-hexane and anhydrous sodium sulfate) and Sigma-Aldrich (St. Louis, MO, USA) (Folin–Ciocalteu’s reagent, gallic acid, catechin, heptahydrate ferrous sulfate, 2,2-diphenyl-1-picrylhydrazyl radical, 2,2t-azino-bis(3-ethylenbenzothiazoline-6-sulfonic acid, Trolox, ferric chloride, 2,4,6-tripyridyltriazine, sodium nitrite, aluminum chloride, sodium acetate, hydroxytyrosol and Supelco 37 FAME Mix). Boric acid 4% was purchased from Panreac (Barcelona, Spain) and methanol from Honeywell International, Inc. (Morris Plains, NJ, USA). Sodium chloride and chloroform were acquired from VWR Chemicals (Alfragide, Portugal). The tocopherol and tocotrienol standards and tocol were obtained from Calbiochem (La Jolla, CA, USA) and Matreya Inc. (State College, PA, USA), respectively. The solvents (HPLC grade) were acquired from Chem-Lab (Zedelgem, Belgium) or Merck (Darmstadt, Germany). A Milli-Q water purification system (Millipore, Bedford, MA, USA) was used to treat and obtain the ultrapure water.

3.2 Sampling and preparation of the olive pomace functional ingredient

The olive pomaces were collected in two-phase olive mills in Portugal during the season 2017/2018. Two olive pomaces from Alfândega da Fé and Valpaços (northeast region) and two from Beja and Ferreira do Alentejo (south) were selected. Olive pomaces were constituted by a mixture of olive cultivars (Table 1).

After homogenization, each pomace (1 kg) was submitted to the process described in the patent international application PCT/IB2018/060111 (2019) [37] to obtain a functional ingredient. A pressing force (200–300 bar) for 30 min was applied to obtain a liquid phase constituted by water and oil. Then, it was centrifuged (5000 rpm; 20 min), and the superior layer separated and lyophilized. In this way, the olive pomace-based samples O1, O2 (from Alfândega da Fé and Valpaços, respectively), O3 and O4 (from Beja and Ferreira do Alentejo, correspondingly) were obtained (Figure 1).

The process was individually applied to each olive pomace sample (Alfândega da Fé, Valpaços, Beja and Ferreira do Alentejo), and the respective olive pomace ingredients (O1, O2, O3 and O4) were obtained.

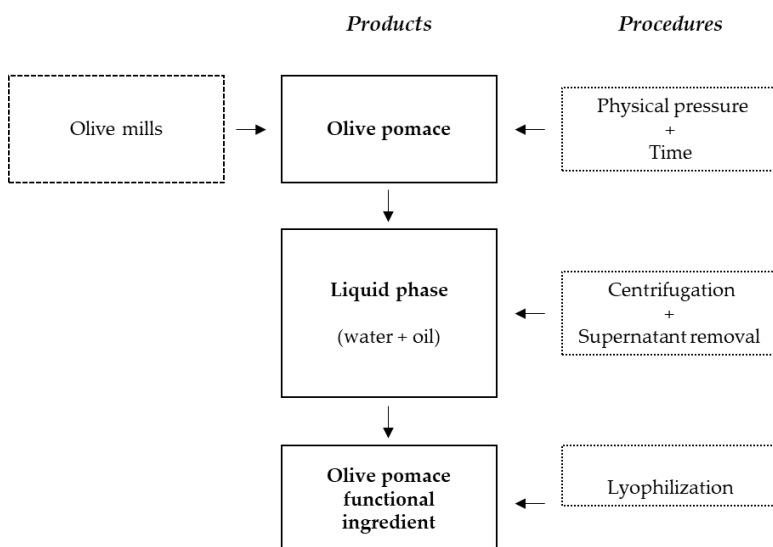


Figure 1. Obtention of the olive pomace functional ingredients.

3.3 Total fat

The total fat was assessed using the Bligh & Dyer method with modifications, according to Manirakiza et al. [38]. The lipidic fraction was extracted with methanol and chloroform, vortexed and deionized water was added. The samples were vortexed again and centrifuged (2000 rpm; 10 min). The lower layer was transferred to a flask, and a second extraction was performed with 10% (v/v) methanol and chloroform, followed by vortexing. Both phases containing fat were mixed, the solvents evaporated and the residue dried (100 °C) until a constant weight. The results were presented as g/100 g.

3.4 Total protein

The total protein was determined by the Kjeldahl method based on standard methods (AOAC 928.08) [39], using 6.25 as the nitrogen conversion factor [40]. The results were presented as g/100 g.

3.5 Ash

The ash content was determined based on the standard methods (AOAC 920.153) (incineration at 500 °C) [39]. The results were expressed as g/100 g.

3.6 *Phytochemicals and antioxidant activity assessment*

The lyophilized samples obtained in Section 3.2 were reconstituted with deionized water. Then, the solutions were vortexed to complete solubilization and centrifuged (5000 rpm; 5 min), followed by filtration throughout the syringe filters (pore size 0.45 μm). The phytochemicals (total phenolics and flavonoids) and antioxidant activity (ferric-reducing antioxidant power, 2,2-diphenyl-1-picrylhydrazyl radical and 2,2'-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid) cation radical scavenging abilities were evaluated by spectrophotometric methods using a Synergy HT Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

3.6.1 *Total phenolics*

The assessment of the phenolics content was performed according to Costa et al. [41] with modifications. Hence, 150 μL of Folin–Ciocalteu's reagent (1:10) and 120 μL of Na_2CO_3 (7.5%, m/v) were added to 30 mL of diluted extracts and standards (gallic acid and hydroxytyrosol) in a microplate. The results were presented in g of gallic acid equivalents (GAE) and hydroxytyrosol equivalents (HE)/100 g (dry weight). For that, two calibration curves using gallic acid (5–100 mg/L; $r=0.9999$) and hydroxytyrosol (2–80 mg/L; $r=0.9999$), respectively, were prepared, and the absorbance was read at 765 nm.

3.6.2 *Total flavonoids*

For the total flavonoid evaluation, catechin was used as the standard (results expressed as g of catechin equivalents (CE)/100 g (dry weight). The standard curve was plotted (2.5–400 mg/L; $r=1$), and the absorbance of the diluted samples was measured at 595 nm according to the method described by Costa et al. [41].

3.6.3 *Ferric reducing antioxidant power (FRAP)*

The ferric reducing antioxidant power was determined using a calibration curve prepared with ferrous sulfate (50–600 $\mu\text{mol/L}$; $r=0.9999$). The FRAP reagent (acetate buffer (0.3 M), TPTZ solution (10 mM) and ferric chloride (20 mM)) were freshly prepared and added (265 μL) to the diluted sample aliquots and standards (35 μL). The absorbance was read at 595 nm and the results expressed as g ferrous sulfate equivalents (FSE)/100 g [41].

3.6.4 *2,2-diphenyl-1-picrylhydrazyl radical (DPPH $\cdot$) scavenging ability (DPPH)*

The samples' DPPH $\cdot$ scavenging ability was evaluated as described by Costa et al. [41] by analyzing the kinetics of the reaction at 525 nm (intervals of 2 min). For that, 270 μL of

a DPPH $\cdot$ solution (6.0×10^{-5} mol/L in ethanol) were added to 30 μ L of diluted extracts. The endpoint was established at 20 min. Trolox was used as a standard to prepare the calibration curve (5.6–101.2 mg/L; $r=0.9979$). The scavenging ability was expressed as g of trolox equivalents (TE)/100 g.

3.6.5 2,2'-azinobis(3-ethylenbenzothiazoline-6-sulfonic acid) cation radical (ABTS $\cdot^+$) scavenging ability (ABTS)

The extracts' scavenging ability against ABTS $\cdot^+$ was determined according to the methodology described by Seiquer et al. [42]. A calibration curve was prepared (0.01–0.1 mg/L; $r=0.9908$) and absorbance read at 730 nm. The results were presented as g of trolox equivalents (TE)/100 g.

3.7 Hydroxytyrosol content analysis by HPLC-DAD-FLD

Aliquots (1 mL) of the lyophilized extracts prepared with deionized water were centrifuged (13,000; 10 min) and filtered using syringe filters (0.45 μ m) before injection into the HPLC-DAD-FLD system (Jasco, Tokyo, Japan). The system was composed of a LC-NetII/ADC hardware interface, a pump (Jasco PU-2089), an automatic sampler (Jasco AS-2057 Plus) and a multiwavelength diode array detector (Jasco MD-2018 Plus) coupled to a fluorescence detector (Jasco FP-2020 Plus, Jasco Co., Tokyo, Japan) and a column thermostat (Jasco CO-2060 Plus) (Jasco, Tokyo, Japan). Hydroxytyrosol was quantified by fluorescence and monitored (λ excitation = 280 nm; λ emission = 330 nm). The gradient elution program used was: 0 min, 5% B; 30 min, 25% B; 50 min, 75% B; 55 min, 100% B; 60 min, 100% B; 63 min, 5% B, being solvent (A) 1% acetic acid and (B) 100% methanol. A chromatographic column (Zorbax-SB-C18; 250 4.6 mm, 5 μ m; Agilent Technologies, Amstelveen, The Netherlands) was used at 20 °C with a flow rate of 1 mL/min and an injection volume of 20 μ L. The hydroxytyrosol content was expressed as g/100 g (dry weight).

3.8 Vitamin E profile by HPLC-DAD-FLD

After extraction of the lipid fraction and sample preparation (*n*-hexane and 50 μ L of 0.1 mg/mL tocol as the internal standard) [43], the solution was injected into an HPLC-DAD-FLD system (Jasco, Tokyo, Japan) equipped with a multiwavelength diode array detector (MD-2015) and a FP-2020 fluorescence detector (Jasco, Tokyo, Japan) (excitation=290 nm; emission=330 nm). A normal phase SupelcosilTM LC-SI column (75 mm x 3.0 mm, 3.0 μ m) (Supelco, Bellefonte, PA, USA) was used to separate the vitamin E vitamers [43]. The vitamer identification was based on the retention time (compared

with the standards) and in its UV spectra. The results were expressed as g/100 g of extract.

3.9 Fatty acids profile by GC-FID

The samples' oil was extracted as described by Alves et al. [43], and the fatty acid methyl esters (FAME) were obtained according to ISO 12966–2:2011 (cold derivatization) [44]. FAME separation was attained in a gas chromatograph (Shimadzu GC-2010 Plus, Tokyo, Japan) coupled to a split/splitless AOC-20i autoinjector (Shimadzu, Tokyo, Japan) and a flame ionization detector (Shimadzu, Tokyo, Japan). A CP-Sil 88 silica capillary column (50 m x 0.25 mm i.d., 0.20- μ m film thickness) (Varian, Middelburg, The Netherlands) was used. The temperature program was the following: 120 °C for 5 min, increased to 160 °C at 2 °C/min, held for 15 min and increased to 220 °C at 2 °C/min. The injector and detector temperatures were 250 and 270 °C, respectively. The FAME retention times were compared with those obtained with a standard mixture (FAME 37, Supelco, Bellefonte, PA, USA). Each fatty acid was expressed as a relative percentage.

3.10 pH

The pH was measured directly in the extracts with pH meter Basic 20+ (Crison, Barcelona, Spain).

3.11 Antimicrobial activity

Different agar-based methods were used to study the antimicrobial activity of the extracts (500 mg/mL). The minimal inhibitory concentration (MIC) was determined by the broth microdilution method of all the positive results obtained after screening. *Staphylococcus aureus* ATCC® 25923, *Escherichia coli* ATCC® 25922 and *Candida albicans* ATCC® 10231 were used to evaluate the antimicrobial activity in the study. Before the antimicrobial activity experiments, the microorganisms were cultured twice in a nutritive agar, such as blood agar (Biomérieux, Marcy-l'Étoile, France). For the antimicrobial activity assays, cell suspensions of 0.5 Mac Farland (CS) were prepared (turbidity scale) in 1 mL of 0.85% NaCl solution (sterilized). Antimicrobial activity screening was performed on the agar through three different methods. A positive result was considered when the presence of a microorganism growth zone of inhibition was greater than the inhibition zone produced only by the solvent (in this case, ultrapure sterile water) [45,46].

3.11.1 Incorporation method

A total of 1 mL of CS was added to a petri dish and 25 mL of melted Mueller–Hinton agar at 50 °C (MH, Liofilchem, Roseto degli Abruzzi, Italy) were added. Circular motions homogenized the mixture, and solidification occurred at room temperature. After solidification, 6 mm diameter cavities were made with sterile disposable tips, followed by cavity sealing by adding Muller–Hinton (10 µL) to the well bottom. After the bottom added agar was solidified, a total of 50 µL of solvent and extract were added to different wells. Plates were incubated at 37 °C for the bacteria (24 h) and yeast (48 h).

3.11.2 Surface spreading method

CS were spread on Muller–Hinton agar by surface scattering with a sterile swab. According to the procedure of Section 3.11.1, the agar wells were filled with the extract and solvent (ultrapure sterile water).

3.11.3 Agar diffusion method

Muller–Hinton agar plates were surface seeded using a swab with CS. The extract and solvent were added (20 µL) to different blank paper disks (sterile) and placed in the seeded agar.

3.11.4 Microdilution method

For the extracts that showed antimicrobial activity in the previously described methods, the solvents with the respective microorganisms, the extract with only TSB (Tryptic Soy Broth, Liofilchem, Roseto degli Abruzzi, Italy) and the extract with the microorganism under study were tested by the microdilution methodology in a 96-well plate, according to the previously described methodology [45].

In the first well of a 96-well plate, 100 µL of extract/solvent were pipetted, with 100 µL of previously diluted 1:150 in concentrated TSB (2) of CS. After, in wells 2–12, TSB (50 µL) and CS (50 µL) diluted (1:150) in 1 TSB were added. Then, in the 1–11th wells, 100 µL were transferred from well to well. The 12th well, containing only TSB and CS, was used as the positive control of the assay. The plates were incubated at the same conditions of Section 3.11.1.

3.12 Statistical analysis

A statistical analysis was performed using IBM SPSS Statistics v.25 (IBM Corp., Armonk, NY, USA). The data were expressed as the mean standard deviation. A one-way ANOVA was used to evaluate the differences between the samples, followed by Tukey's HSD to

make pairwise comparisons between the means. The level of significance for all the hypothesis tests was 0.05.

4. Conclusions

From this work stands out the hydroxytyrosol content that has well-known and remarkable health effects. Hence, the olive pomace functional ingredient can be advantageous as a nutritional profile enhancer. The antibacterial activity determined that the samples can be a natural food preservative against tested Gram-positive and Gram-negative bacteria. As a lower MIC value indicates that less extract is required to inhibit the growth of the microorganism analyzed, O1 presented the highest potential among all the samples. Regarding the chemical profile, the four olive pomace functional ingredients studied presented similar profiles differing in the amount of fat, which was superior in O2, and protein and ash (higher in O4).

Noteworthy, olive pomace extract can be considered an all-in-one and advantageous ingredient, since it presents a mixture of lipidic and hydrophilic bioactive compounds usually not present in other plant extracts.

The balance between environmental sustainability, low-cost and high-yield extraction procedures is challenging. In this research, the use of a green method allowed us to obtain natural, plant-based and chemical-free extracts with antimicrobial activity. Nevertheless, further studies regarding enhancing the phenolic contents in the extracts must be explored to improve the antimicrobial activity. Since some phenolics can be trapped in glycosidic junctions and pomace fibers, the implementation of, for example, a hydrolysis step before pressure can be advantageous.

References

1. Aguilar, C.N.; Ruiz, H.A.; Rios, A.R.; Chávez-González, M.; Sepúlveda, L.; Rodríguez-Jasso, R.M.; Loredó-Treviño, A.; Flores-Gallegos, A.C.; Govea-Salas, M.; Ascacio-Valdes, J.A. Emerging strategies for the development of food industries. *Bioengineered* 2019, 10, 522–537.
2. Kumar, K.; Yadav, A.N.; Kumar, V.; Vyas, P.; Dhaliwal, H.S. Food waste: A potential bioresource for extraction of nutraceuticals and bioactive compounds. *Bioresour. Bioprocess* 2017, 4, 1–14.
3. European Commission. Olive Oil—Detailed Information on the Market Situation, Price Developments, Balance Sheets, Production and Trade. 2020. Available online: <https://ec.europa.eu/info/food-farming-fisheries/farming/facts-and->

figures/markets/prices/price-monitoring-sector/plant-products/olive-oil_en
(accessed on 26 March 2020).

4. Di Giovacchino, L.; Preziuso, S.M.; Di Serio, M.G.; Mucciarella, M.R.; Di Loreto, G.; Lanza, B. Double extraction of olive oil in large oil mills of Southern Italy: Effects on extraction efficiency, oil quality, and economy of the process. *Eur. J. Lipid Sci. Technol.* 2017, 119, 1600161.
5. Nunes, M.A.; Costa, A.S.G.; Bessada, S.; Santos, J.; Puga, H.; Alves, R.C.; Freitas, V.; Oliveira, M.B.P.P. Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid- and water-soluble components. *Sci. Total Environ.* 2018, 644, 229–236.
6. Gullón, P.; Gullón, B.; Astray, G.; Carpena, M.; Fraga-Corral, M.; Prieto, M.A.; Simal-Gandara, J. Valorization of by-products from olive oil industry and added-value applications for innovative eco-foods. *Food Res. Int.* 2020, 137, 109683.
7. Ng, K.R.; Lyu, X.; Mark, R.; Chen, W.N. Antimicrobial and antioxidant activities of phenolic metabolites from flavonoid-producing yeast: Potential as natural food preservatives. *Food Chem.* 2019, 270, 123–129.
8. de Carvalho, B.L.; Salgueiro, M.d.F.; Rita, P. Consumer sustainability consciousness: A five dimensional construct. *Ecol. Indic.* 2015, 58, 402–410.
9. Olszewska, M.A.; Gędas, A.; Simões, M. Antimicrobial polyphenol-rich extracts: Applications and limitations in the food industry. *Food Res. Int.* 2020, 134, 109214.
10. Korukluoglu, M.; Sahan, Y.; Yigit, A. Antifungal properties of olive leaf extracts and their phenolic compounds. *J. Food Saf.* 2008, 28, 76–87.
11. Gyawali, R.; Ibrahim, S.A. Natural products as antimicrobial agents. *Food Control* 2014, 46, 412–429.
12. Mbaveng, A.T.; Sandjo, L.P.; Tankeo, S.B.; Ndifor, A.R.; Pantaleon, A.; Nagdju, B.T.; Kuete, V. Antibacterial activity of nineteen selected natural products against multi-drug resistant Gram-negative phenotypes. *SpringerPlus* 2015, 4, 823.
13. Diallinas, G.; Rafailidou, N.; Kalpaktsi, I.; Komianou, A.C.; Tsouvali, V.; Zantza, I.; Mikros, E.; Skaltsounis, A.L.; Kostakis, I.K. Hydroxytyrosol (HT) analogs act as potent antifungals by direct disruption of the fungal cell membrane. *Front. Microbiol.* 2018, 9, 2624.
14. Xue, J.; Davidson, P.M.; Zhong, Q. Thymol nanoemulsified by whey protein-maltodextrin conjugates: The enhanced emulsifying capacity and antilisterial properties in milk by propylene glycol. *J. Agric. Food Chem.* 2013, 61, 12720–12726.
15. Peralbo-Molina, Á.; Priego-Capote, F.; de Castro, M.D.L. Tentative identification of phenolic compounds in olive pomace extracts using liquid chromatography-tandem

- mass spectrometry with a quadrupole-quadrupole-time-of-flight mass detector. *J. Agric. Food Chem.* 2012, 60, 11542–11550.
16. Cushnie, T.P.T.; Lamb, A.J. Recent advances in understanding the antibacterial properties of flavonoids. *Int. J. Antimicrob. Agents* 2011, 38, 99–107.
 17. Cueva, C.; Moreno-Arribas, M.V.; Martín-Álvarez, P.J.; Bills, G.; Vicente, M.F.; Basilio, A.; Rivas, C.L.; Requena, T.; Rodríguez, J.M.; Bartolomé, B. Antimicrobial activity of phenolic acids against commensal, probiotic and pathogenic bacteria. *Res. Microbiol.* 2010, 161, 372–382.
 18. Stojkovic', D.; Petrovic', J.; Sokovic', M.; Glamočlija, J.; Kukic'-Markovic', J.; Petrovic', S. *In situ* antioxidant and antimicrobial activities of naturally occurring caffeic acid, *p*-coumaric acid and rutin, using food systems. *J. Sci Food Agric.* 2013, 93, 3205–3208.
 19. Alghazeer, R.; Elmansori, A.; Sidati, M.; Gammoudi, F.; Azwai, S.; Naas, H.; Garbaj, A.; Eldaghayes, I. *In vitro* antibacterial activity of flavonoid extracts of two selected Libyan algae against multi-drug resistant bacteria isolated from food products. *J Biosci. Med.* 2017, 5, 26–48.
 20. Yangui, T.; Dhouib, A.; Rhouma, A.; Sayadi, S. Potential of hydroxytyrosol-rich composition from olive mill wastewater as a natural disinfectant and its effect on seeds vigour response. *Food Chem.* 2009, 117, 1–8.
 21. Guo, L.; Gong, S.; Wang, Y.; Sun, Q.; Duo, K.; Fei, P. Antibacterial activity of olive oil polyphenol extract against *Salmonella Typhimurium* and *Staphylococcus aureus*: Possible mechanisms. *Foodborne Pathog. Dis.* 2020, 17, 396–403.
 22. Andrade, J.C.; Morais-Braga, M.F.B.; Guedes, G.M.M.; Tintino, S.R.; Freitas, M.A.; Menezes, I.R.A.; Coutinho, H.D.M. Enhancement of the antibiotic activity of aminoglycosides by alpha-tocopherol and other cholesterol derivatives. *Biomed. Pharmacother.* 2014, 68, 1065–1069.
 23. Yoon, B.K.; Jackman, J.A.; Valle-González, E.R.; Cho, N.-J. Antibacterial free fatty acids and monoglycerides: Biological activities, experimental testing, and therapeutic applications. *Int. J. Mol. Sci.* 2018, 19, 1114.
 24. Zhou, J.; Velliou, E.; Hong, S.H. Investigating the effects of nisin and free fatty acid combined treatment on *Listeria monocytogenes* inactivation. *LWT-Food Sci. Technol.* 2020, 133, 110115.
 25. Klen, T.J.; Vodopivec, B.M. The fate of olive fruit phenols during commercial olive oil processing: Traditional press versus continuous two- and three-phase centrifuge. *LWT-Food Sci. Technol.* 2012, 49, 267–274.

26. Klen, T.J.; Wondra, A.G.; Vrhovšek, U.; Sivilotti, P.; Vodopivec, B.M. Olive fruit phenols transfer, transformation, and partition trail during laboratory-scale olive oil processing. *J. Agric. Food Chem.* 2015, 63, 4570–4579.
27. Tintino, S.R.; Morais-Tintino, C.D.; Campina, F.F.; Pereira, R.L.; Costa, M.d.S.; Braga, M.F.B.M.; Limaverde, P.W.; Andrade, J.C.; Siqueira-Junior, J.P.; Coutinho, H.D.M.; et al. Action of cholecalciferol and alpha-tocopherol on *Staphylococcus aureus* efflux pumps. *EXCLI J.* 2016, 15, 315–322.
28. Bintsis, T. Foodborne pathogens. *AIMS Microbiol.* 2017, 3, 529–563.
29. Denayer, S.; Delbrassinne, L.; Nia, Y.; Botteldoorn, N. Foodborne outbreak investigation and molecular typing: High diversity of *Staphylococcus aureus* strains and importance of toxin detection. *Toxins* 2017, 9, 407.
30. Palmeira, J.D.; Haenni, M.; Metayer, V.; Madec, J.-Y.; Ferreira, H.M.N. Epidemic spread of IncI1/pST113 plasmid carrying the Extended-Spectrum Beta-Lactamase (ESBL) blaCTX-M-8 gene in *Escherichia coli* of Brazilian cattle. *Vet. Microbiol* 2020, 243, 108629.
31. Mota, R.; Pinto, M.; Palmeira, J.; Gonçalves, D.; Ferreira, H. Multidrug-resistant bacteria as intestinal colonizers and evolution of intestinal colonization in healthy university students in Portugal. *Access Microbiol.* 2020, 3, 000182.
32. Rajkowska, K.; Kunicka-Styczyn´ska, A. Typing and virulence factors of foodborne *Candida* spp. isolates. *Int. J. Food Microbiol.* 2018, 279, 57–63.
33. da Silva Dantas, A.; Lee, K.K.; Raziunaite, I.; Schaefer, K.; Wagener, J.; Yadav, B.; Gow, N.A.R. Cell biology of *Candida albicans*–host interactions. *Curr. Opin. Microbiol.* 2016, 34, 111–118.
34. Zoric´, N.; Kopjar, N.; Bobnjaric´, I.; Horvat, I.; Tomic´, S.; Kosalec, I. Antifungal activity of oleuropein against *Candida albicans*—The *in vitro* study. *Molecules* 2016, 21, 1631.
35. Li, X.; Liu, Y.; Jia, Q.; LaMacchia, V.; O'Donoghue, K.; Huang, Z. A systems biology approach to investigate the antimicrobial activity of oleuropein. *J. Ind. Microbiol. Biotechnol.* 2016, 43, 1705–1717.
36. Medina-Martínez, M.S.; Truchado, P.; Castro-Ibáñez, I.; Allende, A. Antimicrobial activity of hydroxytyrosol: A current controversy. *Biosci. Biotechnol. Biochem.* 2016, 80, 801–810.
37. Nunes, M.A.; Costa, A.S.G.; Oliveira, M.B.P.P.; Applicant: University of Porto. Foodstuff Composition Comprising a Derivate of Olive Pomace Patent International Application Number PCT/IB2018/060111, 2019.

38. Manirakiza, P.; Covaci, A.; Schepens, P. Comparative study on total lipid determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and modified Bligh & Dyer extraction methods. *J. Food Compost. Anal.* 2001, 14, 93–100.
39. Official Methods of Analysis, 19th ed.; AOAC International: Rockville, MD, USA, 2012.
40. Tontisirin, K. Chapter 2: Methods of food analysis. In *Food Energy: Methods of Analysis and Conversion Factors: Report of a Technical Workshop*; Food and Agriculture Organization of the United Nations: Rome, Italy, 2003; pp. 7–17.
41. Costa, A.S.G.; Alves, R.C.; Vinha, A.F.; Costa, E.; Costa, C.S.G.; Nunes, M.A.; Almeida, A.A.; Santos-Silva, A.; Oliveira, M.B.P.P. Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chem.* 2018, 267, 28–35.
42. Seiquer, I.; Rueda, A.; Olalla, M.; Cabrera-Vique, C. Assessing the bioavailability of polyphenols and antioxidant properties of extra virgin argan oil by simulated digestion and Caco-2 cell assays. Comparative study with extra virgin olive oil. *Food Chem.* 2015, 188, 496–503.
43. Alves, R.C.; Casal, S.; Oliveira, M.B.P.P. Determination of vitamin E in coffee beans by HPLC using a micro-extraction method. *Food Sci. Technol. Int.* 2009, 15, 57–63.
44. ISO 12966-2:2011—Animal and vegetable fats and oils—Gas chromatography of fatty acid methyl esters—Part 2: Preparation of methyl esters of fatty acids.
45. Almeida, D.; Pinto, D.; Santos, J.; Vinha, A.F.; Palmeira, J.; Ferreira, H.N.; Rodrigues, F.; Oliveira, M.B.P.P. Hardy kiwifruit leaves (*Actinidia arguta*): An extraordinary source of value-added compounds for food industry. *Food Chem.* 2018, 259, 113–121.
46. Palmeira, J.D.; Ferreira, S.B.; de Souza, J.H.; de Almeida, J.M.; Figueiredo, M.C.; Pequeno, A.S.; Arruda, T.A.; Antunes, R.M.P.; Catão, R.M.R. Evaluation of the antimicrobial activity *in vitro* and determination of minimal the inhibitory concentration of hidroalcoholic extracts of angico in strains *Staphylococcus aureus*. *Br. J. Clin. Anal.* 2010, 42, 33–37.

4.2 Inovar no consumo de azeite

M. Antónia Nunes, Joana Lobo, M. Beatriz P.P. Oliveira, Rita C. Alves

REQUIMTE/LAQV, Departamento de Ciências Químicas,
Faculdade de Farmácia da Universidade do Porto

**Com revisão por pares*

- Nunes, M.A., Lobo, J.C., Oliveira M.B.P.P., Alves, R.C. (2021). Inovar no consumo de azeite. *Tecnoalimentar*, 28, 28-31.

Resumo

O presente estudo foi desenvolvido durante a fase de prototipagem de um creme vegetal à base de azeite e funcionalizado com um extrato de bagaço de azeitona, o subproduto maioritário do processamento do azeite. Teve como objetivo desenvolver/melhorar os atributos do produto face ao posicionamento do mercado. Para isso foi desenvolvido e aplicado um questionário constituído maioritariamente por perguntas fechadas.

Os resultados deste estudo permitiram aferir e confirmar atributos do produto, sobretudo as características físicas que o consumidor procura num creme vegetal e a intenção de compra. Reforçou também a preferência do consumidor pelo “natural” e alavancou a utilização de subprodutos no desenvolvimento de novos produtos.

Abstract

This study was developed during the prototyping phase of an olive oil-based vegetable cream functionalized with an olive pomace extract, the major by-product of olive oil processing. The study aimed to develop/improve the product attributes to approach the market. For that, it was developed and applied a questionnaire composed mainly of closed questions.

Data from the survey allowed us to ascertain and confirm the product attributes, especially the physical features that consumers search in a vegetable cream and the buying purpose. Also, it reinforced the consumer preference by the “natural” and supported the use of the by-products to develop new products.

Introdução

O azeite é um produto de elevada qualidade nutricional e com benefícios para a saúde.

Aquando da produção de azeite são geradas grandes quantidades de subprodutos, nomeadamente bagaço de azeitona. Este subproduto é rico em compostos bioativos, como polifenóis, vitamina E, minerais e fibra, com impacto positivo na saúde humana, mas com efeito prejudicial para o meio ambiente (Nunes et al., 2018).

Tendo em conta o interesse nutricional do bagaço de azeitona e o aumento crescente da sua produção, é fundamental encontrar estratégias de valorização. Assim, foi desenvolvido um creme vegetal barrável à base de azeite com incorporação de um extrato de bagaço de azeitona, produto que se denominou Sprelive e que deu origem a um pedido de patente (PCT/IB2018/060111). Pretende-se, igualmente inovar e diversificar as formas de consumo de azeite.

O presente estudo teve como objetivos compreender o consumidor-alvo do Sprelive e melhorar/desenvolver atributos do produto de forma a alinhá-lo no segmento de mercado da saúde e bem-estar.

Material e métodos

Foi desenvolvido e aplicado um questionário simples (13 questões) aos participantes de um evento gastronómico realizado em 2019 no Porto (Portugal) (n=30). O estudo foi realizado com base numa amostra por conveniência uma vez que se pretendia identificar aspetos críticos para o desenvolvimento do produto. O questionário foi distribuído aleatoriamente a indivíduos de ambos os géneros que acordaram no seu preenchimento. Sumariamente os pontos do questionário englobaram a caracterização demográfica (género e escalão etário), e questões fechadas relacionadas com i) Hábitos de consumo de azeite, ii) Saúde e alimentação, iii) Mercado de margarinas e cremes vegetais, iv) Sustentabilidade ambiental e v) Avaliação do novo conceito de creme vegetal para barrar à base de azeite e intenção de compra. O questionário incluiu questões de resposta única e questões de resposta múltipla em número não limitado. De forma a compreender qual o fator decisor preponderante no momento de compra, foi incluída uma questão aberta. A duração média do preenchimento do questionário era de 15 minutos. Para analisar as respostas obtidas foi utilizado o cálculo de frequências absolutas e relativas para as respostas às questões em estudo. Os resultados são apresentados em percentagem (%).

Resultados e discussão

Na Tabela 1 são apresentados os dados referentes à segmentação demográfica (género e escalão etário).

Tabela 1. Perfil demográfico dos participantes.

Característica	%
Género	
Feminino	76,7
Masculino	23,3
Escalão etário (anos)	
≤20	30,0
21 – 30	40,0
31 – 40	16,7
41 – 50	10,0
≥51	3,3

Hábitos e motivações de consumo de azeite

O azeite é amplamente consumido e apreciado a nível mundial, sendo um dos principais pilares da Dieta Mediterrânica. A forte associação entre os efeitos benéficos e a saúde tem levado ao aumento do seu consumo (Nunes et al., 2018).

Para compreender as razões dos hábitos de consumo de azeite, foi colocada a questão “Qual a principal ou principais razões por que consome azeite?” à qual 73% dos participantes respondeu “Sabor” seguido de “Saúde” e “Tradição” (47 e 27%, respetivamente) (Figura 1A), indo de encontro às principais motivações reportadas para o consumo de azeite (saúde e motivações hedonistas) (Peršurić, 2020).

A questão “Costuma utilizar azeite como substituto de manteiga, margarina ou creme vegetal?” teve como objetivo compreender hábitos de utilização de azeite, para além dos mais utilizados em Portugal, como a confeção e tempero de alimentos. Registou-se que 63% não utiliza azeite como substituto de produtos normalmente utilizados para barrar, como a manteiga ou creme vegetais (Figura 1B). Esta questão poderá vir a ser explorada em trabalhos futuros para perceber por que não é utilizado.

Sprelive é um creme vegetal barrável que pretende associar conveniência ao azeite no formato “barrável”. O efeito sinérgico do azeite e do extrato rico em compostos bioativos derivado do bagaço de azeitona faz com que este produto tenha efeitos benéficos para a

saúde. Os resultados observados sugerem que haverá uma boa receptividade para este tipo de produtos à base de azeite.

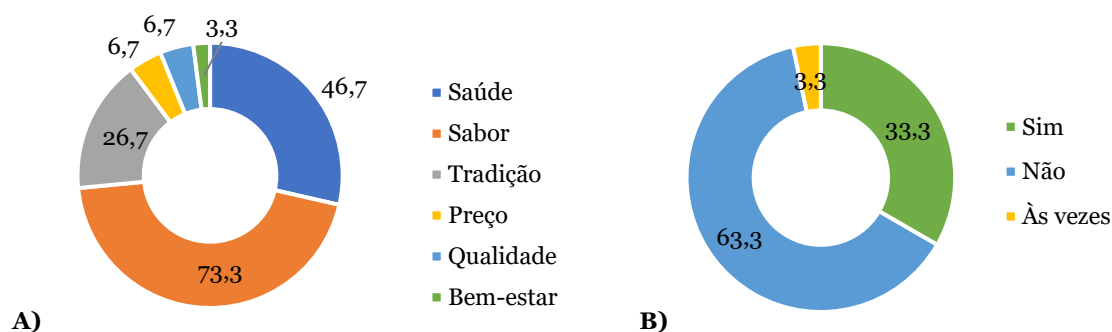


Figura 1. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Qual a principal ou principais razões por que consome azeite?” e B – “Costuma utilizar azeite como substituto de manteiga, margarina ou creme vegetal?”.

Preocupações com a saúde como tendência de mercado

Atualmente, a denominação “saudável” é muito utilizada pelos operadores do mercado e pelo consumidor. No entanto, a percepção do que é “saudável” é ainda subjetiva para os consumidores, considerando que a denominação pode ser atribuída a um produto individualmente e como um todo, ou à presença ou ausência de determinado ingrediente (por exemplo, açúcar, sal, fibra alimentar) ou aditivos (naturais *versus* artificiais) (Di Vita et al., 2020).

O Sprelive pretende ser uma alternativa a outros produtos da mesma categoria, posicionando-se no segmento de mercado da saúde e bem-estar, incluído num padrão alimentar saudável. A questão “Consome um produto alimentar que mesmo que não seja “saboroso” é benéfico para a saúde?” é crucial, porque os produtos funcionalizados com extrato de bagaço de azeitona podem ter características adstringentes devido à presença de compostos antioxidantes como a oleuropeína e hidroxitirosol (Nunes et al., 2016). Neste caso, 57% dos indivíduos responderam que consumiriam um produto alimentar com as características questionadas enquanto 40% declarou que não consumiria e 3% afirmou que talvez o fizesse (Figura 2A). Neste último caso, será importante para o consumidor outros atributos para além do sabor. O facto de 40% notar que um produto saudável terá de ser saboroso, leva a que no desenvolvimento de produtos com bagaço de azeitona venha a ser necessário reduzir a quantidade de extrato, o que pode condicionar as características funcionais do produto, ou que sejam necessários adjuvantes tecnológicos para “mascarar” o sabor adstringente.

A capacidade económica para adquirir alimentos saudáveis *versus* não saudáveis é também um importante determinante para as escolhas alimentares. À questão “Considera que os produtos mais saudáveis são também os mais caros?”, apenas 37% consideram que não (Figura 2B), reforçando que o consumidor percebe os produtos mais saudáveis como mais caros tal como também reportam Headey e Alderman (2019).

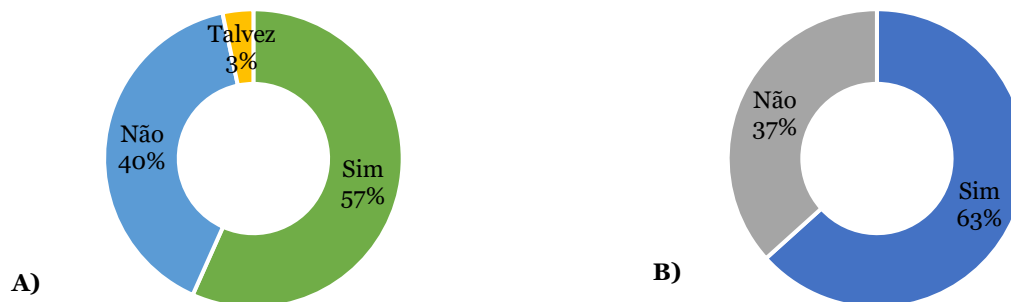


Figura 2. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Consome um produto alimentar que mesmo que não seja “saboroso” é benéfico para a saúde?” e B – “Considera que os produtos mais saudáveis são também os mais caros?”.

Mercado de margarinas e cremes vegetais para barrar

O mercado de margarinas e cremes vegetais para barrar é competitivo principalmente devido à presença de marcas fortes e dominantes. Além disso, é um segmento de rápido crescimento e constante reformulação de produto (Garsetti et al., 2016).

Neste estudo, aquando questionados sobre a satisfação com os cremes vegetais presentes no mercado, 47% indicou que estava satisfeito enquanto 30% não o estão (13,3% dos inquiridos não respondeu a esta questão) (Figura 3A). Considerando que os não-consumidores ou os que estão “mais ou menos” satisfeitos, poderão também constituir um grupo-alvo para o Sprelive, existe potencial de incluir o produto neste segmento de mercado.

As respostas à pergunta “Qual a característica ou quais as características físicas que mais valoriza num creme vegetal? Espalhamento, consistência à temperatura ambiente, brilho, cor, textura?”, permitem aprimorar os atributos do produto. Nesta questão, pedia-se aos inquiridos que indicassem uma ou mais características valorizadas no produto do ponto de vista do consumidor uma vez que todas são tecnologicamente significativas.

Assim, no desenvolvimento do produto devem ser focalizadas as características “Textura”, “Espalhamento” e “Consistência” uma vez que foram as mais referenciadas pelos participantes (Figura 3B).

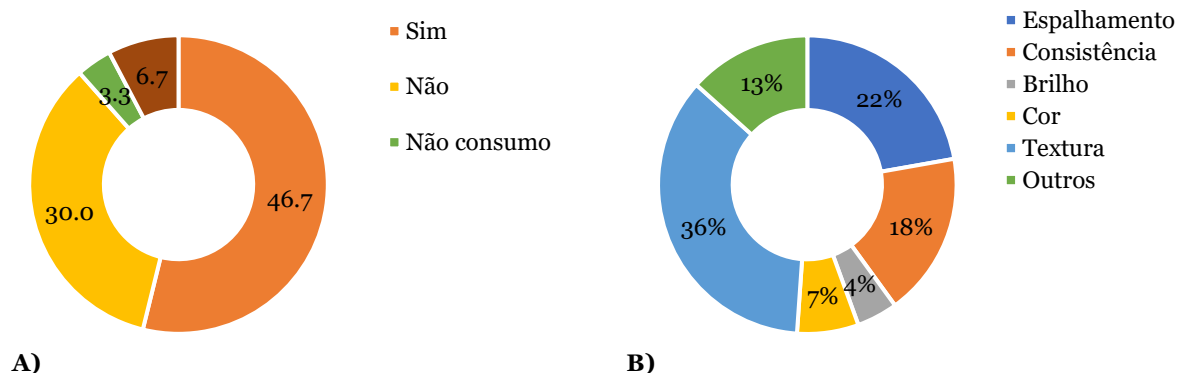


Figura 3. Distribuição das respostas dos inquiridos (%) às perguntas: A – “*Está satisfeito com os cremes vegetais que, neste momento, se encontram no mercado?*” e B – “*Qual a característica ou quais as características físicas que mais valoriza num creme vegetal? Espalhamento, consistência à temperatura ambiente, brilho, cor, textura?*”.

Preocupações com a sustentabilidade ambiental

A extração e utilização de compostos bioativos do bagaço de azeitona, por métodos sustentáveis, leva a que por um lado seja reduzido o impacto ambiental deste subproduto, e por outro seja possível desenvolver novos produtos alimentares funcionais.

À questão “Normalmente, prefere produtos alimentares em que o processo de fabrico e os ingredientes são “amigos do ambiente” e que a pegada ecológica é pequena?”, 90% dos inquiridos respondeu “Sim” (Figura 4A) indo de encontro à megatendência atual que é a preocupação com a sustentabilidade ambiental associada à produção, distribuição e consumo de alimentos (Perito et al., 2020).

Recentemente, foi avaliada a recetividade do consumidor à utilização de subprodutos vegetais da agroindústria, nomeadamente do azeite, no desenvolvimento de produtos (Perito et al., 2020). No presente estudo, observou-se que 93% respondeu positivamente à questão “Consumiria um produto alimentar que incluísse ingredientes naturais obtidos ecologicamente de subprodutos da indústria agroalimentar?”, sendo que 7%, respondeu “Talvez” (Figura 4B). Surpreendentemente nenhum inquirido respondeu que “Não” consolidando, cada vez mais, a possibilidade de utilização de subprodutos no desenvolvimento de novos produtos.



Figura 4. Distribuição das respostas dos inquiridos (%) às perguntas: A - “Normalmente, prefere produtos alimentares em que o processo de fabrico e os ingredientes são “amigos do ambiente” e que a pegada ecológica é pequena?” e B – “Consumiria um produto alimentar que incluísse ingredientes naturais obtidos ecologicamente de subprodutos da indústria agroalimentar?”.

Fatores de decisão mais valorizados na compra de cremes vegetais

O uso de ingredientes naturais pode ser especialmente importante para os consumidores que desejam alimentos mais saudáveis e mais naturais e que dão preferência aos produzidos por métodos sustentáveis.

Para avaliar alguns dos fatores de decisão mais valorizados na compra de cremes vegetais e compreender a intenção de compra do creme vegetal à base de azeite, os participantes responderam às questões “Valoriza mais um produto se tiver aditivos naturais em vez de artificiais?” e “Compraria um creme vegetal para barrar à base de azeite?” (Figura 5).

Relativamente à primeira questão, 97% dos indivíduos respondeu que prefere produtos com aditivos naturais.

O desenvolvimento de novos produtos com adição de extratos bioativos, como forma natural de melhorar o seu perfil nutricional/conservação é desafiante. 74% dos inquiridos respondeu que compraria um creme vegetal à base de azeite e apenas 23% respondeu que não o faria (Figura 5B), o que permite sustentar o avanço do desenvolvimento do Sprelive.

Ainda, o fator “Preço” correspondeu a 80% dos fatores de decisão indicados como mais importantes no momento de compra, seguido da “Qualidade” (33%) (Figura 6). Considerando os ingredientes e processo de fabrico do Sprelive, será relevante determinar preços de produto competitivos para que a população em geral possa usufruir dos benefícios nutricionais deste produto.

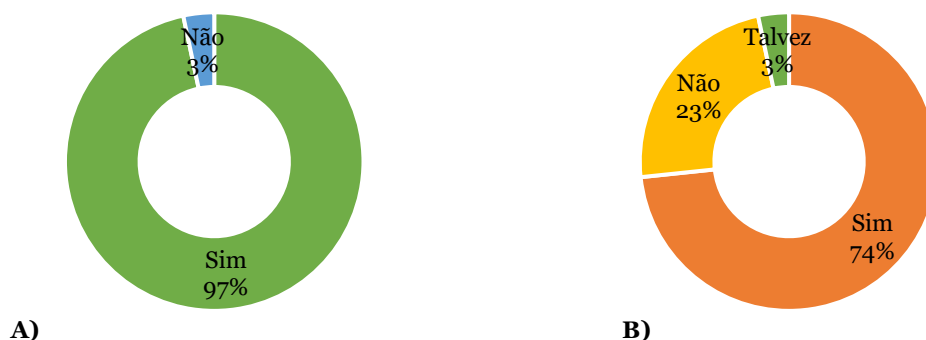


Figura 5. Distribuição das respostas dos inquiridos (%) às perguntas: A – “Valoriza mais um produto se tiver aditivos naturais em vez de artificiais?” e B – “Compraria um creme vegetal para barrar à base de azeite?”.

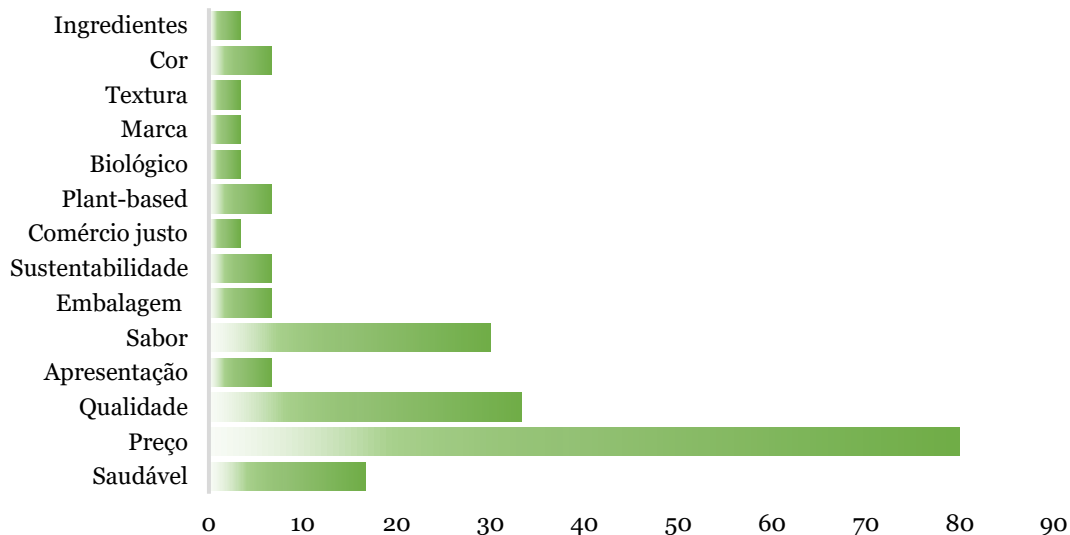


Figura 6. Distribuição das respostas dos inquiridos (%) à pergunta: “Qual o fator ou fatores de decisão mais importante(s) para si no momento de compra? Indique um ou vários.”

Conclusões

A realização de estudos como o apresentado no decorrer do desenvolvimento de produtos pode ser vantajosa porque são de aplicação simples, permitem aferir o processo num determinado momento e levam a uma melhor compreensão das expectativas do consumidor. O Sprelive apresenta um elevado teor de azeite na composição e funcionalidade devido ao extrato de bagaço de azeitona. As características referidas são de interesse para o consumidor pelo que a sua produção e comercialização podem ser vantajosas para o mercado e para a saúde pública. Considerando o produto em estudo, é possível inovar no consumo de azeite indo de encontro às expectativas do consumidor e tendências de mercado.

Bibliografia

- Dii Vita, G., Strano, A., La Via, G., & D'Amico, M. The role of individual knowledge in functional olive oil preferences: does self-coherence lead to different health attributes perception? *Foods*. 9:10 (2020) 1428.
- Garsetti, M., Balentine, D.A., Zock, P.L., Blom, W.A.M., & Wanders, A.J. Fat composition of vegetable oil spreads and margarines in the USA in 2013: a national marketplace analysis. *International journal of food sciences and nutrition*. 67:4 (2016) 372-382.
- Headey, D. & Alderman, H. The relative caloric prices of healthy and unhealthy foods differ systematically across income levels and continents. *The journal of nutrition*. 149:11 (2019) 2020-2033.
- Nunes, M.A., Pimentel, F.B., Costa, A.S.G., Alves, R.C. & Oliveira, M.B.P.P.- Olive by-products for functional and food applications: Challenging opportunities to face environmental constraints. *Innovative food science & emerging technologies*. 35 (2016) 139-148.
- Nunes, M.A., Costa, A.S.G., Bessada, S., Santos, J., Puga, H., Alves, R.C., Freitas, V., & Oliveira, M.B.P.P. – Olive pomace as a valuable source of bioactive compounds: A study regarding its lipid and water-soluble components. *Science of the total environment*. 644 (2018) 229-236.
- Perito, M.A., Di Fonzo, A., Sansone, M., & Russo, C.- Consumer acceptance of food obtained from olive by-products. *British food journal*. 122:1 (2020) 212-226.
- Peršurić, A. Segmenting olive oil consumers based on consumption and preferences toward extrinsic, intrinsic and sensorial attributes of olive oil. *Sustainability*. 12:16 (2020) 6379.

This page was intentionally left in blank.

Chapter 5 – Olive pomace functional ingredient: future developments

Chapter 5 includes preliminary results that are ongoing regarding the chemical characterisation of the olive pomace functional ingredient and a food product fortified using it: the olive oil-based vegetable cream Sprelive.

This page was intentionally left in blank.

5.1 Chemical analysis of a mixed olive pomace functional ingredient

The preliminary results that are presented regard the incorporation of a new olive pomace functional ingredient. It was the result of the mixture of individual pomaces (OFIx). Four olive pomaces with different geographical origins (Alfândega da Fé, Valpaços, Beja and Ferreira do Alentejo) were pooled intended to represent a unique olive pomace sample.

This section briefly presents the methodology used to obtain and characterize OFIx, the preliminary results and conclusions, the ongoing investigation, and the future work that is still required.

5.1.1 Methodology

- Sampling

Four olive pomaces were collected in two-phases olive mills (Portugal). Two olive pomaces are representative of the Northeast region, and the other two are from the South. After homogenisation, pomaces were mixed (25% each) and submitted to the process described in Chapter 3 (Patent pending PCT/IB2018/060111). The mixture of the olive pomaces was transferred into the prototype in fractions of 200 g to obtain the functional ingredient. The extraction was accomplished as follows:

1. A physical force (200-300 bar; 30 min) was applied. The pressing force separates the liquid phase (water and oil) from the solid phase (olive skin, pulp, stone and stones).
2. The process was repeated for 200 g fractions and all the liquid phases jointed.
3. The olive pomace functional ingredient (OFIx) was centrifuged (5000 rpm; 20 min).

The supernatant was removed, lyophilized and its chemical profile evaluated.

- Phytochemicals (total phenolics and flavonoids, hydroxytyrosol) and antioxidant activity assessment

Total phenolics and flavonoids contents and antioxidant activity were determined spectrophotometrically. Phenolics were assessed using the Folin-Ciocalteu method with minor modifications [1]. Flavonoid's content was assessed using the aluminium chloride method [1].

The following methods were used to assess the antioxidant activity: FRAP (ferric reducing antioxidant power), DPPH• (2,2-diphenyl-1-picrylhydrazyl radical) [1] and ABTS•⁺ (2,2'-azinobis (3-ethylenbenzothiazoline-6-sulfonic acid) radical cation) [2] scavenging abilities.

To determine the hydroxytyrosol content, high-performance liquid chromatography was used with a multi-wavelength diode array detector coupled to a fluorescence detector (HPLC-DAD-FLD). The hydroxytyrosol was quantified by fluorescence and monitored at λ excitation=280 nm and λ emission=330 nm [3].

- Lipidic fraction evaluation: total fat, vitamin E and fatty acids profile

The total fat was assessed using the Bligh & Dyer extraction method [4] with modifications.

The vitamin E profile was assessed by HPLC-DAD-FLD. First, a cold extraction method was applied to extract the lipid fraction, using *n*-hexane and tocol (internal standard) [5]. Tocopherols and tocotrienols were identified by comparing compounds retention times with standards and their UV spectra (λ excitation 290 nm; λ emission 330 nm).

Fatty acid methyl esters (FAME) profiles were obtained by a gas chromatograph coupled to a split/splitless auto-injector and a flame ionization detector (GC-FID). First, oil was extracted as described for vitamin E. Then, it was performed a cold methylation according to ISO 12966-2:2011 [6]. The FAME were identified by comparison with a standard mixture (FAME 37).

- Contents of protein and ash

Kjeldahl procedure (6.25 as the nitrogen conversion factor) was used to evaluate the protein content (AOAC 928.08), and ash was assessed after incineration at 500 °C (AOAC 920.153) [7, 8].

5.1.2 Preliminary results and conclusions

Figure 1 shows the lyophilized OFIx, and Table 1 presents its chemical characterisation.

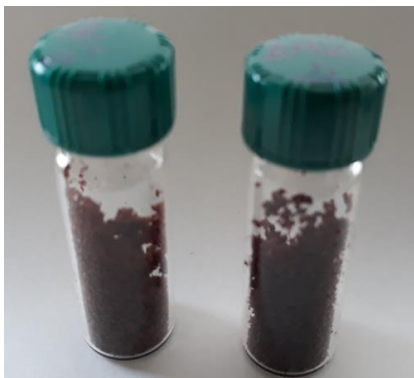


Figure 1. Lyophilized olive pomace functional ingredient (OFIx).

OFlx consists mainly of water-soluble bioactive compounds with recognised benefits for human health, such as hydroxytyrosol, among others. Nevertheless, it also presents 8% of fat in which oleic acid and α -tocopherol stand out (Table 1).

Table 1. Chemical analysis of OFlx: total phenolics, flavonoids, hydroxytyrosol content, antioxidant activity (FRAP, DPPH $\cdot$ and ABTS $^{++}$ scavenging abilities), fatty acids and vitamin E profiles, total fat, total protein and ash (dry weight).

OFlx	
Total phenolics (g GAE/100 g)	2.87 $\pm$ 0.11
Hydroxytyrosol (mg/100 g)	214.89 $\pm$ 8.96
Total flavonoids (g CE/100 g)	2.29 $\pm$ 0.08
Antioxidant activity	
FRAP (g FSE/100 g)	2.28 $\pm$ 0.11
DPPH$\cdot$ scavenging ability (g TE/100 g)	0.96 $\pm$ 0.17
ABTS^{++} scavenging ability (g TE/100 g)	0.19 $\pm$ 0.001
Total fat (%)	8 $\pm$ 0.05
Vitamin E (mg/100 g)	
α-tocopherol (mg/100 g)	1.78 $\pm$ 0.42
α-tocotrienol (mg/100 g)	0.07 $\pm$ 0.00
β-tocopherol (mg/100 g)	0.05 $\pm$ 0.00
γ-tocopherol (mg/100 g)	0.09 $\pm$ 0.01
Fatty acids (relative %)	
C16:0	11.6 $\pm$ 0.04
C18:0	3.7 $\pm$ 0.01
C18:1n9cis	71.7 $\pm$ 0.05
C18:2	10.5 $\pm$ 0.02
Total protein (%)	0.9 $\pm$ 0.02
Ash (%)	11 $\pm$ 0.02

C16:1, C17:0, C20:0, C18:3n3, C20:1n9cis, C22:0, C24:0 < 1%; GAE, gallic acid equivalents; TE, trolox equivalents; CE, catechin equivalents; FRAP, ferric reducing antioxidant power; FSE, ferrous sulfate equivalents; DPPH $\cdot$, 2,2-diphenyl-1-picrylhydrazyl; ABTS, 2,2'-azino-bis(3-ethylenbenzothiazoline-6-sulfonic acid).

The total mineral content is $\approx$ 11%, and protein of $\approx$ 1%. Potassium, magnesium, calcium and other essential minerals are present in olive pomace. Considering the aimed functional properties of OFlx and the high content in total minerals, it will be relevant to determine its mineral profile.

5.1.3 Ongoing investigation and future work

Bioactive compounds, such as the phenolics present in olive pomace, have shown a positive association between its consumption and the reduction of the risk of developing several chronic diseases in humans, including several types of cancer. Therefore, based on the hypothesis that OFIx compounds would exert anticancer activities in breast, pancreatic and colorectal cancer cell lines, the extract's effects on cell viability, proliferation, growth and angiogenesis were measured.

Also, considering the OFIx chemical results obtained, and the ongoing work mentioned above, a more in-depth analysis by UHPLC-ESI-QTOF-MS is in progress in collaboration with the Laboratory of Foodomics of the Institute of Food Science Research (CSIC) in Spain.

Comprehensive identification of minor compounds present in OFIx will allow us to understand better the anticancer activities observed.

5.2 Olive oil-based spread: preparation and composition

The extraction and use of bioactive compounds from the olive pomace by sustainable methods reduce the environmental impact of this by-product. On the other hand, it is possible to develop new food products fortified with these bionutrients because the same phytotoxic compounds (phenolics and fatty acids) are beneficial to human health.

Hence, a new food product was designed, formulated and prototyped. It was hypothesized that the combination of olive oil and a rich extract obtained from olive pomace makes it possible to obtain a functional product that nutritionally stands out and can be an alternative to other products in the same category, positioning itself in the health and well-being market segment.

“Sprelive” is the denomination resultant of the combination of “spread” and “live”, and it was attributed to a functional vegetable cream with a high content of olive oil that incorporates bioactive compounds from olive pomace. In Portuguese, it was named “Olivina”.

Sprelive is an olive oil-based spread with an olive oil content higher than 70% and functionalized with a natural extract obtained from olive pomace. The preliminary results presented are related to the incorporation of OFIx previously characterized.

The formulation and preparation of Sprelive are described. However, the detailed ingredients list and production process are not disclosed due to the ongoing intellectual

property protection. The fundamental premise for Sprelive development is that the olive oil content should be higher than 70% and be spreadable.

The antioxidant characteristics, hydroxytyrosol content and fatty acids profile of two different Sprelive formulations and three selected commercially available vegetable creams were evaluated.

5.2.1 Methodology

- Sampling: formulation and process of production of Sprelive

In Sprelive, the food matrix is constituted mainly by a lipid basis (70%) and an aqueous phase since it was expected that an emulsion could be more suitable to an amphipathic compound such as hydroxytyrosol. The manufacturing process is presented below briefly:

1. Solubilization of lipidic ingredients in the lipid phase.
2. Addition of the OFIx and water-soluble ingredients to the aqueous phase.
OFIx was solubilized in the aqueous phase obtaining a final concentration in the product of 279 mg/100 g, representing 1 mg hydroxytyrosol/100 g of Sprelive.
3. Blending of the oil and aqueous phases to form an emulsion.
4. Cooling.

The development of the formulations had as guiding principles: i) to use an olive oil content the highest possible; ii) to obtain a stable emulsion, after product processing, at room temperature; and iii) follow the “clean label” standards.

Hence, in the development of the formulations were tested several technological improvement agents in different quantities in order to promote and/or improve the stability of the emulsions and texture such as lecithin, citric acid, xanthan gum, salt, pectin, cocoa butter, rice starch, coconut oil, mono and diglycerides, pea protein, modified starch, agar-agar, maltodextrin and lupine flour-based vegetable preparation. It was formulated and prototyped forty preparations, of which were selected the best two considering the visual aspect, textural and firmness features. Two different formulations (A and B) were produced (Figure 2). Control samples (without OFIx) were also produced.

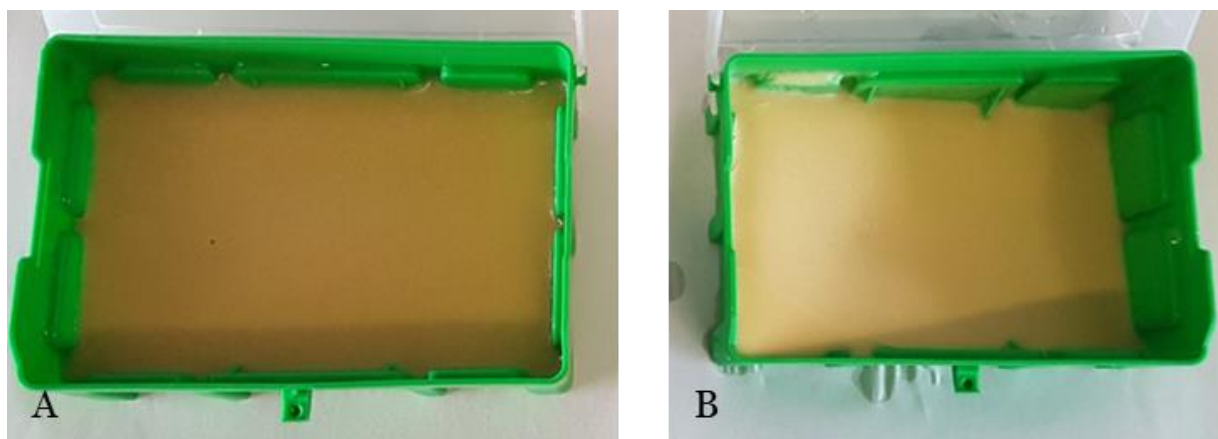


Figure 2. Sprelive with olive pomace functional ingredient (OFIx).
A, formulation A; B, formulation B.

- High-pressure processing

High-pressure processing (HPP) is an innovative non-thermal technology that uses elevated hydrostatic pressures for a few seconds/minutes to inactivate both pathogenic and spoilage vegetative microorganisms [9]. HPP was tested as a preservation method in Sprelive. For that, samples were submitted for 3 min to a 5500 bar.

Table 2 presents the denomination of all samples analysed.

Table 2 . Samples denomination.

Samples	
SAC	Formulation A - without OFI
SAO	Formulation A - with OFI
SAO HPP	Formulation A - with OFI and submitted to HPP
SBC	Formulation B - without OFI
SBO	Formulation B - with OFI
SBO HPP	Formulation B - with OFI and submitted to HPP

Three selected commercially available vegetable creams (CVC1, CVC2, CVC3) were also submitted to the same chemical analysis aiming to compare to Sprelive and explore the potential nutritional advantages of the latter.

Table 3 shows the ingredients of the commercial vegetable creams, and Table 4 presents the proportion (%) of the aqueous and lipidic phases of the products analysed.

Table 3. Ingredient's list of commercial vegetable creams.

Samples	Ingredients
CVC1	Water, oils and vegetable fats (38%) (75% soy, palm, coco in different proportions), modified starch, emulsifiers (mono- and diglycerides of fatty acids, lecithin), salt (0.2%), acidity regulator (citric acid), preservative (potassium sorbate), natural flavour, vitamins (A, D).
CVC2	Vegetable oils (sunflower, extra-virgin olive oil (14%), linseed), water, vegetable fat (karité), emulsifiers (mono- and diglycerides of fatty acids), salt (0.4%), natural flavour, preservative (potassium sorbate), acidity regulator (citric acid), vitamins (A, E, riboflavin and B12), colouring (beta-carotene).
CVC3	Oils and vegetable fats (sunflower (55%), palm), water, buttermilk, salt (1.4%), emulsifiers (lecithin), natural flavours, vitamins (A, D).

Table 4. Proportion (%) between the aqueous and lipidic phases of Sprelive formulations A (SAO) and B (SBO) with OFIx and the commercial vegetable creams.

Samples	Aqueous phase (%)	Lipidic phase (%)
SAO	30	70
SBO	10.63	89.37
CVC1	62	38
CVC2	<i>Not clearly depicted</i>	<i>Not clearly depicted</i>
CVC3	21	79

- Total phenolics, antioxidant activity (FRAP and DPPH• scavenging ability) and hydroxytyrosol content

Total phenolics, antioxidant activity and hydroxytyrosol content assessment, was performed in all samples according to section 5.1.1. The extracts used for analysis were obtained according to Capannesi et al. (2000) [10] with slight modifications. Briefly, 2.5 g of each spread was mixed with 5 mL of *n*-hexane (for the commercial creams, it was used 1.25 g) and vortexed for 15 min. Then 5 mL of a methanol/water (80/20, v/v) solution was added and vortex for 30 min. The hydroalcoholic fraction was separated, and the process was repeated twice. The hydroalcoholic fractions were pooled.

- Fatty acids profile

Likewise, FAME of the samples were also determined according to section 5.1.1. However, first, the samples' fat was extracted by heating 10 g of each spread at 60 °C for 60 min in an oven to separate the oil phase from the aqueous phase. Then, the samples

were centrifuged at 2000 rpm for 5 min to obtain a clear oil phase, which was collected and filtered in an oven at 60 °C using a Whatman paper filter with anhydrous sodium sulphate [11].

- Statistical analysis

Statistical analysis was performed using IBM SPSS Statistics v.27 (IBM Corp., Armonk, NY, USA). The data were expressed as the mean $\pm$ standard deviation. A Student's t-test was used to identify differences between control and fortified or HPP samples. Significance was set at the level of *p*-value less than 0.05. The one-way ANOVA followed by the Scheffe test was used to evaluate the differences among the samples fortified with OFIx and commercial vegetable creams. The level of significance for all the hypothesis tests was 0.05.

5.2.2 Preliminary results and conclusions

The nutritional value of Sprelive is based on two main requirements: the antioxidant and the fatty acid profiles. Hence, the chemical analyses performed, by this time, addressed those two quality parameters. Several questions arose, namely, if the addition of OFIx influenced the antioxidant activity and the hydroxytyrosol contents of the final products and if HPP preserved the chemical features of the Sprelive products. It was also compared the fatty acids profile of Sprelive formulations A (SAO) and B (SBO) (with OFI) and commercial vegetable creams.

Table 5 presents the total phenolics, hydroxytyrosol content and antioxidant activity of Sprelive A and B with/without OFIx and the impact of HPP on Sprelive with OFIx.

Figure 3 presents the total phenolics, hydroxytyrosol content and antioxidant activity of Sprelive formulations A (SAO) and B (SBO) (with OFIx) and the commercial vegetable creams CVC1, CVC2 and CVC3.

Table 5. Total phenolics, hydroxytyrosol content and antioxidant activity (FRAP and DPPH• scavenging ability): comparison between Sprelive formulations A and B with and without the addition of OFIx; and influence of HPP on Sprelive formulations with OFIx.

	Antioxidant activity			
	Total phenolics	Hydroxytyrosol	FRAP	DPPH• scavenging ability
	mg GAE/100 g	mg/100 g	mg FSE/100 g	mg TE/100 g
SAC	20.18 ± 1.76 ^b	1.37 ± 0.14 ^b	15.24 ± 1.32 ^b	13.02 ± 1.19 ^b
SAO	27.77 ± 1.24 ^{aA}	2.16 ± 0.13 ^{aA}	26.27 ± 1.41 ^{aA}	17.71 ± 0.76 ^{aA}
SAO HPP	28.56 ± 3.93 ^A	2.51 ± 0.20 ^A	26.65 ± 1.71 ^A	17.52 ± 1.71 ^A
SBC	15.15 ± 2.29 ^b	1.72 ± 0.14 ^b	16.14 ± 1.84 ^b	16.50 ± 1.56 ^b
SBO	33.22 ± 2.49 ^{aA}	2.36 ± 0.08 ^{aB}	32.68 ± 2.49 ^{aA}	26.83 ± 2.58 ^{aA}
SBO HPP	28.15 ± 0.82 ^B	2.83 ± 0.07 ^A	31.17 ± 2.13 ^A	25.03 ± 1.49 ^A

SAC, Sprelive A control; SAO, Sprelive A with OFIx; SBC, Sprelive B control; SBO, Sprelive B with OFIx. SAO HPP, Sprelive A with OFIx submitted to HPP; SBO HPP, Sprelive B with OFIx submitted to HPP. GAE, gallic acid equivalents; FSE, ferrous sulfate equivalents; TE, trolox equivalents; FRAP, ferric reducing antioxidant power; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical. Data expressed as mean ± standard deviation. Different lower-case letters mean significant differences between samples with/without OFIx. Capital letters mean samples with OFIx and submitted or not to HPP (SAO/SBO, SAO HPP/SBO HPP, respectively).

Bearing in mind that hydroxytyrosol is a specific compound present only in olive and derivatives and in lower amounts in wine [12], this compound was not determined in CVC1 and CVC3 considering their ingredients list.

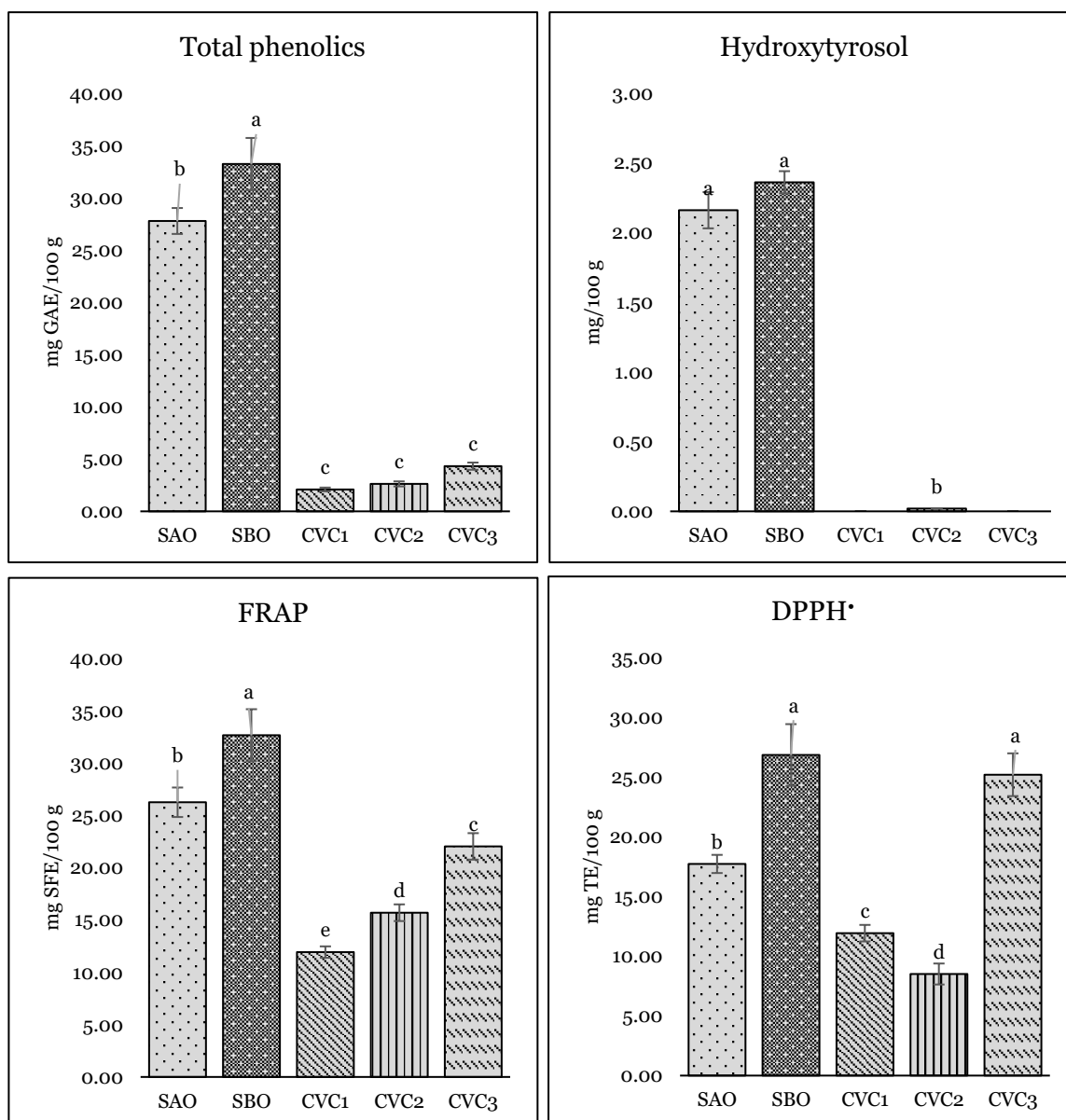


Figure 3. Total phenolics, hydroxytyrosol content and antioxidant activity (FRAP and DPPH• scavenging ability): comparison between Sprelive formulations A and B with OFIx (SAO and SBO) and the commercial vegetable creams CVC1, CVC2 and CVC3.

SAO, Sprelive A with OFIx; SBO, Sprelive B with OFIx. GAE, gallic acid equivalents; FSE, ferrous sulfate equivalents; TE, trolox equivalents; FRAP, ferric reducing antioxidant power; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical scavenging ability. Data expressed as mean $\pm$ standard deviation. Different lower-case letters mean significant differences between samples.

There are vegetable creams available on the market whose composition is a mixture of various vegetable oils, including olive oil (Table 3). However, in these products, the oil content is reduced. Sprelive comprises an oil phase with a high content of olive oil and an aqueous phase that contains a functional ingredient derived from olive pomace. Currently, there is no food product on the market that has these characteristics.

Table 6 presents the fatty acids profile of Sprelive formulations and commercial vegetable creams. Figure 4 shows the saturated (SFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids of the samples.

Developing new food products with botanical extracts as a natural way to improve their nutritional profile and/or shelf life is a challenge and, at the same time, an opportunity for the food industry. Sprelive is a new food product that aims to generate new industrial players and add new lines of business to the olive oil sector.

Currently, its emergent to promote the best use of all resources from olive oil processing, such as the by-products generated that constitutes rich raw materials.

Sprelive will contribute positively to a healthy dietary pattern, increasing the intake of a food recognised for its nutritional properties (olive oil), expanded by adding the olive pomace functional ingredient. In addition, it meets an emerging consumer need: the use of natural ingredients in food formulation. The use of natural ingredients can be especially important for consumers who want healthier and more natural foods and prefer those produced using sustainable methods.

Overall, Sprelive aims to be a food product distinct from others in the same market segment because i) the bioactive compounds present (from olive oil and olive pomace) have beneficial effects on health; ii) it is a product whose main ingredients are obtained naturally, without the use of solvents; iii) it is a healthy alternative to spreadable products on the market, and iv) reduces the environmental impact of the increasing olive oil production.

Table 6. Fatty acids profile (relative %) of Sprelive formulations A and B without the addition of OFIx (SAC and SBC, respectively) and formulations with OFIx (SAO and SBO, respectively); comparison with selected commercially available vegetable creams (CVC1, CVC2, CVC3).

	SAC	SAO	SBC	SBO	CVC1	CVC2	CV3
C6:o	-	-	0.10±0.01	0.10±0.01	-	-	-
C8:o	-	-	1.35±0.10	1.36±0.04	0.12±0.01	-	0.30±0.00
C10:o	-	-	1.02±0.04	1.04±0.01	0.12±0.00	-	0.27±0.01
C12:o	-	-	8.70±0.49	9.04±0.04	1.78±0.02	-	4.07±0.03
C14:o	-	-	3.52±0.17	3.61±0.03	0.76±0.04	-	1.61±0.03
C16:o	12.08±0.02	12.15±0.10	11.70±0.03	11.75±0.05	16.82±0.19	7.28±0.05	16.33±0.09
C16:1	0.81±0.02	0.83±0.03	0.65±0.01	0.65±0.02	-	0.28±0.02	-
C18:o	3.13±0.05	3.11±0.05	3.11±0.03	3.15±0.03	3.72±0.02	14.66±0.06	3.51±0.13
C18:1n9cis	73.61±0.12	73.45±0.16	61.61±0.91	60.92±0.09	31.43±0.12	41.95±0.07	33.88±0.07
C18:2n6cis	8.97±0.07	9.02±0.11	7.04±0.10	7.18±0.09	39.58±0.24	27.38±0.08	39.10±0.08
C20:o	0.32±0.01	0.32±0.03	0.32±0.02	0.31±0.02	-	0.46±0.01	0.20±0.01
C18:3n3	0.83±0.03	0.85±0.05	0.62±0.03	0.62±0.03	5.04±0.02	7.37±0.04	-
C20:1n9cis	0.17±0.01	0.17±0.01	0.16±0.01	0.17±0.01	0.20±0.01	0.08±0.00	-
C22:o	0.09±0.01	0.11±0.01	0.09±0.00	0.09±0.01	0.32±0.03	0.37±0.02	0.53±0.02
C24:o	-	-			0.10±0.01	0.17±0.01	0.22±0.02

SAC, Sprelive A control (without OFIx); SAO, Sprelive A with OFI; SBC, Sprelive B control (without OFIx); SBO, Sprelive B with OFI. Data expressed as mean ± standard deviation. C6:o (caproic acid), C8:o (caprylic acid), C10:o (capric acid), C12:o (lauric acid), C14:o (myristic acid), C16:o (palmitic acid), C16:1 (palmitoleic acid), C18:o (stearic acid), C18:1n9cis (oleic acid), C18:2n6cis (linoleic acid), C20:o (arachidic acid), C18:3n3 (α -linolenic acid), C20:1n9cis (cis-11-eicosenoic acid), C22:o (behenic acid), C24:o (lignoceric acid).

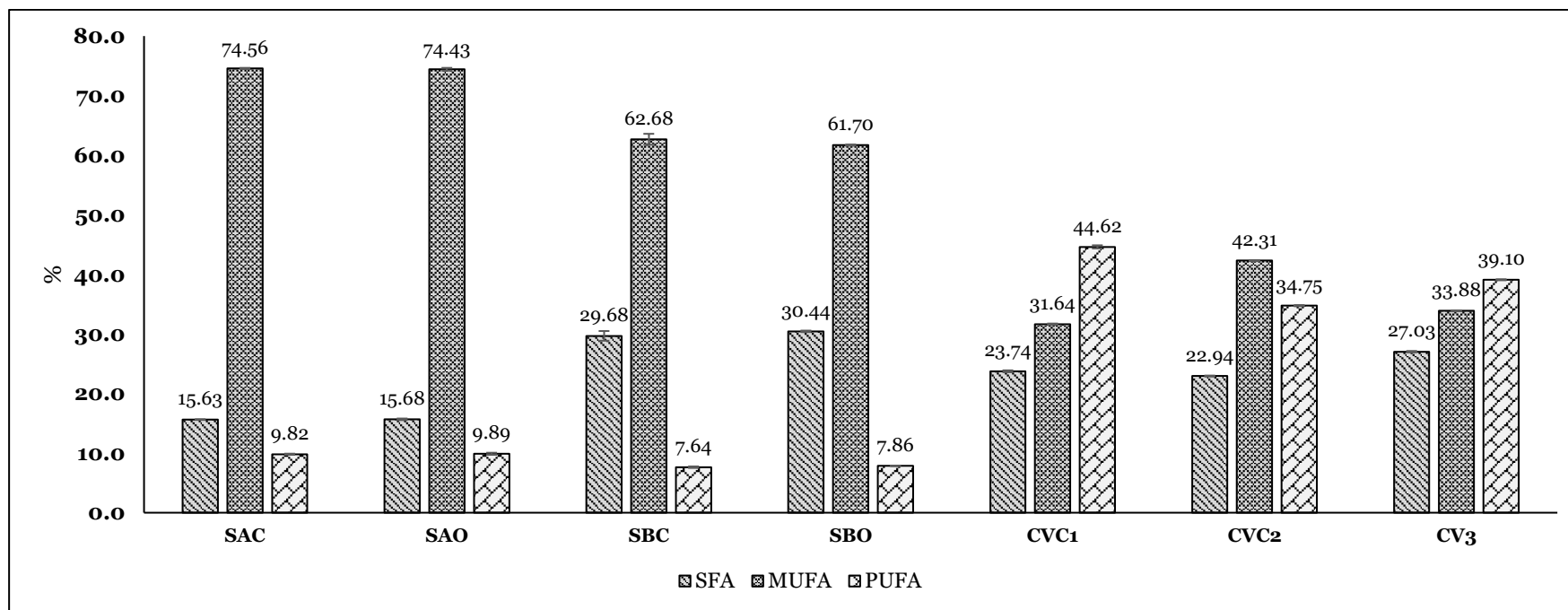


Figure 4. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) (relative %) profiles of Sprelive formulations A and B without the addition of OFI_x (SAC and SBC, respectively) and formulations with OFI_x (SAO and SBO, respectively); comparison with selected commercially available vegetable creams (CVC1, CVC2, CVC3).

SAC, Sprelive A control (without OFI_x); SAO, Sprelive A with OFI; SBC, Sprelive B control (without OFI_x); SBO, Sprelive B with OFI. Data expressed as mean $\pm$ standard deviation.

5.2.3 Ongoing investigation and future work

Further works are still ongoing, and others are needed to conclude this part of the investigation successfully:

- Sprelive rheological and texture properties assessment (viscosity, shear stress, shear rates, firmness, consistency, gumminess, adhesiveness, chewiness, spreadability, colour).
- Emulsion stability evaluation (particle size/distribution, droplet charge/zeta potential).
- Inoculation with pathogenic microorganisms and processing using high pressure.
- Shelf-life assessment.
- Sensory analysis.

References

- [1] Costa ASG, Alves RC, Vinha AF, Costa E, Costa CSG, Nunes MA, et al. Nutritional, chemical and antioxidant/pro-oxidant profiles of silverskin, a coffee roasting by-product. *Food Chem* 2018; 267: 28–35.
- [2] Seiquer I, Rueda A, Olalla M, Cabrera-Vique C. Assessing the bioavailability of polyphenols and antioxidant properties of extra virgin argan oil by simulated digestion and Caco-2 cell assays. Comparative study with extra virgin olive oil. *Food Chem* 2015; 188: 496–503.
- [3] Nunes MA, Palmeira JD, Melo D, Machado S, Lobo JC, et al. Chemical composition and antimicrobial activity of a new olive pomace functional ingredient. *Pharmaceuticals* 2021; 14: 913.
- [4] Manirakiza P, Covaci A, Schepens P. Comparative study on total lipid determination using Soxhlet, Roese-Gottlieb, Bligh & Dyer, and modified Bligh & Dyer extraction methods. *J Food Compos Anal* 2001; 14: 93–100.
- [5] Alves RC, Casal S, Oliveira MBPP. Determination of vitamin E in coffee beans by HPLC using a micro-extraction method. *Food Sci Technol Int* 2009; 15: 57–63.
- [6] ISO 12966-2:2011—Animal and vegetable fats and oils—Gas chromatography of fatty acid methyl esters—Part 2: Preparation of methyl esters of fatty acids.
- [7] Official Methods of Analysis. 19th ed. AOAC International: Rockville USA; 2012.
- [8] Tontisirin K. Chapter 2: Methods of food analysis. In: Food and Agriculture Organization of the United Nations, editor. Food energy: Methods of analysis and conversion factors: report of a technical workshop. Rome; 2003. p. 7-17.
- [9] Pinto CA, Moreira SA, Fidalgo LG, Inácio RS, Barba FJ, Saraiva JA. Effects of high-pressure processing on fungi spores: Factors affecting spore germination and inactivation and impact on ultrastructure. *Compr Rev Food Sci Food Saf* 2019; 19: 553-573.
- [10] Capannesi C, Palchetti I, Mascini M, Parenti A. Electrochemical sensor and biosensor for polyphenols detection in olive oils. *Food Chem* 2000; 71: 553-562.
- [11] Dollah S, Chai KF, Abdulkarim SM, Ghazali HM. Comparative study of table margarine prepared from *Moringa oleifera* seed oil-palm stearin blend and commercial margarines: composition, thermal, and textural properties. *Eur J Lipid Sci Technol* 2020; 122: 1900428.
- [12] Fernández-Mar MI, Mateos R, Garcia-Parrilla MC, Puertas B, Cantos-Villar E. Bioactive compounds in wine: Resveratrol, hydroxytyrosol and melatonin: A review. *Food Chem* 2012; 130: 797-813.

This page was intentionally left in blank.

Chapter 6 – Olive pomace and further valorisation approaches towards the circular economy

Chapter 6 presents other valorisation approaches developed during the period of the PhD work using olive pomace. The works and publications of students from different areas, with the collaboration of the PhD candidate, contributed to the state of the art in the olive pomace scientific field. The outputs are diverse and include a cosmetic product (facial mask) and foodstuff (salty topping and pasta). A brief contextualization, a summary of the work and outputs is presented.

This page was intentionally left in blank.

6.1 Development of a new facial mask using olive pomace: chemical and rheological analysis

Scope and background

- This work was developed in a partnership with Lodz University of Technology (Poland) and the Pharmaceutical Technology Laboratory of the Faculty of Pharmacy of the University of Porto (Portugal) under the scope of the Erasmus programme (2018/2019).
- During the process described in the patent application PCT/IB2018/060111, after centrifugation, a secondary by-product is obtained, the olive pomace paste. As the upper layer is recovered (olive pomace functional ingredient, OFI), initially, it was intended to remove and discharge the olive pomace paste. However, taking the technological properties as a starting point, the chemical profile was assessed and proposed that the olive pomace paste could be explored as an ingredient in cosmetic products.

Purpose

- The study aimed to explore the chemical characteristics of the secondary by-product derived from olive pomace processing (patent application PCT/IB2018/060111). According to those characteristics, a new olive pomace-based cosmetic product was developed – a facial mask.
- The rheological characteristics of the cosmetic product were evaluated over time to determine the product stability.

Key conclusions and novelty

- In this study, a blend of four different olive pomaces (25% of each) - two from Trás-os-Montes and two from Alentejo (Portugal) - constituted the sample.
- The utilization of olive pomace paste allowed the complete recovery of the olive pomace potential. Thus, it accomplishes the “zero waste” strategy associated with the circular economy concepts.
- The results confirmed that the ingredients derived from olive pomace paste are stable, as the facial mask demonstrated lasting stability during the 30 days of analysis period in storage.

- The olive pomace paste has an interesting content in vitamin E and phenolic compounds, which can emphasize its use as a natural ingredient in cosmetic products meeting current consumers trends.

Outputs

- The results were presented in the Master Thesis:
 - Development of a new facial mask using olive pomace: chemical and rheological analysis

Student: Ewa Senderowicz

Work conducted under the supervision of Professor Maria Beatriz Prior Pinto Oliveira and Doctor hab. Engineer Małgorzata Anita Bryszewska with the collaboration of Master Maria Antónia Nunes and Professor Maria Helena dos Anjos Rodrigues Amaral.

6.2 A new and sustainable olive pomace paste ingredient for a topping: influence of heat-treatment processing

Scope and background

- This work was developed to obtain the Degree in Biochemistry (2019/2020) (ICBAS, School of Medicine and Biomedical Sciences of the University of Porto) and in partnership with the Microbiology Laboratory of the Faculty of Pharmacy of the University of Porto (Portugal).
- Nowadays, with the increase of world population, there is a great interest in developing sustainable food products to assure the needs of future generations.
- Using wet olive pomace directly from the olive oil industry is challenging. First, since olives are crushed, olive pomace contains small fragments of olive pits that limit its direct use in food products; also, phenolic compounds such as hydroxytyrosol and oleuropein are responsible for the astringent properties of olive pomace, which restrains it from being used in high quantities in food products.

Purpose

- The study aimed to explore methodologies to remove the olive pits fragments from olive pomace and apply different heat treatments to obtain an olive pomace ingredient microbiological safe and suitable for developing a salty topping.

- The impact of the heat-treatment procedures was evaluated regarding the chemical profile and microbial load.

Key conclusions and novelty

- A new methodology was used to remove the olive pits fragments using a stainless-steel device.
- The heat treatments were efficient in controlling the microbiological load.
- It was possible to select the best heat treatment considering the balance between energy expenditure, chemical profile and product safety regarding the microbiological load.

Outputs

- The results were presented in the Degree Thesis and a scientific paper (*submitted*):
 - A new food ingredient from olive pomace: a preliminary study of the optimal processing conditions

Student: Maria Manuela Sousa

Work conducted under the supervision of Master Maria Antónia Nunes (supervisor) and Professor Maria Beatriz Prior Pinto Oliveira (co-supervisor).

6.3 By-products valorisation: Pasta enrichment with olive pomace and coffee silverskin

Scope and background

- This work was developed under the scope of a master's thesis in Quality Control in the Faculty of Pharmacy of the University of Porto (Portugal) (2020/2021).
- Considering the nutritional characteristics of olive pomace, its application in food products is encouraging. However, it is also a challenge when having in mind the organoleptic features.
- Most of the consumers appreciate pasta. Simultaneously, it has been occurring an opening on the market to diverse and enriched kinds of pasta.
- The olive pits resultant from crushing during olive oil processing is a constraint in final products. Novel methods to remove the olive pits are required.

Purpose

- The study aimed to explore a methodology to remove the fragments of olive pits and its impact on the final nutritional profile.
- To assess the influence of olive pomace and coffee silverskin in pasta on chemical and sensorial features.

Key conclusions and novelty

- Drying, milling and sieving is an alternative and viable method to remove the olive pit fragments from olive pomace and turn it into a safe product without significantly impacting the nutritional profile.
- It is not possible to incorporate amounts higher than 10% of olive pomace in pasta due to the negative impact on the organoleptic features of the final product.
- Incorporating amounts lower than 10% is sufficient to impact the nutritional profile of enriched pasta positively.
- Considering the sensorial analysis, consumers preferred pasta without by-products, followed by pasta with coffee silverskin and olive pomace.
- The incorporation of olive pomace in food products is challenging, and improved formulations are needed.

Outputs

- The results were presented in a Master Thesis:
 - By-product's valorisation: Pasta enrichment with olive pomace and coffee silverskin

Student: Bárbara Oliveira

Work conducted under the supervision of Professor Maria Beatriz Prior Pinto Oliveira (supervisor) and Master Maria Antónia Nunes (co-supervisor).

Chapter 7 –Conclusions and future perspectives

Final reflections are presented in **Chapter 7**, namely, the accomplishing of the purposed objectives at the beginning of the work plan. Finally, and because olive pomace valorisation is an ongoing project, it is presented future perspectives and outlined further work that is still necessary to move towards a Green and Circular Economy in the olive oil sector.

This page was intentionally left in blank.

7.1 Conclusions

At the end of this phase, it is possible to present the partial and general conclusions.

7.1.1 Partial conclusions

- Olive pomace is a source of lipid- and water-soluble compounds. In the lipid fraction, it should be highlighted that the content in MUFA and vitamin E is relevant, mainly in oleic acid and α -tocopherol, respectively, due not only to its biological functions but also to their natural action as preservatives. The phenolic compounds present in the aqueous phase attribute to olive pomace its extraordinary antioxidant activity. Hydroxytyrosol was the main compound identified, representing $\approx 50\%$ of total phenolics.
- The use of the Multi-frequency Multimode Modulated ultrasonic technique, compared to a conventional solid-liquid extraction, allowed a faster and higher recovery of phenolic compounds, being a very effective technique to extract antioxidants from olive pomace.
- Olive pomace is an environmental hazard due to its high content in phenolic compounds, making it phytotoxic. Applying the ecological extraction process described and protected in the patent application PCT/IB2017/053422, it was possible to obtain an extract rich in phenolics. Then, using membrane-assisted processing, the extract was concentrated. The BW30 membrane was found as the most appropriate to be used. The permeate resultant was phenolics-free and contained minerals significant for plant growth, making it suitable for watering.
- Monocultivar olive oils have been increasing on the market. Furthermore, some olive oil producers blend monovarietal oils to attain desired chemical and organoleptic features. Hence, monovarietal olive pomaces are also increasing. The monovarietal pomaces Arbequina, Arbosana, Oliana and Koroneiki were studied. Koroneiki and Arbosana were the richest in total phenolics followed by Arbequina and Oliana. Arbosana pomace presented the highest total vitamin E content.
- NIR spectroscopy and chemometric analysis could differentiate olive pomace cultivars better, even when compared to the reference analytical methodologies applied. The separation obtained using MIR spectra for all cultivars was unclear and indicates that Arbosana pomace is similar to Oliana, which was also shown in the chemical analysis. These two samples are similar and distinguishable from Arbequina and Koroneiki. NIR spectroscopy is better to differentiate olive pomace monocultivars than MIR spectroscopy.

- Blending monovarietal olive pomaces can be a sustainable and advantageous technique compared to the individual use of monocultivar pomaces. The best results were obtained for the following blends: i) 90% Koroneiki, 4.75% Oliana, 3.75% Arbequina, 1.5% Arbosana; ii) 65% Koroneiki, 29% Oliana, 3.25% Arbequina, 2.75% Arbosana; and iii) 85% Koroneiki, 8.75% Arbequina, 3.5% Arbosana, 2.75% Oliana.
- Considering the mixtures analysed, most of the four olive monovarietal pomaces interactions were synergistic and additive when comparing experimental to predicted values. Few antagonistic interactions were observed.
- The green, sustainable and cost-effective method described and protected in the patent application PCT/IB2018/060111 was used to obtain an olive pomace functional ingredient. Additionally, the chemical profile of olive pomace functional ingredient derived from the major Portuguese olive oil production regions (Trás-os-Montes and Alentejo) were compared. There were no significant differences among the four extracts considering the region. Nevertheless, an extract from Trás-os-Montes presented the highest amount of hydroxytyrosol and total phenolics.
- The functional ingredient with a higher hydroxytyrosol content also presented the best minimal inhibitory concentration against *E. coli* and *S. aureus*. None of the extracts showed activity against *C. albicans*.
- Using the olive pomace functional ingredient in food products can be advantageous since it comprises specific lipids (α -tocopherol and oleic acid) and hydrophilic (hydroxytyrosol) compounds, usually not present in other plant extracts which can contribute synergistically to the antimicrobial activity verified.
- Considering the chemical profile and antimicrobial activity, the olive pomace functional ingredient can also be used as a natural nutritional profile enhancer and preservative.
- The developed study during the prototyping phase of the olive oil-based vegetable cream functionalized with the olive pomace functional ingredient allowed to perceive that most consumers do not use olive oil instead of vegetable creams but are receptive to a new form of using olive oil as a spread. Also, more than 90% of consumers surveyed answered that they could consume food products that includes ecologically produced natural ingredients of by-products of the agri-food industry. The preference of the consumers by the “natural” and the use of the by-products to develop new products was reinforced.

- Both olive oil-based vegetable cream formulations functionalized with the olive pomace functional ingredient presented significant higher amounts of total phenolics, hydroxytyrosol and antioxidant activity than the control.
- The fatty acids profile of both Sprelive formulations showed higher content in MUFA compared to the commercial vegetable creams.
- The total phenolics, hydroxytyrosol and MUFA contents and the antioxidant activity of both Sprelive formulations were higher than the commercial vegetable creams.
- Overall, HPP can be a suitable processing method to produce a safe product since no significant differences were found between the control and HPP samples.

7.1.2 General conclusions

The last two pandemic years arose fundamental questions as equal access to health and health care services. Apart from a severe infectious disease, it was shown that vulnerable groups with chronic diseases went through healthcare access constraints. Hence, the necessity of preventing chronic diseases was, once more, reinforced. Ultimately, using natural ingredients derived from by-products will allow the development of functional food products to positively impact the prevention and progression of such diseases (**Chapter 2**). Moreover, the global pandemic exacerbated world hunger and, as never, food security is an emergent topic.

The Sustainable Development Goals of the 2030 Agenda for Sustainable Development advocates to “end hunger, achieve food security and improved nutrition and promote sustainable agriculture” (Goal 2), “ensure healthy lives and promote well-being for all at all ages” (Goal 3), “build resilient infrastructure, promote inclusive and sustainable industrialization and foster innovation” (Goal 9) and a “sustainable consumption and production” (Goal 12). Hence, the research, development and implementation of clean, environmental and economically sustainable processes suitable to the olive oil sector and meeting its needs is mandatory. Otherwise, new processes not fitting in industry needs do not make the bioeconomy move forward. Nevertheless, a scientific basis must continually leverage and support the knowledge transferred to the social and economic sectors.

The use of a natural resource available in high amounts at low cost is an opportunity to develop healthy food and, at the same time, support olive oil producers. This sector is still segmented as there are mostly small and medium companies and a small number of big olive oil producers. Methodologies were proposed to add value to olive pomace and obtain functional ingredients that are an acceptable investment to all olive oil producers.

Its application allows diversifying products offer to industry and expanding the olive oil sector. The establishment of new production lines to process olive pomace in olive mills creates jobs that positively affect the economy and society by improving the regions' sustainability. Moreover, it ensures the sustainable use of natural resources and food production, avoiding an environmental burden (**Chapter 3**).

From the agro-industry point of view, olive pomace is a waste that must be rapidly removed from the olive mills. From a nutritional point of view, olive pomace is an exceptional olive paste with a low-fat content. As olives are rich in bioactive compounds, upcycling olive pomace and developing other products aiming to improve nutrition can increase the diversity of available products on the market. By expanding the number of foods, the market becomes more competitive and affordable to everyone, making easy access to nutritious and safe foods, assuring food security (**Chapter 4**).

Olive pomace is an “old” raw material that has been studied over the years. Developing new products using olive pomace is still a breakthrough in science, olive oil chain and industry. The sensitive balance between olive pomace sustainable processing, its nutritional properties preservation and consumers' acceptance is challenging but must be kept moving forward (**Chapters 5 and 6**).

7.2 Future perspectives and developments

The work presented in this thesis showed that using olive pomace as an ingredient in food products is increasingly advantageous and addresses three major eco-nutrition axes: economy, food and environment. However, further work is required to effectively valorise olive pomace and materialize products.

Therefore, at this moment, points of relevance to further research arise:

- Focusing the chemical profile of olive pomace functional ingredient and aiming to increase the yield of the bioactive compounds, before the pressure procedure, other bio green methods could be applied, namely enzyme-assisted extraction. It is hypothesized that enzymes could release the pomace components to the aqueous phase and increase extractability.
- A wide-ranging knowledge about the yield of the process of obtention of the olive pomace functional ingredient and the chemical profile of each secondary by-product generated is necessary.
- Other methods of preserving the olive pomace functional ingredient besides lyophilization can be explored. The potential organoleptic constraints caused by phenolic compounds can be surpassed, for example, using encapsulation. It is expected that this procedure would simultaneously preserve the bioactive compounds and mask the bitter taste. Chemical analysis should be performed periodically to assess the storage time of the functional ingredient.
- The evaluation of the rheological characteristics of the oil-based vegetable cream formulations is also required.
- Processing methodologies as HPP are beneficial to preserve the antioxidant characteristic of food products. HPP parameters can be studied to eliminate the microbial load and preserve the emulsion stability simultaneously. The chemical and rheological stability of olive oil-based spreads during storage should also be evaluated to understand the HPP's impact on the product.
- Due to pandemic situation only small-scale sensory tests were performed during the prototyping phase of Sprelive. However, a sensory analysis panel must validate the developed products.
- Functional products should be based on scientific data. Therefore, and considering a further long-term step in developing a new functional product, studies regarding

specific health benefits by *in vitro* and *in vivo* assays and clinical studies should be performed.